The Dissertation Committee for Guang Xu Certifies that this is the approved version of the following dissertation:

Elucidation of Mechanisms of Host Immunity against *Orientia tsutsugamushi* in a Newly Developed Murine Model

Committee:

David H. Walker, MD, Mentor, Chair

Donald H. Bouyer, PhD, Co-mentor

Lynn Soong, MD, PhD

Gustavo Valbuena, MD, PhD

Sanjeev K. Sahni, PhD

Daniel H. Paris, PhD

Dean, Graduate School

Elucidation of Mechanisms of Host Immunity against *Orientia tsutsugamushi* in a Newly Developed Murine Model

by

Guang Xu, M.P.H, M.S., B.S.

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Dedication

I would like to dedicate this dissertation to my parents, my sister and my close friends for their continuous support and encouragement throughout the many years I have been in school.

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Elucidation of Mechanisms of Host Immunity against *Orientia tsutsugamushi* in a Newly Developed Murine Model

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Scrub typhus, caused by a Gram-negative obligately intracellular coccobacillus, *Orientia tsutsugamushi*, is a long neglected but important tropical disease. *Orientia tsutsugamushi* causes illness in one million people each year, with an additional 1 billion people at risk. Without appropriate diagnosis and treatment, the disease can cause severe multiorgan failure with a case fatality rate of 7-15%. The current gaps in the knowledge of immunity include the unknown mechanisms of host immunity to *O. tsutsugamushi*. Using an intravenous (i.v.) disseminated infection mouse model, we observed that more $CD8^+$ T cells than $CD4^+$ T cells were present in the spleens of infected mice at 12 days post infection (dpi). We also determined that T_{reg} cells and the proportion of T cells producing IL-10 were significantly increased from 6 dpi, which correlated with the onset of illness, body weight loss, and increased bacterial loads. We further studied $CD8^{-/-}$, MHC $I^{-/-}$ and wild type control (WT) C57BL/6J mice to determine the importance of $CD8^+$ T cells and MHC I molecules. After infection with an ordinarily sub-lethal dose of *O. tsutsugamushi*, all $CD8^{-/-}$ and MHC $I^{-/-}$ mice expired between 12 and 15 dpi, whereas all WT mice survived. Bacterial

loads in the lung, kidney, liver and spleen of $CD8^{-/-}$ and MHC $I^{-/-}$ mice were significantly greater than those in WT mice. Interferon-γ (IFN-γ) and granzyme B mRNA levels in the liver of $CD8^{-/-}$ and MHC $I^{-/-}$ mice were significantly greater than in WT mice. In addition, more severe histopathologic lesions were observed in $CD8^{-/-}$ mice. Finally, adoptive transfer confirmed a major role of activated $CD8^{+}$ T cells, as well as a less effective contribution by activated CD8 T cell-depleted splenocytes, in protection against *O. tsutsugamushi* infection. These studies demonstrated the critical importance of $CD8^{+}$ T cells in the host immune response during *O. tsutsugamushi* infection.

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ARDS	acute respiratory distress syndrome
B6 mice	C57BL/6J mice
BMDCs	bone marrow-derived dendritic cells
$CD8^{-/-}$ mice	B6.129S2-$Cd8a^{tm1Mak}$/J mice
CDC	Centers for Disease Control and Prevention
DIC	disseminated intravascular coagulation
dpi	days post-infection
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FFU	focus forming units
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HMGB1	high-mobility group box 1
IACUC	the Institutional Animal Care and Use Committee
ICT	immunochromatographic test
i.d.	intradermal inoculation
IFA	indirect immunofluorescence assay
IFN-γ	interferon-γ
IIP	indirect immunoperoxidase assay
IL	interleukin
i.p.	intraperitoneal inoculation
i.v.	intravenous inoculation
KO	knockout
LPS	lipopolysaccharide
LTβ	lymphotoxin β
MHC I	major histocompatibility complex class I
MIF	migration inhibition factor
MHC $I^{-/-}$ mice	B6.129P2-$B2m^{tm1Unc}$/J mice
NF-κB	nuclear factor-κB
NK	natural killer

PCR	polymerase chain reaction
PHA	passive hemagglutination assay
SPG	sucrose phosphate glutamate buffer
TGF	transforming growth factor
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
UTMB	The University of Texas Medical Branch
W-F	Weil-Felix test
WHO	World Health Organization
WT	wild type

MAJOR SECTION

Chapter 1 Introduction

Scrub typhus is a serious public health problem in the Asia-Pacific area. The endemic area includes, but not limited to, Korea, Japan, China, Taiwan, India, Thailand, Sri Lanka, and the Philippines (**Figure 1**). Recent studies have identified scrub typhus in South America and Africa (La Scola and Raoult 1997; Groen, Nur et al. 1999; Thiga, Mutai et al. 2015). The causative agent of scrub typhus is *Orientia tsutsugamushi*, an arthropod-borne gram-negative obligately intracellular bacillus (Seong, Choi et al. 2001; Jeong, Kim et al. 2007; Cho, Jun et al. 2010; Kim and Walker 2011; Paris, Shelite et al. 2013; Valbuena and Walker 2013). It threatens one billion people globally, and causes illness in one million people each year (Kelly, Fuerst et al. 2009). . After being bitten by an infected

Figure 1. Worldwide map of countries with reported scrub typhus cases. The "tsutsugamushi triangle" in the Asia-pacific area is the cluster of scrub typhus cases. [Modified from traveltip.org]

vector, a *Leptotrombidium* mite, patients start to exhibit signs of infection such as fever, rash, and an eschar while complaining of non-specific flu-like symptoms approximately 5 to 14 days after the infected chigger bites. In severe cases, multi-organ failure occurs. The case-fatality rate of scrub typhus can be up to 30% if no appropriate treatment is received in time (Varghese, Abraham et al. 2006). Therefore, it is critical to understand the pathogenesis of scrub typhus and corresponding mechanisms of host immune responses.

1.1 NATURAL HISTORY OF SCRUB TYPHUS

Scrub typhus, also known as tsutsugamushi disease, is a severe arthropod-borne gram-negative bacterial infection. The reason for naming the disease scrub typhus is that the vector, *Leptotrombidium* mite, resides in this type of vegetation, where forest is cleared and not maintained (Kuo, Huang et al. 2012). *Tsutsuga* means disease in Japanese, while *mushi* is the Japanese word for mite. Tsutsugamushi disease is endemic covering a large part of the Asia-Pacific area extending from Japan to Pakistan, from the Russian Far East to the north of Australia (Rapmund 1984; Valbuena and Walker 2013). As early as 25 to 225 A.D., diseases with symptoms very similar to scrub typhus appeared as “恙” in ancient Chinese documents. The Chinese word means “stricken with grief” or “an insect sticking to the human beings” (Ito 1967). The first report of the disease could be traced back to at least the 16th century, and the Institute for Infectious Diseases in Tokyo, Japan started investigation of this disease in the early 1890s

(Quintal 1996). The disease was first introduced to the Western world between 1878 and 1879 (Palm 1878; Baelz 1879). Outbreaks of scrub typhus among American servicemen in the Asia-Pacific area during World War II brought this disease to the attention of the Western World (Quintal 1996). Scrub typhus has been a neglected disease for decades, but robust responses from basic research, clinical practice and public health practice can result in prevention and better control of the disease.

There are still many unknowns regarding the mechanisms of pathogenicity and the cell biology of the interaction between this bacterium and its host cell, due to the extra research obstacles of studying an obligately intracellular bacterium (Giengkam, Blakes et al. 2015). The pathogen was first identified as a *Rickettsia* by N. Ogata, a Japanese researcher, in 1929 (Sasa 1967). The bacterium was named *Rickettsia tsutsugamushi* and *R. orientalis* by Nagayo et al. in 1930 (Nagayo, Tamiya et al. 1930; Allen and Spitz 1945). Even though *Orientia* belongs to the Rickettsiaceae family, major differences exist between the two genera, *Rickettsia* and *Orientia*. For instance, *O. tsutsugamushi* does not contain lipopolysaccharide or peptidoglycan, nor has a surrounding electron-lucent zone (Kelly, Fuerst et al. 2009; Rajapakse, Rodrigo et al. 2012). It has a thicker outer cell wall leaflet and distinctive outer membrane proteins. *Orientia* spreads more slowly and buds from the host cytoplasmic membrane (Tamura, Ohashi et al. 1995). In addition, it has strain-specific antigens, which make cross protection much less effective than in infections caused by *Rickettsia* (Lee, Cho et al. 2008; Ge and Rikihisa 2011). It was reclassified as a new genus. *Orientia*,

in 1995 (Ohashi, Tamura et al. 1990; Tamura, Ohashi et al. 1995). *Orientia tsutsugamushi* was the only species in the genus *Orientia* until *O. chuto*, a new species, was identified in an isolate from a patient infected in the United Arab Emirates (Izzard, Fuller et al. 2010).

A substantial number of cases and reinfections have been reported recently, which could result from the antigenic heterogeneity of, reemergence of, and short-lived immunity to *Orientia*. However, both basic research and epidemiologic studies of *O. tsutsugamushi* have been largely neglected after World War II (Lee, Cho et al. 2008; Cho, Cho et al. 2010; Ge and Rikihisa 2011). Multiple studies demonstrated that the long neglected endemic disease has existed in the endemic areas for a while (Berman and Kundin 1973; Ogawa, Hagiwara et al. 2002; Sinha, Gupta et al. 2014; Kwak, Kim et al. 2015; Yang, Liang et al. 2015).

1.2 Transmission of Scrub Typhus

1.2.1 Transmission Cycle

Orientia tsutsugamushi is transmitted to mammalian hosts including humans via the bite of the larval stage of *Leptotrombidium* mites, also known as chiggers (**Figure 2**) (Phasomkusolsil, Tanskul et al. 2012).

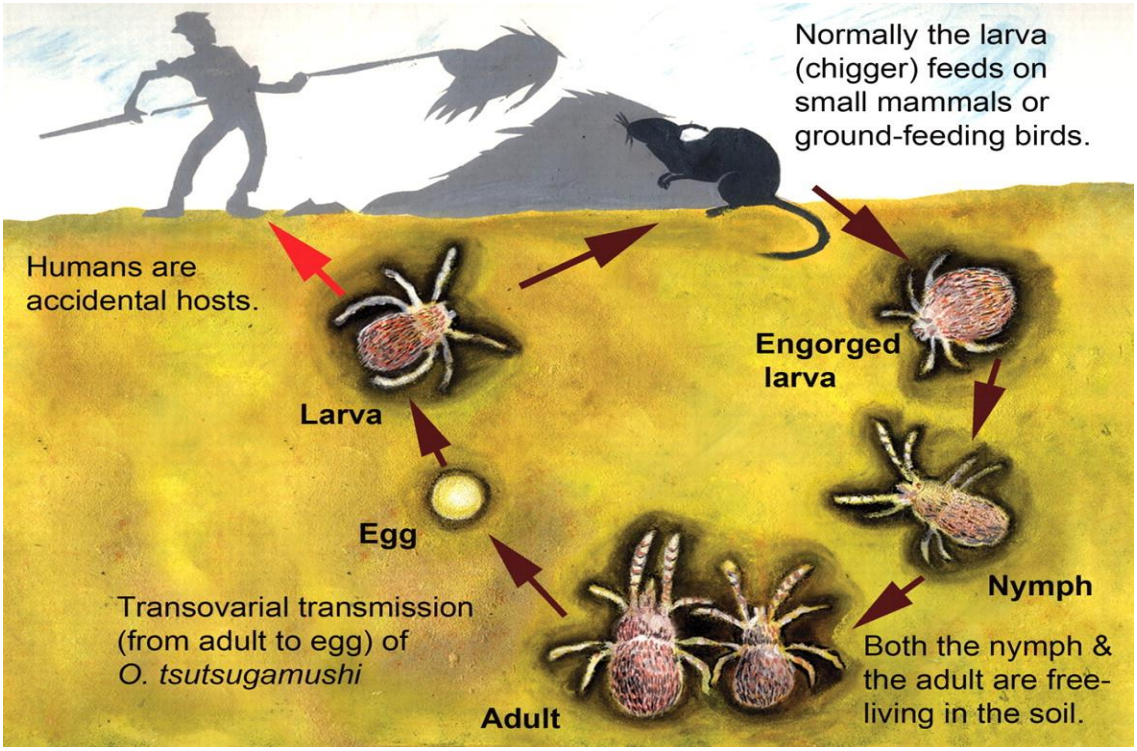


Figure 2. Life cycle of a leptotrombidium mite. Only the larval stage of mite bites and transmits *Orientia* to mammalian hosts (Source: Jeong YJ et al, Radio Graphics 2007)

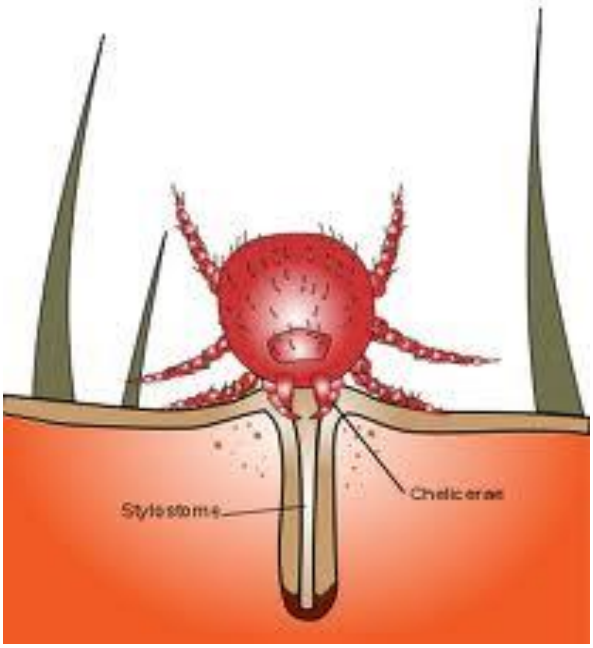


Figure 3. Engorgement of a mite on a mammal host. The engorgement usually lasts 2-4 days. [Modified from http://www.infectionlandscapes.org/2011/06/typhus.html]

Leptotrombidium mites act as the primary reservoirs for *O. tsutsugamushi*. The mites remain infected through their life cycles of larvae, nymph, adult and egg (Jeong, Kim et al. 2007). It has been known that larvae of mites only feed once on a mammal host. The geographic distribution of scrub typhus is determined by the distribution of its vector and reservoir - mites. Humans have been known as accidental hosts (Walker and Fishbein 1991; Kelly, Fuerst et al. 2009).

Chigger usually feeds on thin, tender or wrinkled skin (**Figure 3**). The feeding can last 2 to 4 days (Philip 1948). It was illustrated that instead of piercing the host skin, chiggers indeed take advantage of hair follicles or pores. The mites secrete the liquid to dissolve host tissue around the feeding site, and the mites eat the liquefied tissue. *Orientia tsutsugamushi* has been found in the salivary glands of those infected mites (Mahajan 2005) Transovarial and transstadial transmission are the main mechanisms for maintaining *Orientia* in the mite. It was reported that the bacteria can also be transmitted to mites during co-feeding on an infected human and/or from wild rodents (Walker, Chan et al. 1975; Lerdthusnee, Khlaimanee et al. 2002; Lerdthusnee, Khuntirat et al. 2003; Phasomkusolsil, Tanskul et al. 2009). There have been rare documented occurrences of horizontal transmission of *Orientia* among mites (Traub, Wisseman et al. 1975). During horizontal transmission, a chigger acquires *Orientia* from an infected host, and then passes along the infection through transstadial transmission. Not enough evidence exists to demonstrate that horizontal transmission is an important means of maintenance of *O. tsutsugamushi* in nature (Traub, Wisseman et al. 1975; Walker, Chan et al. 1975;

Frances, Watcharapichat et al. 2000; Lerdthusnee, Khlaimanee et al. 2002). There has been no person-to-person transmission of scrub typhus reported (Jeong, Kim et al. 2007)**.**

1.2.2 Vector of Scrub Typhus

A physician, Keisuke Tanaka in Japan, was the first person who linked the arthropod to scrub typhus in 1899 (Sasa 1967). Brumpt was the first person to name the vector scientifically, *Trombidium akamushi*, referring to Tanaka's drawing in 1910 (Nagayo, Miyagawa et al. 1921; Sasa 1967). Miyajima identified field rodents as the natural host of the mite in 1908, which contributed greatly to the investigation of the vector of scrub typhus (Sasa 1967). In 1916, Nagayo and others proposed a new genus name *Leptotrombidium* for *Trombidium akamushi* after collecting and rearing the engorged larvae of the mites from field voles (Nagayo, Miyagawa et al. 1916; Sasa 1967). Nagayo further identified four additional species of *Trombicula* besides the principal vector from 1919 to 1921: *T. pallida*, *T. palpalis*, *T. intermedia*, and *T. scutellaris* (Nagayo, Miyagawa et al. 1921; Sasa 1967). Two more species of trombiculid mites were placed under the genus *Neotrombicula*: Tanaka *et al.* described and named *Trombicula autumnalis japonica* in 1930, and Philip and Fuller discovered and named *Trombicula tamiyai* in 1950 (Sasa 1967). The outbreak among American servicemen during World War II significantly promoted the research on scrub typhus (Rapmund 1984; Quintal 1996). Since the initial identification, there have been more mites identified as vectors of *O. tsutsugamushi*. For instance, *Leptotrombidium deliense* was reported as the primary vector of *O.*

tsutsugamushi in India. Both *L. deliense* and *L. chiangraiensis* were identified as vectors of the pathogen in Thailand (Kelly, Fuerst et al. 2009). More studies on mites and their engorgement on the host are necessary to better control and prevent scrub typhus in those endemic areas.

1.3 Clinical Features of Scrub Typhus

Scrub typhus is a common acute febrile illness in the Asia-Pacific area. The disease has reemerged in the area in recent years after being neglected for decades. (Watt and Parola 2003; Gupta and Gautam 2004; Mahajan 2005). The public health impact of scrub typhus is critical thanks to its high prevalence in a densely populated area and high case fatality rate without timely treatment. Unfortunately, there are limited options of diagnostic and laboratory tools with imperfect sensitivity and/or specificity (Mahajan 2005). Understanding the clinical features and laboratory tools used for diagnosing scrub typhus will help us understand the fundamental demand of better treatment and prevention of the disease.

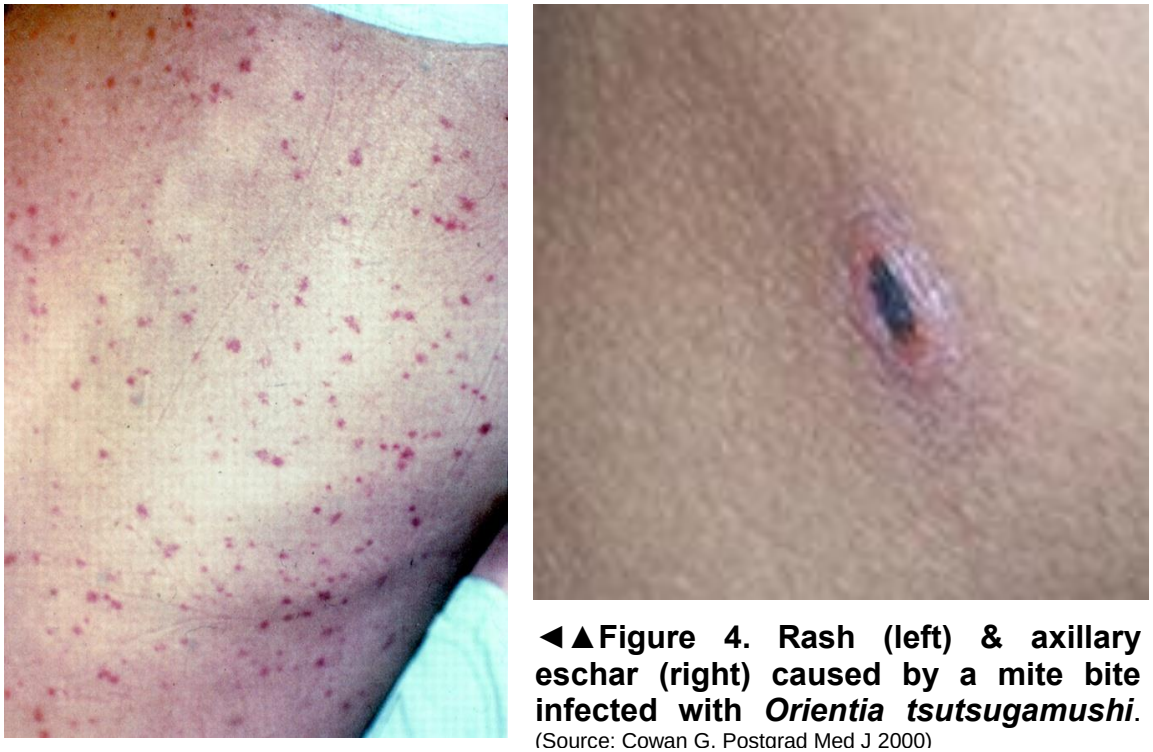
1.3.1 Clinical Manifestations

The symptoms of scrub typhus are usually mild and self-contained, and patients can recover spontaneously within a few days. However, multi-organ failure and deaths could happen among more severe cases (Jeong, Kim et al. 2007). It was reported that case fatality rate of scrub typhus ranges up to 30% in

the pre-antibiotic era (Mahajan 2005). However, the case fatality rate among scrub typhus patients still reached up to 15% if no timely appropriate treatment was received (Varghese, Abraham et al. 2006).

The incubation period for scrub typhus is typically between 10 and 12 days after a bite from an infected mite. There was one report claiming that the incubation period ranges from 6 to 21 days post chigger mite (Jeong, Kim et al. 2007). The common symptoms at the onset of scrub typhus include fever, rash, headache, myalgia, cough, diffuse lymphadenopathy, nausea, vomiting, and abdominal pain (Warrell, Cox et al. 2003; Mahajan 2005; Jeong, Kim et al. 2007). Fever and headache are the most common clinical features among scrub typhus patients, with 95% to 100% of confirmed cases reporting fever as observed in multiple studies (Tsay and Chang 1998; Jamil, Lyngrah et al. 2014). In addition, *Orientia tsutsugamushi* has been found capable of persisting chronically in human patients even when treated with antibiotics (Smadel, Ley et al. 1952; Chung, Lee et al. 2012), but the mechanisms underlying the early and late interactions between the pathogen and the host are still unclear.

One of the most classic clinical features for rickettsial diseases is an eschar at the site of mite feeding. The formation of an eschar first begins as a papule formed at the feeding site (**Figure 4**). The papule eschar then ulcerates and forms a black crust similar to a skin burn from a cigarette (Gupta and Gautam 2004; Mahajan 2005). An eschar is only present in ~60% of scrub typhus patients, which makes it -unreliable for definitive diagnosis of scrub typhus (Kawamura 1995; Tsay and Chang 1998). It was reported that eschar is



◄▲Figure 4. Rash (left) & axillary eschar (right) caused by a mite bite infected with *Orientia tsutsugamushi*. (Source: Cowan G. Postgrad Med J 2000)

more frequently found on Caucasian and East Asian patients, and it's less commonly detected on dark skinned South Asian patients. Approximately eighty percent of eschars develop on the front of the body. In male patients, eschars are primarily within 30 cm below the umbilicus. Lower extremities and anterior chest are the other common locations. Female patients display a different pattern in which the anterior chest is the most prevalent area (Kim, Won et al. 2007).

The other typical clinical features include maculopapular rash and regional lymphadenopathy. In an epidemiological study in Taiwan, rash occurred among about 20% of confirmed scrub typhus patients (Tsay and Chang 1998). The rash normally starts from 5 to 8 days after the onset of fever. Macular or maculopapular rash may appear on the trunk first, i.e., the chest or abdomen, before it spreads to the extremities. It's extremely uncommon to have rash on the

face, palms or soles (Seong, Choi et al. 2001; Mahajan 2005; Jeong, Kim et al. 2007).

Another classic clinical sign, regional lymphadenopathy, emerges at the end of the first week after the onset of scrub typus. The lymph nodes appear swollen and tender in the drainage region of the primary eschar. Generalized lymphadenopathy could follow the regional lymphadenopathy in some cases (Seong, Choi et al. 2001; Jeong, Kim et al. 2007; Raoult 2014).

About twenty percent of the patients experience splenomegaly and hepatomegaly (Jeong, Kim et al. 2007; Jamil, Lyngrah et al. 2014). In addition, *O. tsutsugamushi* infection can cause various other complications, such as jaundice, renal failure, pneumonitis, acute respiratory distress syndrome (ARDS), septic shock, myocarditis, pericarditis and meningoencephalitis (Mahajan 2005; Jeong, Kim et al. 2007; Jamil, Lyngrah et al. 2014). One of the main target organs for *Orientia* is the lung. *Orientia tsutsugamushi* can thus lead to pulmonary complications of variable severity in the host. In severe cases, *Orientia* infection can cause the formation of interstitial pneumonia (Song, Kim et al. 2004; Jeong, Kim et al. 2007). Patients are observed to have elevated serum levels of hepatic enzymes, including aspartate and alanine aminotransferases, and the biliary canalicular marker, alkaline phosphatase (Jeong, Kim et al. 2007). Renal injury happens among roughly 9% of scrub patients (Attur, Kuppasamy et al. 2013; Jamil, Lyngrah et al. 2014; Sun, Kim et al. 2014). In certain cases, patients develop hypoalbuminemia and albuminuria (Mahajan 2005).

Some scrub patients develop neurological injury with various degrees of severity. Headache is the most common cerebral disorder of scrub typhus. Meningitis and/or encephalitis caused by *O. tsutsugamushi* occur in severe illness, which may result in agitation, delirium or even seizures among scrub typhus patients. Focal neurological signs are scarce but have been known to occur. Changes in cerebrospinal fluid similar to those found in viral or tuberculous meningitis have been observed in laboratory tests (Silpapojakul, Ukkachoke et al. 1991; Ben, Feng et al. 1999; Kim, Lee et al. 2000; Seong, Choi et al. 2001).

Scrub typhus patients can develop other non-classic signs. There have been reports of marked hyperemia and even hemorrhage on the conjunctiva during the acute phase of the disease. Hemorrhages and coagulation disorders, mostly gastrointestinal complications, are found among scrub typhus patients. Severely ill patients may also experience mucosal hemorrhage, multiple erosions and ulcers (Kim, Chung et al. 2000; Seong, Choi et al. 2001; Mahajan 2005).

Septic shock can cause multiple organ failure, respiratory failure, and disseminated intravascular coagulation (DIC) among severe scrub typhus cases. Without immediate appropriate treatment, those severe complications can cause fatalities. Absence of eschar, intensive care unit admission and severe complications are associated with a fatal outcome in scrub typhus patients (Lee, Hwang et al. 2009). Early diagnosis contributes effective lifesaving treatment of the disease (Tsay and Chang 1998; Cracco, Delafosse et al. 2000; Thap, Supanaranond et al. 2002; Lee, Hwang et al. 2009).

1.3.2 Diagnosis

Orientia causes a flu-like febrile illness, which makes the quick and correct diagnosis of scrub typhus quite difficult. Generally, its diagnosis depends on the clinical presentation and history of the patient. Presence of an eschar and history of travel to or residence in an endemic area facilitate the diagnosis. An eschar is not ubiquitous among all confirmed patients, and cutaneous lesions from other diseases, e.g., spider bites, leishmaniasis and anthrax, may mislead the clinician. Scrub typhus was reported to be misdiagnosed as malaria, dengue, leptospirosis, meningococcal disease, typhoid, infectious mononucleosis and HIV (Mahajan 2005; Li, Dou et al. 2013; Koraluru, Bairy et al. 2015).

Serological tests and molecular assays are the major laboratory methods of diagnosing rickettsial diseases including scrub typhus. Cross-reactivity of *Orientia* with other rickettsial diseases is rare (Kelly, Wong et al. 1988; Tay, Kamalanathan et al. 2003; Wilkinson, Rowland et al. 2003). The gold standard test for diagnosis of scrub typhus is the indirect immunofluorescence assay (IFA) (Koraluru, Bairy et al. 2015; Lim, Paris et al. 2015). However, the cost and complexity of IFA procedures, which requires extensive training and a biocontainment facility for preparation of antigen, impose a great barrier in developing countries. Even though the assay has been available for quite a long period of time, the application of IFA in clinics in the endemic area is still restricted. In addition, the IFA test is unable to provide accurate diagnostic results at the early stages of infection because the antibodies, parts of adaptive

immunity, are not generated at the early acute infection phase. It is recommended to apply the diagnostic criterion for serological tests that the antibody titer has a 4-fold or greater rise to receive a more reliable and accurate result (Blacksell, Bryant et al. 2007). There are other limitations of IFA, including but not limited to the controversial cut-off antibody titer, particularly in highly endemic regions, subjective determination of results, and imperfect specificity of the test (Lee, Moon et al. 2014; Lim, Paris et al. 2015).

In addition, there are other serological tests including the indirect immunoperoxidase assay (IIP), the Weil-Felix test (W-F), enzyme-linked immunosorbent assay (ELISA), and various commercially available immunochromatographic tests (ICT) (Koh, Maude et al. 2010; Lim, Paris et al. 2015). The W-F agglutination test is based on cross-reaction of antibodies to Proteus mirabilis OXK strain with *O. tsutsugamushi*. The test has been commercially available for a while, but it lacks both specificity and sensitivity, especially the latter, for routine diagnosis (La Scola and Raoult 1997; Shivalli 2016). It was demonstrated that the sensitivity of W-F test is only 50% during the second week of illness (Mahajan 2005; Koraluru, Bairy et al. 2015).

IIP, a modification of the IFA, provides both comparable specificity and sensitivity without the requirement of an ultraviolet microscope for diagnosis of scrub typhus (Yamamoto and Minamishima 1982; Kelly, Wong et al. 1988). Both the IFA and IIP have been used as the reference standard for diagnosing scrub typhus. No significant difference was detected in the accuracy between the two tests, except for one study which claimed that IIP was more sensitive with acute

sera (79.6% for IIP vs. 68.5% for IFA at titer ≥1:400) (Pradutkanchana, Silpapojakul et al. 1997; Coleman, Sangkasuwan et al. 2002).

Commercially available dipstick tests are variants of ELISA. Those assays use either pooled cell lysates of different strains of *O. tsutsugamushi*, recombinant p56 protein, or other outer membrane proteins as the antigen. ELISA and its variants are able to provide sensitive and specific test results. There are some reviews claiming that ELISA and its variants may eventually replace the IFA and IIP assays. Though the sensitivity and specificity of dipstick assays are inferior to ELISA, the commercially available assays are easier to use. Therefore, it could still be employed in underserved areas for rapid diagnoses (Pradutkanchana, Silpapojakul et al. 1997; Coleman, Sangkasuwan et al. 2002; Mahajan 2005)

There is another commercially available kit for early rapid diagnosis, ICT. Similar to ELISA and its variants, ICT also uses the recombinant *Orientia* outer membrane proteins to detect IgG, IgM and IgA antibodies to *O. tsutsugamushi*. ICT has moderate to high sensitivity (~70%) among confirmed scrub typhus patients. The sensitivity further increases with the fever duration. Several studies nevertheless revealed that ICT, similar to the passive hemagglutination assay (PHA), returns a substantial number of false negative results (Lee, Moon et al. 2014). PHA was replaced by ICT due to its lower sensitivity for diagnosis of *O. tsutsugamushi* infection. However, Lim *et al.* demonstrated that the low specificity of IFA IgM causes inaccurate comparisons between IFA and other diagnostic assays. Compared with IFA, ICT IgM instead has comparable

sensitivity and significantly better specificity (Lee, Moon et al. 2014; Lim, Paris et al. 2015).

The PCR assay is the molecular method utilized to diagnose scrub typhus. The functionality of this assay is detecting the bacteria from specimens collected from patients. The target genes are usually the outer membrane proteins of 56 kDa, 47 kDa, and groEL genes (Lim, Paris et al. 2015). The nested PCR was claimed at least by some researchers to be more sensitive than the serological tests (Saisongkorh, Chenchittikul et al. 2004; Paris, Blacksell et al. 2008). The advantage of this molecular biology method is that it is capable of detecting *Orientia* DNA in blood even during the persistent phase of the infection, when no obvious clinical symptoms are observed. The sensitivity of PCRs nevertheless decreases with treatment (Smadel, Ley et al. 1952; Mahajan 2005; Chung, Lee et al. 2012; Koraluru, Bairy et al. 2015)

1.3.3 Prevention and Treatment

No working vaccine has been available for scrub typhus. The reasons attributing to the delay of developing an effective vaccine are: a) enormous antigenic variation observed in different *O. tsutsugamushi* strains; and b) weak and short duration cross protection among different strains. Vaccine efforts are further hampered by the different antigenically divergent strains of *O. tsutsugamushi* in different endemic countries/regions, and/or even different strains in the same location (Sharma 2010; Kuo, Huang et al. 2012; CDC 2015).

Scrub typhus can be effectively treated with the appropriate antibiotics. Early treatment leads to better outcomes, i.e., shorter disease course and lower fatality (Watt and Parola 2003). Oral administering of antibiotics is employed for mild cases, and the injectable route is used for severely ill cases. Similar to the treatment for other rickettsial diseases, doxycycline is one of the most widely used and the most effective antibiotics for treating scrub typhus. For uncomplicated cases, doxycycline is administered orally for a week as treatment. Patients' fever is usually relieved rapidly after antibiotics use. This is even used as a diagnostic method (Liu and Panpanich 2002). A few randomized clinical trials demonstrate that there is no significantly different efficacy among tetracycline, doxycycline, telithromycin, and azithromycin (Liu and Panpanich 2002). Rifampicin was shown to be more effective than tetracycline in patients responding poorly to doxycycline (Mahajan 2005). World Health Organization (WHO) recommends that children or pregnant women use azithromycin. There have been studies claiming that azithromycin and macrolides are as effective as doxycycline or chloramphenicol but without potential side effects. Chloramphenicol has been blamed for causing aplastic anemia in treatment of pregnant women and children (Lee, Lee et al. 2003; Rajapakse, Rodrigo et al. 2011; Chanta and Phloenchaiwanit 2015). There have been reports of antibiotic resistance (Watt, Chouriyagune et al. 1996; Kuo, Huang et al. 2012). Unfortunately, there is still much unknown regarding antibiotic resistance. It is fundamental and urgent to elucidate the mechanisms of poor response in certain

patients, as well as the antibiotic resistance of the pathogen (Watt and Parola 2003; Mahajan 2005; Chogle 2010).

1.4 Epidemiology of Scrub Typhus

Scrub typhus is a life threatening disease that causes illness in one million people each year. It is a serious but long neglected public health problem mainly in the Asia-Pacific area (Watt and Parola 2003). The traditional endemic area of scrub typhus is recognized as "tsutsugamushi triangle" (**Figure 5**). Tsutsugamushi triangle is a region covering over 8 million km^2, from Siberia in the north, Australia in the south, and Pakistan in the west, (Seong, Choi et al. 2001; Izzard, Fuller et al. 2010). One billion people are at risk of *Orientia* infection since the endemic area is densely populated (Kelly, Fuerst et al. 2009). Recent studies have identified scrub typhus in South America and Africa (La Scola and

Figure 5. Distribution of *Orientia* infection. Majority of scrub typhus cases happen in "tsutsugamushi triangle" in the Asia-Pacific area [Modified from http://worldmap.harvard.edu]

Raoult 1997; Groen, Nur et al. 1999; Thiga, Mutai et al. 2015). These new cases outside traditionally endemic areas could result from the long neglect of this disease. Some researchers advocated the progress of globalization and associated travel potentially contributes to the increase of scrub typhus cases outside the “tsutsugamushi triangle” (Kuo, Huang et al. 2012). Both the antigenic and genetic diversity of different *O. tsutsugamushi* strains, and their unclear correlation with virulence for human hosts confound the epidemiological study of *Orientia* infection (Walker and Fishbein 1991).

The disease is more prevalent among outdoor workers, especially field workers in rural areas, who have been confirmed to have a higher risk of acquiring the disease (Kweon, Choi et al. 2009). Rice fields were demonstrated to be an under-appreciated location where the infected mites bite and transmit *O. tsutsugamushi* to the host in the endemic area (Watt and Parola 2003). Tropical weather provides favorable conditions for transmission of the disease. Mites prefer high temperature and high humidity. In temperate climates, the transmission of *O. tsutsugamushi* is more seasonal due to the activity change of chiggers (Matsui, Kramer et al. 2002; Ogawa, Hagiwara et al. 2002; Watt and Parola 2003).

Outbreaks of *O. tsutsugamushi* infection are seasonal and associated with certain types of terrain. *Leptotrombidium* mite, the vector of *O. tsutsugamushi*, preferentially resides in the type of vegetation where the trees have been cut and the land have been left unmanaged (Sharma, Kakkar et al. 2010). This was also the origin of the disease name, scrub typhus (Tamura, Ohashi et al. 1995).

Outbreaks of scrub typhus nevertheless also occur in other terrains, which include semi-desert, sandy beach, dense but disturbed forest glacier slope (Traub and Wisseman 1968; Traub and Wisseman 1974). The Taiwan CDC demonstrated that mites wait at the tips of weeds to attach and engorge on humans or other mammalian hosts. *Orientia tsutsugamushi* simultaneously infect susceptible persons shortly after exposure (WHO 2016). Outbreaks of scrub typhus are usually clustered into small foci. Patients rarely recall being bitten or attacked by a chigger (Watt and Parola 2003; Kuo, Huang et al. 2012).

The reemergence of scrub typhus in the Asia-Pacific area and the appearance of antibiotic resistance as well as discovery of new cases outside endemic areas remind us of the urgency and importance of developing and adopting effective control and preventive measures, especially an effective vaccine, for scrub typhus (Olson, Bourgeois et al. 1980; Brezina 1985; Kazar and Brezina 1991; Sharma 2010; Kuo, Huang et al. 2012).

1.5 HOST-PATHOGEN INTERACTIONS

1.5.1 Pathogenesis

As a long neglected and understudied pathogen, there are still many unknowns and contradictions regarding the pathogenesis of *O. tsutsugamushi* infection and the host immune responses to the infection. During *Orientia* infection of humans, the bacteria infect endothelial cells, macrophages and dendritic cells *in vivo*, and their infection of other cell types is minimal (Rikihisa and Ito 1979; Rikihisa and Ito 1982; Moron, Popov et al. 2001; Walsh, Myint et al.

2001). Paris et al. discovered that dendritic cells are the primary targets in the inoculation site eschar during feeding by an *O. tsutsugamushi*-infected mite (Paris, Phetsouvanh et al. 2012), but during dissemination endothelial cells are the main target of *Orientia* infection (Moron, Popov et al. 2001; Hsu and Chen 2008; Tseng, Yang et al. 2008). The hypothesis for route of spread is that O. tsutsugamushi spreads from the mite feeding site to the regional lymph node via lymphatic vessels, and then disseminates to different organs through the bloodstream (Moron, Popov et al. 2001; Walker, Ismail et al. 2007). Therefore, it is not ideal or accurate to draw conclusions about host immune responses during *O. tsutsugamushi* infection from *in vitro* studies using non-target cells, such as epithelial cells, instead of the main target cells in systemic infection.

1.5.2 Host Immunity

As an obligately intracellular pathogen, it is reasonable to propose that cellular immunity including $CD8^+$ T cells plays critical roles in host defense and clearance of *O. tsutsugamushi* (Palmer, Hetrick et al. 1984; Palmer, Hetrick et al. 1984; Kodama, Kawamura et al. 1987; Soong, Wang et al. 2014). The *in vitro* study by Rollwagen et al. showed that immune splenocytes lysed *Orientia*-infected L929 fibroblasts (Rollwagen, Dasch et al. 1986). In a clinical study, serum concentration of granzymes, interferon-γ (IFN-γ) inducible protein 10, and monokines induced by IFN-γ were elevated in human scrub typhus patients (de Fost, Chierakul et al. 2005). In addition, previous studies of *Rickettsia* infection in our laboratory demonstrated that T lymphocytes not only secrete IFN-γ, but also

provide important immune cytotoxic activity effectors to eliminate rickettsiae (Walker, Olano et al. 2001). Our laboratory confirmed the effectiveness of both $CD4^+$ and $CD8^+$ T lymphocytes, especially $CD8^+$ T cells, in *Rickettsia* infection and clearance through adoptive transfer of immune $CD4^+$ or $CD8^+$ T lymphocytes (Feng, Popov et al. 1997).

During intracellular pathogenic infection, major histocompatibility complex class I (MHC I) is able to regulate both innate and adaptive immunity through presentation of processed foreign peptides (Mu, Tai et al. 2014). Expression of MHC I on nonhematopoietic thymic epithelial cells is essential for $CD8^+$ T cell maturation and survival as well as their effective function (Finelli, Kerksiek et al. 1999; Mu, Tai et al. 2014). The classical MHC I pathway of antigen presentation starts with the degradation of cytosolic and nuclear antigens by the proteasome. The peptides are transported into the endoplasmic reticulum (ER) lumen by transporter TAP, and are then loaded onto MHC I. The Golgi then transports the complexes to the cell surface where $CD8^+$ T cells can recognize them (Peaper and Cresswell 2008; Fiegl, Kagebein et al. 2013). In addition, MHC I is able to bind the receptors on NK cells to induce inhibitory signals (Barao, Alvarez et al. 2011; Tovbis Shifrin, Kissiov et al. 2016). Both innate and adaptive immunity are involved in control of *Orientia* infection, but more systematic studies with appropriate animal models and clinical studies are still lacking (Paris, Shelite et al. 2013; Valbuena and Walker 2013; Soong, Wang et al. 2014).

1.6 Animal Models

The lack of an accurate disease model of scrub typhus fundamentally impeded the necessary studies. *O. tsutsugamushi* causes disseminated endothelial infection and multifocal vasculitis in lung and brain of human patients. Patients suffer interstitial pneumonitis, hepatic damage, encephalitis, and disseminated lymphohistiocytic vasculitis during scrub typhus (Allen and Spitz 1945; Kundin, Liu et al. 1964; Berman and Kundin 1973; Moron, Popov et al. 2001). However, the previously available and still often used model, first employed more than 50 years ago, involves intraperitoneal (i.p.) injection of *O. tsutsugamushi* into mice, which substantially limits the pathogens and disease to the peritoneal cavity (Seong, Choi et al. 2001). Continuous proliferation of *O. tsutsugamushi* in peritoneal mesothelial cells and macrophages, enlargement of the spleen, hepatic lesions, and terminal peritonitis occur in these i.p. infected mice (Catanzaro, Shirai et al. 1976; Ewing, Takeuchi et al. 1978; Oaks, Ng et al. 1985). This widely used i.p. route produces an infection of the peritoneal cavity that results in severe mesothelial cell infection and fatal *Orientia* peritonitis not observed in scrub typhus infection in human patients (Kundin, Liu et al. 1964; Berman and Kundin 1973; Jerrells and Osterman 1981; Jerrells and Osterman 1982; Groves and Kelly 1989). Recently, our laboratory has developed new mouse models for *Orientia* infection, which better mimic the endothelial and macrophage target cells, organ distribution, disease course, pathology and immunology of human patients (Shelite, Saito et al. 2014; Soong, Mendell et al. 2016). We observed human scrub typhus-like disease development, such as disseminated endothelial infection and injury, lymphohistiocytic vasculitis,

interstitial pneumonitis, hepatic damage, and meningoencephalitis with our intravenous (i.v.) and intradermal (i.d.) inoculation models (Shelite, Saito et al. 2014; Soong, Mendell et al. 2016).

1.7 Objectives and Specific Aims

Because of the overlapping clinical manifestations with other common febrile illnesses as well as limitations of current diagnostic methods, the clinical diagnosis of scrub typhus is difficult. The absence of an early diagnosis impedes timely appropriate therapy. There is still a significant gap in understanding of how the pathogen invades, disseminates, and interacts within the host (Paris, Phetsouvanh et al. 2012). The recent reemergence of scrub typhus and appearance of antimicrobial resistance demonstrate the need for the development of effective preventive measures including a vaccine, which requires understanding the mechanisms of the host immune response to the invasion, infection and persistence of *O. tsutsugamushi*. *Orientia tsutsugamushi* can persist chronically in human patients even when treated with antibiotics (Smadel, Ley et al. 1952; Chung, Lee et al. 2012), but the mechanisms underlying the early and late interactions between the pathogen and the host are still unclear.

The long-term goal of this study is to develop new effective preventive measures including vaccines to fight against gram-negative bacteria and antibiotic-resistant pathogens. **The objective** of this study is to determine the mechanisms the mammalian host immune system employs to combat against *O.*

tsutsugamushi infection. We **hypothesize** that $CD8^+$ T cells together with other immune response effectors protect the host from *O. tsutsugamushi* infection, but $CD8^+$ T cells are the most critical to *O. tsutsugamushi* clearance. *Orientia tsutsugamushi* suppress and/or exhaust $CD8^+$ T cells during lethal or persistent infection in the host. Immunoregulation in the host, including but not limited to T_{reg} cells, interleukin 10 (IL-10) and transforming growth factor β (TGF-β), may allow the pathogen to be partially controlled and remain persistent in its mammalian host (Rushbrook, Ward et al. 2005; Easterbrook, Zink et al. 2007; Wingate, McAulay et al. 2009; Johanns, Ertelt et al. 2010; Boettler, Cheng et al. 2012; Ng and Oldstone 2012; Wilson, Kidani et al. 2012).. With our newly developed animal models, we discovered that *O. tsutsugamushi* Karp strain persists in tissues, especially kidneys of mice at 70-84 days post infection. Our laboratory has also discovered that $CD8^+$ lymphocytes are critical for the clearance of *Rickettsia conorii* and *R. australis* (Feng, Popov et al. 1997; Walker, Olano et al. 2001). Our hypothesis is based on the data we and others have previously reported (Feng, Popov et al. 1997; Walker, Olano et al. 2001; Tesh, Siirin et al. 2005; Boettler, Cheng et al. 2012). The **rationale** for this study is that once the research is completed, we will be able to better understand the host immunity during scrub typhus in a mammalian host. It will allow us to develop treatments and prevention measures targeting $CD8^+$ T cells and immune effectors to clear *Orientia* and avoid persistence in the host. This study also will provide foundations to explore and find new treatments for other intracellular pathogens.

We will test our central hypothesis and accomplish our objective by carrying out the following **specific aims**:

Specific Aim 1: Determine the changes in host immunity during *O. tsutsugamushi* infection.

Even though *O. tsutsugamushi* was discovered more than a century ago, the neglect of scrub typhus in recent decades has led to a significant gap of understanding the host immune responses during *O. tsutsugamushi* infection. There have been not enough studies of the host immune responses and their corresponding mechanisms during scrub typhus. In this specific aim, we will determine the changes in the host immune responses during the course of *O. tsutsugamushi* infection in our newly developed murine model. We **postulated** that both cellular and regulatory immunity play important roles in the host immune responses against *O. tsutsugamushi* infection. The regulatory immunity not only controls the inflammation but also enables the pathogen to survive and persist. We examined the changes of proportions of $CD4^+$ T cells, $CD8^+$ T cells, and T_{reg} cells, as well as levels of IL-10 in the mice after infection with *O. tsutsugamushi*. The study determined the major players in the host immunity during *O. tsutsugamushi* infection.

Specific Aim 2: Determine the indispensable role of $CD8^+$ T cells in host immunity against *O. tsutsugamushi*.

$CD8^+$ T cells play important roles in *R. australis* clearance and *R. conorii* persistence in the host when they are deficient (Feng, Popov et al. 1997; Walker,

Olano et al. 2001). We **postulate** that $CD8^+$ T cells are indispensably critical to protection of the host from *O. tsutsugamushi* infection. In those lethal or persistent cases, *O. tsutsugamushi* may suppress and/or exhaust $CD8^+$ T cells and other immune effectors. We compared the survival rate, bacterial loads, and histopathology among wild type controls (WT), CD8 lymphocyte knockout mice, and mice after adoptive transfer of immune $CD8^+$ lymphocytes. The study demonstrated the important role of $CD8^+$ lymphocytes in *Orientia* infection.

Specific Aim 3: Determine the role of MHC I in *O. tsutsugamushi* infection.

MHC I is a key player in cellular immunity as it facilitates the presentation of antigen peptide from antigen presenting cells to $CD8^+$ T cells (Peaper and Cresswell 2008; Fiegl, Kagebein et al. 2013; Mu, Tai et al. 2014). MHC I is also capable of binding and inhibiting NK cells in innate immunity (Barao, Alvarez et al. 2011; Mu, Tai et al. 2014; Tovbis Shifrin, Kissiov et al. 2016). We **proposed** that MHC class I is essential to facilitate $CD8^+$ T cells to protect the host from *O. tsutsugamushi*. The deficiency of MHC I allows the bacteria to remain and propagate in the host without being cleared. We used MHC I knockout mice to determine whether MHC I plays an important role in the host immune responses against *O. tsutsugamushi*. Molecular biology and histopathological analysis were used to examine the effect of MHC I on *O. tsutsugamushi* infection. This study further demonstrated the mechanisms of host immunity in *O. tsutsugamushi* infection.

Chapter 2 Materials and Methods

2.1 ANIMALS

C57BL/6J (B6), B6.129S2-Cd8a^{tm1Mak}/J (CD8$^{-/-}$), and B6.129P2-B2m^{tm1Unc}/J (MHC I$^{-/-}$) mice were purchased from the Jackson Laboratory. Age- and gender-matched, 7-12 week old mice were used in all studies. Experimental mice were housed in the animal biosafety level 3 facility at the University of Texas Medical Branch (UTMB). All procedures and experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of UTMB (Protocols: 9007082 and 1302003) in accordance with Guidelines for Biosafety in Microbiological and Biomedical Laboratories. UTMB operates to comply with the USDA Animal Welfare Act (Public Law 89-544), the Health Research Extension Act of 1985 (Public Law 99-158), the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the NAS Guide for the Care and Use of Laboratory Animals (ISBN-13). UTMB is a registered research facility under the Animal Welfare Act, and has a current assurance on file with the Office of Laboratory Animal Welfare, in compliance with NIH Policy. Mice were inoculated i.v. with ~1.25×10^6 focus forming units (FFU) of *Orientia* as a sub-lethal dose, or ~1.64×10^7 FFU as a lethal dose. All mice were monitored three times each day after infection and euthanized at selected time points.

2.2 BACTERIAL CULTURE

As described previously (Shelite, Saito et al. 2014), we cultivated *O. tsutsugamushi* Karp strain in Vero cells or C57BL/6J mice (Jackson Laboratory,

ME, USA). Briefly, the bacteria were added to T150 cell culture flasks containing confluent monolayers of Vero cells. The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, MA, USA) with 3% fetal bovine serum (FBS, HyClone, PA, USA) and 1% HEPES buffer (Gibco, MA, USA). Smears of infected cells stained with Diff-Quik (Fisher Scientific, MA, USA) were prepared to assess the level of infection when rounded and floating cells were observed between 14 -21 days post-infection. The infected cells were harvested by high speed centrifugation at 22,000× *g* for 45 minutes at 4°C when 80-90% of Vero cells were infected. Sucrose phosphate glutamate buffer (SPG, 218 mM sucrose, 3.8 mM KH_2PO_4, 7.1 mM K_2HPO_4, 4.9 mM monosodium L-glutamic acid) was used to resuspend the pellet. Glass beads were used to release the intracellular bacteria from infected cells, and the supernatant was collected after centrifugation at 700× *g* for 5 minutes. Another high speed centrifugation concentrated the stock, which was stored at -80°C for subsequent use.

The animal passages were performed as previously described (Shelite, Saito et al. 2014). Naive C57BL/6J mice were inoculated i.v. with 10 LD_{50} of *O..tsutsugamushi* Karp strain. The mice were euthanized when they exhibited signs of illness. The livers were aseptically collected and homogenized with cold SPG buffer. Supernatant was collected after two 5-minute centrifugations at 700× *g*. Similar to cell culture-passaged bacteria, a 45-minute high speed centrifugation at 22,000× *g* and 4°C was employed to concentrate the stock. All stocks were stored at −80°C in the biosafety level-3 facility.

2.3 Bacterial Load Determination

Bacterial loads were determined by quantitative real-time PCR (Jiang, Chan et al. 2004). Qiagen DNeasy Kits (Qiagen, Hilden, Germany) were used to extract DNA from the lung, liver, kidney, spleen, and other tissue samples. We used the *O. tsutsugamushi* 47-kDa gene to identify the bacteria and determine the bacterial loads (Jiang, Chan et al. 2004). The primers were OtsuF630 (5'-AACTGATTTTATTCAAACTAATGCTGCT-3') and OtsuR747 (5'-TATGCCTGAGTAAGATACGTGAATGGAATT-3') (Integrated DNA Technologies, IA, USA). The conditions of qPCR were 3 minutes at 94°C, and 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds. We used total nanogram (ng) of DNA per μL and/or a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, (F, 5'-CAACTACATGGTCTACATGTTC-3'; R, 5'-CTCGCTCCTGGAAGATG-3', IDT) of the same sample to normalize the concentration of the bacterial loads (Jiang, Chan et al. 2004; Shelite, Saito et al. 2014).

2.4 Flow Cytometry

Flow cytometric analysis was carried out with a BD LSRII Fortessa cell analyzer (BD Bioscience, CA, USA) to enumerate and characterize the lymphocyte subpopulations and particular cytokine-containing cells during *Orientia* infection. The data were processed and analyzed with FlowJo 10 (FlowJo LLC, OR, USA). The spleens of *Orientia*-infected mice or uninfected control mice were collected and homogenized with phosphate buffered saline.

Samples were exposed to cell stimulation cocktail (eBioscience, CA, USA) and GolgiPlug protein transport Inhibitor (BD Bioscience, CA, USA) at 37°C for 4 hours. The samples were further stained using Live/Dead cell stain (Near IR, Life Technologies), Fc CD16/32 block (BD Bioscience, CA, USA), eFluor 450-labeled anti-CD3, PE-Cy7-labeled anti-CD25 (eBioscience, CA, USA), Alexa Fluor (AF) 700-labeled anti-CD4, AF 488-labeled anti-CD8a, PE-CF594-labeled anti-FoxP3, and PE-labeled anti-IL-10 (BD Bioscience, CA, USA).

2.5 Determination of IFN-γ and granzyme B mRNA Levels

Quantitative reverse transcriptase PCR (qRT-PCR) was used to analyze the mRNA levels of cytokines and chemokines (Table 1) (Soong, Wang et al. 2014). The tissues were collected and placed in RNALater (Ambion, MA, USA) at 4°C for 24 hours before being stored at -80°C. RNeasy minikits (Qiagen, Hilden, Germany) and RNase-free DNase (Qiagen, Hilden, Germany) were used to

Table 1. Primers of murine genes for qRT-PCR

GAPDH	Forward 5'-TGGAAAGCTGTGGCGTGAT-3'
	Reverse 5'-TGCTTCACCACCTTCTTGAT-3'
IFN-γ	Forward 5'-ATGAACGCTACACACTGCATC-3'
	Reverse 5'-CCATCCTTTTGCCAGTTCCTC-3'
TNF-α	Forward 5'-ATAGCTCCCAGAAAAGCAAGC-3'
	Reverse 5'-TTGGTCCTTAGCCACTCCTTC-3'
CXCL-10	Forward 5'-CCAAGTGCTGCCGTCATTTTC-3'
	Reverse 5'-GGCTCGCAGGGATGATTTCAA-3'
Bcl-2	Forward 5'-ATGCCTTTGTGGAACTATATGGC-3'
	Reverse 5'-GGTATGCACCCAGAGTGATGC-3'

extract and purify total RNA. iScript cDNA synthesis kits (Bio-Rad Laboratories, CA, USA) were used to synthesize cDNA. The abundance of target genes was determined by qRT-PCR with Bio-Rad CFX 96 Real-Time System and SYBR Green Supermix (Bio-Rad Laboratories, CA, USA). The PCR incubations were 3 minutes at 95°C, and 40 cycles of 95°C for 10 seconds, 60°C for 10 seconds, followed by 10 seconds at 72°C. Dissociation melting curves were obtained to confirm the purity of final products. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative abundance of mRNA levels with GAPDH as housekeeping gene denominator (Livak and Schmittgen 2001; Soong, Wang et al. 2014).

2.6 HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

The tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Hematoxylin and eosin were used to stain the tissue sections (5 μm thickness). All slides were reviewed and scored by four scientists trained in histopathology. We used a 0 to 4 scoring system (0, normal; 1, cellular infiltration presence; 2, multi-focal lesions; 3, no more than 2 areas of necrosis; 4, more than 2 foci of necrosis in one slide) to assess the histopathology in the liver. Tissue sections from the experimental animals were stained with ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Darmstadt, Germany) following the manufacturer's protocol with hematoxylin used instead of methyl green in the counterstaining step. The scoring system for apoptosis staining was a 0 to 5 point scale (0, no apoptosis; 1, no more than 10 apoptotic staining positive cells per 100x field; 2, between 11 and 20 apoptotic cells per 100x field;

3, between 21 and 30 apoptotic cells per 100x field; 4, between 31 and 40 apoptotic cells per 100x field; 5, more than 40 apoptotic cells per 100x field).

2.7 Adoptive Transfer of Splenocytes

In this experiment, a sub-lethal dose (1.25×10^6 FFU) of *O. tsutsugamushi* Karp strain and the same dose given as a booster were inoculated into C57BL/6J mice 27 days and 7 days, respectively, before the spleen of the donor mouse was harvested (Feng, Popov et al. 1997; Walker, Olano et al. 2001). The spleens of uninfected mice of the same age were used to prepare nonimmune control cells. $CD8^+$ T lymphocytes were then isolated by the negative magnetic $CD8^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). We also collected the CD8 T cell-depleted splenocytes from the column following the manufacturer's instructions. One spleen-equivalent of separated $CD8^+$ T cells or CD8 T cell-depleted splenocytes (~10^7 cells) was transferred to recipient B6 mice via the i.v. route. After 24 hours, all recipient mice were challenged i.v. with an ordinarily lethal dose (10 LD_{50}: 8.25×10^7 FFU) of *O. tsutsugamushi*. The negative magnetic selection generated more than 91% purity of $CD8^+$ T cells with less than 0.5% $CD4^+$ T cells. There were 20%-27% $CD4^+$ T cells and less than 2% $CD8^+$ T cells among the CD8 T cell-depleted splenocytes.

2.8 Statistical Analysis

Results are presented either as mean ± SD or as median and range, as indicated. Analyses were performed with independent samples T test. $P < 0.05$

was considered to be statistically significant. GraphPad Prism 5.0 and SPSS 18.0 were used for statistical calculations.

Chapter 3 Changes of Host Immunity during *Orientia* Infection

3.1 INTRODUCTION

Due to its long neglect, it is still unclear how the host immunity responds to *O. tsutsugamushi* infection. The **objective** of this aim is to determine the changes of the mammalian host immunity during *O. tsutsugamushi* infection. Our **hypothesis** is that both cellular and regulatory immunity play important roles in the host immune responses against *O. tsutsugamushi* infection. The regulatory immunity not only controls the inflammation but also enables the pathogen to survive and persist. To determine the host immune responses during *Orientia* infection, we inoculated three groups of C57BL/6J mice i.v. with PBS, a sub-lethal dose, or a lethal dose of *O. tsutsugamushi* Karp strain. We then used flow cytometry to analyze the proportional changes of $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+CD25^+FoxP3^+$ Treg cells, as well as IL-10 producing $CD3^+$ T cells in the spleen during the course of *O. tsutsugamushi* infection in our i.v. mouse model. The **rationale** for the work is to determine the major players in host immunity during *O. tsutsugamushi* infection.

As are other bacteria in Rickettsiaceae family, *Orientia tsutsugamushi* is a gram-negative obligately intracellular coccobacillus. Previous studies demonstrated the changes of host immunity during *Rickettsia* infection (Feng, Popov et al. 1994; Feng, Popov et al. 1997; Walker, Olano et al. 2001). As mentioned in Chapter 1, there are, nevertheless, major differences between the *Rickettsia* genus and *Orientia* genus. A complete analysis of the host immune responses against *O. tsutsugamushi* infection is necessary and urgent. The

study of this specific aim demonstrated the key participants in host immunity during scrub typhus infection. The results provided a foundation for future studies of the mechanisms of host immunity against the obligately intracellular pathogen, which will benefit the development of new preventive measures including a vaccine.

3.2 RESULTS

3.2.1 Host Immunity during Acute *O. tsutsugamushi* Infection

All mice challenged with a lethal dose were either sacrificed when moribund or died by 12 days post-infection (dpi). On 6, 12 and 18 dpi, the spleens were collected and processed for flow cytometric analysis as described above. The percentages of neither $CD4^+$ T cells nor $CD8^+$ T cells from both sublethal and lethal dose of *O. tsutsugamushi*-challenged mice were significantly greater than in PBS-inoculated control mice on 6 dpi (**Figure 6**). However, we observed that both infected groups had considerably increased spleen size after 12 dpi reflecting increased cellular content. Our flow cytometry data further demonstrated that more $CD8^+$ T cells than $CD4^+$ T cells were induced in infected mice at 12 and 18 dpi (**Figure 6B and C**). We also determined that the proportion of T_{reg} cells and T cells producing IL-10 were increased from 6 dpi, but the proportions of both of them were reduced after 12 dpi (**Figure 7**). Our data showed that the adaptive immune response after *Orientia* infection was skewed toward $CD8^+$ T cell development, and regulatory immune responses were induced at an early stage. These results suggested that $CD8^+$ T cells may play

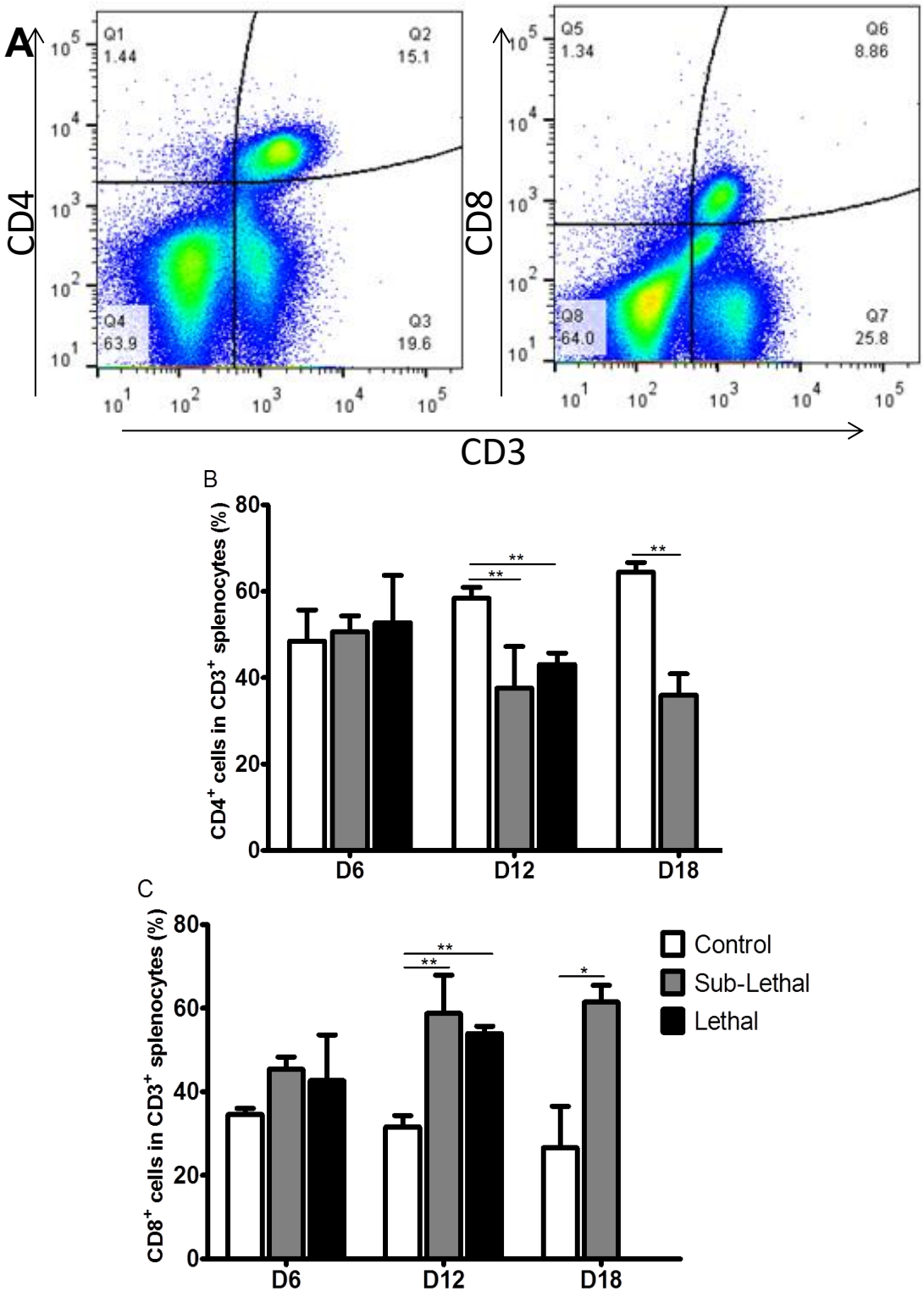


Figure 6. Levels of T cell subsets in *Orientia*-infected mice. Using the gating strategy (**A**), we observed that more $CD8^+$ T cells (**C**) than $CD4^+$ T cells (**B**) were induced in wild type C57BL/6J (WT) mice at 12 days post sub-lethal or lethal *Orientia* infection. PBS was used as control inoculation; data are expressed as mean ± SD. *, $p<0.05$; **, $p<0.01$.

important roles in host immunity against *Orientia* infection.

3.2.2 Host Immunity during Persistent *O. tsutsugamushi* Infection

In addition to study of the host immunity changes during acute *O. tsutsugamushi* infection, we examined the host immune responses in the persistent infection phase. At 76 dpi and 90 dpi, we sacrificed and processed the spleen of sub-lethal dose challenged mice and uninfected control mice as before. Flow cytometric analysis demonstrated that the trend of disparate percentages of $CD4^+$ T cells, $CD8^+$ cells and T_{reg} cells, and levels of IL-10-producing cells continued as in the acute infection phase (**Figure 8**), but most of the differences between *Orientia*-infected and uninfected mice were not statistically significant.

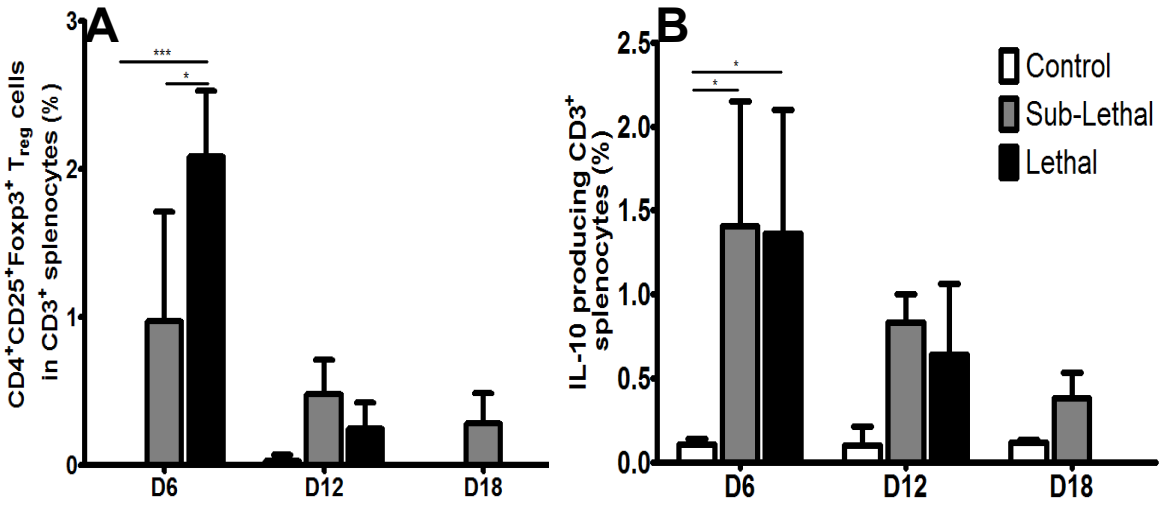


Figure 7. Levels of T_{reg} cells and IL-10-producing T cells in *Orientia*-infected mice. From day 6 after *Orientia* infection, more $CD4^+CD25^+FoxP3^+$ T_{reg} cells (**A**) and IL-10 producing $CD3^+$ T cells (**B**) were detected in both sub-lethal and lethal dose challenged mice than in uninfected control mice. The levels of both T_{reg} cells and IL-10 producing $CD3^+$ T cells peaked on day 6. Data are expressed as mean ± SD. *, $p<0.05$; ***, $p<0.001$.

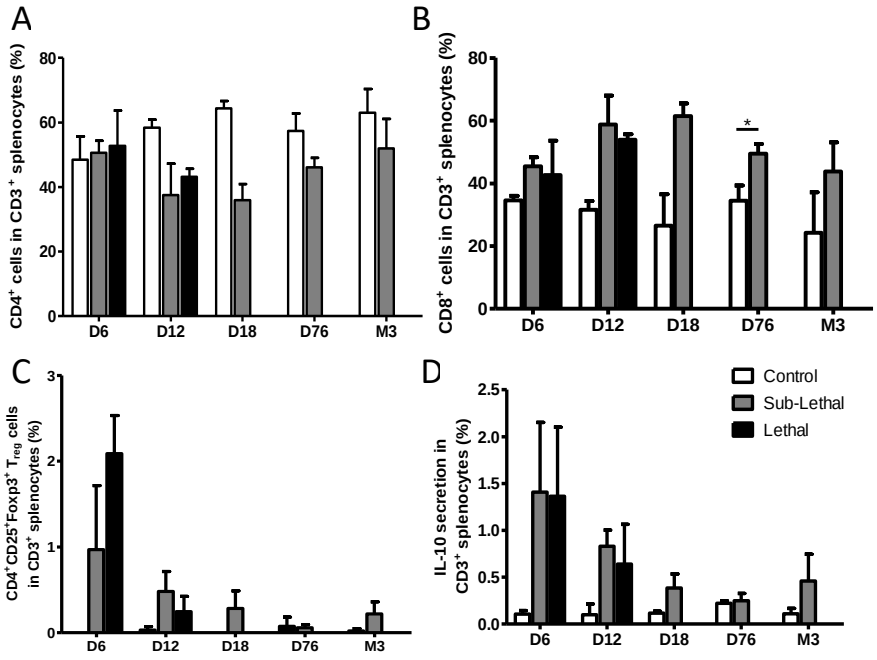


Figure 8. Levels of T cell subsets and IL-10-producing T cells in mice persistently infected with *Orientia*. Using the same gating strategy as before, we observed that there were still more $CD8^+$ T cells (**B**) but not $CD4^+$ T cells (**A**) in infected mice than uninfected control at 76 dpi. There was not a statistically lower percentage of $CD4^+$ T cells (**A**), T_{reg} cells (**C**) or IL-10-producing cells (**D**) between infected and uninfected mice at 76 dpi and 90 dpi. PBS was used as control inoculation, data are expressed as mean ± SD. *, $p<0.05$; **, $p<0.01$.

The only significant disparity of proportion we observed was the $CD8^+$ T cells between sub-lethally infected and naive mice at 76 dpi (**Figure 8B**). This study further confirmed the indisputable involvement of $CD8^+$ T lymphocytes in the *O. tsutsugamushi* infection including the early persistent stage. Other effectors and cytokines in the host immune systems are also actively involved in *O. tsutsugamushi* infection.

3.3 DISCUSSION

We discovered previously that host immunity is skewed towards T_h 1 responses from 12 dpi until 3 months post-infection (Soong, Mendell et al. 2016). Our flow cytometry data determined that in the acute *Orientia* infection phase, more $CD8^+$ T cells than $CD4^+$ T cells were present in the spleen of infected mice after 12 dpi. We also found that $CD4^+CD25^+Foxp3^+$ T_{reg} cells and levels of IL-10-producing T cells increased significantly from 6 dpi to 18 dpi, which paralleled the change in body weight and increased bacterial loads.

In the persistent infection stage, we observed the infected hosts continued the trend of their immune response but became less robust. For instance, the size of the spleen and liver in infected mice became smaller though they were still larger than that in uninfected control. We still observed a significantly greater percentage of $CD8^+$ T cells in the spleen of *O. tsutsugamushi*-infected mice than that in uninfected controls. Differences in other effectors of the host immunity, such as $CD4^+$ T cells, T_{reg} cells, and IL-10-producing T cells, did not reach statistical significance in infected mice compared to control mice. These studies contribute to the foundation of understanding the dynamic changes of host immunity during *O. tsutsugamushi* infection, which led us to postulate that $CD8^+$ T cells play key roles in the host immunity against *O. tsutsugamushi* infection.

Chapter 4 The Indispensable Function of $CD8^+$ T Cells during *Orientia* Infection

4.1 INTRODUCTION

The mechanisms how *Orientia* can persist in hosts even after treatment with doxycycline are unclear. The **objective** of this aim is to determine whether the $CD8^+$ lymphocyte is critical to immune clearance of *Orientia* infection. We **hypothesize** that $CD8^+$ T cells are indispensably critical to protection of the host from *O. tsutsugamushi* infection. In those lethal or persistently infected cases, *Orientia tsutsugamushi* may suppress and/or exhaust $CD8^+$ T cells and other immune effectors. We tested our working hypothesis by using the **approach** of comparing the survival rates, bacterial loads and histopathology after ordinarily sublethal *O. tsutsugamushi* infection between the $CD8^+$ T cell knockout mice and wild type controls. We also carried out adoptive transfer of immune $CD8^+$ lymphocytes to further examine the effects of $CD8^+$ lymphocytes on *Orientia* clearance. The **rationale** for the work is to determine how *O. tsutsugamushi* interacts with $CD8^+$ T cells to invade and infect the host. It helps us better understand the host immune responses during *O. tsutsugamushi* infection including its persistence.

O. tsutsugamushi, an obligately intracellular gram-negative bacterium, is classified as a separate genus in the Rickettsiaceae family (Seong, Choi et al. 2001). As mentioned above, there is diversity among different *Orientia* strains. The lack of long lasting cross protection results in reinfection in patients (Romeo 1946; Philip 1948; Kuwata 1952). Further studies confirmed that heterologous

immunity is only short lived while homologous immunity lasts longer before waning (Valbuena and Walker 2013). Persistence of *O. tsutsugamushi* was found in 1949 when researchers detected the bacteria in guinea pigs' urine after recovery from acute infection (Kouwenaar and Esseveld 1949). Our laboratory and others also observed persistence in different animal models. The mechanisms behind this are not understood.

As our preliminary studies indicate that, *O. tsutsugamushi* persists predominantly in the host's kidney at 70-84 dpi. We found that kidneys from all of the i.d. infected mice contained bacteria detected by real-time PCR while only 3 out of 12 lung samples had detectable bacteria at that time (**Figure 9**). We also demonstrated that the *Orientia* were viable by infecting mice with the homogenized kidneys and lung. The mice inoculated via the highly sensitive i.p. route with homogenized kidney began to exhibit signs of illness at 10 dpi, but those inoculated with lung homogenate did not. Upon necropsy, the gross pathology of the mice was identical to control animals inoculated with a known bacterial dose. During the acute infection phase, the bacteria invade several tissues, and lung is the main target during this phase. As a member of family Rickettsiaceae, *O. tsutsugamushi* shares several common characteristics with

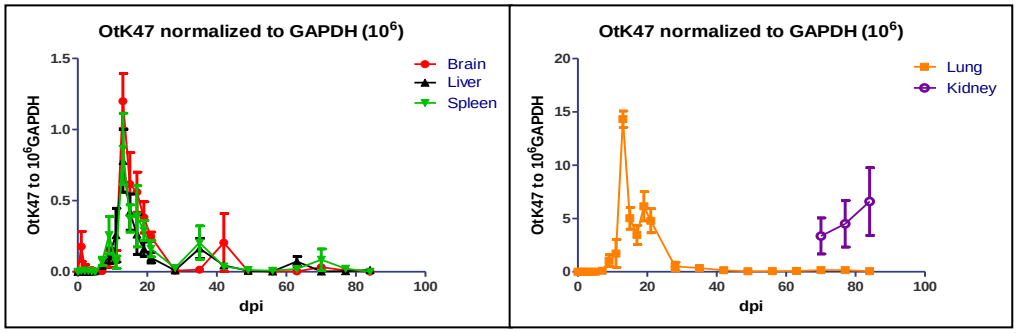


Figure 9. ***Orientia*** **bacterial loads in different tissues from 1 to 84**

Rickettsia. Previous studies in our laboratory demonstrated that $CD8^+$ T lymphocytes not only secrete IFN-γ, but also provide important immune effectors to eliminate rickettsiae directly (Walker, Olano et al. 2001). Our laboratory confirmed the function of $CD8^+$ T lymphocytes during *Rickettsia* infection and clearance through adoptive transfer of $CD8^+$ T cells to depleted mice (Feng, Popov et al. 1997). This led to our hypothesis that $CD8^+$ T lymphocytes are critical to protect the host from *Orientia* infection and persistence. *Orientia tsutsugamushi* may induce the down-regulation and exhaustion of $CD8^+$ T lymphocytes after invasion of the host, which allows organisms to stay in the host without being cleared by host immunity. In this aim, we used approaches that have already been widely used, which include comparing the survival rates and bacterial loads between infected $CD8^+$ T cell knockout mice (Jackson Laboratory: B6.129S2-$Cd8^{atm1Mak}$/J) and infected WT mice. We then used adoptive transfer of $CD8^+$ T cells to further verify the role of $CD8^+$ T cells. The studies help us determine the mechanism that host immunity uses to fight against *O. tsutsugamushi*. The results facilitate better understanding of the fundamental roles of $CD8^+$ T cells in host immunity.

4.2 RESULTS

4.2.1 $CD8^{-/-}$ mice lost more weight and were more susceptible to *O. tsutsugamushi* than WT mice

We first infected both $CD8^{-/-}$ mice and aged-matched WT mice i.v. with one LD_{50} of *O. tsutsugamushi* Karp strain. Both groups of mice maintained stable

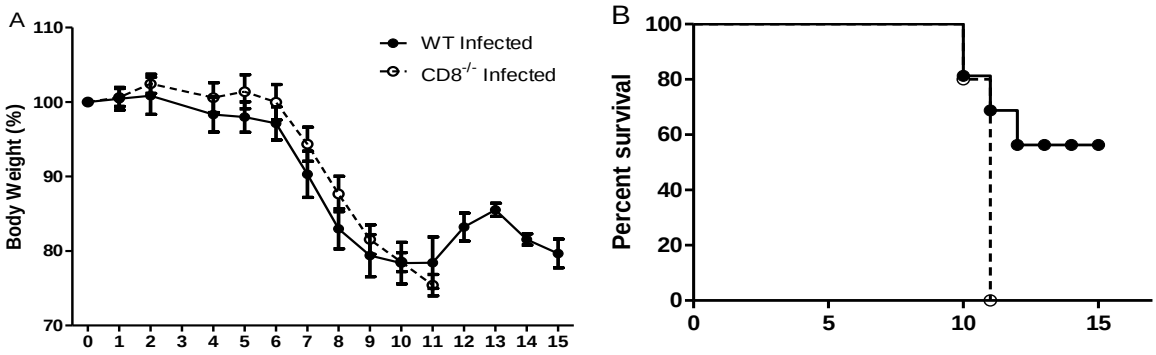


Figure 10. Body weight change and survival of CD8$^{-/-}$ and WT mice infected with one LD_{50} of *O. tsutsugamushi*. Mice began losing weight 7 dpi concurrent with signs of illness (**A**). Half of the WT mice (solid circles) expired between days 10 and 12 while all CD8$^{-/-}$ mice (open circles) expired or were moribund by 11 dpi (**B**).

weight until 6 dpi (**Figure 10A**). We observed signs of illness including hunched back, ruffled fur, erythema, and eye secretions that coincided with weight loss beginning at 7 dpi. CD8$^{-/-}$ mice lost more weight than their WT counterparts at 11 dpi (**Figure 10A**) and became moribund or had expired by day 11 post-infection while, as expected, only half of the WT mice expired (**Figure 10B**). The WT survivors began to gain weight after 11 dpi, and maintained their weight after 14 dpi (**Figure 10A**).

To better understand the effects of CD8^{+} T cells during *O. tsutsugamushi* infection, we repeated the experiment with a sub-lethal i.v. dose challenge of *O. tsutsugamushi* Karp strain. Similar to the one LD_{50} dose-challenged mice, both sub-lethal dose of *O. tsutsugamushi* challenged WT and CD8$^{-/-}$ mice showed similar weight change and other signs of sickness during the early stage. Both groups of infected mice in this study began losing weight on 9 dpi, and CD8$^{-/-}$ mice continued losing weight until they became moribund or expired (11-15 dpi)

while WT mice ceased losing weight on 11 dpi and recovered (**Figure 11A and B**).

We further used quantitative real-time PCR to determine the bacterial loads in different organs of the sub-lethally infected $CD8^{-/-}$ mice and WT mice. There were significantly higher bacterial loads in the lung, kidney, liver, and spleen of $CD8^{-/-}$ mice than in WT mice on day 12 of infection (**Figure 11C**). These studies indicated that $CD8^{-/-}$ mice were more susceptible to *Orientia* infection.

4.2.2 Deficiency of $CD8^{+}$ T cells resulted in increase of IFN-γ and granzyme B mRNA levels in the liver of infected mice

To further understand the host immune responses during *Orientia* infection, we employed qRT-PCR to determine the mRNA levels of cytokines among different organs in infected $CD8^{-/-}$ mice and WT mice. The mRNA levels of the important pro-inflammatory cytokine IFN-γ were significantly higher in the liver of infected $CD8^{-/-}$ mice than in the infected WT controls at 12 dpi (p value=0.0037, **Figure 12A**). Significantly higher mRNA levels of IFN-γ were observed in both infected $CD8^{-/-}$ and WT mice than in corresponding uninfected mice (WT: p=0.0137; $CD8^{-/-}$: p=0.0013, **Figure 12A**). There were no significant differences in the IFN-γ mRNA levels in the spleen, kidney and liver of the uninfected knockout (KO) and WT mice. Both groups of infected mice had higher levels of IFN-γ mRNA in the lung than those in uninfected mice. Our qRT-PCR studies demonstrated that the granzyme B mRNA levels in the liver of infected

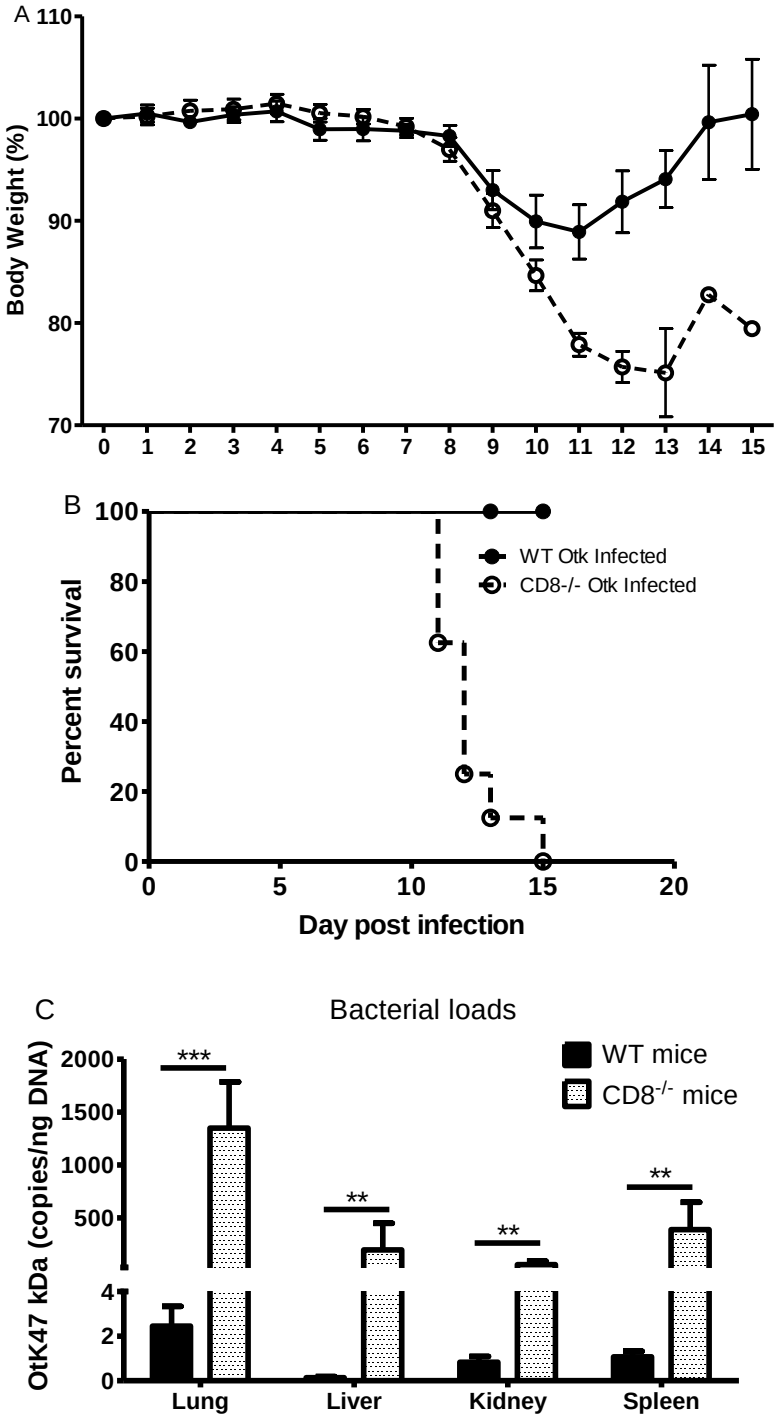


Figure 11. Body weight change, survival, and bacterial loads of CD8$^{-/-}$ mice and WT mice infected with *O. tsutsugamushi*. Weight change (**A**) and survival curve (**B**) of mice following challenge with a sub-lethal dose of *Orientia.* None of the WT mice expired while all CD8$^{-/-}$ mice expired or were moribund between 11 and 15 dpi (**B**). QPCR demonstrated that CD8$^{-/-}$ mice infected with a sub-lethal dose of *O. tsutsugamushi* had significantly higher bacterial loads in the lung, liver, kidney, and spleen than WT mice at 12 dpi (**C**). *, p<0.05; **, p<0.01; ***<0.001.

CD8$^{-/-}$ mice were significantly higher than in the infected WT mice at 12 dpi (p=0.0092, **Figure 12B**). Infected CD8$^{-/-}$ mice had significantly greater mRNA levels of granzyme B than uninfected CD8$^{-/-}$ mice (p=0.0048, **Figure 12B**). Granzyme B is secreted by CD8^{+} T lymphocytes and natural killer (NK) cells to mediate apoptosis of cells infected with intracellular pathogens and tumor cells (Salti, Hammelev et al. 2011).

4.2.3 CD8$^{-/-}$ mice infected with *O. tsutsugamushi* had more severe liver damage

Although we observed more cellular infiltrations in the lung, liver, kidney (**Figure 13A**), and heart of WT mice than in matched CD8$^{-/-}$ mice, the infected CD8$^{-/-}$ mice had a more severe liver histopathology score than WT control mice (Pathology score 2.70 vs 1.92, p<0.05, **Figure 13B**). The livers of infected CD8$^{-/-}$ mice also showed steatosis. We further used an Apoptosis Detection Kit to

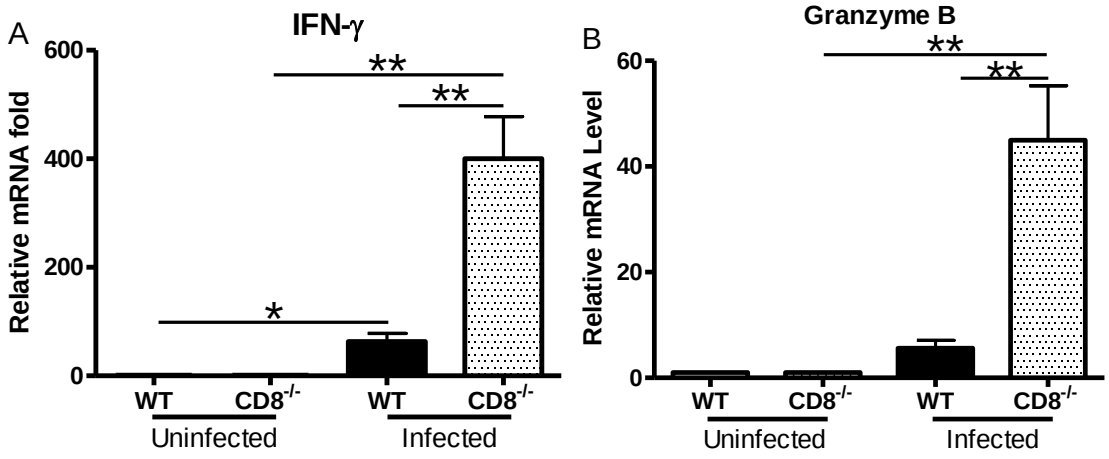


Figure 12. Gene expression in the liver of CD8$^{-/-}$ and WT mice infected with *O. tsutsugamushi*. There was significantly greater IFN-γ (**A**) and granzyme B (**B**) mRNA in the liver of infected CD8$^{-/-}$ mice than in the WT mice at 12 dpi. Data are shown as mean ± SD in each group and presented as relative mRNA levels with the $2^{-\Delta\Delta Ct}$ of housekeeping genes normalization method. *, p<0.05; **, p<0.01.

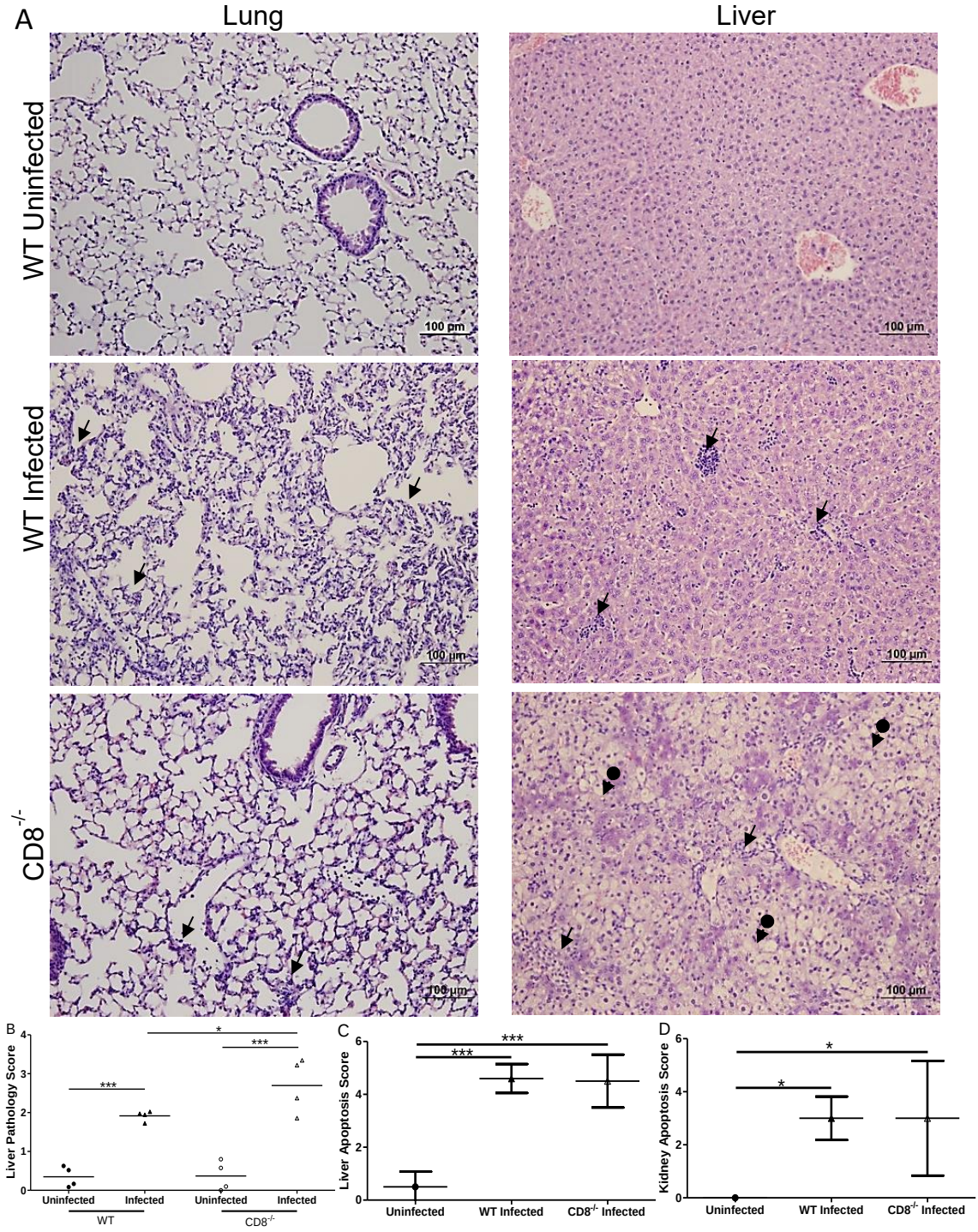


Figure 13. Histopathological comparison of CD8$^{-/-}$ and WT mice infected with *O. tsutsugamushi*. More cellular infiltrations (arrows) were found in the lung and liver (**A**, mag: 100x) of WT mice than CD8$^{-/-}$ mice. The liver of CD8$^{-/-}$ mice also showed more necrosis and steatosis (arrows with circle end). Higher liver pathology scores indicating injury were observed in infected WT and CD8$^{-/-}$ mice (**B**). The pathology score of infected CD8$^{-/-}$ mice was greater than that of infected WT mice (**B**). There were no differences of apoptosis in the liver (**C**) and kidney (**D**) between infected WT and CD8$^{-/-}$ mice, but significantly greater apoptosis was observed in all mice after *Orientia* infection. *, $p<0.05$; ***, $p<0.001$.

determine the difference in apoptosis during *Orientia* infection between CD8$^{-/-}$ mice and WT mice. The immunohistochemical method clearly demonstrated that there were more apoptotic cells in the liver and kidney of all infected mice than in the uninfected control mice (**Figure 13C and D**). However, the apoptosis scores in liver and kidney of infected WT and CD8$^{-/-}$ mice were not statistically different. In addition, mRNA levels of *Bcl*-2, which is an anti-apoptotic molecule, were similar in the liver between infected CD8$^{-/-}$ mice and WT mice (**Figure 14**).

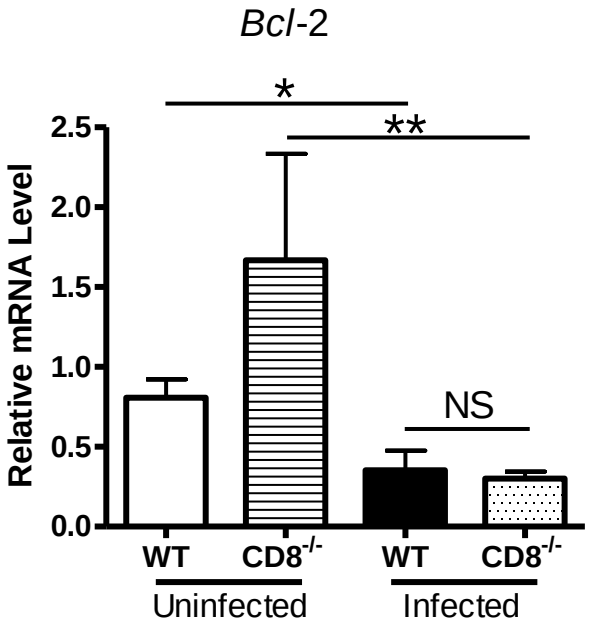


Figure 14. *Bcl*-2 mRNA levels in the liver of CD8$^{-/-}$ and WT mice infected with *O. tsutsugamushi*. There were significantly lower mRNA levels of *Bcl*-2 in the liver of infected WT and CD8$^{-/-}$ mice than in the corresponding uninfected mice. No difference was detected between infected WT and CD8$^{-/-}$ mice at 12 dpi. Data are shown as mean $\pm$ SD in each group and presented as relative mRNA levels with the $2^{-\Delta\Delta Ct}$ of housekeeping genes normalization method. *, p<0.05; **, p<0.01.

4.2.4 Adoptive transfer of activated CD8$^+$ T lymphocytes or CD8 T cell-depleted splenocytes protected mice against *Orientia* infection

To further evaluate the roles of CD8$^+$ T lymphocytes and other immune cells in scrub typhus, immune or naive CD8$^+$ T cells or CD8 T cell-depleted splenocytes were adoptively transferred to WT C57BL/6J mice. All mice that received naive CD8$^+$ T cells or naive CD8 T cell-depleted splenocytes expired between 8 and 9 dpi (**Figure 15C and D**). Half of the mice that received immune CD8$^+$ T cell-depleted splenocytes expired between 13 and 14 dpi (**Figure 15D**). The onset of signs of illness was delayed when compared to mice that received naive CD8 T cell-depleted splenocytes. All mice that received immune CD8$^+$ T cells survived (**Figure 15C**). Compared to the other groups, these mice began showing signs of illness later, and their illness was much less severe. These mice lost ~12% or less of body weight, while the recipients of immune CD8 T cell-depleted splenocytes lost ~13% of body weight during infection after challenge with 10 LD_{50} of *O. tsutsugamushi* (**Figure 15A and B**). Mice that received nonimmune CD8$^+$ T cells or CD8 T cell-depleted splenocytes lost ~20% of their body weight. Immune CD8$^+$ T cells were highly protective, and CD8 T cell-depleted splenocytes provided less, but significant, protection against the lethal dose challenge of *O. tsutsugamushi* with delayed onset and 50% survival. Even though CD8$^+$ T cells are critical and provide more effective protection, some other non-CD8$^+$ immune cells also contribute to protection in *O. tsutsugamushi* infection.

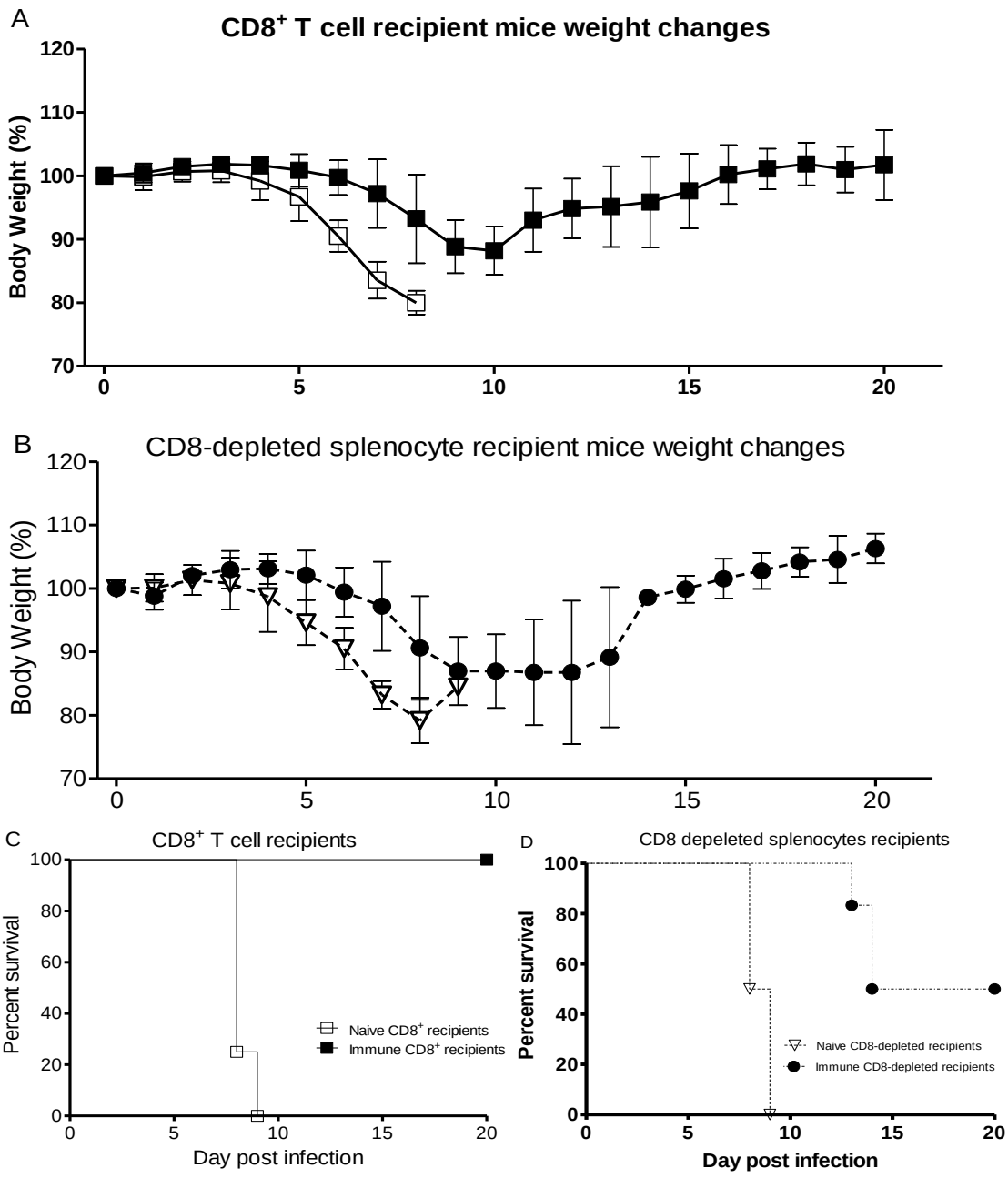


Figure 15. Body weight change and survival of WT mice adoptively transferred i.v. with different populations of splenocytes after *O. tsutsugamushi* infection. Naive $CD8^+$ T cell recipient mice (open squares) began losing weight at 4 dpi with onset of other signs of illness at 6 dpi (**A**), and they all expired between 8 dpi and 9 dpi (**C**). Immune $CD8^+$ T cell-recipient mice (solid squares) had delayed onset of illness that was milder, and they lost less weight between 7 dpi and 10 dpi (**A**). They all survived (**C**). We observed that naive CD8-depleted splenocyte recipient mice (open triangles, **B**) shared a similar course as the naive $CD8^+$ T cell recipient group (solid square). Half of immune CD8-depleted splenocyte-recipient mice (solid circles, **B** & **D**) expired between 13 dpi and 14 dpi.

4.3 Discussion

After being administered a sub-lethal dose of *O. tsutsugamushi*, $CD8^{-/-}$ mice started showing signs of sickness earlier, and lost more weight than their age-matched WT mice. Most $CD8^{-/-}$ mice expired by 13 dpi except 1 that expired on 15 dpi, while all infected WT mice survived. Bacterial loads in the lung, kidney, liver and spleen of $CD8^{-/-}$ mice were significantly and dramatically higher than those in WT mice. IFN-γ and granzyme B mRNA levels in the liver of $CD8^{-/-}$ mice were significantly higher than in WT mice, though KO mice had higher bacterial loads and mortality rates (100% vs 0%) than their WT counterparts. IFN-γ is produced by multiple cell types including NK cells, natural killer T cells, $CD4^+$ T cells, and $CD8^+$ T cells (Schoenborn and Wilson 2007). Both $CD8^+$ T lymphocytes and NK cells produce granzyme B to mediate apoptosis in target cells (Salti, Hammelev et al. 2011). Although $CD8^{-/-}$ mice have a deficient cytotoxic T cell response, other immune response cells still function properly.

We observed greater immune-inflammatory cellular infiltration in different tissues of wild-type mice than in $CD8^{-/-}$ mice, but there were more multi-foci lesions and necrosis in $CD8^{-/-}$ mice. Our results suggested that the host pro-inflammatory immune responses provided more protection than pathogenic effects. Apoptosis staining of the liver demonstrated that *Orientia* infection caused significantly more apoptosis in both $CD8^{-/-}$ and WT mice, but there was no statistically significant difference between $CD8^{-/-}$ and WT mice.

The adoptive transfer experiment provided critical analysis of the functions of different T lymphocytes and a mixure of other immune response cells including $CD4^+$ T cells, macrophages, NK cells, and neutrophils

in scrub typhus. Our studies demonstrated that not only immune $CD8^+$ T lymphocytes but also other immune response cells provide protection to the naive recipient mice from scrub typhus, even though the protection by immune $CD8^+$ T cells is more efficient than other immune response cells.

Chapter 5 Determine the Role of MHC I in *O. tsutsugamushi* Infection

5.1 Introduction

It is still incompletely known how $CD8^+$ T cells and host immunity respond to *O. tsutsugamushi* infection. The **objective** of this aim is to determine whether MHC I participates in host immunity against *O. tsutsugamushi* infection. We **hypothesize** that MHC class I is essential to facilitate $CD8^+$ T cells to protect the host from *O. tsutsugamushi* and that deficiency of MHC I allows the bacteria to remain and propagate in the host without being cleared. We tested this hypothesis by examining the differential survival of MHC $I^{-/-}$ and WT mice after *O. tsutsugamushi* infection. We used similar methods as described above in Chapter IV to examine the effects of MHC I on cytokine levels, bacterial loads, and histopathology in *O. tsutsugamushi* infection. The **rationale** for the proposed studies was to more comprehensively understand the mechanisms employed by the host to defend against and clear *O. tsutsugamushi* infection. It helps provide a foundation to develop new and effective control of scrub typhus and infections with other intracellular pathogens.

MHC I plays multiple roles in host immunity, including facilitating the presentation of processed antigen peptides to $CD8^+$ T cells, and recognizing and binding the receptors on NK cells, such as killer Ig-related receptor (in humans), Ly49s (in mice), and CD94/NKG2A (both humans and mice) to prevent attacking and killing of the host's own cells (Peaper and Cresswell 2008; Barao, Alvarez et al. 2011; Fiegl, Kagebein et al. 2013; Mu, Tai et al. 2014; Tovbis Shifrin, Kissiov et al. 2016). Our preliminary studies demonstrated that homologous immunity

against *O. tsutsugamushi* can be sustained for over a year. Those mice immunized with a sub-lethal dose of *O. tsutsugamushi* Karp strain survived lethal-dose challenge with the same strain of *O. tsutsugamushi* even 1 year after the primary infection immunization. Our results were consistent with the finding of homologous immunity in humans. Previous studies have confirmed that heterologous immunity against scrub typhus, caused by a different strain of *O. tsutsugamushi*, is short-lived (MacMillan, Rice et al. 1985). It was found that the concentrations of many cytokines and chemokines, both proinflammatory ones and immunoregulatory ones, are elevated in human and animal infections. Transcripts of lymphotoxin β (LTβ), tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-10, IFN-γ, TGF-β1 and migration inhibition factor (MIF) are increased on day 2 post infection, peak on day 4-6, and persist thereafter (Koh, Yun et al. 2004; Kramme, An le et al. 2009). More studies are necessary to fully understand the mechanisms of host immunity and the roles of $CD8^+$ T cells and MHC I.

The results from this specific aim together with those from the previous aims elucidate the mechanisms and a key effector of the mammalian host immune response against *O. tsutsugamushi* infection. The information can provide potential targets and immune mechanisms for both *O. tsutsugamushi* and other intracellular pathogens

5.2 RESULTS

5.2.1 MHC $I^{-/-}$ mice had increased severity of illness and greater susceptibility to *O. tsutsugamushi* infection

To further investigate the role of $CD8^+$ T cells and the upstream pathway, we used an ordinarily sub-lethal dose of *O. tsutsugamushi* Karp strain to infect MHC $I^{-/-}$ mice and WT C57BL/6J mice via i.v. inoculation. Similar to the results of $CD8^{-/-}$ mouse studies, both groups of mice maintained a stable weight until 8 dpi (**Figure 16A**). We observed signs of illness including hunched back, ruffled fur, erythema, and ocular secretions coincident with the onset of weight loss on day 9. MHC $I^{-/-}$ mice became more severely ill than WT C57BL/6J mice after 9 dpi. One MHC $I^{-/-}$ mouse expired on 10 dpi, and the rest became moribund or expired on 11 and 12 dpi. None of the infected C57BL/6J control mice became moribund, and they gained weight after 10 dpi (**Figure 16A and B**).

Quantitative real-time PCR determination of bacterial loads in organs of MHC $I^{-/-}$ mice and WT mice revealed significantly greater bacterial loads in the lung, kidney, liver, and spleen of MHC $I^{-/-}$ mice than in the WT mice at 11 dpi (**Figure 16C**). We concluded that MHC $I^{-/-}$ mice were more susceptible to *Orientia* infection.

5.2.2 MHC $I^{-/-}$ mice infected with *O. tsutsugamushi* had greater mRNA levels of IFN-γ, granzyme B, and CXCL-10 in the liver

We used qRT-PCR to determine the levels of IFN-γ, granzyme B, and CXCL-10 mRNA in different organs of infected MHC $I^{-/-}$ mice and WT mice. Similar to $CD8^{-/-}$ mice, infected MHC $I^{-/-}$ mice had a significantly higher level of IFN-γ mRNA in the liver than infected WT control mice at 11 dpi ($p=0.0204$, **Figure 17A**). We also observed that infected MHC $I^{-/-}$ mice had significantly

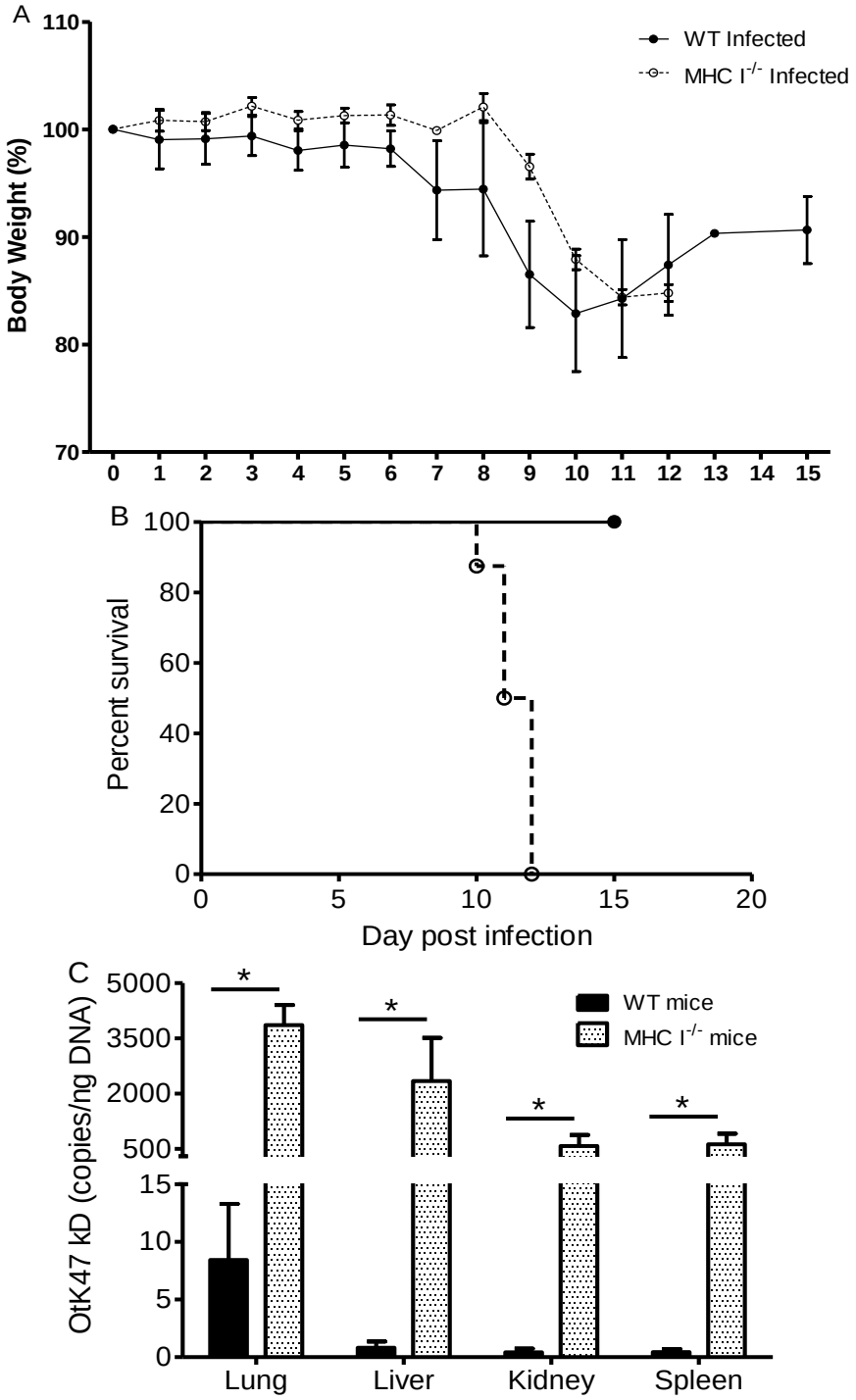


Figure 16. Body weight change, survival and bacterial loads of MHC $I^{-/-}$ and WT mice infected with *O. tsutsugamushi*. Both WT (solid circles) and MHC $I^{-/-}$ mice (open circles) shared the same weight loss trend until 11 dpi, when WT infected mice, but not MHC $I^{-/-}$ infected mice, began to recover and gain weight (**A**). All infected MHC $I^{-/-}$ mice expired or became moribund between 10 and 12 dpi (**B**). MHC $I^{-/-}$ mice had significantly greater bacterial loads in the lung, liver, kidney, and spleen than WT mice at 11 dpi (**C**). *, $p<0.05$.

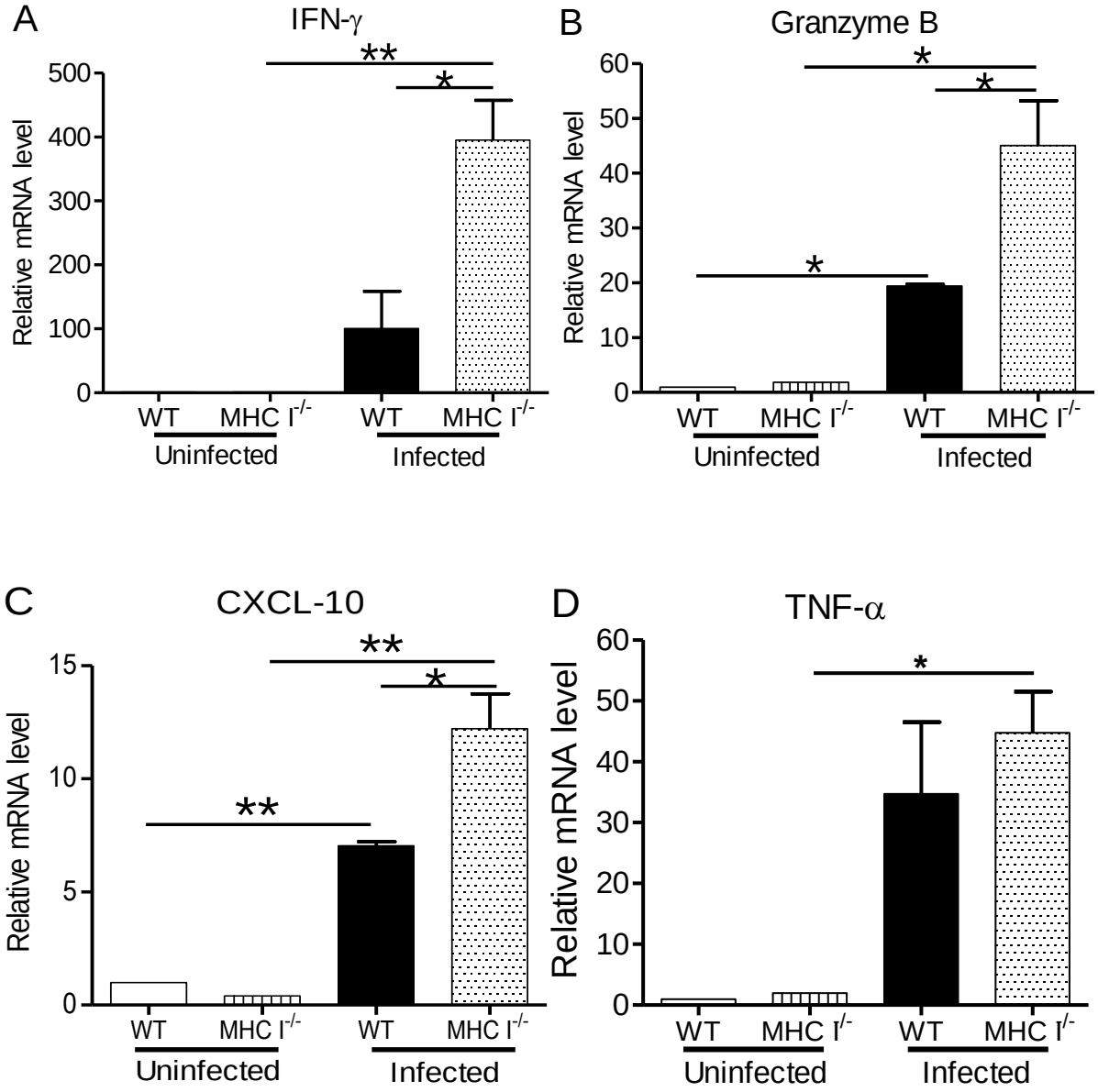


Figure 17. Gene expression in the liver of MHC $I^{-/-}$ mice and WT mice infected with *O. tsutsugamushi*. There were significantly higher levels of IFN-γ (**A**), granzyme B (**B**) and CXCL-10 (**C**) mRNA in the liver of infected MHC $I^{-/-}$ mice than in the infected WT mice at 11 dpi. No difference of the mRNA levels of TNF-α was observed (**D**). Infection induced greater mRNA levels of those cytokines in the liver of both WT and MHC $I^{-/-}$ mice. Data are shown as mean ± SD in each group and presented as relative mRNA levels with the $2^{-\Delta\ \Delta Ct}$ of housekeeping genes normalization method. *, $p<0.05$; **, $p<0.01$.

higher mRNA levels of IFN-γ than in uninfected MHC $I^{-/-}$ mice (p=0.0078, **Figure 17A**). The granzyme B mRNA levels in the infected MHC $I^{-/-}$ mice, similar to the trend with $CD8^{-/-}$ mice, were higher than those in infected WT mice (p=0.0447, **Figure 17B**). Infected MHC $I^{-/-}$ mice had significantly greater mRNA levels of granzyme B than their uninfected controls (p=0.0116, **Figure 17B**). CXCL-10, induced by IFN-γ, is a monokine that can recruit primed T lymphocytes to the site of inflammation (Whiting, Hsieh et al. 2004; Ross, Strieter et al. 2012). CXCL-10 mRNA was significantly higher in the liver of infected MHC $I^{-/-}$ mice than in the WT control mice at 11 dpi (p=0.0041, **Figure 17C**). No significant differences were observed between infected MHC $I^{-/-}$ and WT control mice in the levels of TNF-α mRNA in the liver (**Figure 17D**). Infection induced greater mRNA levels of those cytokines in the liver than uninfected controls (**Figure 17**).

5.2.3 MHC $I^{-/-}$ mice infected with *O. tsutsugamushi* had more apoptosis in the liver and kidney

A greater number of foci of cellular infiltrations were observed in the lung, liver, kidney (**Figures 18 and 19**) and heart (data not shown) of WT mice than in matched MHC $I^{-/-}$ mice. The same scoring system as used previously revealed that infected MHC $I^{-/-}$ mice had higher, but not statistically significantly different, liver pathology scores than WT mice (2.78 vs 2.29, p=0.10, **Figure 18B**). Both groups of *Orientia*-infected mice had significantly higher pathology scores than their uninfected counterparts. In contrast with $CD8^{-/-}$ mice, infected MHC $I^{-/-}$ mice had

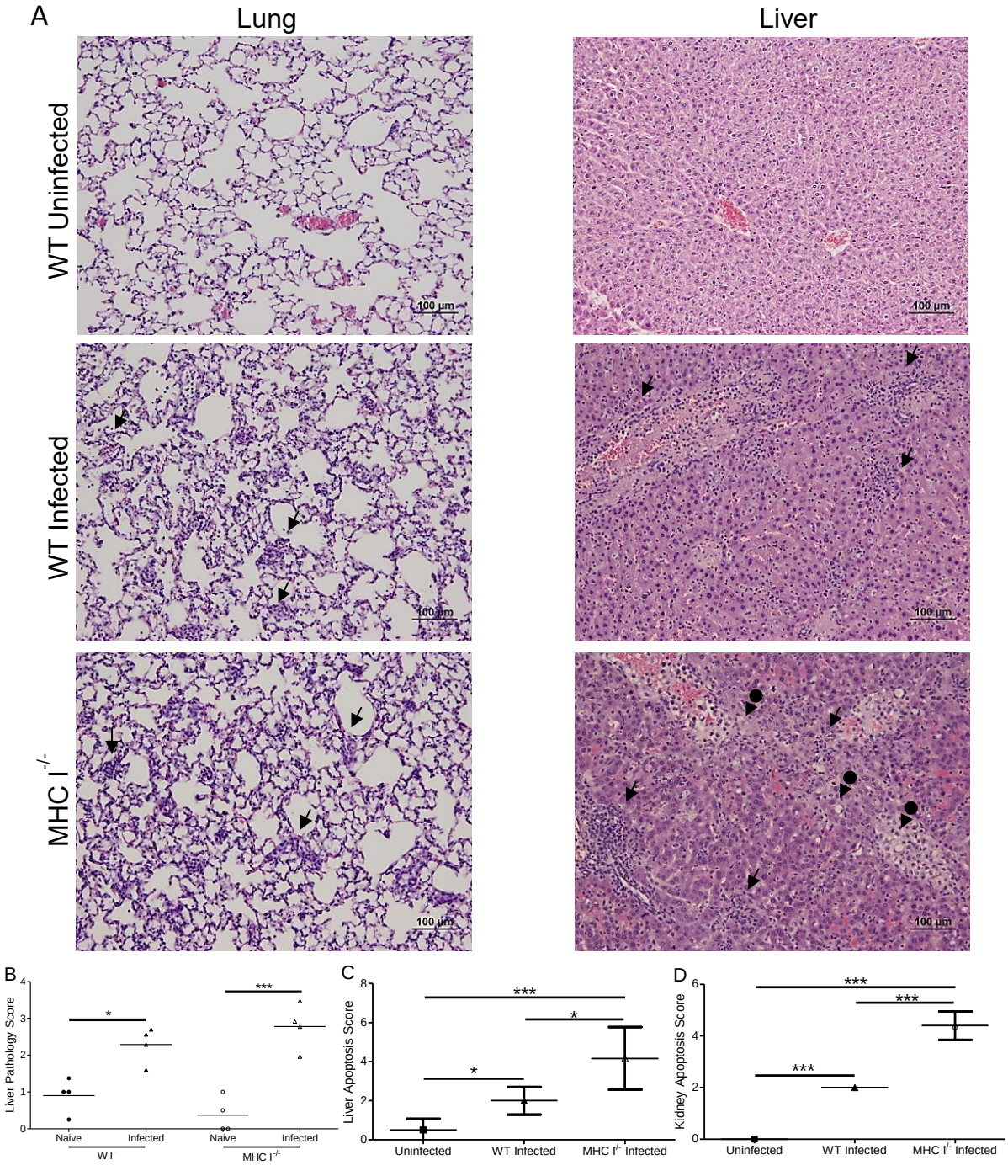


Figure 18. Histopathological comparison of MHC $I^{-/-}$ mice and WT mice infected with *O. tsutsugamushi*. Foci of inflammation (arrows), including infiltration of macrophages and lymphocytes, were observed in infected mice (**A**, mag: 100x). Many apoptotic cells were observed in the liver of MHC $I^{-/-}$ mice, possibly neutrophils. The livers of MHC $I^{-/-}$ mice also showed more necrosis and steatosis (arrows with circle end). Higher scores indicating greater injury were observed in the livers of infected mice (**B**). There were significantly more apoptotic cells in the liver (**C**) and kidney (**D**) of MHC $I^{-/-}$ mice than their WT counterparts. All infected mice had more apoptosis than uninfected mice. *, $p<0.05$; ***, $p<0.001$.

significantly greater apoptotic scores than infected WT mice in both liver and kidney (p=0.02, **Figure 18C & D**).

5.3 Discussion

Similar to the outcomes of infected CD8$^{-/-}$ mice, MHC I$^{-/-}$ mice were sicker and more susceptible than WT control mice to *O. tsutsugamushi*. All MHC I$^{-/-}$ mice became moribund or expired between 10 and 12 dpi. None of the WT mice became moribund, and they started recovery from 10 dpi. MHC I$^{-/-}$ mice had

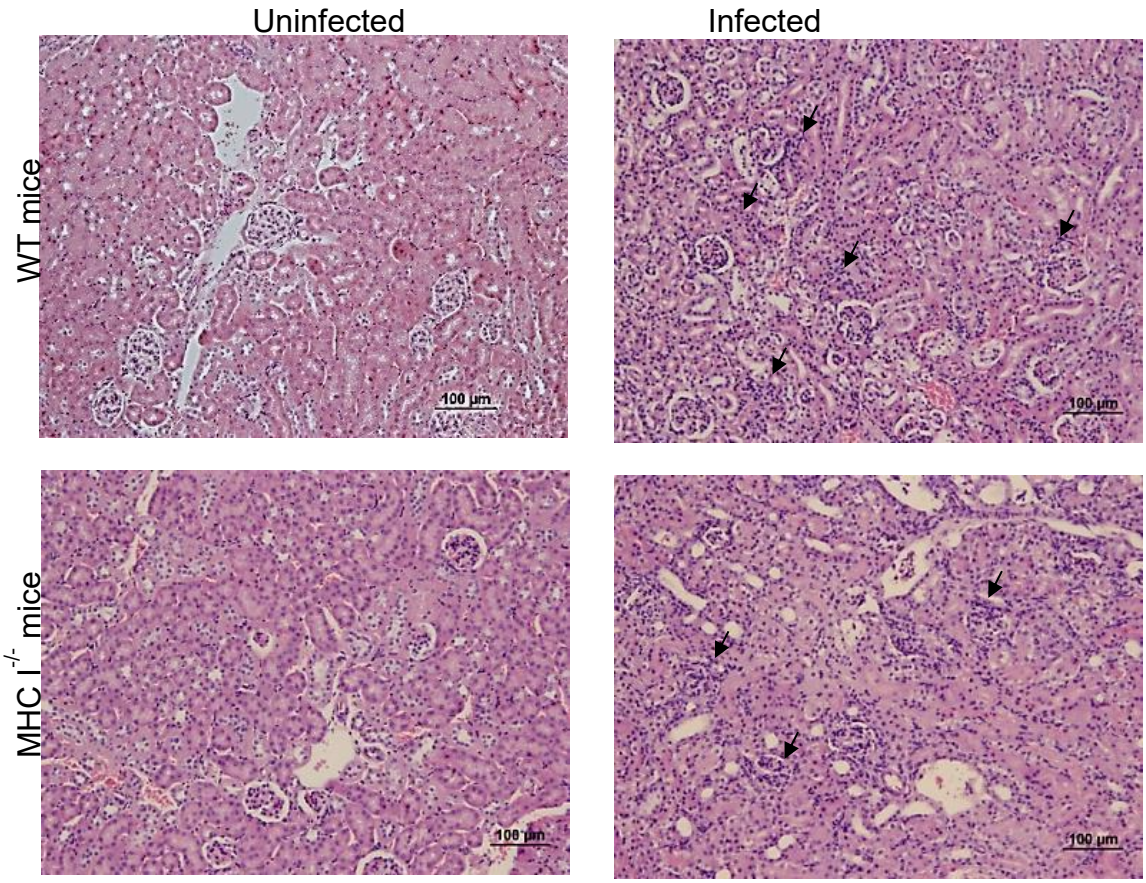


Figure 19. Histopathological comparison of kidney of MHC I$^{-/-}$ and WT mice infected with *O. tsutsugamushi*. Fewer foci of inflammation (arrows), including infiltration of macrophages and lymphocytes, were observed in MHC I$^{-/-}$ mice.

significantly greater bacterial loads in lung, kidney, liver and spleen than in the WT mice. Our quantative RT-PCR determined that mRNA levels of IFN-γ, granzyme B, and CXCL-10 were statistically higher in the liver of MHC $I^{-/-}$ mice than in the WT mice. As we mentioned in last Chapter, the causes behind these phenomena could be the multiple sources of IFN-γ and granzyme B production *in vivo* (Schoenborn and Wilson 2007; Salti, Hammelev et al. 2011).

In histopathological analysis, we demonstrated the difference in histopathology between MHC $I^{-/-}$ and WT mice after an ordinarily sub-lethal dose of *O. tsutsugamushi* infection. In general, infected MHC $I^{-/-}$ mice had more steatosis and necrosis while the liver of WT mice displayed more cellular infiltration. The pathology scores for the liver of MHC $I^{-/-}$ mice were higher than those in WT mice, but were not statistically significant. All infected mice had a greater apoptosis score than their uninfected counterparts. Different from the results in the studies of $CD8^{-/-}$ mice, there were more apoptotic cells in the liver and kidney of infected MHC $I^{-/-}$ mice compared to infected WT mice.

Chapter 6 Summary and Future Directions

6.1 Summary and Discussion

Orientia tsutsugamushi, a gram-negative obligately intracellular cocco-bacillus, is the causative agent of scrub typhus, which is a serious public health problem in the Asia-Pacific area (Kelly, Fuerst et al. 2009; Cho, Jun et al. 2010). There is still a significant gap in understanding how the pathogen invades, disseminates, and interacts within the host (Paris, Phetsouvanh et al. 2012). We discovered that host immunity was skewed towards T_h 1 responses from 12 dpi until 3 months post infection (Soong, Wang et al. 2014; Soong, Mendell et al. 2016). Our flow cytometry data determined that a significantly greater proportion of $CD8^+$ T cells was induced in the spleen of infected mice than in the uninfected mice at 12 dpi and thereafter; meanwhile the proportion of $CD4^+$ T cells was significantly reduced in the spleen of infected mice compared to uninfected controls. We also found that $CD4^+CD25^+Foxp3^+$ T_{reg} cells and IL-10-producing T cell levels were increased significantly from 6 dpi, which paralleled the body weight change and bacterial loads. During the persistent infection phase, those changes of host immune responses continued but became milder. We observed that there was still a significantly greater proportion of $CD8^+$ T cells induced in the spleen of mice infected with *Orientia* Karp strain than that in uninfected control mice at 76 dpi. There was no statistical difference among the proportion of $CD4^+$ T cells, T_{reg} cells, and IL-10-producing T cells between the infected and uninfected mice at the persistent stage.

We further used $CD8^{-/-}$ mice, MHC $I^{-/-}$ mice and their WT B6 control counterparts to determine the roles of $CD8^{+}$ T cells and MHC I molecules. After being administered an ordinarily sub-lethal dosage of *O. tsutsugamushi*, both $CD8^{-/-}$ mice and MHC $I^{-/-}$ mice started showing signs of sickness earlier, and lost more weight than their age-matched WT mice. Most $CD8^{-/-}$ mice expired by 13 dpi except 1 expired on 15 dpi, while all WT mice survived. All MHC $I^{-/-}$ mice with the same *Orientia* dosage expired by 12 dpi. Bacterial loads in the lung, kidney, liver and spleen of $CD8^{-/-}$ mice and MHC $I^{-/-}$ mice were significantly higher than those in WT mice.

In our studies, IFN-γ and granzyme B mRNA levels in the liver of *O. tsutsugamushi*-infected $CD8^{-/-}$ mice and MHC $I^{-/-}$ mice were significantly higher than in infected WT mice, though both groups of KO mice had higher bacterial loads and mortality rates upon sublethal challenge (100% vs 0%) than their WT counterparts. IFN-γ is produced by multiple cell types including natural killer cells, natural killer T cells, $CD4^{+}$ T cells, and CD8 T^{+} cells (Schoenborn and Wilson 2007). Both $CD8^{+}$ T lymphocytes and NK cells produce granzyme B to mediate apoptosis in target cells (Salti, Hammelev et al. 2011). We also observed higher mRNA levels of CXCL-10 in the liver of infected MHC $I^{-/-}$ mice than in infected WT mice or uninfected MHC $I^{-/-}$ mice. Secreted by several cell types including endothelial cells, CXCL-10 is an important chemokine for T cell accumulation under the influence of IFN-γ (Brissette, Kees et al. 2013; Antonelli, Ferrari et al. 2014). The increase of CXCL-10 mRNA levels in the liver of infected mice could be the response of infected endothelial cells to *O. tsutsugamushi*. Higher levels

of IFN-γ in MHC $I^{-/-}$ mice could induce higher levels of CXCL-10 than in WT mice. Although both groups of KO mice have a deficient cytotoxic $CD8^+$ T cell response, other immune response cells are present and may have compensatory changes in their functional levels. We hypothesize that other non-$CD8^+$ immune cells compensated by increased secretion of pro-inflammatory cytokines and granzyme B. Our studies demonstrated that even though there were enhanced levels of IFN-γ and granzyme B from other immune response cells, the host immune system nevertheless failed to control and eliminate the bacteria.

However, it is still unclear which of the exact pathways of the host immune system are activated and functioning during *O. tsutsugamushi* infection. One common pathway involves the pathogen recognition Toll-like receptor (TLR) system. *Orientia tsutsugamushi*, without lipopolysaccharide (LPS) that is commonly expressed on the surface of gram-negative bacteria, is recognized by TLR2 and TLR4 (Biragyn, Ruffini et al. 2002; Janardhanan, Joseph Martin et al. 2013; Gharaibeh, Hagedorn et al. 2016). The ligation of TLRs can further activate transcription factors, such as nuclear factor (NF)-κB via MyD88- and/or TRIF-dependent pathway (Kawai and Akira 2006; Gharaibeh, Hagedorn et al. 2016). In vitro studies also confirmed the activation of NF-κB after *O. tsutsugamushi* infection(Cho, Seong et al. 2000; Kim, Lee et al. 2010). There is still a fundamental gap for understanding the mechanism of TLR 2/4 activation by *O. tsutsugamushi* without a LPS. Recent studies demonstrated that TLR signaling can be activated without pathogen by endogenous molecules, such as high-mobility group box 1 (HMGB1), heat shock protein (HSP) 60 and HSP70

(Ohashi, Burkart et al. 2000; Asea, Rehli et al. 2002; Yu, Wang et al. 2006; Zurolo, Iyer et al. 2011; Ibrahim, Armour et al. 2013). One clincal study in India observed greater mutation rates of TLR2, TLR4 and HSP70 genes among scrub typhus patients (Janardhanan, Joseph Martin et al. 2013). These preliminary data demonstrated the participation of these pathways and effectors, but they did not provide any clear mechanisms behind this. It is still too premature to conclude any significant roles of TLR2/4, HSP70, HMGB1, or other effectors in different signaling pathways without more analytical studies.

Histopathological results demonstrated greater immune-inflammatory cell infiltration in different tissues of WT mice than in $CD8^{-/-}$ mice and MHC $I^{-/-}$ mice, but increased hepatic multifocal lesions and necrosis in $CD8^{-/-}$ mice and MHC $I^{-/-}$ mice. We postulate that the increased cellular infiltration was part of the host immune response to control the intracellular bacterial infection. Our results suggested that the host immune responses contribute more to protective roles than causing pathologic effects. These findings were consistent with previous studies on *O. tsutsugamushi* and other rickettsiae (Feng, Popov et al. 1997; Walker, Olano et al. 2001; Hauptmann, Kolbaum et al. 2016; Tsai, Chang et al. 2016). This study suggests that host mortality upon lethal-dose challenge was more a result of *O. tsutsugamushi* pathogenicity rather than immunopathologic effects. Both higher apoptotic score and downregulation of the anti-apoptotic gene *Bcl*-2 mRNA levels confirmed that *Orientia* infection induced apoptosis in the host regardless of whether mice were WT or deficient in CD8 or MHC I (Shelite, Liang et al. 2016). *Bcl*-2 and other members in its family are critical for

controlling the mitochondrial pathway of apoptosis after being activated by p53 tumor suppressor protein (Kiraz, Adan et al. 2016). It could be a host defense mechanism that the balance of apoptosis was skewed towards pro-apoptotic pathways instead of anti-apoptotic pathways after *O. tsutsugamushi* infection. We further demonstrated that there was more apoptosis in the MHC $I^{-/-}$ mice than WT mice and $CD8^{-/-}$ mice. Our data support the hypothesis that NK cells in MHC $I^{-/-}$ mice may induce more apoptosis in the tissue after *Orientia* infection because MHC-I also functions as an inhibitor of NK cells' cytotoxicity *in vivo* (Markel, Lieberman et al. 2002; Mahmoud, Tu et al. 2016). More studies are necessary to understand the mechanisms behind this.

Our adoptive transfer experiment provided direct evidence of the contribution of $CD8^+$ T lymphocytes and the mixture of the non-CD8 immune cells including potentially $CD4^+$ T cells, macrophages, NK cells, NKT cells, and/or neutrophils in immunity to scrub typhus. Our studies demonstrated that in addition to immune $CD8^+$ T lymphocytes other non-CD8 immune cells also provide some protection against scrub typhus, even though the protection by immune $CD8^+$ T cells was more effective. Neither naive $CD8^+$ T cells nor naive $CD8^+$ cell-depleted splenocytes provided any protection. Our results utilizing the i.v. route of infection support the study of Hauptmann et al (with the i.p. and footpad challenge routes) (Hauptmann, Kolbaum et al. 2016), which found a critical role for $CD8^+$ T cells, but our study also implicates the contribution of other non-$CD8^+$ immune cells in protection against *O. tsutsugamushi*. Given that i.p. and footpad inoculations do not mimic the human infection as the i.v. and i.d.

models do (Shelite, Saito et al. 2014; Soong, Mendell et al. 2016), validating the role of immune cells by using a well-developed mouse model and high-purity $CD8^+$ T cells (91% purity) in this study is important for our understanding of host immunity to *Orientia* infection of disseminated endothelial cells. Chigger feeding results in intradermal, not subcutaneous inoculation, which footpad inoculation represents.

The utilization of inaccurate disease models of scrub typhus fundamentally impedes the necessary studies to understand host-pathogen interactions. *Orientia tsutsugamushi* causes disseminated endothelial infection and multifocal vasculitis in lung and brain of human patients. Patients suffer interstitial pneumonitis, hepatic damage, encephalitis, and disseminated lymphohistiocytic vasculitis during scrub typhus (Allen and Spitz 1945; Berman and Kundin 1973; Moron, Popov et al. 2001). However, the historic animal infection with *O. tsutsugamushi*, first employed more than 50 years ago, involves i.p. injection of *O. tsutsugamushi* into mice, which substantially limits the pathogens and lesions to the peritoneal cavity (Kundin, Liu et al. 1964; Seong, Choi et al. 2001). Continuous proliferation of *O. tsutsugamushi* in mesothelial cells and peritoneal macrophages, enlargement of the spleen, hepatic lesions, peritonitis, and limited dissemination occur in these i.p. infected mice (Kundin, Liu et al. 1964; Catanzaro, Shirai et al. 1976; Ewing, Takeuchi et al. 1978; Oaks, Ng et al. 1985; Shelite, Saito et al. 2014). This widely used i.p. route produces an infection of the peritoneal cavity that results in fatal *Orientia* peritonitis not observed in scrub typhus infection in human patients (Berman and Kundin 1973). The report of

Hauptmann used this less appropriate mouse model in their $CD8^+$ T cell adoptive transfer studies. Recently, our laboratory has developed new i.v. and i.d. inoculation mouse models for *Orientia* infection, which better mimic the pathogen distribution, pathology and immunology of human scrub typhus patients, namely accurately reflecting the i.d. site of chigger feeding inoculation of *O. tsutsugamushi* and hematogenous dissemination, respectively. We also observed persistence of *O. tsutsugamushi* in the kidney of our i.d. and i.v. inoculated mice, which has been reported in human cases but not other murine animal models (Smadel, Ley et al. 1952; Chung, Lee et al. 2012). Our data regarding the histopathology and cytokines in relevant organs such as lung and liver confirmed that the i.v. and i.d. inoculated mouse models developed in our laboratory are more appropriate for studies representing systemic disseminated and mite-inoculated scrub typhus, respectively (Shelite, Saito et al. 2014; Soong, Wang et al. 2014; Soong, Mendell et al. 2016). The appropriate animal models that can mimic characteristics of human cases of scrub typhus can facilitate the understanding of the pathogenesis of the disease, and the roles and mechanisms of host immunity during the infection.

In summary, we have demonstrated that $CD8^+$ T cells play a critical protective role in the host immune response against *O. tsutsugamushi* infection in our hematogenously disseminated endothelial cell and macrophage target murine model of scrub typhus. However, we also determined that other immune response cells in addition to $CD8^+$ T cells contribute protective effects during scrub typhus. Our animal models and experimental design more appropriately

mimicked the development of disease and host immunity; therefore, our results are an important contribution to the understanding of the host immune responses during *O. tsutsugamushi* infection. Our studies proved that $CD8^+$ T cells together with other immune response cells protect the host from *O. tsutsugamushi* infection by eliminating intracellular organisms from endothelial cells and macrophages. We also further demonstrated that pro-inflammatory cytokines, such as IFN-γ and granzyme B, cannot control and clear the intracellular bacteria in the absence of $CD8^+$ T cells or MHC I. Although there were significantly greater cellular infiltrations in infected WT mice, our histopathologic analysis determined that $CD8^{-/-}$ mice had a higher pathology score indicating greater tissue damage than in WT mice and MHC $I^{-/-}$ mice. Further studies including survival of mice deficient in $CD4^+$ T cells and/or other immune cells, during scrub typhus will determine the protective mechanisms in greater detail. The understanding of these mechanisms will facilitate development of innovative management of *O. tsutsugamushi* infections by targeting stimulation of immunity mediated by $CD8^+$ T cells and other host immune components.

6.2 Future Directions

There are still a lot of unknowns in the mechanisms of host immunity against *O. tsutsugamushi* and the interaction between the host and the intracellular pathogen, including persistence of *O. tsutsugamushi*. Previous studies demonstrated that *Orientia tsutsugamushi* is capable of inducing autophagy and then escaping from cellular autophagy, especially in DCs (Choi,

Cheong et al. 2013; Ko, Choi et al. 2013). T_{reg} cells, IL-10 and TGF are known to contribute greatly to the persistence of intracellular pathogens in the host (Rushbrook, Ward et al. 2005; Easterbrook, Zink et al. 2007; Wingate, McAulay et al. 2009; Johanns, Ertelt et al. 2010; Boettler, Cheng et al. 2012; Ng and Oldstone 2012; Wilson, Kidani et al. 2012). Based on these preliminary data and the findings in our project, we hypothesize that that *O. tsutsugamushi* can induce but evade autophagy to avoid fusion with the lysosome in DCs allowing the bacteria to invade in the host. Once it is inside the mammalian host, *O. tsutsugamushi* can induce immunoregulatory pathways including regulatory $CD4^+$ T cells (T_{reg}) via increasing levels of IL-10 and TGF-β to suppress $CD8^+$ T cell responses in the host.

One of the future directions could be determining the roles of autophagy in DCs during *O. tsutsugamushi* infection. One could compare the changes in bone marrow-derived dendritic cells (BMDCs) after *Orientia* infection among starvation-induced, IFN-γ- or lithium-induced, or autophagy inhibitor wortmannin-treated groups. Starvation is known to induce autophagy (Abdulrahman, Khweek et al. 2011). The autophagy inhibitor wortmannin targets type III PI3K hVPS34 to block the earliest stages of autophagosome formation (Gutierrez, Master et al. 2004). BMDCs could be infected with *O. tsutsugamushi* for 2 h first, and then incubated for 2 h in complete medium in the presence or absence of IFN-γ, lithium or wortmannin, or in starvation medium. One would use Western blot to determine endogenous LC3-I, LC3-II and LAMP2 protein levels, and also employ flow cytometry to analyze the activation status of DCs with or without autophagy

after *Orientia* infection. MHC II, CD40, CD80 and CD86 antibodies are used to stain DCs.

The second study could be determining the roles of autophagy in *O. tsutsugamushi* infection and persistence. One could compare the survival rates, bacterial loads in different tissues, and histopathology in mice after autophagy is induced or inhibited and in control mice after *Orientia* infection. One could use our new animal models with lethal or sublethal doses of *O. tsutsugamushi*. The mice would receive IFN-γ, lithium or wortmannin before *Orientia* infection. Three mice from each group would be sacrificed on each of days 0, 6, 12 and 18, and 3 months post infection for collection of serum, lung, liver, spleen, kidney, and draining lymph nodes. A portion of spleen and draining lymph nodes would be studied by flow cytometric analysis. Part of the collected tissues would be assayed for bacterial loads by real-time PCR, while the remaining tissue would be fixed in 10% formalin for histologic study. We would determine whether altering the autophagy level *in vivo* changes the outcome of *O. tsutsugamushi* infection.

Another direction could be studies of roles of regulatory immunity during *Orientia* persistence. One could determine whether IL-10 deficiency affects *O. tsutsugamushi* infection in the host. One could compare the survival rates, bacterial loads in different tissues, and histopathology between *Orientia*-infected IL-10 knockout mice and wild type control mice. C57BL/6 J- IL-10 knockout mice are available from Jackson Laboratories. Mice would be infected as described in the previous study. As mentioned above, we would use flow cytometry to

examine the intracellular levels of IL-10. Mice would be observed daily for morbidity and mortality. Two mice from each group would be sacrificed on each of days 0, 6, 12 and 18, and at 3 months post infection for collection of serum, lung, liver, spleen, kidney, and brain. A portion of the collected tissues would be assayed for bacterial loads by real-time PCR, while part of the tissue would be used for histologic study. Whether altering the IL-10 level *in vivo* can upregulate $CD8^+$ T cells during persistent infection with *O. tsutsugamushi* would be determined. The study would determine whether IL-10 is a key effector to prevente clearance of *O. tsutsugamushi* during the persistent phase of infection.

In order to understand the role of TGF-β in *O. tsutsugamushi* persistence, we would compare the survival rates, bacterial loads in different tissues, and histopathology between TGF-β-depleted mice and wild type control mice after *Orientia* infection. SB 431542, a TGF-β receptor I blocker, could be used to block the TGF-β pathway. The small molecule would be dissolved in DMSO and administered i.p. every 3 days starting from the day before infection. DMSO would be given as a control (Boettler, Cheng et al. 2012). Flow cytometry could be used to examine the levels of cells with intracytoplasmic TGF-β as before. Mice would be observed daily for morbidity and mortality. Two mice from each group would be sacrificed on days 0, 6, 12 and 18, and 3 months post infection for collection of serum, lung, liver, spleen, kidney, and brain. Portions of collected tissues would be used for bacterial load determinations by real-time PCR, while the remaining part of the tissue could be fixed in 10% formalin for histologic study. We could determine whether changes in TGF-β levels *in vivo* can prevent

the clearance of *O. tsutsugamushi* by $CD8^+$ T cells during persistent infection. The study would determine whether TGF-β has a key role in persistence of *O. tsutsugamushi*.

To understand the role of T_{reg} cells in *O. tsutsugamushi* persistence, we would compare the survival rates, bacterial loads in different tissues, and histopathology between T_{reg} cell-deficient mice and wild type control mice after *Orientia* infection. We would use scurfy mice with $Foxp3^{sf}$ mutation from Jackson Laboratory. Scurfy mice develop an X-linked lymphoproliferative disease due to defective T cell tolerance. Flow cytometry could be used to examine the levels of T_{reg} cells as before. Mice would be observed daily for morbidity and mortality. Two mice from each group would be sacrificed on days 0, 6, 12, 18, and at 3 months post infection for collection of serum, lung, liver, spleen, kidney, and brain. Portions of collected tissues would be used for bacterial load determinations by real-time PCR, while the remaining part of the tissue could be fixed in 10% formalin for histologic study. We would also compare changes in $CD8^+$ T lymphocyte concentrations with T_{reg} cells levels. This study would determine the functions of T_{reg} cells in host immunity against *Orientia* infection.

The next study could be determining the mechanisms of T_{reg} cell-mediated *O. tsutsugamushi* persistence in the host. We could determine whether T_{reg} cell-mediated *Orientia* persistence is CTLA-4 dependent or IL-2 dependent. We would compare the bacterial loads and histopathology in different tissues of CTLA-4-deficient mice, IL-2-deficient mice and wild type mice after *O. tsutsugamushi* infection. We would collect serum and tissue similarly as before at

different time points including both acute and persistent infection phases. Quantitative real-time PCR, histopathology, and immunofluorescence staining would be used to determine the bacterial loads and lesions in the infected mice. We could determine whether the CTLA-4 pathway and/or IL-2 pathway play important roles in T_{reg} cell-induced immunosuppression during persistent *O. tsutsugamushi* infection. We could also compare the changes of T_{reg} cell levels with levels of IL-10 and TGF-β during *Orientia* infection. The study could help to determine the mechanisms of T_{reg} cell-mediated *O. tsutsugamushi* persistence in the host.

Overall, there are still a lot of unknowns in the mechanisms of host immunity against *O. tsutsugamushi* infection. Our studies that determined the critical roles of the $CD8^+$ T cell and its pathways provide a foundation of understanding the complex interactions between the host and the intracellular pathogen. More research is necessary and urgent to comprehensively understand the immune mechanisms, and develop preventive measures including a vaccine.

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Vita

Guang Xu was born on May 31, 1984 in Jiaxing, China to Dongliang Xu and Huiming Zhu. He acquired a Bachelor's degree cum laude in Pharmacy at Zhejiang Chinese Medical University (ZJMU) in 2006. During his study at ZJMU, he researched preclinical pharmacodynamics studies of new anti-osteoporotic, anti-diabetic and anti-microbial drugs under the direction of Dr. Guiyuan Lv. Guang Xu obtained his Master's degree in Pharmacology at Shanghai Jiao Tong University in 2009. He studied the roles of 5-HT_{2C} receptor, NEP and α4β2/α7 nicotine receptors during Alzheimer's disease. Guang Xu worked as clinical trial assistant intern at Health Science Global Business Unit of Oracle Corporation between 2009 and 2010. He was also invited by Shanghai Scientific and Technical Publishers to review and edit The Encyclopedia of Chinese Natural Products. The project was sponsored by the National Natural Science Foundation of China.

Guang enrolled at Graduate School of Biomedical Science, the University of Texas Medical Branch in 2010 and joined the Experimental Pathology Graduate Program in 2011. He was competitively awarded McLaughlin Pre-doctoral Fellowship and several travel awards to national conferences including the annual meetings of the American Society of Tropical Medicine and Hygiene, and the American Society for Rickettsiology. Guang received The Edward S. Reynolds, Jr., MD Experimental Pathology Graduate Scholarship Endowment, and Recognition Award for The Microcirculation in Acute Infections Symposium in 2015; and The Mariann Blum, Ph.D. Endowed Presidential Scholarship, and UTMB Department of Pathology's 22nd Annual Trainee Research Day: The Edward S. Reynolds Award in 2016. In 2015, Guang Xu enrolled in the Master of Public Health Graduate Program at UTMB. He received his MPH degree in August, 2016. He received UTMB Public Health Symposium Poster Award in 2016.

Education

B.S., Pharmacy (cum laude), June 2006, Zhejiang Chinese Medical University, Hangzhou, Zhejiang Province, China

M.S., Pharmacology, March 2009, Shanghai Jiao Tong University, Shanghai, China

M.P.H., Epidemiology, August 2016, The University of Texas Medical Branch, Galveston, Texas, U.S.A.

Publications

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Permanent address: 424 10th St.
Apt 10
Galveston, TX 77550

This dissertation was typed by Guang Xu