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Involvement of Toll-like Receptor 2 in Recognition of *Orientia tsutsugamushi* by the Innate Immune System

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Dedicated to
my beloved mother and father
and to my beloved wife Ala'a
and my sweet son Abd-Alrahman

Table of contents

1 Introduction 1

2 Literature review 3

2.1 Scrub typhus **3**
2.1.1 Epidemiology, clinical features, diagnostic and treatment. **3**
2.1.2 Host cell range, intracellular trafficking and replication of *O. tsutsugamushi* **4**
2.1.3 Immune response against *O. tsutsugamushi* **5**
2.1.4 Animal models **6**
2.1.5 Composition of the cell envelope of *O. tsutsugamushi* **7**

2.2 TLRs and immunity **8**
2.2.1 TLR signaling pathways **8**
2.2.2 Structure of TLR2 Ligands **9**
2.2.3 Role of TLRs in infectious diseases **10**

2.3 Role of phagocytes in antimicrobial defense **11**
2.3.1 Dendritic cells (DCs) **11**
2.3.2 Macrophages **12**
2.3.3 Monocytes **12**
2.3.4 Neutrophil granulocytes **13**
2.3.5 Antimicrobial mechanisms of phagocytes **13**

3 Materials and methods 16

3.1 Materials **16**

3.1.1 Devices **16**

3.1.2 Consumable materials **17**

3.1.3 Chemicals and reagents **18**
3.1.3.1 Reagents for cell biological work: **18**
3.1.3.2 Reagents for molecular biology work: **19**
3.1.3.3 Reagents for chemical work: **20**
3.1.3.4 Antibodies **20**

3.1.4 Culture media, buffers and stock solutions **21**
3.1.4.1 Culture media, buffers and stock solutions for cell biology and animal experimental work: **21**
3.1.4.2 Buffers and stock solutions for molecular biological work: **23**
3.1.4.3 Buffers and stock solutions for protein chemical work: **23**

3.1.5 Nucleic acids: 23
3.1.6 Cell lines, bacteria and animals: 24
3.1.7 Software 24

3.2 Methods 25

3.2.1 Methods in cell biology 25
3.2.1.1 Cell culture 25
3.2.1.2 Preparation of infectious and L929 cells control inoculum 25
3.2.1.3 Immunofluorescence stains 25
3.2.1.4 Quantification of infectious inoculum - immunofocus assay 27
3.2.1.5 Purification of *O. tsutsugamushi* from infected cell cultures 27
3.2.1.6 Preparation of cell suspensions from mouse spleen and lymph nodes 28
3.2.1.7 Cell counting 28
3.2.1.8 Flow cytometry 28
3.2.1.9 Stimulation and inhibition mechanisms of 232 and 261 macrophages 29
3.2.1.10 Transfection and stimulation of human embryonic kidney cells 293 29
3.2.1.11 Isolation and stimulation of primary DC culture 30

3.2.2 Methods in molecular biology 30
3.2.2.1 DNA purification 30
3.2.2.2 Determination of DNA concentrations 31
3.2.2.3 Quantitative real-time PCR (qPCR) 31

3.2.3 Methods for biochemical analyses 32
3.2.3.1 Cytokine ELISA 32
3.2.3.2 Proteinase K, polymyxin B, H_2O_2 and NaOH treatments 33

3.2.4 Animal Experiments 33
3.2.4.1 Infection of mice with *O. tsutsugamushi* 33
3.2.4.2 Retrieval of mouse organs for qPCR 34
3.2.4.3 Clinical scores and survival curves after i.p. infection 34

3.2.5 Statistics 35

4 Results 36

4.1 Identification of TLR2 as a receptor for *O. tsutsugamushi* by *in vitro* screening 36
4.1.1 *O. tsutsugamushi* stimulates TLR2- but not TLR4-transfected HEK293 cells 36
4.1.2 Chemical composition of the TLR2 ligands 38

4.2 Requirement of TLR2 for bacterial growth control and proinflammatory responses to *O. tsutsugamushi* 41
4.2.1 Replication of *O. tsutsugamushi* in macrophages 41
4.2.2 *O. tsutsugamushi* triggers NF-κB signaling depending on ligation of TLR2 42
4.2.3 TLR2-dependent cytokine production by macrophages 44
4.2.4 Cytokine production by BMDCs 45
4.2.5 TLR2-dependent signaling is required for intracellular growth control of *O. tsutsugamushi* 46
4.2.6 Inhibition of *O. tsutsugamushi* growth by recombinant TNF-α in macrophages 48

4.3 Influence of TLR2 deficiency on murine *O. tsutsugamushi* infection **50**
4.3.1 Recruitment of phagocytic host cells to regional lymph nodes **50**
4.3.2 Monocytes have a higher infection rate in $TLR2^{+/+}$ mice **51**
4.3.3 TLR2 deficiency does not alter the dissemination pattern of *O. tsutsugamushi* to internal target organs upon s.c. infection **53**
4.3.4 $TLR2^{-/-}$ mice are protected from lethal i.p. infections with *O. tsutsugamushi* **55**

5 Discussion **57**

5.1 TLR2 involvement in *O. tsutsugamushi* recognition **57**

5.2 Chemical properties of the TLR2 ligand of *O. tsutsugamushi* **59**

5.3 Activation of NF-κB by *O. tsutsugamushi* via TLR2 .... **60**

5.4 TLR2-deficient macrophages and BMDCs are inferior producers of pro-inflammatory cytokines **61**

5.5 TLR2-deficient macrophages support increased proliferation of *O. tsutsugamushi* **62**

5.6 Monocyte and neutrophil recruitment during *O. tsutsugamushi* infection **64**

5.7 Subcutaneous (s.c.) infection model **66**

5.8 Intraperitoneal (i.p.) infection model **67**

6 Conclusion **71**

7 Summary **72**

8 Zusammenfassung **74**

9 References **77**

Abbreviations

AP-1	activation protein1
APC	antigen presenting cell
BCG	Bacille Calmette Guérin
BM	bone marrow
BMDC	bone marrow-derived dendritic cell
BSA	bovine serum albumin
BSL	biosafety level
CCL	CC-chemokine ligand
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
ddH_2O	double distilled water
DAP	diaminopimelic acid
DMSO	dimethyl sulfoxide
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
GM-CSF	granulocyte-monocyte colony-stimulating growth factor
H_2O_2	hydrogen peroxide
HBS	hepes buffered saline
HEK cells	human embryonic kidney cells
HK	heat killed
HRP	horse radish peroxidase
HSP	heat shock protein
i.p.	intraperitoneal
IDO	indoleamine-2,3-dioxygenase
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase

IRAKs	IL-1 receptor-associated kinase
ISGs	interferon-stimulated gene
IVCs	individually ventilated cages
JNK	c-Jun N-terminal kinase
L.N.	lymph node
LPS	lipopolysaccharides
LTA	lipoteichoic acid
MAPK	mitogen-activated protein kinase
MCP	macrophage chemoattractant protein
ME	mercaptoethanol
MHC	major histocompatibility complex
MIP1α	macrophage inflammatory protein 1 α
MDP	muramyl dipeptide
MPS	mononuclear phagocyte system
MyD88	myeloid differentiation primary response protein 88
NaOH	sodium hydroxide
NF-κB	nuclear factor-kappa B
NHP	nonhuman primate
NK cell	natural killer cell
NLR	NOD-like receptor
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
PFA	paraformaldehyde
PAMP	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PG	peptidoglycan
PGTP2-RL	*Porphyromonas gingivalis* synthetic lipopeptides
PMN	polymorphonuclear leukocytes
PRR	pattern recognition receptor
qPCR	quantitative PCR
RLRs	retinoic acid-inducible gene (RIG)-I-like receptors
RNS	reactive nitrogen species

ROS	reactive oxygen species
RT	room temperature
rTNF- α	recombinant TNF-α
s.c.	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SNP	single nucleotide polymorphism
TIP DCs	TNF−α and inducible nitric oxide (iNOS)-producing DCs
TIR	Toll–interleukin-1 receptor
TLR	Toll-like receptor
TMB	tetramethylbezidin
TNF	tumor necrosis factor
VV	vaccinia virus

1 Introduction

Scrub typhus is a disease caused by the Gram-negative bacterium *Orientia* (*O.) tsutsugamushi*. It is widely distributed in a part of the world known as the "tsutsugamushi triangle" which covers Asia and the Pacific region including Northern Australia [1]. An estimated one billion people are at risk of acquiring the disease within this endemic area, and the incidence is believed to be around one million new cases every year [2]. Phagocytic cells such as dendritic cells, macrophages, monocytes and neutrophils are the most frequently identified host cells in both humans and mice, especially at the site of pathogen entry [3].

Long before adaptive immunity is induced, an immediate antibacterial defense is provided by the innate immune system. The first critical step is recognition of microbial pathogens by innate immune cells such as macrophages and dendritic cells. These cells express a variety of pattern-recognition receptors (PRRs), which recognize evolutionarily conserved structures in pathogens. Up to now, several classes of PRRs have been recognized such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [4, 5].

The protective role of TLRs has been recognized to play a role in many infections caused by a diverse set of pathogens, including bacteria, viruses, parasites and fungi [6-9]. TLR2 and TLR4 are the most important receptors for recognition of pathogen surface structures. *In vivo*, infections with many bacteria, including *Rickettsia spp.*, are controlled by TLR2- or TLR4-dependent pathways [10-12]. While, a deleterious role of TLR2 has been shown in some infection models such as *Plasmodium* (*P.*) *berghei* and *Pseudomonas (P.) aeruginosa* [13, 14].

The role of innate immune responses mediated by sensing receptors such as TLRs and NLRs in response to *O. tsutsugamushi* is presently unknown. Moreover, little is known about how innate signaling shapes the early events of infection with *O. tsutsugamushi*. The goal of this study was to identify the innate receptors involved in recognition of surface structures of *O. tsutsugamushi*, and to elucidate their role in the experimental scrub typhus mouse model.

The following questions guided the experimental investigations:

- Which receptors are involved in recognition of *O. tsutsugamushi* surface structures?
- What is the chemical composition of the *O. tsutsugamushi* ligand?
- Which phagocytic cell types are recruited to the regional lymph node early after subcutaneous infection with *O. tsutsugamushi*, and does recognition by innate receptors influence this recruitment?
- Which phagocytic cells are infected in the lymph node early after subcutaneous infection with *O. tsutsugamushi*, and is pathogen dissemination to internal organs dependent on innate receptor signaling?
- Is the presence of the innate receptor for *O. tsutsugamushi* required for protection against severe intraperitoneal infection?

2 Literature review

2.1 Scrub typhus

Scrub typhus is a vector-borne zoonotic disease [15], also known as *tsutsugamushi* disease. The name is derived from two Japanese words: tsutsuga (something small and dangerous) and mushi (creature) [16]. This acute, febrile, infectious illness is caused by *Orientia* (formerly *Rickettsia*) *tsutsugamushi (O. tsutsugamushi)*. The disease is typically characterized by focal or disseminated vasculitis and perivasculitis in many organs including lung, liver, heart, spleen, and central nervous system [1]. In fact, delayed treatment can lead to severe multi-organ failure with mortality rates ranging from 1 to 50%, depending on the *Orientia* strain and immune competence of patients [17].

2.1.1 Epidemiology, clinical features, diagnostic and treatment.

O. tsutsugamushi is an obligate intracellular bacterium. It is classified as a separate genus in the Rickettsiaceae family [18]. Phylogenetic analysis based on 16S rRNA gene sequence homology suggested that *O. tsutsugamushi* is a member of Gram-negative bacteria [19]. *O. tsutsugamushi* is unique among other rickettsia-related species due to the presence of several antigenic variants [20] and a lack of peptidoglycan (PG) and lipopolysaccharide (LPS) [21]. Even with the large number of serotypes (including Karp, Gilliam, Kato, Kawasaki, and Kuroki), there is sufficient crossreactivity with antigens from major serotypes with other strains, which allows serologic diagnosis with the indirect fluorescent antibody test [20].

Scrub typhus, the disease caused by *O. tsutsugamushi*, is endemic to a part of the world named the “tsutsugamushi triangle” (Figure 1) [1]. This area covers a large part of Eastern Asia and the Western Pacific region [1] and naturally forms a triangle bordered by Japan and far-Eastern Russia in the North, the Northern part of Australia in the South and Pakistan in the West [22].

Figure 1. Map demonstrating the geographic areas where scrub typhus is endemic (black) [1].

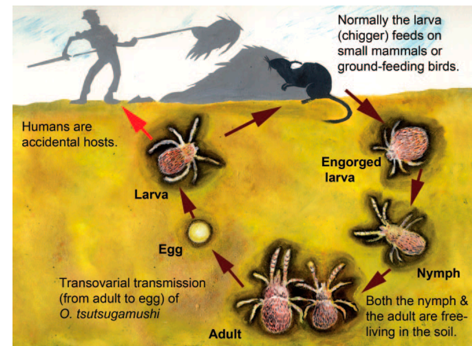

Figure 2. Diagram elucidates the life cycle of a *Leptotrombidium* mite [1].

In 1878 was the first description of the disease in Japan [23]. It is transmitted to humans and rodents by the bite of an infected larval-stage trombiculid mites, e.g. *Leptotrombidium deliense* [24]. The mites have a four-stage lifecycle: egg, larva, nymph and adult (Figure 2) [1], but only larval stages (chigger) can transmit the disease to humans and other vertebrates because the other life stages do not feed on vertebrates [25, 26].

The clinical features of scrub typhus vary in severity from mild and self-limiting to fatal as a result of the virulence of the infecting strain [27]. Thus the average annual fatality rate of scrub typhus in Japan was reported to be as high as 50% before antibiotics were introduced [28]. Antibiotics such as tetracycline and chloramphenicol are effective agents against scrub typhus and fever resolves within 24 hours after treatment in most patients [1].

The diagnosis is usually made on the basis of rising serum antibody titers. A variety of serologic assays are available. The Weil-Felix agglutination test lacks sensitivity but is easy to perform in less developed areas [29]. The indirect immunofluorescence test is more sensitive and specific than the Weil-Felix test [29]. More recently developed assays, including the immunoperoxidase test and the polymerase chain reaction (PCR), have also been used successfully to diagnose acute infection with *O. tsutsugamushi* [30-32].

2.1.2 Host cell range, intracellular trafficking and replication of *O. tsutsugamushi*

At the beginning of the infection *O. tsutsugamushi* infects myeloid cells including Monocytes, macrophages and polymorphonuclear leukocytes (PMNs) in the inoculation eschar [3, 33, 34].

Endothelial cells lining the vasculature have also been shown to be infected by *O. tsutsugamushi*, but the mechanisms and kinetics of endothelial infection are essentially unknown [33]. *O. tsutsugamushi* internalization into host cells involves a specific interaction between bacteria and host cells [35]. *O. tsutsugamushi* first attaches to target cells by the interaction of surface molecules such as TSA56 [36] and ScaC [37] with host cell receptors such as Syndecan-4 [38] and fibronectin [37, 38]. It has been proposed that this interaction facilitates bacterial entry [36] via induce endocytosis or phagocytosis [39]. This process could be triggered by integrin α5β1and is mediated by clathrin [40, 41]. Subsequent, downstream signaling leads to cytoskeletal reorganization. Afterwards *O. tsutsugamushi* escapes from the endo-/phagosome and replicates in the host cytoplasm via binary fission [42]. Budding of *O. tsutsugamushi* from the cell surface of the infected cells is observed after 2-3 days following infection [42]. The released bacterium is covered by host cell membrane. which may be lost, leaving naked *O. tsutsugamushi* to invade other cells [33, 43].

2.1.3 Immune response against *O. tsutsugamushi*

The immune system has different mechanisms to protect the host from pathogens. Innate mechanisms encompass cellular components (e.g. macrophages, eosinophils, neutrophils, natural killer (NK) cells), and humoral factors (e.g. complement, proinflammatory cytokines such as interleukins, interferons, and tumor necrosis factor (TNF)-α) [44].

During this first line of defense antigen-presenting cells such as dendritic cells (DCs) and macrophages encounter antigen and get activated. These cells are responsible for the initiation of adaptive immunity by presenting antigen in major histocompatibility complex (MHC) II. Adaptive immunity involves T and B lymphocytes and counters not only a high specific response but also immunological memory [44]. Both CD8+ cytotoxic T-lymphocytes (CTL) and CD4+ type 1 helper T (Th1) play a central role in cell-mediated immunity against pathogens, especially intracellular bacteria [45]. They are activated by antigen-presenting cells (APC) such as macrophages and DCs that present antigenic peptides to CTL and Th cells via MHC class I and II, respectively [46].

A number of studies in mice have shown that Th1cells are important in immunity to *O. tsutsugamushi* [47, 48]. Adoptively transferred IFN-γ-producing Th1 cells have demonstrated to mediate protection against *O. tsutsugamushi in vivo* [49]. Moreover, IFN-γ secreted by the murine Th1 subset of CD4+ T cells are able to inhibit *O. tsutsugamushi* growth in

macrophages and in fibroblasts *in vitro* [50-52]. in addition TNF-α inhibits the growth of *O. tsutsugamushi* in human endothelial cells [53]. However, CTL play a dominant role in the elimination of intracellular pathogens, such as *Rickettsia* or *Listeria (L.) monocytogenes*, while the Th cells play a role in defense against bacteria located in vacuolar compartments, such as *Mycobacterium* or *Salmonella* [54].

The humoral immune response is also important in protection against scrub typhus. Antibodies if present early in the disease can play a protective role [55]. More specifically, antibodies to the homologous strain confer partial protection, while heterologous antibodies do not [56]. On the other hand, it was demonstrated that anti-*O. tsutsugamushi* antibodies increased the uptake of *O. tsutsugamushi* by professional phagocytes such as PMNs [57] and macrophages [58], but not nonprofessional phagocytes such as endothelial cells and fibroblasts [18]. These results show collectively that antibacterial control of *O. tsutsugamushi*, like other obligate intracellular microbes, involves both cellular and humoral immunity [18].

Unfortunately, there is very little information about the nature of the innate immune response in *O. tsutsugamushi* infection. Recently, *Cho et al.* reported that NOD1 senses an *O. tsutsugamushi* component in endothelial cells and activates the downstream pathway of nuclear factor-kappa B (NF-κB) during *O. tsutsugamushi* infection, which ends with production of interleukin (IL)-32 [59]. In contrast to *O. tsutsugamushi*, TLRs have been shown to play an important role in innate immunity response against other *Rickettsia* species. Previous studies have demonstrated that *R. africae* and *R. akari* are capable of inducing cellular activation through TLR2 and TLR4 [12, 60]. Moreover, *Jordan et al.* reported the important of TLR2 for immune response and protection against *R. conorii* infection [10].

2.1.4 Animal models

Many factors influencing susceptibility have been identified, including *O. tsutsugamushi* strain, infectious dose, and host [61, 62]. The first experiments of *O. tsutsugamushi* were carried out by Hayashi using non-human primates (NHPs) and guinea pigs [63]. Subsequently, the majority of studies have used the intraperitoneal (i.p.) route to infect mice with *O. tsutsugamushi*. I.p. infection of mice causes prominent localized infection of the peritoneum and the serosal lining and generally serves as a test for susceptibility against *O. tsutsugamushi* [43]. In contrast, studies using the subcutaneous (s.c.) inoculation route are much closer to the natural dermal infection as occurring after mite bites. While an eschar is

not formed at the site of inoculation, s.c. infection is marked by early accumulation of bacteria in the lymph node and subsequent systemic pathogen spread [64]. Unlike i.p. infected mice, s.c. infected mice generally do not succumb to the infection with *O. tsutsugamushi*, independently of their genetic background. Moreover, they are even protected against subsequent rechallenge via the i.p. route. S.c. infection is therefore a model for establishment of immunity under close-to-natural infection conditions. However, susceptibility largely depends on the genetic backgrounds e.g. Balb/c more susceptible than C57BL/6 mice [65].

NHPs such as rhesus macaques have also been used. These NHPs develop a disease resembling human scrub typhus when infected s.c. with low mortality. These clinical symptoms include formation of an eschar at the inoculation site, fever and lymphadenopathy [66, 67].

2.1.5 Composition of the cell envelope of *O. tsutsugamushi*

A previous study has shown that the composition of the cell envelope of *O. tsutsugamushi* differs substantially from that of the other rickettsiae. *O. tsutsugamushi* lacks both PG and LPS [21]. More specifically, *Amano et al.* reported that the cell wall of *O. tsutsugamushi* does not contain muramic acid, glucosamine, heptose, 2-keto-3-desoxy-octonate (KDO), and hydroxyl fatty acid which are the constituents of bacterial PG and LPS. *Hanson* reported that the cell wall contains only the major strain-variable 56-kDa protein as well as the antigenically variable 110-, 47-, and 25-kDa proteins [68]. At present, five immunodominant proteins of *O. tsutsugamushi* are characterized in human with molecular weights of 22kDa, 47kDa, 56kDa, 58kDa, and 110kDa [69]. Among all antigens, the 56-kDa protein is often recognized by both human and animal host immune systems during infection [70]. It is structurally and functionally nearly identical to the eukaryotic 60-kDa heat shock protein (Hsp) [71], suggesting that it is a suitable diagnostic antigen and vaccine candidate [70]. In contrast to *O. tsutsugamushi,* other *Rickettsia* species do contain LPS and PG in their cell wall as shown by the presence of KDO, heptose, gluocosamine and muramic acid in *R. typhi* [72] and diaminopimelic acid in *R. prowazekii* and *R. typhi* [73].

2.2 TLRs and immunity

Innate immunity is builds the first line of defense against invading pathogens and is rapidly activated after encounter of pathogen components. Innate immune cells such as DCs, macrophages and PMNs can recognize the evolutionarily conserved structures of pathogens by a set of pattern recognition receptors (PRRs). These molecules are necessary components for the survival of the pathogen and are called pathogen-associated molecular patterns (PAMPs) [4, 5]. So far, several classes of PRRs have been identified, e.g. TLRs and NLRs. The term TLR was coined owing to their homology to the *Drosophila* Toll protein. TLRs can recognize a wide range of structural components of pathogens leading to induction of innate immune responses [74]. These include double-stranded RNA (TLR3), LPS (TLR4), flagellin (TLR5), CpG motifs in unmethylated bacterial DNA (TLR9) and peptidoglycan, lipoteichoic acid and lipoproteins (TLR2) [75]. Among NLR family NOD1 recognizes diaminopimelic acid (ie-DAP) while NOD2 recognizes the muramyl dipeptide (MDP) fragment of PG. Ligation of both TLR and NLR family members generally leads to a signaling cascade culminating in NF-κB activation and subsequent production of important mediators of inflammation such as cytokines and chemokines [75].

The activation of innate immune response is not important only for the eliminate of pathogens early in the infection, but also to initiate and shape pathogen-specific adaptive immunity [76].

2.2.1 TLR signaling pathways

In general, TLR ligation may initiate the activation of two distinct signaling pathways: the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway and the TIR domain-containing adapter inducing IFNβ (TRIF) pathway [77].

The MyD88-dependent pathway is triggered by all TLRs except TLR3. This pathway leads to the activation of kinase cascades ending with phosphorylation and degradation of inhibitor of NF-κB (IκB), allowing NF-κB to enter the nucleus and resulting in production of inflammatory cytokines such as IL-12, TNF-α and IL-6. Furthermore, the MyD88-dependent pathway can also activate mitogen-activated protein kinases (MAPKs), such as p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), which can influence both transcription of inflammatory genes via phosphorylation of the activation protein 1 (AP-1) and mRNA stability of those transcripts (Figure 3) [44, 77].

The TRIF pathway mediates the induction of IFN-β. TRIF-dependent signaling is utilized by TLR3 which is expressed in endosomal vesicles, and by TLR4 which is mainly expressed on the cellular surface (Figure 3) [77]. A recent study found that TRIF could also play a role in TLR2-mediated signaling, as shown in *Borrelia burgdorferi* infection [78].

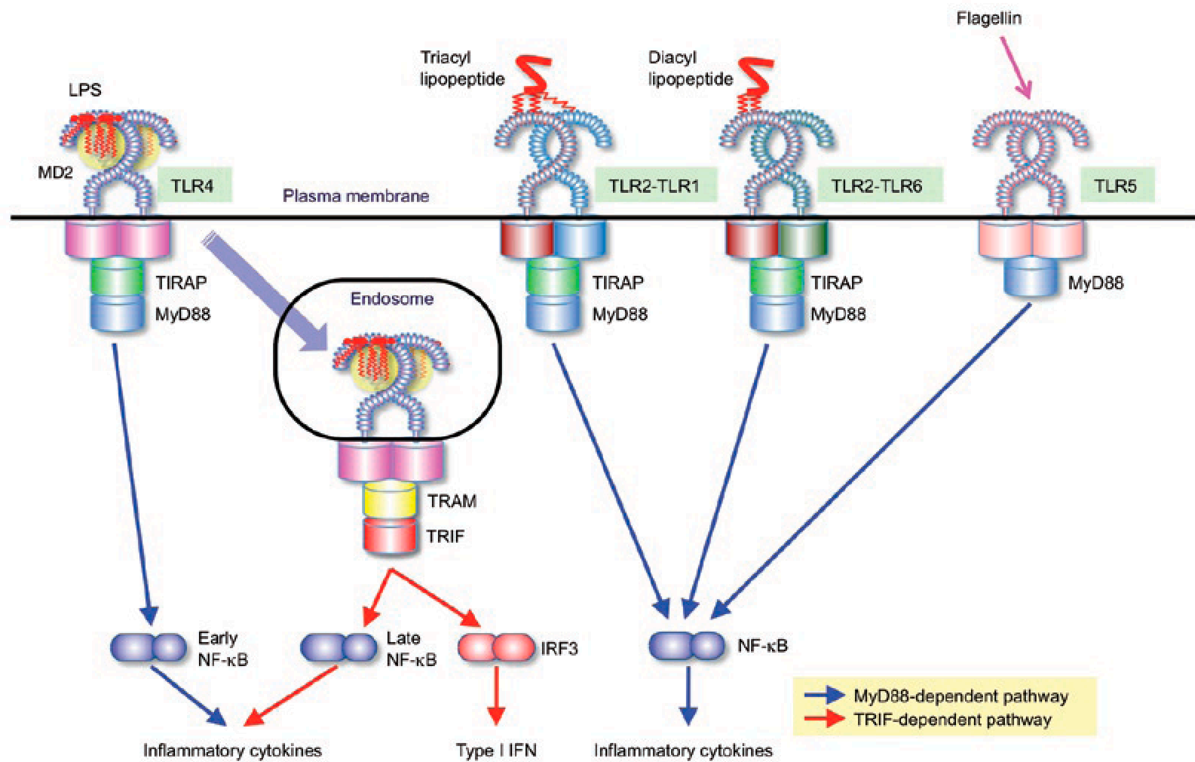

Figure 3. Overview of TLR signaling pathways [79].

2.2.2 Structure of TLR2 Ligands

TLR2 is triggered by a variety of microbial products such as lipoproteins, lipoteichoic acids, peptidoglycans, zymosans, lipomannans, phenol-soluble modulins, and hyaluronans [80]. Moreover, TLR2 is a distinctive member of the TLR family because it can form heterodimers with other TLRs, specifically TLR1 and TLR6. These heterodimers can bind to lipopeptides or lipoproteins in bacterial membranes [81]. Lipoproteins and lipopeptides which are functionally and structurally diverse bacterial proteins are covalently bound to the membrane by two or three attached lipid chains [82]. Lipoprotein recognition requires interaction of accessory proteins such as CD14 and CD36 [81]. Recently *Zähringer et al.* reviewed that TLR2 is likely recognizing a particular molecular structure by lipoproteins/lipopeptides [80]. This structure is critical for TLR2 recognition by lipoprotein/lipopeptide and is characterized by a highly conserved thioether structure, as it was identified in the mycoplasmal lipopeptide

R-MALP-2 [80]. The synthetic lipopeptides Pam_2CSK_4 and Pam_3CSK_4 imitate the properties of lipoproteins. The acylated cysteine group on synthetic lipopeptide N termini appears to play a role as the main immune stimulatory motif [83, 84].

2.2.3 Role of TLRs in infectious diseases

In order to investigate the role of TLRs in pathogenesis of infection or immunological defense, several approaches have been used. Most researchers have used mice deficient in individual TLRs [85]. *In vitro* approaches were also used widely, involving both murine and human macrophages or dendritic cells and human peripheral blood mononuclear cells (PBMCs) [86].

The protective role of TLRs has been recognized to play a role in infection caused by a diverse set of pathogens, including bacteria, viruses, fungi and parasites. TLR2-deficient mice are more susceptible than wild-type mice to certain viruses, such as vaccinia virus (VV) and cytomegalovirus (CMV) [6]. As far as fungal infections are concerned, TLR2 was also shown to play a major role in protection against *Penicillium (P.) marneffei* [7]. TLR2 equally has a protective role in infections with the Gram-positive bacteria *Staphylococcus aureus* [8] and *Streptococcus pneumoniae* [87]. Furthermore, TLR2-deficient mice challenged with *L. monocytogenes* are more susceptible than wild-type mice and show increased bacterial numbers within the liver and a reduced survival rate [88]. On the other hand, a deleterious role for TLR2 has been shown in some infection models. For example, in murine malaria with *P. berghei* a pathological role for TLR2 and TLR9 has been demonstrated whereas the absence of TLR2 or TLR9 increased resistance to cerebral malaria-related mortality [13]. Moreover, TLR2 deficiency increased resistance to *P. aeruginosa* pneumonia [14].

TLR4-deficient mice are more susceptible to infection with *Salmonella typhimurium* [89] and is also essential in the control of *Mycobacterium* (*M.*) *tuberculosis* infection [90] although another study showed that TLR2-deficient mice are less resistant to high-dose challenge with *M. tuberculosis* [9]. Furthermore, TLR4-deficient mice are found to be more susceptible to *Candida albicans* infection [91]. Among *Rickettsia* infections, previous studies demonstrated a protective role of TLR4 in *R. conorii* while both TLR2 and TLR4 plays a protection role in *R. akari* [10-12].

2.3 Role of phagocytes in antimicrobial defense

The mononuclear phagocyte system (MPS) consists of DCs, monocytes and macrophages, and is implicated in the control of many immunologically mediated processes such as inflammation, infection, autoimmunity and cancer.

2.3.1 Dendritic cells (DCs)

DCs are tissue-resident phagocytes and potent APCs, even more potent than macrophages. They play an important role in the orchestration of immune responses [92]. DCs are derived from bone marrow progenitor cells and encompass a complex lineage of cells [93]. Upon infection, DCs interact with foreign antigen and undergo a maturational program, including the increased surface expression of MHC and co-stimulatory molecules, such as CD40, CD80, and CD86. They subsequently migrate to lymph nodes where potent interactions with T cells initiate the adaptive immune response [94]. DCs express a set of PRRs on the surface that can specifically interact with PAMPs, including the mannose receptors, C-type lectins and TLRs [95, 96]. Depending on the type of PAMP they are exposed to, DCs are able to shape the adaptive T cell response rather towards a Th1 phenotype via production of IL-12 or towards a Th2 phenotype via production of IL-4 [92]. Thus, DCs play an important role in linking the innate and adaptive immunity through their unique expression patterns of TLR-mediated recognition and cytokine production [96].

At present, there is limited knowledge about the role that DCs might play in *O. tsutsugamushi* infection. However, a recent study employing eschar skin biopsies from scrub typhus patients showed that *O. tsutsugamushi* infects dermal DCs and monocytes rather than endothelial cells which are considered the primary target cell for *O. tsutsugamushi* [3]. More importantly, the authors suggested that the infection of DCs and macrophages may offer a potential route for dissemination of *O. tsutsugamushi* from the initial site of infection to the rest of body [3]. This interpretation was reinforced by another study showing that *O. tsutsugamushi* decelerates DC migration and escapes from degradation by autophagy in bone marrow-derived DCs (BMDCs) [97]. Migratory DCs, which are intended to kill pathogens and carry pathogen-derived antigens to the regional lymph node, may thus be exploited by *O. tsutsugamushi* as replication reservoirs, before systemic dissemination occurs. In addition by infecting APCs such as DCs *O. tsutsugamushi* may modulate host immune response [3].

2.3.2 Macrophages

Macrophages are classified as tissue-resident phagocytes that specialize in the capture and clearance of damaged cells as well as invading pathogens. Macrophages secrete pro-inflammatory molecules in response to microbial infection and thus have an essential role in host defense [98]. *O. tsutsugamushi* invades humans via the skin upon mite biting and primarily infects myeloid cells in the inoculation eschar [3]. Once the infection becomes systemic, endothelial cells will be infected [33]. However, monocytes and macrophages are believed to be secondary targets in all organs [33]. Early *in vitro* experiments showed that macrophages could kill *O. tsutsugamushi* after activation by cytokines present in supernatants of activated splenocytes [99]. Also, recombinant TNF-α inhibited *O. tsutsugamushi* growth in macrophages *in vitro* [53]. Furthermore, *O. tsutsugamushi* is able to infect and multiply in peritoneal macrophages *in vivo* [99, 100]. Tissues from human scrub typhus patients contained *O. tsutsugamushi* in macrophages from the liver and the spleen [8]. A recent study showed that *O. tsutsugamushi* is able to replicate within human monocyte-derived macrophages and strongly enhances transcription of interferon-stimulated gene (ISGs), apoptosis-related genes and genes encoding inflammatory mediators. It was shown that *O. tsutsugamushi* infection also induces the release of interferon (IFN)-β and inflammatory cytokines [101].

2.3.3 Monocytes

Monocytes are mononuclear phagocytes circulating in the blood and differentiate to macrophages and DCs in peripheral tissues [102]. Monocytes are characterized by different phenotypic subsets as assessed by the expression of surface markers. Human monocytes consist of two subsets on the basis of surface CD14 and CD16 expression: $CD14^{++}$ $CD16^{-}$ inflammatory (also known as classical) and $CD14^{+}$ $CD16^{+}$ resident (also known as non-classical) monocytes [103]. In the mouse $CCR2^{+}$ $CX3CR1^{lo}$ $Ly6C^{hi}$ monocytes are classified as inflammatory monocytes while resident monocytes are defined as $CCR2^{-}$ $CX3CR1^{hi}$ $Ly6C^{lo}$ [103, 104]. The inflammatory monocytes are essential in the immune response against infectious pathogens. These cells take part in antibacterial immune responses by production of pro-inflammatory cytokines and contribute to the rise of TNFα- and inducible iNOS-producing (TIP) DCs during infection [98]. The non-classical subset of monocytes patrols the blood circulation and promotes tissue healing [98]. A recent study demonstrated that *O.*

tsutsugamushi replicates in human monocytes isolated from healthy donors and that infection alters the expression of thousands of host genes [105]. In this study, the type I IFN response and the expression of an M1-associated cytokines suggested an important role for monocytes in the regulation of the immune response [105]. All of the before mentioned cells are not only effectors of immunity, but also considered as a secondary targets of *O. tsutsugamushi* infection in humans [33].

2.3.4 Neutrophil granulocytes

Neutrophils which are known also as PMNs are a crucial part of the innate immune system and play an important role in the early defense against pathogens. They are the most abundant subpopulation of peripheral blood leukocytes. These cells are known to act as phagocytic cells and are recruited very fast to sites of tissue damage and infection following signals such as IL-8, IFN-γ and C5a in a process called chemotaxis. They release antimicrobial and inflammatory mediators that permit recruitment and activation of other effector cells, leading to control of infection by clearance of the pathogen and initiation of the adaptive immune response [106]. At the site of infection, neutrophils bind and ingest the pathogen through a process called phagocytosis, resulting in release of reactive oxygen species and lytic enzymes into the pathogen-containing phagosome. Neutrophils are furthermore equipped to discharge cytoplasmic granules containing antimicrobial enzymes into the extracellular space which helps to combat extracellular pathogens [107].

Neutrophils are found to play an essential role in many infectious diseases. In lung infection with *Chlamydia (C.) pneumoniae* neutrophils are recruited to the lung as one of the first events of infection [108]. In *M. bovis* infection, neutrophils are not only recruited to sites of infection, but are also able to shuttle Bacille Calmette Guérin (BCG) to the draining lymph node [109]. Moreover, neutrophils are the first cells arriving to the site of infection in *Leishmania major* and starting phagocytosis process [110].

2.3.5 Antimicrobial mechanisms of phagocytes

Phagocytic cells are equipped with several antimicrobial mechanisms to internalize and destroy pathogens. Phagosome formation and maturation after uptake of pathogens is a key process in killing invading pathogens. However, certain pathogens possess strategies to

overcome these defense mechanisms and avoid phagosomal degradation. Some of these mechanisms include inhibition of phagosomal maturation (e.g. *M. tuberculosis* [111]) or enzyme-mediated escape from the phagosome (e.g. *L. monocytogenes* and *Rickettsia spp.* [112, 113]). This section summarizes important antimicrobial mechanisms of phagocytes.

One significant antimicrobial mechanism is the production of reactive nitrogen species (RNS). The enzyme iNOS is responsible for the production of nitric oxide (NO•) radicals which are needed for the formation of RNS [114]. RNS production and its relevance for intracellular pathogens has been studied widely in macrophages [115]. E.g., iNOS$^{-/-}$ mice are more susceptible to *L. monocytogenes* than wild-type mice. [116]. Moreover, another study demonstrated the role of NO in killing of *L. monocytogenes* in infected macrophages but only in the presence of IFN-γ. This effect was prevented by adding NO inhibitors such as aminoguanidine [117]. In addition, *Feng et al*. reported that hepatocytes and endothelial cells can kill intracellular *R. conorii* through iNOS-dependent mechanisms [118].

Another effective antimicrobial mechanism is the production of reactive oxygen species (ROS). ROS such as superoxide anion (O_2^-), hydroxyl radical (HO•) and hydrogen peroxide (H_2O_2) are produced mainly in neutrophils [115, 119]. The importance of ROS in the defense against pathogens is emphasized in people with chronic granulomatous disease, where the infection is recurrent and can lead to death [120]. Both ROS and RNS were found to play an important role in controlling infection with *Francisella (F.) tularensis* where mice deficient in production of RNS or ROS demonstrated a higher susceptibility [121]. Moreover, both H_2O_2-dependent mechanisms and NO synthesis were found to contribute to inhibition of *R. conorii* replication [118]. Tryptophan deprivation is another antimicrobial defense mechanism. Macrophages produce indoleamine-2,3-dioxygenase (IDO), which degrades the essential amino acid tryptophan [122]. Several studies have demonstrated the role of tryptophan depletion in pathogen inhibition in infections with *C. psittaci*, *Toxoplasma gondii (T. gondii)* and *R. conorii* [118, 123, 124]. A recent study has also demonstrated a role for IDO in *O. tsutsugamushi*-infected patients [125].

Autophagy is an intracellular process that contributes to cellular homeostasis. It is characterized by sequestration of cellular components in double-membrane vesicles that combine with lysosomes [115]. For several years, autophagy has been suggested to play a role as an antimicrobial defense mechanism against many bacteria infections, such as *M. tuberculosis* and streptococci [126, 127]. Interestingly, the degradation of *O. tsutsugamushi* in

autophagosomes seems to play a minor role in antimicrobial defense as *O. tsutsugamushi* was shown to actively escape from autophagy [97, 128].

3 Materials and methods

3.1 Materials

3.1.1 Devices

Device	Manufacturer
Centrifuges	
Biofuge pico	Heraeus Instruments, Hanau
Megafuge 1.0 and 1.0 R	Heraeus Instruments, Hanau
Centrifuge 3-16 K	Sigma, Osterode am Harz
CO_2 incubators	Memmert, Schwabach
	Binder, Tuttlingen
	Heraeus, Hanau
Flow cytometer	
FACS-Aria III	Becton Dickinson, Heidelberg
Accuri C6	Becton Dickinson
heating block	Eppendorf, Hamburg
Homogenizer (Precellys 24)	Peqlab, Erlangen
Laminar Flow Hoods	BDK Luft und Reinraumtechnik, Sonnenbühl
Light Cycler 480 II	Roche Diagnostics, Risch, Switzerland
Liquid nitrogen container	Air Liquide, Marne la Vallée, France
Magnetic stirrer	Heidolph, Schwabach
Microscopes	
inverted microscope	Hund, Wetzlar
Fluorescence microscope, Axioskop 2 plus	Zeiss, Jena
Confocal laser scanning microscope	Olympus, Japan
pH meter	WTW, Weilheim
Pipettes	Eppendorf, Hamburg
	Thermo Scientific, Waltham, USA
ELISA plate reader (MRX II)	Dynex Technologies, Berlin

Device	Manufacturer
Portable pipet-aid	Hirschmann, Eberstadt
	Integra Biosciences, Fernwald
Refrigerators and Freezers	Liebherr, Bulle, Switzerland
	Bosch, Gerlingen
Scale	
Analytical Lab Scale	Sartorius AG, Göttigen
Spectrophotometer (NanoDrop 2000c)	Thermo Scientific, Wilmington, USA
Vortexers	Bioblock Scientific, Illkirch, France
	VWR, Darmstadt
Water bath	GFL, Burgwedel
γ-irradiation device	STS, Braunschweig

3.1.2 Consumable materials

Material	**Producer**
Buttoned cannulas (1428 a'LL)	Acufirm, Ernst Kratz, Dreieich
Cannulas (18, 20, 27 gauge), sterile	Braun, Melsungen
Cell culture flasks (25 cm^2, 75 cm^2, 175 cm^2), sterile	Greiner Bio-One, Frickenhausen
Cell culture plates, sterile	
96-well, round bottom	Greiner Bio-One, Frickenhausen
24-well, flat bottom	
6-well, flat bottom	
6-well, flat bottom, ultra-low attachment	
Cell scrapers	TPP, Trasadingen, Switzerland
Cell strainers (Cell Strainer, 100 µm), sterile	BD Falcon, Heidelberg
Cell strainers (CellTrics, 30 µm)	Partec, Görlitz
Centrifuge tubes (15 ml, 50 ml), sterile	Sarstedt, Nümbrecht
Cover slip (round, 12 mm diameter)	Roth, Karlsruhe
Cryotube, sterile	Sarstedt, Nümbrecht
ELISA plate (96-well MICROLON flat bottom)	Greiner, Frickenhausen
FACS-tubule (5 ml Polystyren)	Sarstedt, Nümbrecht
Filter tips, Biosphere, sterile	Sarstedt, Nümbrecht

Material	Producer
Glass bottles (SCHOTT)	Schott, Mainz
Glass Capillary Tubes, sterile	Brandt, Wertheim
Immunofluorescence slides, 12-well	Thermo Scientific, Braunschweig
Microscope slides (Mattrand)	Engelbrecht GmbH, Edermünde
Neubauer counting chambers	Hecht-Assistent, Sondheim
Pasteur pipettes (3.4 ml)	Roth, Karlsruhe
PCR-reaction plates (96-well, 384-well)	Roche Diagnsotics, Risch, Switzerland
Petri dishes, 92 mm, sterile	Sarstedt, Nümbrecht
Pipette tips	Sarstedt, Nümbrecht
Pipettes (5 ml, 10 ml, 25 ml), sterile	Sarstedt, Nümbrecht
Precellys tubes (1.4/2.8 mm)	Peqlab, Erlangen
Reaction tubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf, Hamburg
Sterile filter (0.22 µm und 0.45 µm), sterile	Sarstedt, Nümbrecht
Surgical disposable scalpels , sterile	Braun, Melsungen
Syringes (1 ml, 5 ml), sterile	Braun, Melsungen
Vacuum Filter Units (Stericup), sterile	Millipore, Bedford, USA

3.1.3 Chemicals and reagents

Unless otherwise mentioned, all the standard chemicals used were purchased from Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Darmstadt) or Sigma-Aldrich (Deisenhofen).

3.1.3.1 Reagents for cell biological work:

Reagent	Producer
Collagenase D	Roche Diagnostics, Risch, Switzerland
DAPI	Sigma, Deisenhofen
DNAse I	Sigma, Deisenhofen
F_c block	BNI, Hamburg
Fetal calf serum (FCS)	Gibco/Invitrogen, Karlsruhe
Gentamicin	Sigma, Deisenhofen
HEPES	PAA, Linz, Ausria
Ketamin (Ketavet)	Pharmacia, Berlin
L-Glutamin	PAA, Linz, Austria

Reagent	Producer
RPMI 1640 culture medium without L-Glutamin	PAA, Linz,Austria
Tetramethylbenzidine (TMB) for *Immunofocus* assay	Mikrogen, Neuried
TNF-α (mouse, recombinant)	Millipore, Billerica, USA
Triton X-100	Sigma, Deisenhofen
Trypan blue	Serva, Heidelberg
Trypsin	PAA, Pasching, Austria
Xylazine (Rompun)	Bayer, Leverkusen

3.1.3.2 Reagents for molecular biology work:

Reagent	Producer
10× Buffer for DNA Polymerase HotStarTaq	Qiagen, Hilden
Alkaline Phosphatase (Shrimp)	MBI Fermentas, St. Leon-Rot
Ampoules water	Fresenius Kabi, Bad Homburg
Bovine serum albumin (BSA)	Roche Diagnsotics, Schwetzerland
Deoxyribonucleosidtriphosphate (GeneAmp dNTP-Mix)	Applied Biosciences, Carlsbad, USA
DMEM	Gibco, Eggenstein
DNA Polymerase HotStarTaq	Qiagen, Hilden
HBS (Hepes buffered saline) (2×)	Fulka, Busch, Switzerland
Penicillin/ Streptomycin	Gibco/BRL GmbH, Eggenstein
Polyfect	Qiagen, Hilden
pREP9	Invitrogen, Karlsruhe,Germany
QIAamp DNA Mini Kit	Qiagen, Hilden
SYBR Green I	Invitrogen, Darmstadt

3.1.3.3 Reagents for chemical work:

Reagent	Producer
EDTA	Sigma, Deisenhofen
ELISA Kits	
(DuoSet)	R&D Systems, Wiesbaden
(CytoSet^{TM})	Invitrogen, Frederick, USA
Hydrogen peroxide (H_2O_2)	Merck, Darmstadt
Polymyxin B	Sigma-Aldrich, Deisenhofen
Proteinase K	Qiagen, Hilden
Sodium hydroxide (NaOH)	Merck, Darmstadt
TMB for ELISA	Roth, Karlsruhe
Tween 20	Serva, Heidelberg

3.1.3.4 Antibodies

Table 3.1.3.1: Antibodies for FACS-Analysis

Antibodies	Clone	Dilution	Producer
anti-CD11b-PerCP-Cy5.5	M1/70	1:200	BD, Heidelberg
anti-Ly6C-FITC	HK1.4	1:100	BioLegend, USA
anti-Ly6G-APC	1A8	1:100	BioLegend, USA

Antibodies for ELISA

All enzyme-linked immunosorbent assays (ELISAs) for detection of murine IL-6 and TNF-α were performed using the ELISA Kits (DuoSet) from R&D Systems, Wiesbaden.

All ELISAs for detection of human IL-8 were performed using the ELISA Kits (CytoSet^{TM}) from Invitrogen, Frederick, USA.

Table 3.1.3.2: Antibodies for Immunfluorescence

Antibody	Clone	Manufacturer
Alexa Fluor® 568 Goat Anti-Rabbit IgG (H+L)		Invitrogen, Karlsruhe, Germany
anti-56 kDa (*O. tsutsugamushi*)	2F2	BNI, Hamburg
anti-mouse IgG2a-DyLight 488	polyclonal	Dianova, Hamburg
NF-κB	E498	Cell Signaling Tec., Danvers, USA

3.1.4 Culture media, buffers and stock solutions

Unless otherwise mentioned, all buffers and stock solutions were diluted with double distilled water (ddH_2O). The pH of the buffers was adjusted if necessary with HCl or NaOH. Sterile needed solutions were autoclaved, sterile filtered (pore size 0.22 µm) or prepared from sterile reagents. FCS was incubated for 30 min at 56°C for inactivation of complement, and stored at -20°C until use.

3.1.4.1 Culture media, buffers and stock solutions for cell biology and animal experimental work:

Medium/buffer/ stock solution	Composition	pH
Collagenase / DNAse-solution	1:100 collagenase stock solution 10 µg/ml DNAse I in $R5F^{-G}$	
Collagenase solution	1:100 Collagenase stock solution in $R5F^{-G}$	
Collagenase stock solution	20 mg/ml Collagenase D stored at -20°C	
Cryomedium	60% FCS 40% dimethyl sulfoxide (DMSO)	
Erythrocyte lysis	10% 0.17 M TRIS pH 7,4 90% 0.17 M NH_4Cl	

Medium/buffer/ stock solution	Composition	pH
FACS-buffer	1% FCS	
	0.01% NaN_3, in PBS	
Formalin solution	4% Formalin	
Ketamin/Xylazin solution	12 mg/ml Ketamin	
	1.6 mg/ml Xylazin, in PBS	
Methyl Cellulose medium	1:3 Methyl Cellulose solution	
	2:3 $R10F^{-G}$	
Methyl Cellulose solution	16.8 g Methyl Cellulose	
	in 600 ml water	
	autoclaved and stored at 4°C	
Paraformaldehyde 1% (IIF)	1% Paraformaldehyde	7.4
Paraformaldehyde 4% (FACS)	4% Paraformaldehyde	7.4
PBS (10x)	137 mM NaCl	7.4
	2,7 mM KCl	
	100 mM Na_2HPO_4	
	2 mM KH_2PO_4	
RPMI-Media:	500 ml RPMI 1640-Medium 50 ml	
$R10F^{DC}$	50 ml FCS	
	5 ml L-Glutamin (100 mM)	
	5 ml Sodium Pyruvate 1mM	
	2,5 ml Gentamicin (10 mg/ml)	
	500 µl β-Mercaptoethanol (ME) stock	
	(stock: 100 ml 0, 9% NaCL + 70 l ME)	
RPMI-Media:	500 ml RPMI 1640-Medium without	
$R5F^{+G}$ / $R5F^{-G}$ / $R10F^{-G}$/ $R10F^{+G}$	Glutamin	
	50 ml FCS (100 ml für $R10F^{-G}$-Medium)	
	10 ml HEPES (1 M)	
	10 ml L-Glutamin (200 mM)	
	2.5 ml Gentamicin (10 mg/ml) (für $R5F^{-G}$)	
	500 µl β-Mercaptoethanol	
Trypan blue solution	2% Trypan blue (50x) in PBS	

3.1.4.2 Buffers and stock solutions for molecular biological work:

Medium/buffer/stock solution	Composition	pH
Sybr Green solution	1:1000 Sybr Green I in DMSO	

3.1.4.3 Buffers and stock solutions for protein chemical work:

Medium/Buffer/Stock solution	Compostion	pH
ELISA stop solution	2 M H_2SO_4	
ELISA substrate solution	12 ml ELISA-Substrate buffer	
	200 µl ELISA-TMB-solution	
	1.2 µl 30% H_2O_2	
ELISA substrate buffer	100 mM NaH_2PO_4	
ELISA TMB solution	30 mg TMB	
	In 5 ml DMSO	
ELISA wash buffer	1x PBS containing 0.05% (w/v) Tween 20	
PBS-Tween	0.1% Tween 20 in PBS	

3.1.5 Nucleic acids:

Table 3.1.5.1: Oligonucleotides

All oligonucleotides were purchased from TIB Molbiol, Berlin

Designation	Sequence
traD-fw	CACAACATCCAAATGTTCAG
traD-rv	GCACCATTCTTGACGAAA

3.1.6 Cell lines, bacteria and animals:

Table 3.1.6.1: Cell lines

Description	Origin
232 macrophages, immortalized cell line from C57BL/6 mice	Dr. D. T. Golenbock, University of Massachusetts Medical School, Worcester MA, USA
261 macrophages, immortalized cell line from TLR2$^{-/-}$ mice	Dr. D. T. Golenbock, University of Massachusetts Medical School, Worcester MA, USA
HEK293	Dr. Holge Heine, Forschungszentrum Borstel, Germany
L929 mouse fibroblasts	DSMZ, Braunschweig

Table 3.1.6.2: Bacteria

Bacterial strain	Origin
Orientia tsutsugamushi Karp	Dr. J. Stenos, Division of Veterinary and Biomedical Science, Murdoch University, Murdoch, Australia

Table 3.1.6.3: Mouse strains

Mouse strain	Origin
C57BL/6 N	Charles River, Sulzfeld
TLR2 $^{-/-}$	Ludwig-Maximilians University, Munich

3.1.7 Software

Software	Hersteller
CFlow 1.0.227.1	Becton Dickinson, Heidelberg
GraphPad Prism 5.04	GraphPad Software, La Jolla, USA

3.2 Methods

3.2.1 Methods in cell biology

Unless otherwise mentioned, all centrifugation steps were performed for 5 minutes at 1,200 rpm at room temperature (RT).

3.2.1.1 Cell culture

All cell lines and BMDCs generated for *ex vivo* stimulation were maintained and stimulated under sterile conditions. All cells were cultured at 37°C and 5% CO_2 in a humidified incubator. Uninfected L929 cells, 232 and 261 macrophages were maintained in $R5F^{+G}$, and were split twice in a week. L929 cells infected with *O. tsutsugamushi* were maintained continuously in culture by help of technical assistance. One day before infection, L929 cells were γ-irradiated at 3000 rad and cultured in $R5F^{-G}$. For the infection, two to three weeks old infected L929 cells were detached with a cell scraper, removed by pipetting and one part of the infected cell suspension were added to the new confluent monolayer of L929 cells. The cells were centrifuged for 30 min at 800 rpm or left without centrifugation for 1 h. Subsequently, the supernatant was removed and the cells were cultured in new $R5F^{-G}$. The medium was changed once after three days, after that it was not replaced.

3.2.1.2 Preparation of infectious and L929 cells control inoculum

Infectious inocula or L929 cells control incocula were produced from infected L929 cells or from uninfected L929 cells respectively. The cells from four 75 cm^2 flasks were pooled, centrifuged, and resuspended in 28 ml $R5F^{-G}$. 0.5 ml aliquots of the cell suspension were placed in cryotubes, and mixed with 0.5 ml of cryomedium per tube, and quickly frozen in liquid nitrogen.

3.2.1.3 Immunofluorescence stains

Intracellular staining of infected J774 macrophages

J774 macrophages were harvested from 5 midsize flasks with trypsin-treatment and washed with PBS. The cells were counted and $2.2x10^7$ cells re-suspended in 2.2 ml $R10F^{-G}$ medium. A 3 *Orientia* stocks were washed (centrifugation: 13000 rpm, 5 min) with PBS then combined and re-suspend in 100 µl $R10F^{-G}$ medium and pipetted to the cells. Then the cells were shaken in 1.5 ml tube vertically on Eppi-shaker at low speed (500 rpm) for 30 min at 37°C. The cells

were washed 2 times with PBS (centrifugation: 1200 rpm, 5 min) and resuspended in 33 ml R10F^{-G} medium. Then cells were distributed in 6-wells plates (low adherent) (2x10^6 cells /3 ml to each well). 2×10^6 cells of a continuous *O. tsutsugamushi*-infected or uninfected culture were detached from 6-well cell culture plates (low adherent) with pipetting up and down at indicating time, then washed with PBS in 1.5 ml tubes and pelleted. The cells were resuspended in 1 ml PBS, and then 20 µl of the suspension was added on each well of the immunofluorescence slides. The slides were dried under the bench and fixed with cold acetone for 10 min. Each slide well was incubated with 20 µl anti-56 kDa mAb (diluted 1:10,000 in PBS) for 1 h at 37°C degree in humidity chamber. The cells were washed three times with PBS and incubated for 1 h with the DNA – marker DAPI (diluted 1:2,000 in PBS), and the anti-mouse IgG-DyLight 488 (diluted 1:100 in PBS) at 37°C in humidity chamber. The cells were then washed three times, and the fluorescence microscope was used at 100 × magnification for capturing the pictures.

Intracellular staining of NF-κB in 232 and 261 macrophages

Glass cover slips (12 mm diameter) in 24-well tissue culture plates were seeded with 2×10^5 232 or 261 macrophages. 24 h later, the cells were infected with live *O. tsutsugamushi*, or stimulated with the indicated concentrations of LPS or Pam_3CSK_4. After the indicated time, the cover slips were fixed for 30 min using the 4% paraformaldehyde (PFA) fixative. A 30 µl drop of blocking buffer (1X PBS containing 2% fetal calf serum (FCS) and 0.1% Triton X100) was placed on a clean parafilm (same for primary and secondary antibodies), then the cover slips were placed, with cells facing down, on the drop (excess fluid was drained from the cover slips beforehand, in order not to dilute the antibody further) for 20 min at RT. The cover slips were gently lifted and washed by rinsing for a few seconds by gently plunging repeatedly in a beaker of PBS, then incubated with a 30 µl drop of primary antibody (anti-56 kDa mAb was diluted 1:5,000 and anti-NF-κB was diluted 1:100) diluted in blocking buffer for 30 min at 37°C and then washed. Subsequently, the cells were incubated with a 30 µl drop of secondary antibody (anti-mouse IgG Alexa 488 and anti-rabbit IgG Alexa 568) diluted in blocking buffer for 30 min at 37°C and then washed. The cover slips were incubated for 5 min with DAPI-containing blocking buffer (dilution of DAPI 1:2,000), and finally washed with PBS. Then the cover slips were mounted in a small drop (5–10 µL) of Prolong Gold Anti-fade

on a clean glass slide. After the mounting medium had hardened (optimally overnight), the confocal microscope was used at 100x magnification for capturing the pictures.

3.2.1.4 Quantification of infectious inoculum - immunofocus assay

The infectivity of the inocula was determined *in vitro* by the immunofocus assay. Briefly, L929 cells were γ-irradiated at 3,000 rad and seeded in 24-well plates (4×10^5 cells per well in 1 ml $R10F^{+G}$). The cells were cultured overnight. The next day, a cryovial of inoculum was thawed rapidly in the hand, and then washed by gently adding $R5F^{-G}$. 1/20 of inoculum suspension was taken and filled up to 10 ml with $R5F^{-G}$. Four serial 1:4 dilutions were made in $R5F^{-G}$. The medium was removed from the plates, and 200 µl of the inoculum dilutions were added per well. A quadruplicate of each dilution was used. The plate was centrifuged for 30 min at 800 rpm in order to ensure an effective infection. The medium was removed; the wells were washed twice with $R5F^{-G}$ and cultured for 14 days in methylcellulose medium. Then, the medium was removed. The plates were washed once with PBS and then fixed for 1 to 24 h in 4% formalin. Afterwards, the infected spots in the cellular monolayer were stained. For this reason, the plates were incubated with various reagents, and washed with PBS prior to each step at least once. The cells were permeabilized for 30 min with 0.5% Triton X-100, then blocked for 1 h at RT with blocking buffer. The *O. tsutsugamushi*-infected cells were labeled with anti-56kDa mAb (diluted 1:2000) for 1 h, and then incubated for 1 hour with POD-conjugated anti-mouse mAb (1:2000 in PBS). After two washes, cells were incubated with TMB substrate until blue spots were clearly visible. Finally the reaction was washed twice in ddH_2O. The spots were counted and plotted against the inoculum dilution. Regression curves were calculated by single-phase logarithmic association. The proportion of an inoculum, which had given rise to 50 infectious spots per well, contains the infectious dose 50 *spot-forming units (sfu)*.

3.2.1.5 Purification of *O. tsutsugamushi* from infected cell cultures

O. tsutsugamushi Karp was propagated in Vero or L929 cells. 2 to 3 weeks post infection; the cells were harvested in culture medium using a cell scraper and pelleted by centrifugation at 4,000 rpm for 30 min. The cell pellet was resuspended with 11 ml of $R5F^{-G}$ and transferred into 15 ml centrifuge tubes. The resuspended cells were disrupted via rocking with sterile glass beads (diameter 3 mm) for 5 min at 1,400 rpm at RT using a Thermo mixer. The suspension was transferred into new 15 ml centrifuge tubes without glass beads. The

suspension was treated for 1 h at 25 kHz, and 100% power in the ultrasonic bath, the temperature of the ultrasonic bath was controlled in order not to exceed 37°C by adding ice. The suspensions were resuspended and centrifuged for 1 h at 1200 rpm. The supernatant was transferred to new 15 ml centrifuge tube, and the bacteria were pelleted at 4000 rpm for 30 min, then washed in PBS and resuspended in the required volume. Heat-inactivated *O. tsutsugamushi* was prepared by heating a bacterial preparation for 30 min at 56°C. The heat-killed *O. tsutsugamushi* was prepared by heating a bacterial preparation for 10 min at 95°C. The purified amount of *O. tsutsugamushi* form 75 cm^2 culture flask was used to stimulate 50 wells in 24-well cell culture plate (this concentration was considered as the highest one and denoted by (1), the second and third dilution were prepared by a 10 fold serial dilution and denoted (0.1) (0.01) respectively).

3.2.1.6 Preparation of cell suspensions from mouse spleen and lymph nodes

The spleen and lymph nodes were crushed between 2 glass slides in 4 ml $R5F^{-G}$ and the cells were pelleted. Cells from lymph nodes were counted and used directly. Splenocytes were initially treated with 5 ml of erythrocyte lysis buffer for 5 min at RT, and then washed three times in $R5F^{-G}$, followed by cell counting.

3.2.1.7 Cell counting

10-50 µl of the cell suspension was diluted in trypan blue depending on cell density in an appropriate ratio, and the vital, colorless cells scattered in four great squares (composed of 4x4 small squares, corresponding to 0.1 mm^3) were counted in a Neubauer counting chamber.

3.2.1.8 Flow cytometry

FACS ARIA-based sorting of stained lymph node cell populations

Mice were infected s.c. bilaterally. Popliteal lymph node suspensions from two mice were pooled in 15 ml tube and pelleted. The cell pellets were fixed with 5 ml -20°C cold methanol (methanol was added with vortexing) and incubated for 1 h at least at -20°C. Then the cells were washed twice with FACS buffer, and then incubated with 500 µl F_c block for 10 min at 4°C. The cells then were stained with 500 µl of the required antibody in F_c block for 1 h at 4°C (required antibodies were indicated before in the chapter of materials). The cells were then washed once in FACS buffer and resuspended in FACS buffer. The required cell

populations were sorted on FACS Aria at 4°C in PBS. The pathogen loads in the sorted populations were determined by qPCR.

Surface staining of single cell suspensions for measurement by Accuri C6 flow cytometer

1×10^6 cells were placed in FACS tubes. The tubes were centrifuged, and the cells were incubated with 50 µl F_c block for 10 min at 4°C. The cells then were stained with 50 µl of the diluted surface staining antibody in F_c block for 1 h at 4°C (required antibodies were indicated before in materials chapter). Subsequently, the cells were washed once with FACS buffer. Finally the cells were resuspended in 200 µl FACS buffer. The measurement was made by Accuri C6 flow cytometer.

3.2.1.9 Stimulation and inhibition mechanisms of 232 and 261 macrophages

2×10^5 232 or 261 macrophages were seeded in 24-well plates directly or on cover slips (12 mm diameter). The cells were grown in $R5F^{-G}$ overnight. The next day, macrophages were infected with freshly purified cell-free *O. tsutsugamushi*, stimulated with heat-inactivated *O. tsutsugamushi*, LPS, or Pam_3CSK_4. Then the 24-well plates were centrifuged for 30 min at 800 rpm in order to ensure an effective infection. All conditions were set in quadruplicate. At various times after stimulation, either the supernatant was used for cytokine ELISA, or DNA was extracted from infected cells to determine the bacterial load. The cover slips were taken for NF-κB translocation staining. In case of the rTNF-α assay, next to the stimulation step of cells with fresh *O. tsutsugamushi* and centrifuged for 30 min at 800 rpm, the supernatant was removed and the cells were treated with (0.1, 1, 10, 30 or 100 ng/ml) of rTNF-α in $R5F^{-G}$. At various times cells were harvested, and *O. tsutsugamushi* was quantified by qPCR.

3.2.1.10 Transfection and stimulation of human embryonic kidney cells 293

Transfected human embryonic kidney (HEK) 293 cells were a kind gift from Dr. Holger Heine (Borstel, Germany) and provided as described in [129]. Briefly, HEK293 cells were plated at a density of $5\text{x}10^4$/ml in 96-well plates. As medium, DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS was used. The next day, using Polyfect as transfection reagent according to the manufacturer’s protocol, cells were transiently transfected with pREP9 plasmids into which flag-tagged versions of human TLR2 and TLR4 had been cloned. Cells received antibiotic-free medium afterwards. After 24 h, cells were washed and infected with live *O. tsutsugamushi*, or stimulated with heat-

inactivated *O. tsutsugamushi,* LPS, Pam_3CSK_4 or rTNF-α for another 18 h. In some experiments, crude *O. tsutsugamushi* extracts and Pam_3CSK_4 were pre-incubated with different chemical or enzymatic compounds (Proteinase K, polymyxin B, H_2O_2 or NaOH) then the HEK293 cells were stimulated with the treated stimuli. Finally, the concentration of IL-8 in cell culture supernatants was quantified by ELISA.

3.2.1.11 Isolation and stimulation of primary DC culture

Murine bone marrow cells were isolated from femurs and tibias, and differentiated into myeloid DCs by culturing at $2x10^6$ cells/ml in $R10F^{DC}$. 10% culture supernatants of Ag 8653 myeloma cells were used as the source of granulocyte monocyte colony-stimulating growth factor (GM-CSF). Non-adherent cells were harvested between days 6-8. DCs (BMDC) were plated in 96-well microculture plates (at $2x10^5$ cells/well) and infected with fresh *O. tsutsugamushi*, or stimulated with heat-inactivated *O. tsutsugamushi*, LPS, Pam_3CSK_4 or L929 cell lysate. After 18 h of stimulation, the supernatants were harvested for cytokine determination.

3.2.2 Methods in molecular biology

For molecular biology basically ampoule water was used.

3.2.2.1 DNA purification

DNA extraction was carried out using the "QiaAmp DNA Mini Kit". All washing steps were performed in duplicate to be sure that the PCR inhibitors were washed out well. Elution was carried out basically in 50 µl EB buffer.

Cell culture samples and sorted cell populations were extracted according to the manufacturer's protocol, "DNA Purification from Blood or body fluid". In each case, 200 µl of sample material were used.

For DNA extraction from solid organs, suspensions were prepared as described in section 3.2.4.2. 80 µl of prepared suspension were incubated overnight with 100 µl ATL buffer and 20 µl proteinase K at 56°C. The next steps were performed according to the manufacturer's protocol, "DNA Purification from Tissues".

3.2.2.2 Determination of DNA concentrations

DNA concentrations from undiluted DNA extracts were measured using the NanoDrop photometer at a wavelength of 260 nm.

3.2.2.3 Quantitative real-time PCR (qPCR)

qPCRs were used in this study for quantification of pathogen-specific DNA. The routinely used traD-PCR in this study was developed by Dr. Matthias Hauptmann (BNI, Hamburg).

All reagents except the DNA samples (master mix) were prepared on ice, and 8 µl master mix were used for each reaction performed in a 96-well or 384-well qPCR plate (Table 3.2.2.1).

Table 3.2.2.1: PCR master mixes

The values refer to the volumes required for a sample.

Reagent	Volume
Ampoule H_2O	5.4
10x polymerase buffer (Qiagen)	1
dNTP (10 mM)	0.2
Primer traD fw (10 µM)	0.6
Primer traD rv (10 µM)	0.6
SYBR Green	0.1
BSA	0.05
Hotstar taq (5 U/µl)	0.05

2 µl of the DNA samples were added in a concentration of 10 ng/µl DNA. Duplicates were performed. In each plate, a linearized plasmid standard, which contained a known copy number of the target gene, was measured in quadruplicates. All qPCRs were carried out with the following temperature cycle.

Table 3.2.2.2: PCR Reaction conditions

Number of cycles	Temperature	Time
1x	95°C	15 min
	94°C	10 sec
45x	58°C	15 sec
	72°C	20 sec
1x	60-75°C	0.6°C/sec

The evaluation was performed according to the "Absolute Quantification Analyses Using the Second Derivative Maximum" method with respect to the external standard curve generated

by measuring the titrated standard plasmid in quintuplicate. The number of traD copies measured in every individual sample was converted into the number of *O. tsutsugamushi* genome copies per sample, dividing by the correction factor of 21.6 detectable traD alleles per genome. The results are expressed as genome copies per microgram of total DNA.

3.2.3 Methods for biochemical analyses

3.2.3.1 Cytokine ELISA

Cytokines in cell culture supernatants were quantified by sandwich ELISA. It was carried out with the reagents of "DuoSet ELISA kits" from R&D Systems (for IL-6 and TNF-α) or "CytoSet^{TM} kits" from Invitrogen (for IL-8).

For "DuoSet ELISA kits" from R&D

The antibodies, standards and reagents were diluted according to the manufacturer's protocol. Subsequently, 96-well ELISA plates were coated with 50 µl per well of the diluted capture antibody and incubated overnight at 4°C. The plates were then washed three times in wash buffer, incubated for 2 h at RT with 100 µl per well of the blocking buffer (in some experiments, the plates were kept at -20°C until used). The plates were washed three times and the wells were filled with 50 µl of the diluted standards in duplicates or with 40µl of culture supernatants in triplicate. Then the plates were incubated for 2 h at RT or overnight at 4°C. The plates were then washed three times and filled with 50 µl per well of diluted detection antibody and incubated 2 h at RT. Then the plates were washed three times, before they were incubated for 20 min with 50 µl per well of diluted streptavidin-horse radish peroxidase (HRP). Subsequently, the plates were washed three times and incubated with 100 µl per well TMB substrate. After about 15 min, the reaction was stopped by the addition of 25 µl of stop solution. The extinction was measured at 450 nm in the plate photometer.

For "CytoSet^{TM} kits" from Invitrogen

The levels of IL-8 in the cell cultures supernatants of HEK293 cells were quantified using the commercial "CytoSet^{TM}" ELISA kits from Invitrogen. All steps were performed according to the manufacturer’s standard protocol.

3.2.3.2 Proteinase K, polymyxin B, H_2O_2 and NaOH treatments

O. tsutsugamushi crude extract was first heat-inactivated for 10 min at 95°C. Then the extract and Pam_3CSK_4 were pre-incubated with different chemical compounds as follows. Proteinase K (was diluted in 30 mM Tirs-Buffer Ph 8.0) treatment was performed by incubation with 0.1 mg/ml proteinase K for 30 min at 37°C followed by inactivation for 15 min at 70°C. Polymyxin B (was diluted in PBS) treatment was performed by incubation with 1mg/ml polymyxin B for 1 h at 37°C. NaOH (was diluted in ddH_2O) treatment was performed by incubation with 10 mM NaOH for 1 h at RT followed by neutralization with 37% (v/v) HCl. H_2O_2 (was diluted in PBS) treatment was performed with 1% H_2O_2 for 6 h at 37°C.

3.2.4 Animal Experiments

The animal experiments were approved by the Hamburg Ministry of Health and Consumer Protection (approval no. 74/09) and carried out in accordance with the requirements of the Animal Welfare Act. The animals were housed in BNI in individually ventilated cages (IVCs) under animal biosafety level (BSL)-3 conditions. Cages with infected animals were opened exclusively under BSL3 conditions. At the beginning of each experiment, mice were 7-12 weeks old.

3.2.4.1 Infection of mice with *O. tsutsugamushi*

Infectious inocula or L929 cells controls were rapidly thawed in the hand, subsequently 1 ml $R5F^{-G}$ was added slowly to each tube. Subsequently, the cell suspension was transferred into 15 ml centrifuge tube and 8 ml of $R5F^{-G}$ were added slowly. The cells were washed twice with 10 ml of PBS (centrifugation for 5 min at 1,200 rpm) and pelleted in 1.5 ml tube (centrifugation for 5 min at 8,000 rpm). Then the cells were resuspended with 1 ml of PBS in 1.5 ml reaction tube. For s.c. injection mice were anesthetised with 10 µl/g body weight of (ketamine/xylazine) i.p. Once the mice showed no foot reflexes, a 50 µl of inoculum were injected with a 27 gauge needle into the right footpad.

For i.p. injection of mice, infected cells were washed twice as mentioned above. Then the cells were pelleted in a 15 ml tube (centrifugation for 5 min at 1,200 rpm) and resuspended with 5 ml of PBS in a 15 ml tube. 500 µl of inoculum was injected i.p. with using a 27 gauge needle.

3.2.4.2 Retrieval of mouse organs for qPCR

The mice were injected first i.p. with 15 µl/g body weight of anesthetic solution (ketamine/xylazine). When the mice showed no reflexes, the abdomen was opened and the inferior vena cava was cut. Subsequently, the diaphragm was opened. After that, the chest was cut and the right heart ventricle was pierced with an 18 gauge needle. A button cannula was inserted through the hole in the right ventricle and was moved to the entrance of the pulmonary artery. Subsequently, the lungs were flushed through the main pulmonary artery with 3-5 ml PBS. The lungs were first removed, followed by the other organs. The removed organs were stored in 6-well plates in $R5F^{-G}$ on ice.

Preparation of organs for the determination of the bacterial load

Lung, spleen, brain, right popliteal lymph node, kidney and liver were taken. Then the organs were cut in small pieces and 25-50 mg of spleen or 50-100 mg of other organ tissues were collected in Precellys tubes. 500 µl of PBS were added and the tissues were lysed (2×15 sec at 6600 rpm) using a homogenizer (Precellys). Subsequently, the samples were processed as described in section (3.2.2.1.)

3.2.4.3 Clinical scores and survival curves after i.p. infection

After i.p. infection, mice were monitored daily for survival and clinical scores. Clinical scores were the sum of clinical symptoms for the severity of the disease, based on the fur and body condition of mouse per day (Table 3.2.4.1)

Table 3.2.4.1: Clinical score for mice

clinical score	ruffled fur	Condition
0	None	no signs
1	between ears	tiredness
2	on back	tiredness, distended abdomen
3	all over	pain, hunched posture
4	-	Dead

3.2.5 Statistics

Statistical analysis of all experimental data was performed using the software GraphPad Prism 5.02. Data are expressed as the mean ± standard error of the mean (SEM) or standard deviation (SD), and the statistically significant difference between groups was determined by one-way ANOVA, two-way ANOVA or Student's unpaired *t*-test. A p value < 0.05 was considered significant. For survival comparison between treatment groups, the Log-rank (Mantel-Cox) Test was used.

4 Results

4.1 Identification of TLR2 as a receptor for *O. tsutsugamushi* by *in vitro* screening

TLRs are primary sensors of microbial pathogens that activate innate immune cells and play a role in development of adaptive immune responses [12]. However, the role of TLRs in *O. tsutsugamushi* recognition and activation of host cells remains poorly understood. First, the role of TLR2 and TLR4, the two major TLRs involved in the recognition of bacterial surface structures, in the recognition of *O. tsutsugamushi* was examined.

4.1.1 *O. tsutsugamushi* stimulates TLR2- but not TLR4-transfected HEK293 cells

HEK293 cells were transiently transfected with human TLR2, TLR4, NOD1 and NOD2 as described in Materials and Methods and shown in (Figure 4.1.1 A). After 24 h, cells were infected with the indicated relative amounts of infectious (alive) *O. tsutsugamushi* (100, 10 and 1 µl from a freshly purified bacterial suspension), stimulated with heat-inactivated *O. tsutsugamushi* (100, 10 and 1 µl, containing equal amounts of bacteria as the alive samples), LPS from *S. typhimurium* (100, 10 and 1 ng/ml), synthetic lipopeptide Pam_3CSK_4 (100, 10, and 1 µM), or recombinant TNF-α. The release of IL-8 in the culture supernatant was determined after 18 h of culture by using sandwich ELISA.

The result showed that only TLR2-transfected HEK293 cells produce IL-8 following challenge with both, alive and 56°C-heat-inactivated *O. tsutsugamushi* in a dose-dependent fashion (Figure 4.1.1 B). Pam_3CSK_4, a synthetic lipopeptide ligand of TLR2, was used as the positive control. As expected, Pam_3CSK_4 induced IL-8 production. IL-8 production induced by heat-inactivated *O. tsutsugamushi* was approximately 2-fold higher than the response induced by live *O. tsutsugamushi* (Figure 4.1.1 B). It was observed that *O. tsutsugamushi* heat-killed at 95°C for 10 minutes elicited an even higher IL-8 response when compared to *O. tsutsugamushi* inactivated at 56°C for 30 minutes (data not shown).

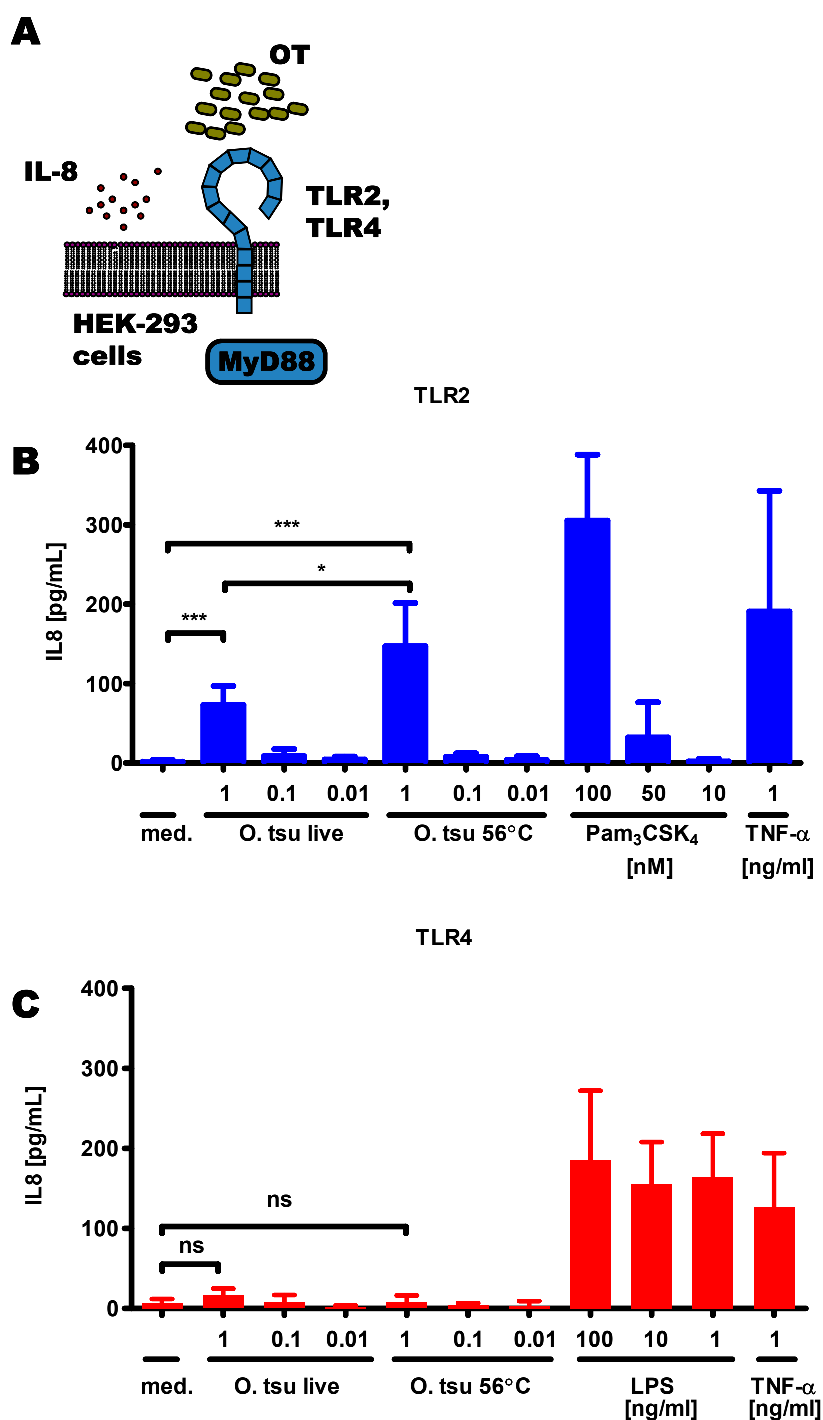

Figure 4.1.1: Induction of IL-8 release in transiently TLR2- but not TLR4-transfected HEK293 cells. HEK293 cells were transiently transfected with TLR2 or TLR4. After 24 h, cells were infected with indicated amounts of live *O. tsutsugamushi,* or stimulated with heat-inactivated *O. tsutsugamushi*, LPS from *S. typhimurium*, or synthetic lipopeptide Pam$_3$CSK$_4$ for 18 h. IL-8 contents of the supernatants were analyzed by sandwich ELISA. **(A)** Experimental design. **(B)** TLR2 transfection. **(C)** TLR4 transfection. Data shown are combined results of two independent experiments (mean ± SD, ns: not significant. * $p < 0.05$, *** $p < 0.001$ as determined by one-way ANOVA with Bonferroni post correction test, comparing the indicated groups).

It was also tested whether *O. tsutsugamushi* could ligate TLR4 in TLR4-transfected HEK293 cells. The results showed that TLR4-transfected HEK293 cells could not produce IL-8 following challenge with neither alive *O. tsutsugamushi* nor heat-inactivated *O. tsutsugamushi*. TLR4-transfected HEK293 cells responded only to *S. typhimurium* LPS, the natural ligand of TLR4 (Figure 4.1.1 C). NOD1- or NOD2-transfected HEK293 cells also could not produce IL-8 following challenge with heat-inactivated *O. tsutsugamushi* (data not shown). In summary, *O. tsutsugamushi* could induce IL-8 production by HEK293 cells only upon transfection with TLR2. Moreover, the induction of IL-8 by heat-killed *O. tsutsugamushi* was higher than the induction by live *O. tsutsugamushi*.

4.1.2 Chemical composition of the TLR2 ligands

In the previous section it was shown that *O. tsutsugamushi* was only recognized via TLR2, not via TLR4. Recognition by TLR2 is not restricted to a defined chemical structure, since a large number of structurally unrelated molecules have been reported as ligands [80]. Thus, the chemical composition of the TLR2 ligand identified in *O. tsutsugamushi* was further analyzed.

TLR2 ligands are commonly glycolipids, glycoproteins, lipoproteins or lipopeptides [80]. In order to further characterize the molecular structure sensed by TLR2, simple chemical or enzymatic degradation procedures were performed on whole *O. tsutsugamushi* extract (degradation of proteins: proteinase K; hydrolysis of lipid esters: NaOH; thioether oxidation in lipopeptides: H_2O_2). Polymyxin B which degrades LPS was used as a control.

The reaction was investigated by comparing two stimuli with and without the specific treatment, the *O. tsutsugamushi* whole cell extract heat inactivated at 95°C for 10 min and the synthetic lipoprotein Pam_3CSK_4. Both *O. tsutsugamushi* antigen and Pam_3CSK_4 were treated with one of the four treatment agents (Proteinase K, polymyxin B, H_2O_2 and NaOH), or mock-treated in buffer only. Afterwards, the TLR2-transfected HEK293 cells were incubated with the chemically modified stimuli. The release of IL-8 in the culture supernatant was determined after 18 h of culture by ELISA.

Proteinase K digestion of heat-inactivated *O. tsutsugamushi* reduced the activity of TLR2 by over 75%, while the digestion of Pam_3CSK_4 reduced its activity non-significantly (Figure 4.1.2 A). This strongly suggests that the TLR2 activating component of *O. tsutsugamushi* contains a protein or peptide structure. H_2O_2 treatment reduced TLR2 activation of both heat-

inactivated *O. tsutsugamushi* and Pam_3CSK_4 almost completely (by over 99%). This finding suggests that a lipid thioether structure also partakes in recognition of *O. tsutsugamushi* by TLR2 (Figure 4.1.2 B). Similarly, NaOH treatment reduced TLR2 activation of both heat-inactivated *O. tsutsugamushi* and Pam_3CSK_4 almost completely (by over 95%), implying fatty acids as parts of the TLR2-activating component(s) of *O. tsutsugamushi* (Figure 4.1.2 C). In contrast, as was expected, treatment with polymyxin B did not reduce the TLR2 activating capacity of both heat-inactivated *O. tsutsugamushi* and Pam_3CSK_4 (Figure 4.1.2 D). These results suggest that the main TLR2 ligand of *O. tsutsugamushi* could be a lipopeptide. However, the pronounced sensitivity to proteinase K treatment, which typical lipopeptides such as Pam_3CSK_4 do not show, could support the presence of an additional protein ligand for TLR2 in *O. tsutsugamushi*. A summary of the results is presented in Table 4.1.2.

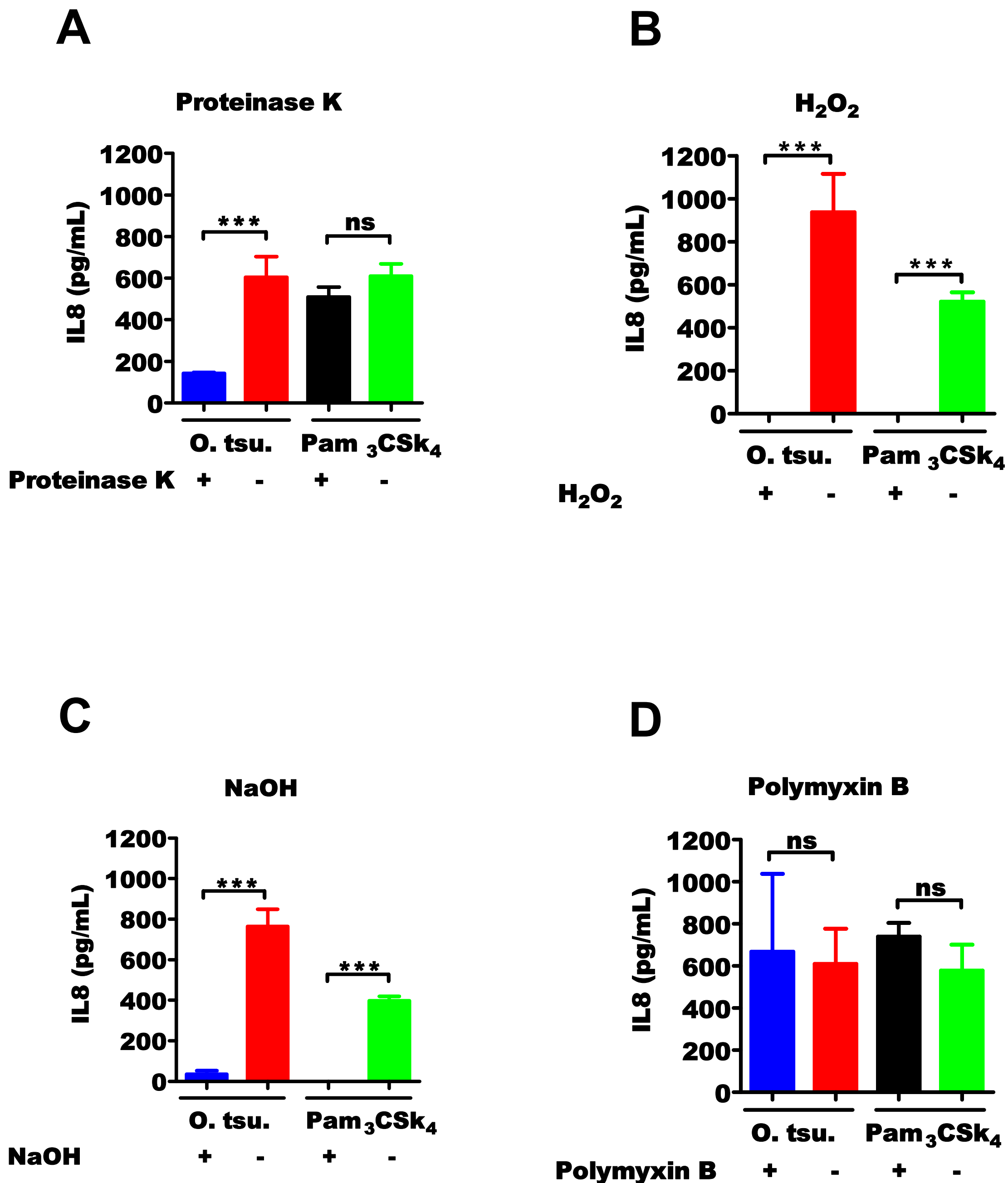

Figure 4.1.2: IL-8 release by TLR2-transfected HEK293 cells in response to chemically or enzymatically modified stimuli

IL-8 release by TLR2-transfected HEK cells in response to stimulation by heat-inactivated *O. tsutsugamushi* or Pam_3CSK_4 with (+) and without (-) treatment with 100 µg/ml of Proteinase K for 30 min at 37°C **(A)**, 1% of H_2O_2 for 6 h at 37°C **(B)**, 0.5 mol/l of NaOH for 1 h at 56°C **(C)** or 10 µg/ml of Polymyxin B for 30 min at 37°C **(D)**. Data shown are representative results of one of two independent experiments (mean ± SD, ns: not significant, *** $p < 0.001$ as determined by one-way ANOVA with Bonferroni post correction test, comparing the indicated groups).

Treatment	Sensitivity	Interpretation
Proteinase K	+	Protein or peptide compound
H_2O_2	+++	Lipid thioether compound
NaOH	++	Lipid ester compound
Polymyxin B	-	No LPS

Table 4.1.2
The table summarizes the sensitivity of the *O. tsutsugamushi* TLR2 ligand towards specific chemical or enzymatic treatments.

4.2 Requirement of TLR2 for bacterial growth control and proinflammatory responses to *O. tsutsugamushi*

Previous studies have shown that *O. tsutsugamushi* invades humans through mite biting and replicates in endothelial cells and macrophages [18, 59]. Macrophages can act as host cells for intracellular pathogens, but may also acquire antibacterial effector functions, killing a wide range of commensal and pathogenic bacteria.

In the previous section it was shown that HEK293 cells recognize *O. tsutsugamushi* after overexpression of TLR2. Next, it was questioned whether TLR2 is required by phagocytes to sense the presence of *O. tsutsugamushi*. To this end, either primary BMDC from C57BL/6 wild type or TLR2-deficient mice, or macrophage cell lines derived from these mouse strains (WT: 232; $TLR2^{-/-}$: 261) were used.

4.2.1 Replication of *O. tsutsugamushi* in macrophages

As indicated before, *O. tsutsugamushi* can survive and replicate in macrophages, although they are potent effector cells against many pathogens. Bacterial growth in J774 and RAW macrophages cell lines was investigated in this section.

J774 macrophages grown in 6-well plates (low adherent) were infected with *O. tsutsugamushi in vitro*. The macrophages were harvested at the indicated times and were stained by indirect

immunofluorescent antibody staining, using the 2F2 monoclonal antibody against the immunodominant 56 kD *O. tsutsugamushi* surface antigen. For the control experiment, macrophages received only fresh medium. As shown in (Figure 4.2.1), *O. tsutsugamushi* (green color) had multiplied within J774 macrophages until day 8 p.i. The *O. tsutsugamushi* 56 kD signal was detected immediately p.i. Soon after infection, the bacteria were aggregated at one pole of the infected cell. Notably, the signal for *O. tsutsugamushi* antigen was low at day 2 p.i. but recovered at day 3 p.i., possibly indicating a transient drop in surface protein expression. Growth of *O. tsutsugamushi* in RAW macrophages revealed similar results (data not shown). Thus, *O. tsutsugamushi* is able to replicate efficiently in macrophages *in vitro*.

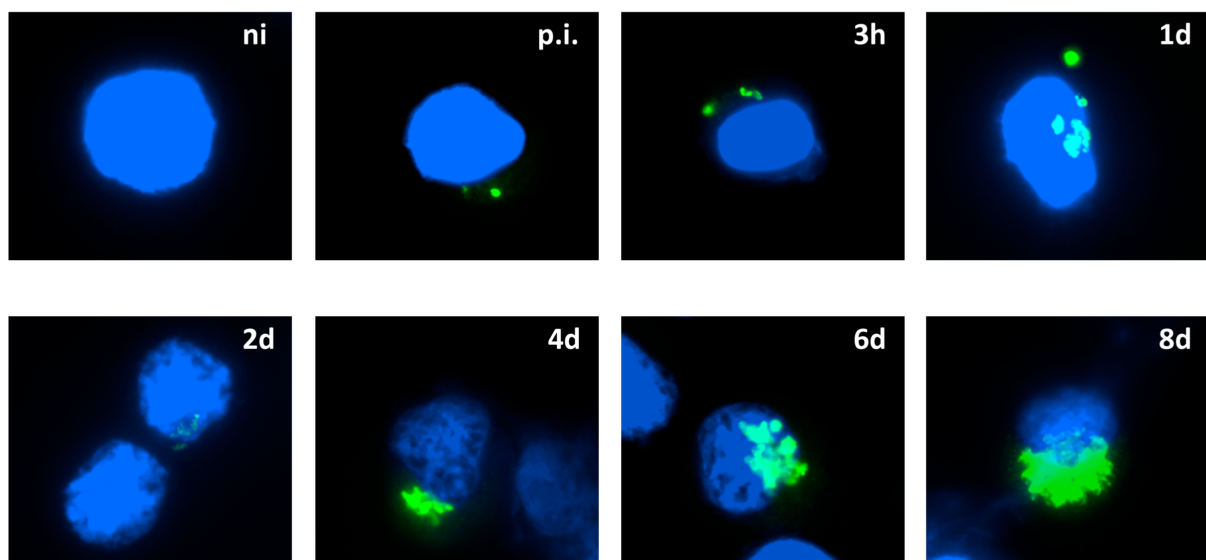

Figure 4.2.1: Kinetics of O. *tsutsugamushi* replication in J774 macrophage
J774 macrophages were infected with *O. tsutsugamushi in vitro*. Using the 2F2 monoclonal antibody against the immunodominant 56kD *O. tsutsugamushi* surface antigen, the infection course was monitored for 8 days by immunofluorescence labeling. DNA was stained with DAPI. Images were taken with a 100x magnification lens. ni, not infected; p.i., post infection. *O. tsutsugamushi* in green and DAPI nuclear counterstain in blue.

4.2.2 *O. tsutsugamushi* triggers NF-κB signaling depending on ligation of TLR2

Ligand recognition by TLRs leads to the recruitment of various TIR domain-containing adaptors such as MyD88. This recruitment of adaptors triggers the cascade of signaling pathway and ultimately the activation of transcription factors such as NF-κB [130]. It was shown that NF-κB activation follows infection of J774A.1 macrophages with *O. tsutsugamushi* [131]. Therefore the next step was to answer the question whether it is the

TLR2-dependent recognition of pathogen structures that leads to activation of the NF-κB pathway.

Following infection of 232 (WT) macrophages with freshly prepared *O. tsutsugamushi*, there was a time-dependent increase in the translocation of cytoplasmic NF-κB into the nucleus (Figure 4.2.2 A), whereas the TLR2-deficient 261 macrophages infected with *O. tsutsugamushi* did not exhibit NF-κB nuclear translocation (Figure 4.2.2 A). Likewise, only the TLR2-competent 232 macrophages showed NF-κB translocation in response to treatment with Pam_3CSK_4, while the 261 macrophages remained unresponsive. LPS stimulation resulted in nuclear translocation of NF-κB in both cell lines, and cells treated with medium alone did not exhibit nuclear NF-κB nuclear translocation (data not shown).

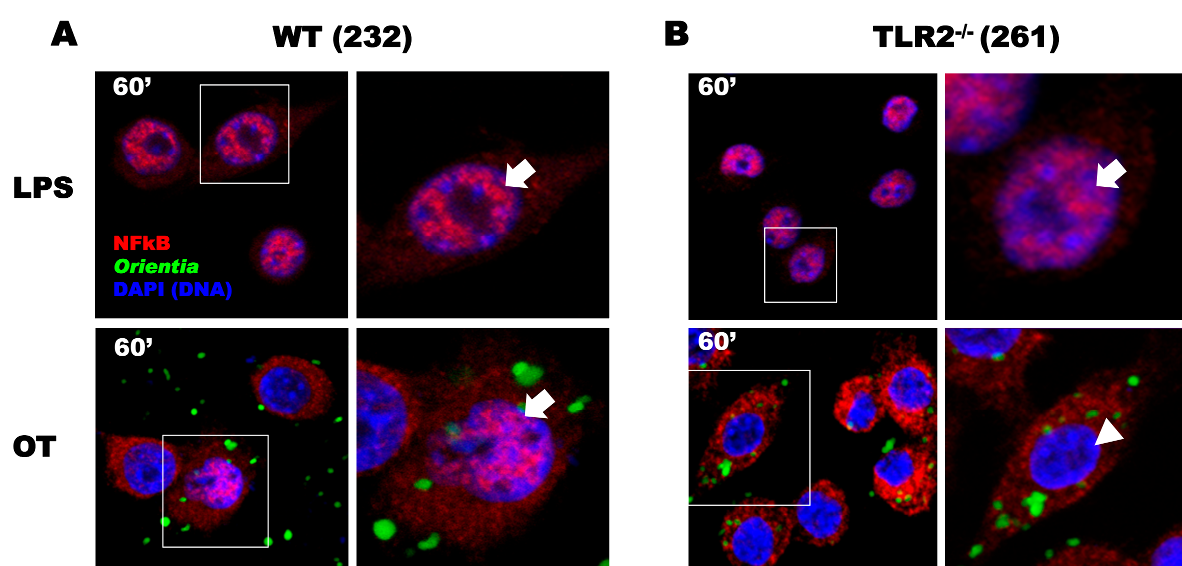

Figure 4.2.2: *O. tsutsugamushi* triggers NF-κB signaling depending on ligation of TLR2
Murine 232**(A)** or 261**(B)** macrophages were seeded on glass cover slips in 24-well plates at a concentration of $2x10^5$/well and infected with live *O. tsutsugamushi* freshly purified from cell culture, or stimulated with *S. typhimurium* LPS, Pam_3CSK_4 or medium only. Infection was synchronized by centrifugation at 130xg. At 15, 30, 60, 120 and 240 min p.i., cover slips were fixed with paraformaldehyde, labeled by immunofluorescence for *O. tsutsugamushi* 56 kD surface protein (green) and NF-κB (red), and counter stained with DAPI (blue). Arrows indicate NF-κB translocation into cell nuclei, while arrowheads indicate nuclei with deficient NF-κB labeling.

When calculating the percentage of cells with nuclear localization of NF-κB in *O. tsutsugamushi* infected 232 (WT) cells, we observed that maximal levels of nuclear translocation of NF-κB were reached 60 min p.i. and remained high through 120 min (Figure 4.2.3). Interestingly, almost no 261 macrophages showed residual, i.e. TLR2-independent translocation of NF-κB within the first four hours p.i., suggesting that no other innate

receptors play a relevant role in early NF-κB activation after infection with *O. tsutsugamushi*. These results indicated that *O. tsutsugamushi* triggers NF-κB translocation to the macrophage nucleus depending on ligation of TLR2.

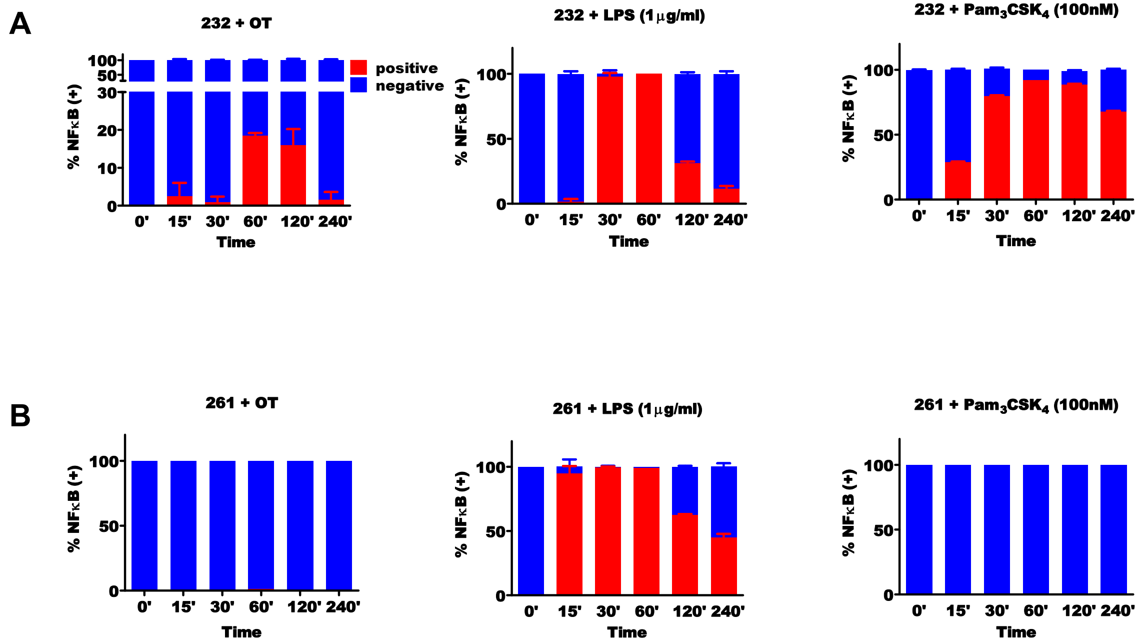

Figure 4.2.3 TLR2-dependent translocation of NF-κB after infection with *O. tsutsugamushi* is highest between 60 and 120 min p.i.
Graphical representation of NF-κB translocation, depicted as the percent of nuclei with nuclear localization of NF-κB, in 232 (WT) macrophage **(A)** or 261 (TLR$^{-/-}$) **(B)**, following *O. tsutsugamushi* infection or stimulation with LPS or Pam$_3$CSK$_4$. For each time point, nuclear localization of NF-κB in 100 nuclei was enumerated independently by two microscopers. Data shown are combined results of two independent experiments (mean ± SD).

4.2.3 TLR2-dependent cytokine production by macrophages

In the previous section it was shown that TLR2 plays a role in innate recognition of *O. tsutsugamushi*. This section describes how 232 (WT) and 261 (TLR2$^{-/-}$) macrophages react to *O. tsutsugamushi* by cytokine production *in vitro*.

TNF-α was measured in the supernatant of macrophage cultures infected with live or heat-inactivated *O. tsutsugamushi* for 24 h. Cell culture medium alone was used as a negative control, *S. typhimurium* LPS (1 µg/ml) and Pam$_3$CSK$_4$ (1µM) were used as positive controls. As can be seen in (Figure 4.2.4), both live and heat-inactivated *O. tsutsugamushi* induced the production of TNF-α in 232 (WT) cells. In contrast to 232 cells, lower amounts of TNF-α were produced by 261 (TLR2$^{-/-}$) cells in response to *O. tsutsugamushi* (Figure 4.2.4). These

results indicated that macrophages need TLR2 to secrete TNF-α in response to *O. tsutsugamushi in vitro.*

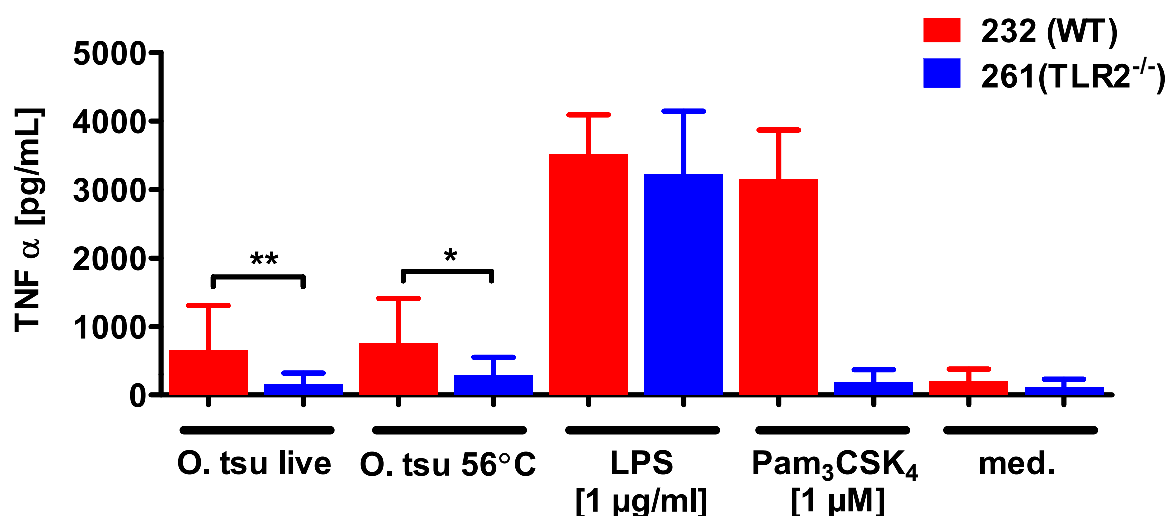

Figure 4.2.4: Cytokine production by macrophages infected with *O. tsutsugamushi*
Murine C57BL/6 WT (232) or TLR2$^{-/-}$ (261) macrophage cell lines were infected with live *O. tsutsugamushi* or stimulated with 56°C-inactivated *O. tsutsugamushi*, 95°C-inactivated *O. tsutsugamushi*, LPS or Pam$_3$CSK$_4$ for 24 h. The concentrations of TNF-α were determined in the supernatant by sandwich cytokine ELISA. Data shown are combined results of two independent experiments (mean ± SD; * $p < 0.05$, ** $p < 0.01$ as determined by Student's *t*- test).

4.2.4 Cytokine production by BMDCs

In the previous section it was shown that macrophages need TLR2 to secrete TNF-α in response to *O. tsutsugamushi in vitro*. *In vivo*, the even more specialized cells for antigen recognition, uptake and presentation are dendritic cells, rather than macrophages [92]. In order to further characterize the role of TLR2 in a professional APC, BMDCs were prepared *ex vivo* from C57BL/6 and TLR2$^{-/-}$ mice and infected with *O. tsutsugamushi*.

TNF-α and IL-6 in the supernatant of both BMDCs cultures infected with live or heat-inactivated *O. tsutsugamushi* for 24 h were measured. As can be seen in (Figure 4.2.5), both live and heat-inactivated *O. tsutsugamushi* induced the production of TNF-α and IL-6 in C57BL/6 BMDCs (WT). In contrast to WT BMDCs, lower amounts of TNF-α production were observed when TLR2$^{-/-}$ BMDCs had been infected by live or stimulated by heat-killed bacteria (Figure 4.2.5). Interestingly, significantly higher levels of cytokines were produced when BMDCs had been infected by heat-inactivated *O. tsutsugamushi*, compared to live *O. tsutsugamushi*. These results indicated that BMDCs need TLR2 to secrete pro-inflammatory cytokines in response to *O. tsutsugamushi.*

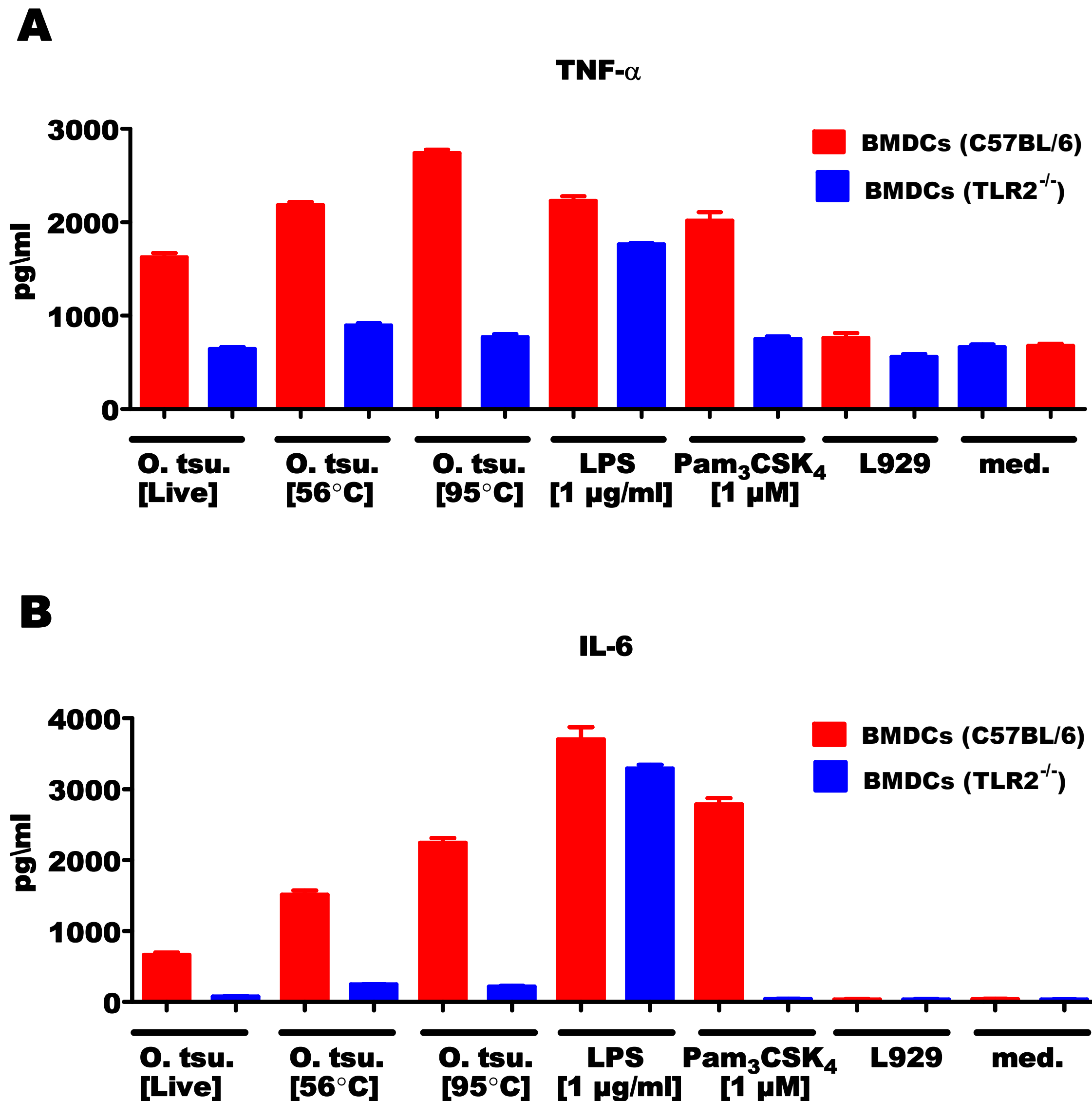

Figure 4.2.5: Cytokine production by BMDCs infected with *O. tsutsugamushi*
BMDCs isolated from C57BL/6 or TLR2$^{-/-}$ mice were infected with live *O. tsutsugamushi* or stimulated with 56°C-or 95°C-inactivated *O. tsutsugamushi*, LPS or Pam$_3$CSK$_4$ for 24 h. The concentrations of TNF-α **(A)** and IL-6 **(B)** were determined in the supernatant by sandwich cytokine ELISA. Data shown are representative results of one experiment (mean ± SEM).

4.2.5 TLR2-dependent signaling is required for intracellular growth control of *O. tsutsugamushi*

The failure to recognize and respond to microbial structures by TLRs can lead to increased susceptibility to infection [132]. In this section it was thus questioned whether TLR2 deficiency increases the intracellular replication of *O. tsutsugamushi* in macrophage cell lines *in vitro*. To this end, two macrophages cell lines 232 (WT) or 261 (TLR2$^{-/-}$) were infected

with live *O. tsutsugamushi.* Immediately p.i., 1, 2, 3, 4 and 6 days p.i. and cells were harvested. *O. tsutsugamushi* gene copies were determined by qPCR.

A measurable replication of *O. tsutsugamushi* was found in both 232 (WT) and 261 (TLR2$^{-/-}$) by 3 days p.i. After this time point, the replication rates were higher in 261 (TLR2$^{-/-}$) than in 232 (WT) cells. By 6 days p.i., the total number of bacteria had increased about 22-fold in 261 (TLR2$^{-/-}$) as shown in (Figure. 4.2.6). In contrast, the total number of bacteria had increased only about 10-fold in 232 (WT) (Figure 4.2.6). This result indicated that *O. tsutsugamushi* replicated more efficiently in TLR2-deficient macrophages.

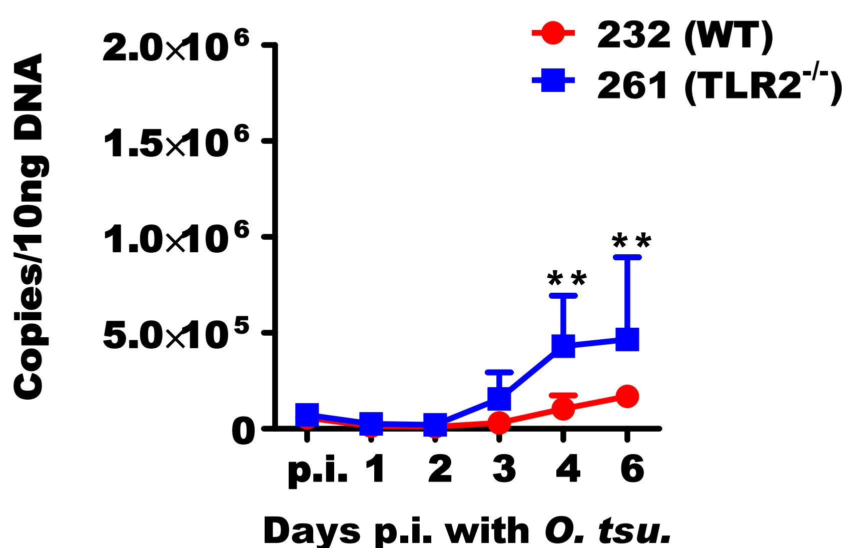

Figure 4.2.6: *O. tsutsugamushi* grows more efficiently in TLR2$^{-/-}$ (261) macrophages
Murine macrophages were seeded in 24-well plates at a concentration of $2x10^5$/well and infected with live *O. tsutsugamushi*. Immediately p.i., 1, 2, 3, 4 and 6 days p.i., cells were harvested. *O. tsutsugamushi* gene copies were determined by qPCR. Data shown are combined results of two independent experiments (mean ± SD, ** $p<0.01$ as determined by two-way ANOVA with Bonferroni post correction test, comparing the indicated groups).

4.2.6 Inhibition of *O. tsutsugamushi* growth by recombinant TNF-α in macrophages

It was shown in the previous sections that wild type macrophages and DCs, being potential host cells, were major producers of TNF-α following infection with *O. tsutsugamushi*, while TLR2-deficient cells produced significantly lower amounts of this cytokine. TNF-α has been shown before to inhibit the growth of *O. tsutsugamushi* Karp in mouse macrophages [53]. It was hypothesized that decreased TNF-α production by $TLR2^{-/-}$ macrophages could be in part responsible for enhanced growth of *O. tsutsugamushi*. To that end it was tested whether rTNF-α is able to equally reduce the growth of infective *O. tsutsugamushi* in 232 and 261 macrophages.

$2x10^5$ 232 or 261 cells/well were infected with live *O. tsutsugamushi* in 24-well plates and treated with various concentrations of rTNF-α. At indicated time point (p.i., 24 h, 48 h, 72 h, and 96 h) cells were harvested, and *O. tsutsugamushi* was quantified by qPCR.

After 96 h rTNF-α reduced the growth of infectious *O. tsutsugamushi* in a dose-dependent manner, a 2.7 fold decrease in 232 cells could be achieved by treatment with rTNF-α in concentrations of 100ng/ml (Figure 4.2.7). Infected 261 macrophages treated with TNF-α showed a similar dose-dependent reduction of bacterial growth on day 4 p.i. (Figure 4.2.7). Moreover, rTNF-α treatment reduced the growth of infectious *O. tsutsugamushi* in a similar manner in both 232 (WT) and 261 ($TLR2^{-/-}$) macrophage cell lines, although the 261 ($TLR2^{-/-}$) cells showed a higher bacterial growth without rTNF-α treatment (Figure 4.2.8).

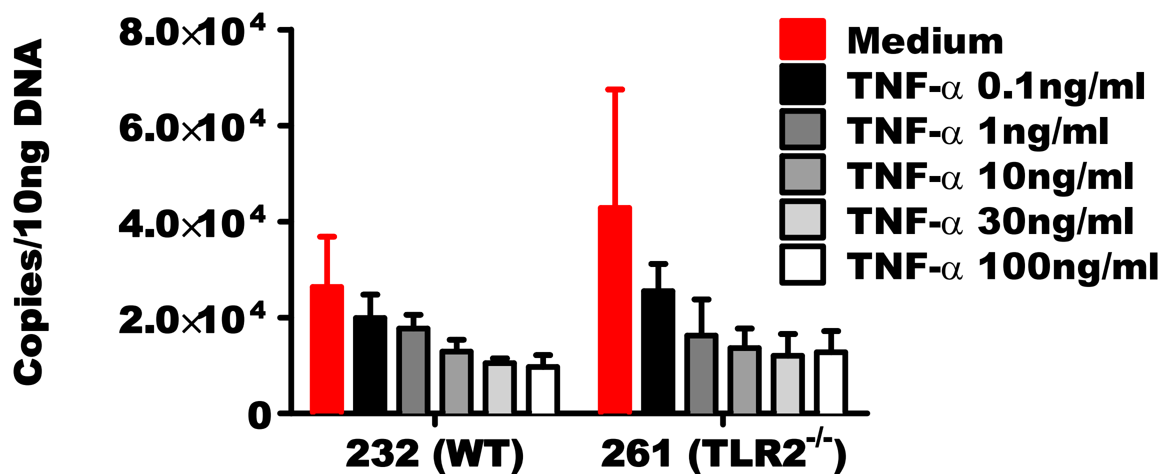

Figure 4.2.7 ***O. tsutsugamushi*** **growth inhibition by rTNF-α as measured by qPCR**
232 and 261 macrophage cells were infected with live *O. tsutsugamushi* in 24-well plates. The growth medium contained rTNF-α in indicated concentrations. 96 h p.i. samples were collected and bacterial copy numbers were determined. Data shown are combined results of two independent experiments (mean ± SD).

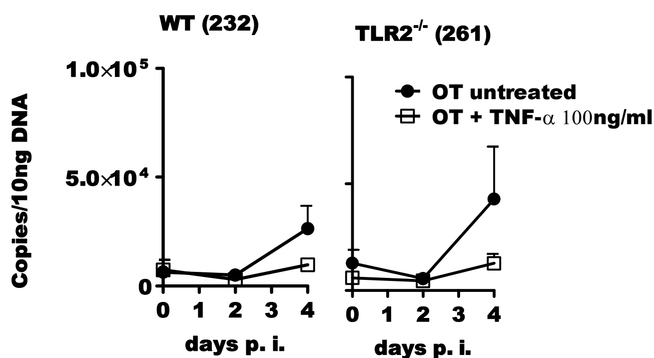

Figure 4.2.8 ***O. tsutsugamushi*** **inhibition by rTNF-α as measured by qPCR.**
232 and 261 macrophages were infected with live *O. tsutsugamushi* in 24-well plates. Cells were left either untreated or treated with rTNF-α (100 ng/ml). Samples were collected at the indicated time points for determination of bacterial copy numbers by qPCR. Data shown are combined results from two independent experiments (mean ± SD).

4.3 Influence of TLR2 deficiency on murine *O. tsutsugamushi* infection

In many infections, the presence of TLR ligands influences the outcome of infection [9, 10, 133]. The common phenotype of TLR deficiency is an enhanced susceptibility towards infection. The importance of TLR receptors in immunity to *O. tsutsugamushi* has not yet been investigated. Therefore the role of TLR2 in protection against *O. tsutsugamushi* infection was analyzed. We used TLR2$^{-/-}$ mice and compared the results to WT C57BL/6 mice, following i.p. (partially lethal) or s.c. (non-lethal) inoculation with *O. tsutsugamushi*.

4.3.1 Recruitment of phagocytic host cells to regional lymph nodes

Phagocytic cells do not only have a critical role in antimicrobial defense during the early stages of infection. In many infections, the recruitment of certain phagocytes such as inflammatory monocytes and neutrophils is required for pathogen drainage into the regional lymph node or beyond [134-136]. It was thus investigated whether recruitment of neutrophils or inflammatory monocytes to the regional lymph node is affected in the context of TLR2 deficiency. C57BL/6 and TLR2$^{-/-}$ mice were infected s.c. with 5,000 *sfu* of *O. tsutsugamushi*. Mice were sacrificed at day 1, 3, and 5 p.i. Lymph node cell suspension was stained for monocytes (Ly6C^{hi} CD11b^{hi}) and neutrophils (Ly6G^{hi} CD11b^{hi}) and analyzed by flow cytometry.

The absolute number of neutrophils (Ly6G^{hi} CD11b^{hi}) cells in the lymph node suspension measured by flow cytometry did not differ between both genotypes at any time point, suggesting that neutrophils are attracted to the regional lymph node irrespective of TLR2-mediated signaling (Figure 4.3.1 A). The absolute number of inflammatory monocytes (Ly6C^{hi} CD11b^{hi}) was similar at 1, 3 and 5 days p.i. (Figure 4.3.1 B), although TLR2-deficient mice showed a tendency towards lower monocyte numbers. These results suggest that inflammatory monocytes (Ly6C^{hi} CD11b^{hi}) are attracted with a similar efficiency to the regional lymph node in TLR2-competent and TLR2-deficient mice.

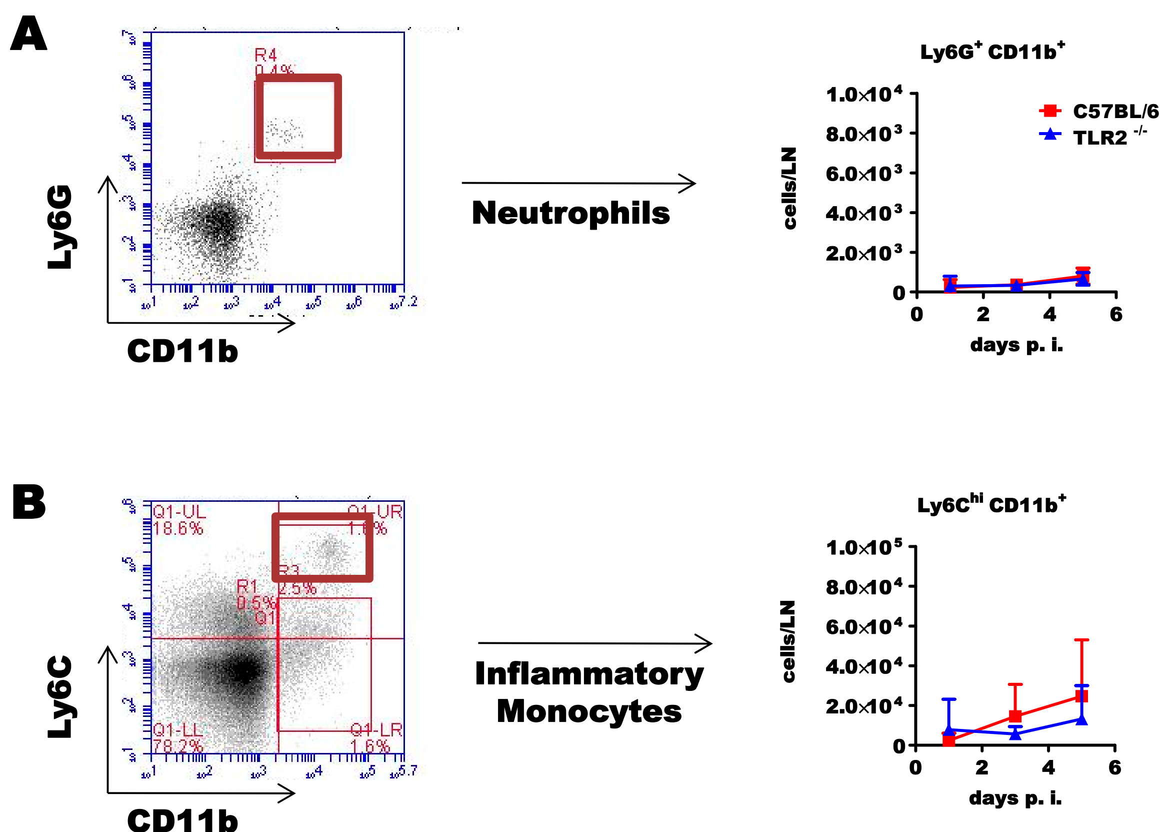

Figure 4.3.1: Neutrophil and monocyte recruitment in infected C57BL/6 and TLR2$^{-/-}$ mice
C57BL/6 and TLR2$^{-/-}$mice were infected s.c. with *O. tsutsugamushi*. Mice were sacrificed at day 1, 3, and 5 p.i. Absolute numbers of neutrophils (Ly6G^{+} CD11b^{+}) **(A)** and monocytes (Ly6C^{+} CD11b^{+}) **(B)** per lymph node (L.N.) were determined by FACS. Data shown are combined results from two independent experiments (mean ± SD (n=3)).

4.3.2 Monocytes have a higher infection rate in TLR2$^{+/+}$ mice

It was shown in the last section that inflammatory monocytes are attracted at much higher numbers to the regional lymph node compared to neutrophils (Figure 4.3.1). Although a significant difference between monocyte numbers was not demonstrated until day 5 p.i., a tendency towards lower numbers of inflammatory monocytes was observed in TLR2-deficient mice.

Both neutrophils and inflammatory monocytes have been ascribed to function as primary host cells responsible for pathogen transport to regional lymph nodes [134, 137-140]. Therefore, the next step was to determine to which degree both cell types are infected with *O. tsutsugamushi.* To that end, the bacterial loads of neutrophils and monocytes in regional

lymph nodes were measured in WT C57BL/6 and TLR2$^{-/-}$ mice after s.c. infection with *O. tsutsugamushi*.

To determine the degree of infection in potential target cells, flow cytometric sorting approaches with subsequent quantification of the pathogen in the sorted cell populations using qPCR were done. C57BL/6 and TLR2$^{-/-}$ mice were infected s.c. bilaterally into the hind footpads with *O. tsutsugamushi*. Mice were sacrificed at day 5 p.i., and the left and right popliteal lymph nodes were removed. Lymph node cell suspensions from two mice were pooled and fixed with methanol. The cells were then stained with the antibody conjugates Ly6G-APC, Ly6C-FITC and CD11b-PerCP/Cy5.5. Neutrophils (Ly6G^{+} CD11b^{+}) (P1) and monocytes (Ly6C^{+} CD11b^{+}) (P3) were sorted by FACS Aria (Figure 4.3.2 A). The pathogen loads in the sorted populations were determined by qPCR.

WT C57BL/6 mice had significantly greater numbers of *O. tsutsugamushi* in monocytes compared to neutrophils in the regional lymph nodes (Figure 4.3.2 B). The numbers of *O. tsutsugamushi* in neutrophils did not differ between WT C57BL/6 and TLR2 deficient mice (Figure 4.3.2 B). However, the bacterial load was significantly higher in WT C57BL/6 monocytes compared to monocytes of TLR2-deficient mice (Figure 4.3.2 B).

In conclusion, inflammatory monocytes in the regional lymph node tolerate a much higher bacterial load than neutrophils. This suggests that they are more potent in phagocytosis of *O. tsutsugamushi* in that stage and could thus play a more important role in early antibacterial defense compared to neutrophils. It cannot be concluded from this experiment if they act as host cells to support replication of *O. tsutsugamushi* or whether they are responsible for the transport of *O. tsutsugamushi* to regional lymph nodes. The results also suggest that the bacterial load of inflammatory monocytes recirculating to the draining lymph nodes depends on signaling via TLR2.

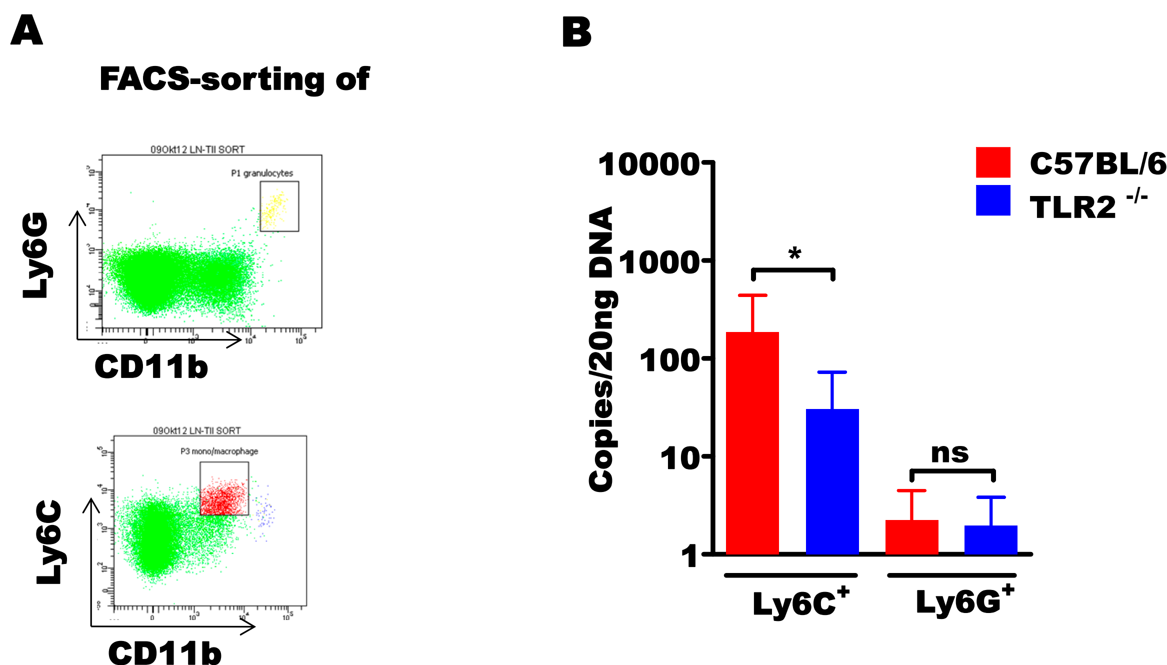

Figure 4.3.2: Monocytes, but not neutrophils show a high bacterial load
C57BL/6 and $TLR2^{-/-}$ mice were infected s.c. bilaterally into the hind footpad with *O. tsutsugamushi*. Mice were sacrificed at day 5 p.i. Four L.N. cell suspensions from two mice were pooled and stained with the antibody conjugates Ly6G-APC, Ly6C-FITC and CD11b-PerCP/Cy5.5 **(A)** Neutrophils ($Ly6G^+$ $CD11b^+$; P1) and monocytes ($Ly6C^+$ $CD11b^+$; P3) were sorted by FACS Aria. **(B)** The pathogen load in the sorted populations was determined by qPCR. Data shown are combined results of two independent experiments (mean ± SD, ns: not significant * $p < 0.05$ as determined by one-way ANOVA with Bonferroni post correction test, comparing the indicated groups (n=6)).

4.3.3 TLR2 deficiency does not alter the dissemination pattern of *O. tsutsugamushi* to internal target organs upon s.c. infection

Since TLR2-deficient inflammatory monocytes, a cell type with high migratory capacity that is able to recirculate between inflamed tissue and the blood circulation, exhibited a lower bacterial load, it was investigated next if dissemination of *O. tsutsugamushi* follows different kinetics in TLR2-deficient mice.

The i.p. inoculation of *O. tsutsugamushi* results in an intense localized infection of the peritoneal lining [43], but not a significant disseminated endothelial infection, which is typical for human scrub typhus [33]. In contrast, s.c. inoculation produces nonlethal disseminated infection [141]. S.c. infection also more closely resembles the natural transmission by mite vectors via the skin. For this reason, the more physiological s.c. infection model was used to investigate the role of TLR2 in controlling growth and dissemination of *O. tsutsugamushi* in host organs.

C57BL/6 and TLR2$^{-/-}$ mice were infected s.c. in the right hind footpad with 5,000 *sfu* of *O. tsutsugamushi*. To determine bacterial loads by qPCR, the lymph node, heart, brain, spleen, lung and liver of mice were harvested on days 7, 14, and 21 after infection.

There were no differences in organs loads of wild-type C57BL/6 and TLR2$^{-/-}$ mice after s.c. infection (Figure 4.3.2). Only in popliteal lymph nodes, a tendency towards higher bacterial loads early in infection (7 days p.i.) was observed in the WT C57BL/6 mice compare to TLR2$^{-/-}$ mice. The lung, brain, liver and heart showed a maximum bacterial load at day 14 p.i. in both mice types, while the lymph node and spleen showed that at day 7 p.i. These data suggest that in s.c. infection, the absence of TLR2 has no obvious influence on pathogen growth, disease course or antibacterial effector mechanisms.

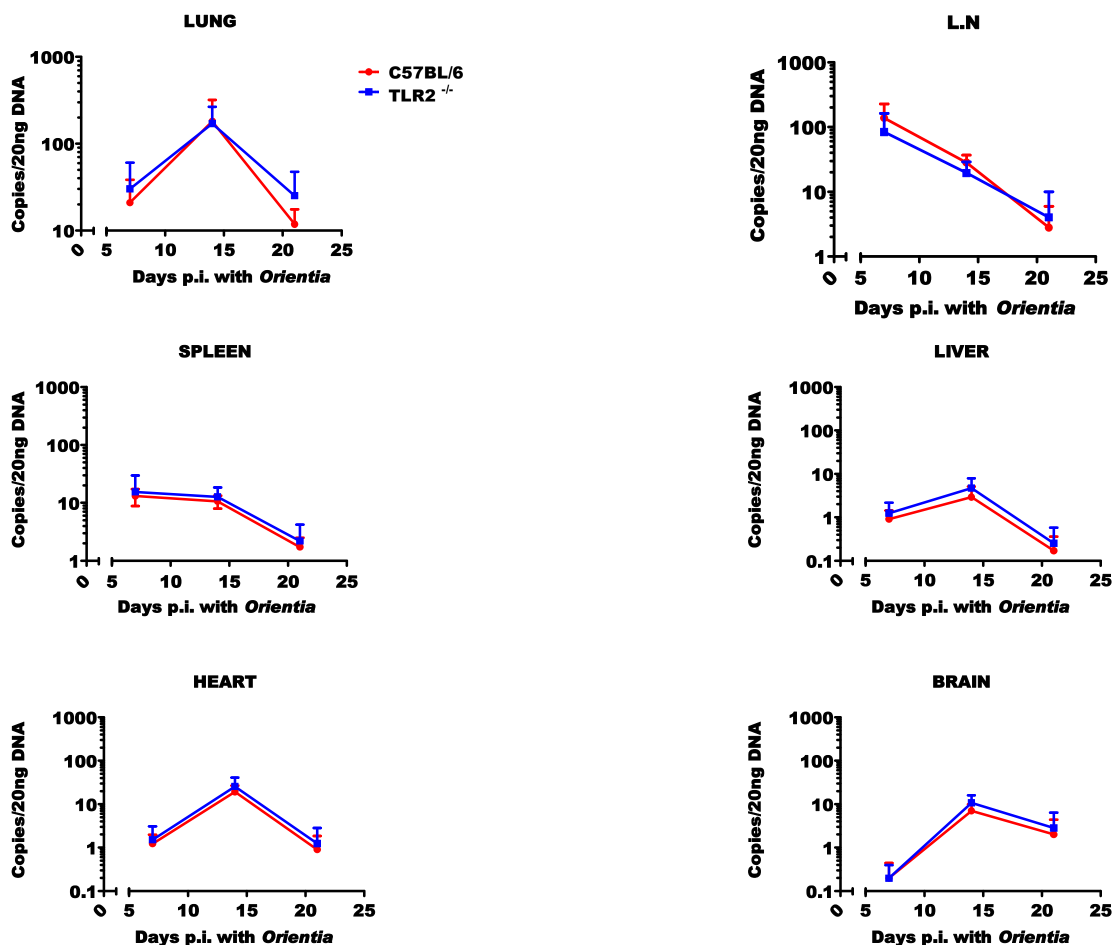

Figure 4.3.3: TLR2 does not influence the dissemination and replication of *O. tsutsugamushi* in host organs following a s.c. infection
C57BL/6 and TLR2$^{-/-}$mice were infected s.c. with *O. tsutsugamushi*. Mice were sacrificed at day 7, 14, and 21 p.i. *O. tsutsugamushi* load in L.N., heart, brain, spleen, lung and liver of mice was determined by qPCR. Data shown are combined results of two independent experiments (mean ± SD, (n=3)).

4.3.4 TLR2$^{-/-}$ mice are protected from lethal i.p. infections with *O. tsutsugamushi*

In this section, the role of TLR2 in the protective response against i.p. *O. tsutsugamushi* infection was studied by using gene targeted mice lacking TLR2 (TLR2$^{-/-}$). This mouse strain was established on the genetic background of C57BL/6 mice. C56BL/6 mice are relatively resistant to i.p. infection with *O. tsutsugamushi* compared to other mouse strains [142]. This way, comparisons to transgenic strains that show either lower or higher degrees of susceptibility to *O. tsutsugamushi* are possible [143].

C57BL/6 and TLR2$^{-/-}$ mice were infected i.p. with 5.000 *sfu O. tsutsugamushi*. During the infection period, the clinical score and survival curve were recorded. Clinical scores were the sum of clinical symptoms for the severity of the disease, based on the fur and body condition of the mice (Table 3.2.4.1). The WT C57BL/6 mice showed first clinical signs by day 6 p. i. After that the mice became gradually sicker and were seriously ill by day 11. At this time, increased amounts of ascites were present. The mice then gradually recovered afterwards and appeared normal with no ascites by day 17 (Figure 4.3.4 A). Surprisingly, the TLR2$^{-/-}$ mice showed a less intensive illness with a delay of the onset and termination of the clinical signs (Figure 4.3.4 A). The ascites observed in TLR2$^{-/-}$ mice by day 11 was comparable to WT mice. WT C57BL/6 mice displayed a moderate degree of susceptibility to *O. tsutsugamushi* infection with a mortality rate of 45% (Figure 4.3.4 B). In contrast, TLR2$^{-/-}$ mice displayed resistance to *O. tsutsugamushi* infection, with respective survival rates of 88% (Figure 4.3.1 B). In summary, TLR2$^{-/-}$ mice are largely protected against lethal i.p. infections with *O. tsutsugamushi*, compared to the wild type. Thus, pro-inflammatory innate signaling triggered by TLR2, rather than conferring protection, contributes to a lethal outcome in the mouse model of severe i.p. infection with *O. tsutsugamushi*.

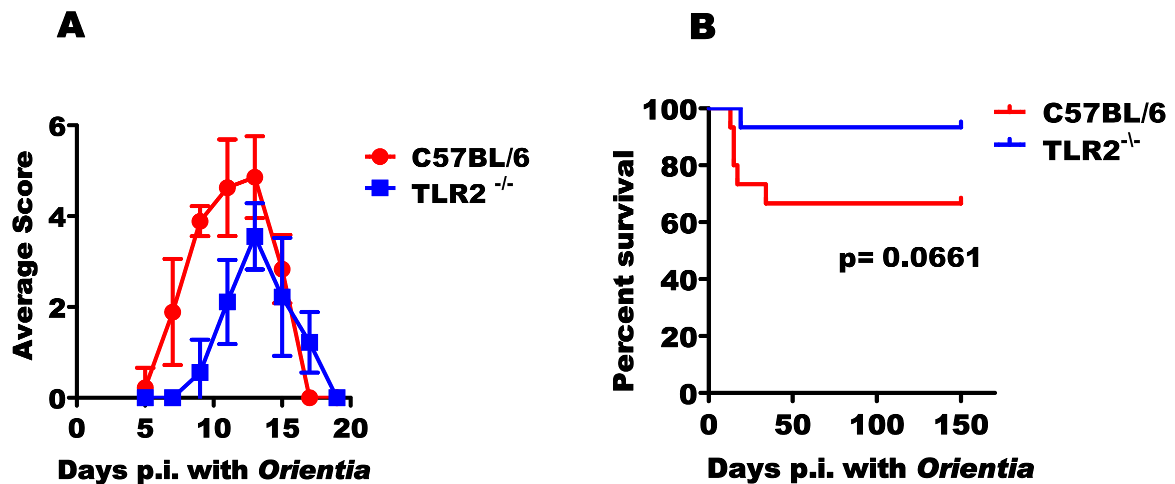

Figure 4.3.4: I.p. infection with *O. tsutsugamushi* results in reduced clinical signs and better survival curve in TLR2$^{-/-}$ mice

(A) Clinical scores of C57BL/6 and TLR2$^{-/-}$ following i.p. infection with 5.000 *sfu.* Data shown are combined results of two independent experiments (mean ± SD, (n=4)). **(B)** Survival curve of C57BL/6 and TLR2$^{-/-}$ following i.p. infection with 5.000 *sfu*. Survival was monitored for 22 weeks. Data shown are combined results of two independent experiments (mean ± SD, p= 0.0661 as determined by Log-rank (Mantel-Cox) test (n=15)).

5 Discussion

The role of innate immune responses mediated by sensing receptors in response to *O. tsutsugamushi* has not been analyzed in the literature. The goal of this study was to investigate the role of TLRs such as TLR2 and TLR4, which can recognize mainly structural bacterial surface molecules, such as lipopeptides, LPS or flagellin [75], and NLR family proteins in infection with *O. tsutsugamushi*. The goal was to be achieved by identification of receptors for *O. tsutsugamushi* using HEK293 cells overexpressing a number of candidate receptors, and by analysis of in receptor-deficient *in vivo* and *in vitro* models. This study shows for the first time that TLR2 is involved in *O. tsutsugamushi* recognition and mediates activation of the transcription factor NF-κB. The data also demonstrate that TLR2-dependent mechanisms control *O. tsutsugamushi* growth in infected macrophages. Surprisingly, i.p. infected TLR2$^{-/-}$ mice showed a less intensive illness and a better survival compared to C57BL/6 mice, indicating that TLR2-dependent signaling may be associated with pathogenesis in severe infections.

5.1 TLR2 involvement in *O. tsutsugamushi* recognition

In this study, several lines of evidence indicated involvement of TLR2 but not TLR4 in recognition of *O. tsutsugamushi* by the host cell. First, transfection-based complementation of TLR2/4- negative HEK293 cells with TLR2, but not TLR4, showed responsiveness to heat-inactivated and live *O. tsutsugamushi*, as judged by IL-8 production, while NOD1- or NOD2-transfected HEK293 cells also could not produce IL-8 following challenge with heat-inactivated *O. tsutsugamushi* (data not shown). In contrast to this finding *Cho et al.* reported the possible contribution of NOD1 and IL-32 to *O. tsutsugamushi* infection in ECV304 cells [59]. In the present study, an inactivated bacterial antigen preparation was used, so that possible NOD ligands might have been degraded. This could lead to low production of cytokines, while NOD1 might recognize only live *O. tsutsugamushi.* On the other hand, several previous studies in *Rickettsia* have demonstrated a role for both TLR2 and TLR4 in recognition and activation of different cells. For example, it has been reported that *R. africae* induces the activation of TLR4 on endothelial cells [60], *R. conori* needs TLR4 ligation for

activation of NK cell cytotoxicity and IFN-γ production [10, 11], and heat-killed *R. akari* triggers cells activation via TLR2 or TLR4 [12].

A previous study has shown that the composition of the cell envelope of *O. tsutsugamushi* differs substantially from that of the other rickettsiae, lacking both LPS and PG [21]. In this study, *Amano et al.* reported that the structure of *O. tsutsugamushi* cell wall did not contain muramic acid, glucosamine, heptose, KDO, and hydroxyl fatty acid, which are the constituents of bacterial PG and LPS. *Hanson* reported that the cell wall contained only the major strain-variable 56-kDa protein as well as the antigenically variable 110-, 47-, and 25-kDa proteins [68]. Compared to *O. tsutsugamushi,* other *Rickettsia* species have been shown to contain LPS and PG in their cell wall. In that course, the presence of the PG constituents muramic acid in *C. burnetii* and *R. typhi* [72] and the NOD1 ligand diaminopimelic acid in *R. prowazekii*, *R. typhi*, *Rochalimaea quintana*, and *C. burnetii* [73] was reported.

It is interesting to note also that a higher level of IL-8 was produced when TLR2-transfected HEK293 cells were stimulated with heat-inactivated *O. tsutsugamushi*, compared to stimulation with live *O. tsutsugamushi*. A stronger pro-inflammatory stimulation with inactivated bacteria was also observed in wild type macrophage cell lines, or BMDCs. A heat stable component that induces the secretion of cytokines has been observed before [144], presumably it is the ligand for TLR2. Some bacteria as *Listeria* organisms are more efficient at stimulating TNF-α when they are alive [145], whereas other intracellular pathogens as *Brucella* fail to induce TNF-α when they are alive [146].

It remains unclear why live *O. tsutsugamushi* bacteria are less stimulatory. Possibly, *O. tsutsugamushi* has mechanisms by which the live bacteria suppress the production of inflammatory cytokines for their own survival. *Kim et al.* suggested that the suppression of TNF-α production by *O. tsutsugamushi* in J774 macrophages is mediated by IL-10 [131]. This suppressive effect of IL-10 has been shown to play a role in other infection with bacteria such as *M. tuberculosis* [147] and *Yersinia* [148, 149]. In the light of the present study, it is thinkable that the suppression described by *Kim et al.* is directed against the pro-inflammatory response induced by the TLR2 ligand of *O. tsutsugamushi*. Alternatively, TLR2 ligands such as membrane-associated lipoproteins may be located with their receptor-binding sites towards the inner part of the membrane and thus the ligands may be masked in viable *O. tsutsugamushi*, while demasked upon thermal inactivation. In Gram-negative bacteria, mature lipoproteins are localized to various sites within the cell wall; attached to the cytoplasmic

membrane or the extracellular or peripheral side of the outer membrane [150]. For example, it has been reported that *Escherichia coli* has more than 90 lipoproteins and most of these are located at the periplasmic face of the outer membrane, while the rest are located at the periplasmic face of the inner membrane [151]. In Gram-positive bacteria, lipoproteins are anchored to the extracellular surface of the cytoplasmic membrane. Also in *M. tuberculosis* cell wall they are located to the unique mycolate-based lipid layer and are more easily accessible for TLR recognition [150]. It is not known in which part of the membrane *O. tsutsugamushi* ligands are located. Possibly they reside in the inner membrane and are dissolved upon thermal treatment allowing recognition by TLRs.

5.2 Chemical properties of the TLR2 ligand of *O. tsutsugamushi*

TLR2 plays an important role in recognition of pathogen cell wall components such as glycolipids in *T. cruzi* [152], lipoproteins in *E. coli* [153], or lipopeptides in *Mycoplasma fermentans* [154, 155]. Stimulation of TLR2-transfected HEK293 cells with selectively degraded *O. tsutsugamushi* cell wall extracts suggested that the TLR2 ligand of *O. tsutsugamushi* could be a lipopeptide. The treatment of *O. tsutsugamushi* cell wall extracts with H_2O_2, a process being known as thioether oxidation, abolished completely the TLR2 activity. The thioether present in the characteristic N-terminal motifs of all lipoproteins or lipopeptides (e.g. MALP-2) can be destroyed by this reaction converting the N-terminal cystein-thioether substructure into TLR2-inactive sulfoxide derivatives [156]. A similar result was shown, after alkaline hydrolysis with NaOH. These results show that the TLR2 ligand might contain lipid structures [80].

Moreover, digestion of *O. tsutsugamushi* cell wall extracts with proteinase K reduced the activity of TLR2 by over 75%. Proteinase K is a broad-spectrum serine protease that was first isolated from the fungus *Tritirachium album* Limber. Proteinase K is unspecific, the predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids [157, 158]. This result strongly suggests that the TLR2 activating component of *O. tsutsugamushi* contains a protein or peptide structure. Proteinase K treatment may not have been complete; it is also possible that *O. tsutsugamushi* contains two types of TLR2 ligands, one is susceptible, the other one is resistance to Proteinase K digestion.

Proteinase K sensitivity could speak for a membrane protein such as the ligand for TLR2 in *Francisella.* It could also suggest the presence of a Proteinase K-sensitive lipopeptide structure. Although it was noted that the majority of lipopeptides is resistant to proteases, *Thakran et al*. suggested that *F. tularensis* may have two different TLR2 agonists, one being resistant to digestion with protease and that could possibly be a lipopeptide, and a second ligand which is susceptible to protease treatment and that could be an integral membrane protein [159]. Finally Polymyxin B, which is a cationic, cyclic peptide antibiotic, known to inhibit biological activities of LPS through the binding of the lipid A moiety [160] was used as a control. The pretreatment of *O. tsutsugamushi* cell wall extracts with polymyxin B did not reduce the TLR2 activating capacity. Taken together these results suggest that the TLR2 ligand of *O. tsutsugamushi* could be a lipopeptide, while the presence of a second ligand, possibly of protein nature, is not excluded.

5.3 Activation of NF-κB by *O. tsutsugamushi* via TLR2

In this study we have first shown the role of TLR2 in recognition of *O. tsutsugamushi*. Therefore the next step was to investigate whether the NF-κB signaling pathway is activated via TLR2 in macrophage cell lines. TLR2 ligation triggers the MyD88-dependent intracellular signaling pathway, common to all TLRs except TLR3 [86]. This pathway induces activation of NF-κB, leading to cytokine and co-stimulatory molecule expression [130, 161]. The cascade also involves MAPKs which, by AP-1 induction, influences both transcription of inflammatory genes and mRNA stability of those transcripts [162].

We observed that in 232 (WT) macrophages, peak levels of nuclear translocation of NF-κB were reached 60 min p.i. and remained high through 120 min. Interestingly, almost no 261 ($TLR2^{-/-}$) macrophages showed residual, i.e. TLR2-independent translocation of NF-κB within the first four hours p.i., suggesting that no other innate receptors play a relevant role in early NF-κB activation after infection with *O. tsutsugamushi*.

It is shown for the first time in this study that *O. tsutsugamushi* triggers NF-κB translocation to the macrophage nucleus depending on ligation of TLR2. Signals from other receptors such as NOD1 are unlikely to play an important role in NF-κB activation within the first 4 h p.i. This result was unexpected because a recent study by *Cho et al.* demonstrated that NOD1

sensed an *O. tsutsugamushi* component in endothelial cells, and suggested that NOD1 activated the downstream pathway of NF-κB, and lead to IL-32 production [59]. On the other hand many previous studies have demonstrated the involvement of *O. tsutsugamushi* in activation of NF-κB [131, 163, 164]. Of note, almost 100% of 232 (WT) macrophages responded to treatment with soluble TLR2 ligand Pam_3CS_4K, while with fresh *O. tsutsugamushi* only 20% became positive. This finding could be related to the fact that LPS and Pam_3CS_4K are soluble molecules, and their concentration was sufficient to stimulate all cells. In contrast, *O. tsutsugamushi* as a complex antigen may have to be taken up by individual cell before NF-κB signaling is triggered. This could explain why only 20% of cells in infected experiments were positive for NF-κB.

5.4 TLR2-deficient macrophages and BMDCs are inferior producers of pro-inflammatory cytokines

The first line of evidence, using TLR-transfected HEK293 cells, indicated involvement of TLR2 in recognition of *O. tsutsugamushi*. The second line of evidence showed that deficiency of TLR2 significantly decreased secretion of TNF-α by murine macrophages stimulated with live or heat-inactivated *O. tsutsugamushi*, similarly with Pam_3CSK_4, a TLR2 agonist. This study is the first demonstration that *O. tsutsugamushi* engages TLR2 for eliciting cytokine production in murine macrophages. Previous studies on other infectious agents have demonstrated an important role of TLR2 in production of TNF-α and IL-6 by macrophages. *Takeuchi et al.* demonstrated impaired TNF-α production in response to Gram-positive bacterial cell walls in TLR2-deficient macrophages [165]. In murine macrophages, *Campos et al.* demonstrated that TLR2-mediated signaling results in IL-12 and TNF-α production in *T. cruzi* infection [166]. In peritoneal macrophages, the absence of TLR2 showed a statistically significant reduction in expression of TNF-α and IL-6 in response to infection with *C. trachomatis* [167].

In order to further characterize the role of TLR2 in a professional APC, we prepared BMDCs *ex vivo* from WT C57BL/6 and $TLR2^{-/-}$ mice and infected them with *O. tsutsugamushi*. DCs are the most potent APCs, even more than macrophages. They play a critical role in inducing and shaping adaptive immune responses [92]. DCs express a set of PRRs on the surface that can specifically interact with PAMPs, including the mannose receptors, c-type lectins and

TLRs [95, 96]. Thus DCs play an important role in linking the innate and adaptive immunity through their unique expression patterns of TLRs and cytokine production [96]. TLR ligation induces DC maturation characterized by up-regulated expression of MHC class II and the CD86/CD80 co-stimulatory family molecules resulting in enhanced antigen presentation and cytokine secretion [168, 169].

Stimulation of TLR2$^{-/-}$ BMDCs with live or heat-inactivated *O. tsutsugamushi,* as well as with Pam_3CSK_4, resulted in lower induction of IL-6 and TNF-α. The results of BMDCs confirm the results obtained in murine macrophages discussed above. These results indicate that also DCs need TLR2 to secrete IL-6 and TNF-α in response to *O. tsutsugamushi ex vivo.* A previous study demonstrated that stimulation of TLR2$^{-/-}$ BMDCs with *Porphyromonas gingivalis* synthetic lipopeptides (PGTP2-RL) as well as with Pam_3CSK_4, resulted in almost no induction of IL-6, TNF-α, IL-10 or IL-12p40, indicating that PGTP2-RL activates BMDCs to induce these cytokines in a TLR2-dependent fashion [170].

5.5 TLR2-deficient macrophages support increased proliferation of *O. tsutsugamushi*

The 3rd line of evidence showing that TLR2 is involved in recognition of *O. tsutsugamushi* was indicated by the response of two macrophages cell lines, 232 (WT) or 261 (TLR2$^{-/-}$), to infection with live *O. tsutsugamushi*. This was done to investigate the role of TLR2 deficiency in intracellular replication of *O. tsutsugamushi*. Our results argue strongly for an important role of TLR2-induced innate immunity, since *O. tsutsugamushi* replicated more efficiently in 261 (TLR2$^{-/-}$) macrophages. These data suggested that innate immune mechanisms triggered by TLR2 ligation diminish *O. tsutsugamushi* proliferation *in vitro*.

TLRs play an important role in recognition of conserved microbial structures known as PAMPs. The failure to recognize and respond to microbial structures by TLRs can lead to increased susceptibility to infection [132]. To explain the observations that *O. tsutsugamushi* growth is reduced in 232 (WT) murine macrophages compared to 261 (TLR2$^{-/-}$) macrophages, three possible defense mechanisms that kill these microorganisms may play a role.

First, TLR2-competent macrophages produced significantly greater amounts of TNF-α. Previous studies demonstrated that TNF-α inhibits the growth of *O. tsutsugamushi* in mouse

fibroblasts and macrophages [50, 53]. A number of studies have established the role of TNF-α in the inhibition of intracellular growth of bacteria [53, 171, 172]. As described above, wild type macrophages and DCs, being potential host cells, were major producers of TNF-α following infection with *O. tsutsugamushi*, while TLR2-deficient cells produced significantly lower amounts of this cytokine. rTNF-α reduced the growth of infectious *O. tsutsugamushi* in a dose-dependent manner in 232 cells. Infected 261 macrophages treated with TNF-α showed a similar dose-dependent reduction of bacterial growth on day 4 p.i. Our results argue that decreased TNF-α production by $TLR2^{-/-}$ macrophages could be in part responsible for the enhanced growth of *O. tsutsugamushi*.

Second, a differential induction of apoptosis in TLR2-sufficient and $TLR2^{-/-}$ cells may contribute to the intracellular growth control, similar to apoptosis-dependent killing shown for *M. tuberculosis* [173]. Both pro- and anti-apoptotic effects have been reported for *O. tsutsugamushi*. The controversy among reports may be due to the differences in host cells, bacterial strains and infection stages [15]. For instance, it has been reported that apoptotic death of macrophages and lymphocytes occurs in the spleen and lymph nodes of mice infected with *O. tsutsugamushi* strain Karp but not Gilliam [174]. Additionally, apoptosis was also demonstrated in J774 macrophage cells infected with *O. tsutsugamushi* [175]. Currently, it is not yet known whether host cell apoptosis is a mechanism of host defense or rather beneficial for pathogen survival. It has been reported that NF-κB activation by *Rickettsia* infection is related to the inhibition of apoptosis in endothelial cells and fibroblasts [176]. Further study of apoptosis modulation in *O. tsutsugamushi*-infected cells by NF-κB activation is needed.

Third, one of the mechanisms by which the macrophages cells can destroy invading bacteria is the respiratory burst. It is due to increased activation of NAD(P)H-oxidase localized in the plasma membrane [177] or the phagosome. Following phagocytosis, there is increased activity of the hexose monophosphate (HMP) shunt in the cytosol, elevated oxygen consumption, and production of superoxide radicals and hydrogen peroxide [178]. Myeloperoxidase is an enzyme found in the azurophilic granules of mammalian neutrophils and also identified in human monocytes [179], it is also believed to be involved in augmenting the cytotoxic activity of H_2O_2 [180]. However, the role of the respiratory burst in *O. tsutsugamushi* infection is unknown.

Of note, we did not observe production of NO in both macrophage cell lines in response to the *O. tsutsugamushi* infection (data not shown) indicating that nitric oxide (NO) is either not elicited, or its production actively suppressed by *O. tsutsugamushi*. A previous study that compared NO production elicited by Kuroki and Karp strains of *O. tsutsugamushi* demonstrated a significant level of NO production in macrophages infected with the avirulent strain Kuroki, but not with the virulent strain Karp [100]. In sum, it was shown that TLR2 ligation is an important mediator in limiting intracellular *O. tsutsugamushi* proliferation.

5.6 Monocyte and neutrophil recruitment during *O. tsutsugamushi* infection

Phagocytes such as monocytes/macrophages and neutrophils possess potent antimicrobial effectors mechanisms against intra- and extracellular pathogens. They respond to TLR-related danger signals [181]. In the present study, it was investigated whether the recruitment of neutrophils and inflammatory monocytes to the regional lymph node following s.c. infection with *O. tsutsugamushi* is dependent on TLR2, by infecting WT C57BL/6 and $TLR2^{-/-}$ mice. The number of neutrophils ($Ly6G^{hi}$ $CD11b^{hi}$) did not differ between both mice types at any time point, suggesting that neutrophils are attracted to the regional lymph node irrespective of TLR2-mediated signaling. Monocytes ($Ly6C^{hi}$ $CD11b^{hi}$) appeared at much higher number in the lymph node compared to neutrophils. Although the difference in the number of monocytes in WT C57BL/6 and $TLR2^{-/-}$ mice was not significant, a tendency to lower numbers in $TLR2^{-/-}$ mice was visible. These results suggest that monocyte recruitment is not directly associated with TLR-related danger signals and that other signals may play a role. These data are consistent with previous observations by *Torres et al.* who demonstrated that the recruitment of mononuclear cells and neutrophils is not impaired in the absence of TLR2 in hepatic microabscesses after *L. monocytogenes* infection [133].

On the other hand, chemokines play a pivotal role in monocyte recruitment to the site of injury. For example CC-chemokine ligand 2 (CCL2; also known as macrophage chemoattractant protein-1 (MCP1)) and CCL7 (also known as MCP3) are CC-chemokines that bind to CCR2 and mediate $LY6C^{hi}$ monocyte recruitment [182]. A previous study demonstrated an increase in the level of mRNAs for certain chemokines such as macrophage inflammatory proteins 1α/β (MIP1α/β), MIP2, and MCP1 in J774 macrophage cells infected

with *O. tsutsugamushi* [163]. From a model of ischemic stroke it was reported that TLR2 signaling is needed for adequate recruitment of circulating monocytes, presumably related to a selective decrease in MCP-1 in $TLR2^{-/-}$ mice [183]. Although chemokine production was not measured in the present study, a relationship between TLR2 signaling and monocyte recruitment was not found in our *O. tsutsugamushi* infection model.

In *L. monocytogenes* infection, two signaling pathways are required for monocyte emigration from the bone marrow during the early stage of infection, both, type I IFN and MyD88 signals [104]. We observed TLR2-independent recruitment of monocytes to the regional lymph nodes, so probably TLR-unrelated factors contribute to recruitment of monocytes such as type I IFNs [101]. A previous study demonstrated that the murine CMV and VV induced type I IFN expression by $LY6C^{hi}$ monocytes, this process requires TLR2 stimulation by viral ligands in endosomes of virus-infected cells [6].

Monocyte recruitment is important for defense against a vast range of pathogens for example *L. monocytogenes* [137], *Brucella melitensis* [184], *M. tuberculosis* [138], *Aspergillus fumigatus* [134], *T. gondii* [139] *and Blastomyces dermatitidis* [140]. However, several results point to the cooperation of monocytes and neutrophils in the elimination of intracellular pathogens such as *T. gondii* [136, 181, 185-187].

In this study, it was demonstrated that WT C57BL/6 mice had 100-fold greater numbers of *O. tsutsugamushi* in monocytes compared to infected neutrophils in the regional lymph node, indicating that inflammatory monocytes rather than neutrophils are responsible for the phagocytosis and possibly the transport of *O. tsutsugamushi* to regional lymph nodes. Moreover, it was found unexpectedly that the bacterial load in WT C57BL/6 monocytes was significantly higher compared to monocytes of TLR2-deficient mice, suggesting that the bacterial load of inflammatory monocytes depends on signaling via TLR2. These results seem to contradict the data presented in chapter 4.2.5 where *O. tsutsugamushi* replicated more efficiently in 261 ($TLR2^{-/-}$) macrophages than 232 (WT) macrophages.

The higher bacterial load in *in vitro* infected macrophages must be related to replication, because both 232 and 261 macrophages were infected with the same dose of bacteria. However, bacterial loads determined in host cells *ex vivo* may not necessarily reflect actively replicating bacteria. A higher load may also be caused by enhanced phagocytosis, with high numbers of degraded intracellular bacteria. A previous study demonstrated that ligation of

TLRs enhanced antigen capture by DCs [188]. TLR2 may thus ensure a more efficient phagocytosis of *O. tsutsugamushi* by inflammatory monocytes in the lymph node. This enhanced bacterial uptake could thus mask the suppressive effect of TLR2 on bacterial growth that was demonstrated *in vivo*.

Yet, it remains unclear whether *O. tsutsugamushi* is also able to replicate more efficiently in TLR2-deficient monocytes *in vivo*. To investigate this, another experimental approach to determine the viability of monocyte-associated bacteria is needed, e.g. inflammatory monocytes could be isolated in order to determine the infectivity of intracellular bacteria in an immunofocus plaque test. Further experiments will also have to clarify whether monocyte recruitment supports or reduces systemic dissemination of *O. tsutsugamushi*.

5.7 Subcutaneous (s.c.) infection model

It was shown that the inflammatory monocytes displayed a high migratory capacity and are able to recirculate between inflamed tissue and the blood circulation [104]. Therefore, it was investigated next if the different bacterial load of inflammatory monocytes in TLR2-deficient and wild type mice would lead to a difference in organ dissemination, following s.c. (non-lethal) inoculation with *O. tsutsugamushi*. S.c. infection more closely resembles the natural transmission by mite vectors via the skin. For this reason, the more physiological s.c. infection model was used to investigate the role of TLR2 in controlling growth and dissemination of *O. tsutsugamushi* in host organs.

Although inflammatory monocytes contained much lower bacterial number in $TLR2^{-/-}$ mice, it was found that there were no differences in organs loads of WT C57BL/6 and $TLR2^{-/-}$ mice after s.c. infection. Only in popliteal lymph nodes, a tendency towards higher bacterial loads early in infection (7 days p.i.) was observed in the WT C57BL/6 mice compared to $TLR2^{-/-}$ mice. The pathogen in the early phase of the infection invaded the draining lymph node, but afterwards spread systemically. At day 14 p.i., the pathogen load was maximal in the lung, brain, liver and heart, while the lymph nodes and spleen showed the maximum at day 7 p.i. Three weeks after infection, only a low level of pathogen DNA could be detected in all organs. Similar to what was observed in by *Keller et al.* (manuscript submitted) in BALB/c mice, a clear tropism for lung tissue was also shown in C57BL/6 mice in the present study [64].

In many infections, the presence of TLRs influences the outcome of infection. Previous studies demonstrated a role of TLR2 and TLR4 in protection against *Rickettsia* infection [10-12]. *Jordan et al.* reported that TLR4-deficient mice had significantly greater numbers of rickettsiae in the brain and lungs on days 3 and 5 p.i. [10]. Other studies demonstrated that TLR2-deficient mice are more susceptible to infection of many infectious agents. For example, after infection with *L. monocytogenes*, the bacterial burden was increased in the liver [133], or in both liver and spleen [189] in TLR2-deficient mice. However, the data of the present study suggest that in s.c. infection with *O. tsutsugamushi*, the absence of TLR2 has no obvious influence on pathogen growth, disease course or antibacterial effector mechanisms.

5.8 Intraperitoneal (i.p.) infection model

Although s.c. infection also more closely resembles the natural transmission by mite vectors via the skin, the i.p. inoculation of *O. tsutsugamushi* has often been used as a model of severe infection, resulting in an intense localized infection of the peritoneal lining [43]. Therefore, the role of TLR2 in the protective response against i.p. *O. tsutsugamushi* infection was studied. Surprisingly, the TLR2$^{-/-}$ mice showed a less intensive illness with a delay of the onset and termination of the clinical signs and a better survival compared to C57BL/6 mice. Although the difference survival curves was statically not significant (P= 0.06), there was a clear tendency towards protection in TLR2$^{-/-}$ mice with a respective survival rate of 88%, compared to 45% in WT C57BL/6 mice. A larger number of mice might be needed to obtain statistically significant results.

The absence or presence of TLRs has been associated with both pathological and protective immune responses [190]. The protective role of TLRs has been recognized to play a role in infection caused by a diverse set of pathogens, including bacteria, viruses, parasites and fungi. For example, *Jordan et al.* reported that TLR4-deficient mice had an overwhelming rickettsial infection and showed decreased protection [10]. Moreover, respiratory infection models of TLR2$^{-/-}$ mice with *Mycoplasma* and *Mycobacterium* showed a protective role for TLR2 [86]. Relating to *M. tuberculosis* infection, one observation might be interesting for our infection model. The importance of TLR2 and TLR4 may depend on the dose used for challenge. In response to the low-dose infection with *M. tuberculosis*, the survival was identical in mutant and control mice. However, high-dose infection with *M. tuberculosis* revealed TLR2-, but not TLR4-defective mice to be more susceptible than control mice [9]. Moreover, the observation

period reported in the present study did not extend beyond day 90, so it is not excluded that there might be a difference in the survival rate later in the course of *O. tsutsugamushi* infection.

In viruses, TLR2 has a protective role against VV and CMV [6]. In fungi, TLR2 was also shown to play a major role in protection against *P. marneffei* [7]. In contrast, in some infection model a deleterious role for TLR2 has been shown. For example, in experimental malaria after infection with *P. berghei*, a pathological role for TLR2 and TLR9 has been demonstrated, whereas the absence of TLR2 or TLR9 increased resistance to cerebral malaria-related mortality [13].

In this study we have demonstrated that $TLR2^{-/-}$ mice are largely protected against lethal i.p. infections with *O. tsutsugamushi*, compared to the wild type. This suggests that TLR2-mediated danger signals could induce overwhelming immune responses and thus contribute to the development of immunopathology. Previous studies have demonstrated different mechanisms to explain the pathological role of TLR2 in infection. In protozoan infections such as *Toxoplasma, Entamoeba* and *Leishmania* infections, TNF-α induced via TLR2 may be related to pathology seen with these infections [86]. It has furthermore claimed that the severe disease state in leishmaniasis could be associated with TLR2-driven Th2 responses [191]. Other groups suggested a mechanism for decreased susceptibility of TLR2 KO mice infected with *Yersinia enterocolitica* or *Candida albicans*. Accordingly, a stronger Th1-type cytokine response due to diminished production of IL10 during infection is responsible for this protective effect [148, 192].

Previous studies suggested a novel role for IL-27 in regulating innate immunity and neutrophil function during septic peritonitis. IL-27 mediated the attraction of neutrophils to the peritoneal cavity [193]. IL-27 subunits are produced by DCs and macrophages after TLR ligation and may act very early in Th1-mediated immunity [194]. *Fukuhara et al.* reported a significant increase of neutrophil influx into the peritoneal cavity following i.p. infection with the virulent Karp strain of *O. tsutsugamushi,* which was absent in infection with the avirulent Kuroki strain. Only the virulent Karp strain leads to a high degree of mortality in BALB/c mice [100]. Possibly TLR2-mediated signals could induce an IL-27-dependent influx of neutrophils to the peritoneal cavity and thus cause an immunopathological reaction.

It is accepted that TLRs have potent effects on phagocytic cells. However, several groups indicated that the adjuvant effects of certain TLR agonists may also be attributed to the activation of TLRs and the TLR adapter molecule MyD88 directly in T cells [53]. It is not excluded that the difference in survival in $TLR2^{-/-}$ mice is related to TLR2 expression on T cells. Further studies are needed to assess the role of TLRs in T cells.

Indeed, the fact that TLR2-deficient mice are much more susceptible to certain infections suggests that TLR2 polymorphisms in humans may impair host response to a given spectrum of microbial pathogens [195]. More than 175 single nucleotide polymorphisms (SNPs) have been reported for the human TLR2 gene located on chromosome 4q32 [196]. TLR2 polymorphisms influence the susceptibility of patients to several infections such as leprosy and tuberculosis infection [195, 197].

A number of genetic association studies suggest that TLR polymorphisms may be associated with susceptibility to different infectious diseases such as TLR2 and TLR4 [198]. For example, SNPs within the TLR2 gene have been shown to increase the incidence of certain infectious diseases, such as tuberculosis [197, 199]. In humans, impaired TLR2 signaling caused by homozygosity for R753Q polymorphism contributes to CMV infection after liver transplantation [86]. In malaria, no TLR2 polymorphisms are associated with *P. falciparum* infections [200]. However, in Ghana, SNPs D299G and T399I were associated with severe malaria [201]. In Lyme disease, an Arg753Gln polymorphism of TLR2 gene protects from the development of late disease stage [202]. A recent study on *R. akari* demonstrated that the presence of the R753Q TLR2 or D299G TLR4 polymorphisms resulted in a significant loss of the capacities of the respective TLRs to mediate NF-κB reporter activation in HEK293T transfectants [12]. Whether TLR2 polymorphisms play a role for protection or susceptibility to *O. tsutsugamushi* in humans has not yet been investigated. Further studies are worthwhile to address the role of TLR2 polymorphisms in human *O. tsutsugamushi* infection.

In summary, this study reveals for the first time that TLR2 is involved in recognition of *O. tsutsugamushi in vitro*. This finding is supported by several lines of evidence, including the ability of *O. tsutsugamushi* to stimulate TLR2- but not TLR4-transfected HEK293 cells. Moreover, the deficiency of TLR2 significantly decreased secretion of TNF-α by murine macrophages stimulated with *O. tsutsugamushi*. Our results also argue strongly for an important role of TLR2-induced innate immunity, taking into account the ability of O. *tsutsugamushi* to replicate more efficiently in 261 ($TLR2^{-/-}$) macrophages. However, TLR2

has no protective role *in vivo*, but rather it might mediate an inflammation-induced immunopathology. Moreover, TLR2 has no influence on pathogen dissemination kinetics *in vivo*. Further studies are needed to determine the precise mechanism of TLR2-dependent damage in *O. tsutsugamushi*-induced immunopathogy *in vivo*.

6 Conclusion

This study shows for the first time that TLR2 is the major receptor responsible for innate recognition of *O. tsutsugamushi*. Ligation of TLR2 mediates activation of NF-κB in macrophages. The data also demonstrate that TLR2-dependent mechanisms control *O. tsutsugamushi* growth in infected macrophages.

In the subcutaneous inoculation mouse model of scrub typhus, which closely mimics natural transmission, TLR2 was shown to have no influence on pathogen dissemination kinetics and the recruitment of inflammatory monocytes and neutrophils. Possibly, TLR2-mediated danger signaling is redundant in *O. tsutsugamushi* infection, and other transduction pathways such as type I interferon-dependent mechanisms are able to compensate for the loss of TLR2-dependent signaling.

In the more severe intraperitoneal infection, the presence of TLR2 surprisingly did not protect from lethal courses, but rather predisposed for more severe symptoms and higher death rates. In that course, the present data suggest the development of a TLR2-mediated immunopathology in severe infections with *O. tsutsugamushi*.

The results of this study contribute to a better understanding of the innate immune response against *O. tsutsugamushi*. However, further studies are needed to determine the precise role of TLR2 in *O. tsutsugamushi*-induced immunopathogy *in vivo.*

7 Summary

Scrub typhus, an infectious disease caused by *O. tsutsugamushi*, is widely distributed in Asia and the Pacific region including Northern Australia, with more than one billion people at risk of contracting the disease and probably more than one million new cases every year. *O. tsutsugamushi*, which was classified as a separate genus in the Rickettsiaceae family, is an obligate intracellular bacterium that is transmitted via the skin by mite bites. Little is known about the early immunological events of *O. tsutsugamushi* infection. Particularly, the role of immediate innate immune responses induced by pattern recognition receptors such as toll-like receptors (TLRs) and NOD-like receptors in response to *O. tsutsugamushi* remains poorly understood.

The goal of this study was to identify an innate receptor responsible for recognition of bacterial surface structures of *O. tsutsugamushi* and to investigate its role in a mouse infection model. First, an *in vitro* overexpression system for innate receptors was used in order to test reactivity with *O. tsutsugamushi*. HEK293 cells transiently transfected with human TLR2, TLR4, NOD1 or NOD2, were stimulated or infected with *O. tsutsugamushi* and the production of IL-8 was measured in cell culture supernatants. It was thus shown that TLR2, but not TLR4, NOD1 or NOD2, has a role in recognition of both inactivated and live *O. tsutsugamushi*. The TLR2 ligand of *O. tsutsugamushi* was heat-stable and showed complete sensitivity to treatment with hydrogen peroxide and sodium hydroxide, and partial sensitivity to proteinase K, suggesting the presence of two different TLR2 ligands in *O. tsutsugamushi*, possibly a lipopeptide and a protein.

The requirement of TLR2 for the induction of pro-inflammatory responses was investigated in the murine macrophage cell lines 232 (C57BL/6 wild-type) and 261 (TLR-deficient). It was found that *O. tsutsugamushi* activated the NF-κB signal transduction pathway depending on the presence of TLR2. TLR2 deficiency increased the intracellular replication of *O. tsutsugamushi* in macrophage cell lines *in vitro*. This increase in replication in TLR2-deficient macrophages was correlated with significantly lowered levels of TNF-α production, indicating that decreased TNF-α production by TLR2-deficient macrophages could be in part responsible for enhanced growth of *O. tsutsugamushi*. In consistence with these results, treatment with recombinant TNF-α reduced the growth of infectious *O. tsutsugamushi* in a

concentration-dependent manner to a similar extent in both 232 and 261 macrophage cell lines.

Finally, the influence of TLR2-dependent signaling on the kinetics of *O. tsutsugamushi* dissemination was investigated *in vivo*. Infection was initiated by subcutaneous inoculation, thus mimicking the natural transmission of *O. tsutsugamushi* via the skin. TLR2-deficient mice and C57BL/6 wild types showed no significant differences in the recruitment of inflammatory monocytes and neutrophils to the regional lymph node during the first week of infection. On a cellular level, at day 5 post infection, a significantly lower bacterial load was found in TLR2-deficient inflammatory monocytes compared to wild type cells, while neutrophils had very low bacterial loads in both mouse strains. Although TLR2-deficient macrophages supported more efficient bacterial growth *in vitro*, it is possible that the higher bacterial load in wild type monocytes *in vivo* reflects enhanced TLR2-mediated bacterial uptake rather than replication. The suppressive effect of TLR2 on bacterial replication may thus be masked *in vivo* by enhanced uptake.

The *O. tsutsugamushi* loads in the lymph node and other target organs during the 3-week course of infection did not differ between TLR2-deficient and wild type mice. All animals survived the infection. Thus, TLR2-mediated pathogen recognition influenced neither the recruitment of monocytes and neutrophils nor the bacterial dissemination *in vivo* or overall survival following s.c. infection. Possibly, TLR2-mediated danger signaling is redundant *in vivo* during *O. tsutsugamushi* infection and can be compensated by other innate signaling pathways.

Upon intraperitoneal inoculation of *O. tsutsugamushi*, which is more severe and potentially lethal infection, surprisingly TLR2 deficiency did not predispose for overwhelming infection, but even ameliorated the severity of symptoms and protected mice from lethal outcomes. This unexpected finding suggests the development of a TLR2-mediated immunopathology in severe, highly replicative infections with *O. tsutsugamushi*.

This study shows for the first time that TLR2 serves as an innate receptor for *O. tsutsugamushi*. Instead of contributing significantly to antibacterial immunity *in vivo*, as it is known from infections with other pathogens, TLR2-dependent signals rather deteriorate the course of infection.

8 Zusammenfassung

Die Rolle von Toll-like-Rezeptor 2 bei der Erkennung von *Orientia tsutsugamushi* durch das angeborene Immunsystem

Das Tsutsugamushifieber, eine durch *Orientia (O.) tsutsugamushi* hervorgerufene Infektionserkrankung, ist endemisch in weiten Teilen Asiens und des pazifischen Raums sowie in Nordaustralien. Für mehr als eine Milliarde Menschen besteht das Risiko, sich zu infizieren; die Inzidenz liegt vermutlich bei mehr als einer Million Fälle jährlich. *O. tsutsugamushi*, eine eigene Art innerhalb der Familie der Rickettsiazeen, ist ein obligat intrazelluläres Bakterium, das beim Biss bestimmter Milbenarten durch die Haut übertragen wird. Zu den frühen immunologischen Ereignissen der Infektion mit *O. tsutsugamushi* ist wenig bekannt. Insbesondere fehlen Erkenntnisse über die durch das angeborene Immunsystem hervorgerufenen Immunantworten, die nach Erkennung mikrobieller Strukturen durch konservierte Rezeptoren der Toll-like-Rezeptor (TLR)- und NOD-like-Rezeptor-Familien ausgelöst werden.

Das Ziel der vorliegenden Arbeit war die Identifizierung eines Rezeptors des angeborenen Immunsystems, der für die Erkennung bakterieller Oberflächenstrukturen von *O. tsutsugamushi* verantwortlich ist, und die Charakterisierung seiner Rolle für den Infektionsverlauf im Mausmodell. Zunächst wurde *in vitro* in einem Expressionssystem für TLR- und NLR-Rezeptoren die Reaktivität mit *O. tsutsugamushi* untersucht. Es wurden dazu HEK293-Zellen verwendet, die transient mit humanem TLR2, TLR4, NOD1 oder NOD2 transfiziert waren. Nach Stimulation oder Infektion mit *O. tsutsugamushi* wurde die Konzentration von Interleukin (IL)-8 im Überstand mittels ELISA gemessen. Es wurde so gezeigt, dass TLR2, aber nicht TLR4, NOD1 oder NOD2, an der Erkennung sowohl inaktivierter wie lebender Bakterien beteiligt ist. Der TLR2-Ligand von *O. tsutsugamushi* war hitzestabil und zeigte sich komplett sensibel auf Behandlung mit Wasserstoffperoxid und Natriumhydroxid, sowie teilweise sensibel auf Behandlung mit Proteinase K, was für das Vorkommen zwei unterschiedlicher TLR2-Liganden, einem Lipopeptid und einem Protein, spricht.

In den Mausmakrophagen-Linien 232 (C57BL/6 Wildtyp) und 261 (TLR2-Defizienz) wurde untersucht, ob TLR2-abhängige Signale notwendig sind für die Induktion proinflammatorischer Immunantworten. Es zeigte sich, dass *O. tsutsugamushi* in Abhängigkeit von TLR2-Ligation den NF-κB-Signalweg aktiviert. TLR2-Defizienz führte zudem zu einer stärkeren intrazellulären Vermehrung von *O. tsutsugamushi in vitro*. Diese stärkere Vermehrung was assoziiert mit signifikant erniedrigter Produktion von TNF-α, so dass die geringere Produktion dieses Zytokins durch TLR2-defiziente Makrophagen teilweise für das stärkere Wachstum verantwortlich sein könnte. In beiden Zell-Linien ließ sich das Wachstum durch Behandlung mit rekombinantem TNF-α konzentrationsabhängig auf vergleichbare Werte reduzieren.

Schließlich wurde *in vivo* der Einfluss TLR2-abhängiger Signale auf die Disseminierungskinetik von *O. tsutsugamushi* untersucht. Die Infektion erfolgte durch subkutane Inokulation, was der natürlichen Übertragung von *O. tsutsugamushi* über die Haut sehr nahekommt. Die Rekrutierung inflammatorischer Monozyten und neutrophiler Granulozyten in die regionalen Lymphknoten während der ersten Woche der Infektion zeigte keine Unterschiede zwischen C57BL/6-Wildtypen und TLR2-defizienten Mäusen. Auf zellulärer Ebene wurde an Tag 5 nach Infektion eine signifikant niedrigere Bakterienlast in TLR2-defizienten inflammatorischen Monozyten im Vergleich zu Wildtyp-Zellen gefunden, während die Bakterienlast Neutrophiler in beiden Mausstämmen sehr gering war. Obwohl *O. tsutsugamushi in vitro* besser in TLR2-defizienten Makrophagen replizierte, ist es möglich, dass die höhere Bakterienlast in Wildtyp-Monozyten eher bedingt ist durch eine TLR2-abhängig verstärkte Aufnahme von Bakterien als durch ihre Replikation. Der supprimierende Effekt von TLR2 auf die Replikation könnte daher *in vivo* durch eine erhöhte Aufnahme von Bakterien maskiert werden.

Die Gesamtlasten im Lymphknoten und anderen Zielorganen unterschieden sich nicht signifikant während des 3-wöchigen Verlaufs der Infektion. Alle Tiere überlebten die Infektion. Die TLR2-vermittelte Erkennung von *O. tsutsugamushi* hatte also weder Einfluss auf Rekrutierung von Monozyten oder Neutrophilen noch auf die systemische Verbreitung der Bakterien *in vivo* oder das Überleben nach subkutaner Infektion. Möglicherweise sind TLR2-abhängige Signalwege nicht unverzichtbar für die bakterielle Erkennung *in vivo* und können durch andere Signalwege der angeborenen Immunantwort kompensiert werden.

Nach intraperitonealer Infektion mit *O. tsutsugamushi*, einem Modell für schweren und potenziell tödlichen Verlauf der Infektion, führte die TLR2-Defizienz überraschenderweise nicht zu einer unkontrollierten Infektion, sondern milderte sogar die Schwere der Symptome und schützte die Mäuse vor tödlichen Verläufen. Diese unerwartete Beobachtung legt nahe, dass es bei schweren *O. tsutsugamushi*-Infektionen mit hohen Bakterienlasten zur Entwicklung einer TLR2-abhängigen Immunpathologie kommen kann.

Diese Arbeit zeigt zum ersten Mal, dass TLR2 als Rezeptor für *O. tsutsugamushi* fungiert. Anstelle aber, wie aus Infektionen mit anderen Erregern bekannt, eine entscheidende Rolle in der Entstehung antibakterieller Immunität zu spielen, verstärken TLR2-vermittelte Signale eher die Schwere des Infektionsverlaufs.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Hamburg, den 26 April 2013
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