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Committee:

<Supervisor's name, Degrees>, Supervisor or Mentor

<Member's Name, Degrees >, or Co-Supervisor, Chair

<Member's Name, Degrees >

<Member's Name, Degrees >

<Member's Name, Degrees >

<Member's Name, Degrees >

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Dean, Graduate School

Autophagy and Inflammation During Rickettsial Infection

by

Jeremy Bechelli, B.A., M.S.

Dissertation

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Dedication

I dedicate my dissertation work to my family and friends who helped me reach this achievement. The constant support of my parents Deborah and Michael Bechelli, and my brother John Bechelli, made my dream of being a microbiologist a reality. I am forever grateful for the sacrifices you made so that I could attend college and pursue my dreams. None of this would be possible without the encouragement of Michael Mazanec. You have been a constant support system from the moment I started this journey and have helped me throughout the entire process. Your belief in me has been a driving force in the completion of this work. I am forever indebted to you for helping my dream happen. For all of your sacrifices -- thank you!

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Autophagy and Inflammation During Rickettsial Infection

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Rickettsiae are obligately intracellular bacteria that replicate in the cytoplasm of host cells. In my dissertation, I have studied the induction of autophagy by rickettsiae as well as the role of autophagy in the pathogenesis of rickettsioses *in vitro* and *in vivo*. I found that autophagy is induced in murine bone marrow macrophages (BMMs) upon infection with *R. australis* and bacteria are specifically targeted by autophagosomes. Using BMMs from *Atg5*$^{flox/flox}$ Lyz-*Cre* and *Atg5*$^{flox/flox}$ mice, I showed that rickettsia-induced autophagy is *Atg5*-dependent. Additionally, deletion of autophagy gene *Atg5* in *Atg5*$^{flox/flox}$ Lyz-*Cre* BMMs significantly reduced bacterial replication compared to *Atg5*$^{flox/flox}$ littermates. Furthermore, rickettsial loads in liver, lung and spleens of autophagy-deficient *Atg5*$^{flox/flox}$ Lyz-*Cre* animals were significantly less compared to *Atg5*$^{flox/flox}$ littermates, suggesting that autophagy in macrophages promoted rickettsial replication *in vivo*. *R. australis*-infected BMMs of *Atg5*$^{flox/flox}$ Lyz-*Cre* mice secreted significantly higher levels of several proinflammatory cytokines

including IL-1α, IL-6 and TNF-α and the inflammasome-dependent IL-1β when infected with rickettsiae, suggesting that autophagy suppresses the induction of these inflammatory cytokines. To investigate the regulation of autophagy induced by rickettsiae, I examined the mammalian target of rapamycin (mTOR), a master regulator of autophagy, during infection of BMMs with *R. australis*. Rickettsiae induced mTOR phosphorylation and activation of the downstream protein P70S6K, a measurement of mTORC1 activation. Furthermore, treatment with mTOR inhibitors, rapamycin and PP242, which induce autophagy, significantly promoted rickettsial replication in BMMs compared to untreated controls. Additionally, I showed for the first time that rickettsiae not only infect, but also replicate in human macrophages *in vitro*. Interestingly, I demonstrated that rickettsiae did not induce autophagy in human endothelial cells at time points up to 48 hours post infection. Inhibition of autophagy by treatment with 3-MA did not affect rickettsial replication in endothelial cells compared to untreated controls. My studies suggest that rickettsiae induce autophagy at the early stage of infection in mouse macrophages to escape the bactericidal effect possibly mediated by inflammatory cytokines including IL-1β. These data also suggest that rickettsiae interact with the autophagy system in cell-type specific mechanisms.

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List of Abbreviations

3- MA	3-Methyladenine
4E-BP1	4E binding protein 1
ACTB	β-actin
AG	Ancestral group
ABSL3	Animal biosafety level-3
ASC	Apoptosis-associated speck-like protein containing a carboxy-terminal CARD
Atg	Autophagy-related genes
AMBRA1	Autophagy/beclin-1 regulator-1
BSL3	Biosafety level 3
BMMs	Bone marrow macrophages
BCV	*Brucella*-containing vacuole
CRP	C-reactive protein
B6	C57BL/6 mouse strain
COX-2	Cyclooxygenase 2
DAMP	Danger-associated molecular pattern
DEPTOR	DEP domain-containing mTOR-interacting protein
DAB	Diaminobenzidine
ER	Endoplasmic reticulum
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fig.	Figure
FKBP12	FK506 binding protein 1A 12kDa
FAK	Focal adhesion kinase
GSBS	Graduate School of Biomedical Sciences
G-CSF	Granulocyte colony-stimulating factor
GAS	Group A Streptococcus
H&E	Hematoxylin and eosin
HO-1	Heme oxygenase -1
HCV	Hepatitis C virus
HCMV	Human cytomegalovirus
HUVECs	Human umbilical vein endothelial cells
IgG	Immunoglobulin
IFN	Interferon
IL-	Interleukin
i.v.	Intravenous
ISF	Israeli spotted fever
LAP	LC3-asociated phagocytosis
LAMP-2	Lysosome-associated membrane protein 2
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
MIP	Macrophage inflammatory protein

MHC-I	Major histocompatibility complex
mSin1	Mammalian stress-activated protein kinase-interacting protein 1
mTOR	Mammalian target of rapamycin
MSF	Mediterranean spotted fever
LC3	Microtubule-associated protein 1 light chain 3
MAP	Mitogen-activated protein
MCP-1	Monocyte chemoattractant protein
MOI	Multiplicity of infection
MyD88	Myeloid differentiation factor 88
NK	Natural killer cell
NO	Nitric oxide
NLR	NOD-like receptor
NF-κB	Nuclear factor κB
NOD	Nucleotide-binding oligomerization domain
ANOVA	One-way analysis of variance
OmpA (B)	Outer membrane protein A or B
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern-recognition receptors
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol-3-kinase
PLD	Phospholipase D
pfu	Plaque forming units
PCR	Polymerase chain reaction
p.i.	Post infection
QTT	Queensland tick typhus
RAW	RAW264.7 (Mouse macrophage cell line)
Ra	*Rickettsia australis*
Rap	Rapamycin
Raptor	Regulatory-associated protein of mTOR
S6RP	Ribosomal protein S6
S6K	S6 ribosomal kinase
SCV	Salmonella-containing vacuole
SFG	Spotted fever group
T_H1	T helper 1
T_H2	T helper 2
mTORC1	Target of rapamycin complex 1
Tel2	Telomere maintenance 2
TLR	Toll-like receptor
TGF-β	Transforming growth factor-β
TRG	Transitional group
TBS	Tris-buffered saline
TSC	Tuberous sclerosis complex
TNF	Tumor necrosis factor
TG	Typhus group
ULK1	Unc-51-like kinase-1

UTMB	University of Texas Medical Branch
WT	Wild type

CHAPTER 1: GENERAL INTRODUCTION

1.1 Rickettsiae and rickettsial diseases

1.1.1 Introduction. The Gram-negative α-proteobacteria in the family Rickettsiaceae, order *Rickettsiales*, genus *Rickettsia* are small pleomorphic obligately intracellular bacteria approximately 0.3 μm in diameter and 1–4 μm long. They are classified into four major groups made up of the typhus group (TG), the ancestral group (AG), the transitional group (TRG), and the spotted fever group (SFG) (Merhej and Raoult, 2011) (Gillespie, Beier, Rahman et al., 2007). While rickettsiae have been identified in several different animal reservoirs (Caspi-Fluger, Inbar, Mozes-Daube et al., 2012) (Perlman, Hunter and Zchori-Fein, 2006), the SFG rickettsiae are among the oldest known vector-borne diseases spread by several genera of ticks (Parola, Paddock and Raoult, 2005).

The Australian subcontinent had experienced four rickettsial agents (*Rickettsia prowazekii*, *R. typhi*, *Orientia tsutsugumushi* and *Coxiella burnetii*) prior to the discovery of Queensland tick typhus caused by *R. australis* (Graves and Stenos, 2009). *Rickettsia australis* is a member of the SFG that was first identified in 1946 in Queensland, Australia and became the fifth rickettsial agent to cause human disease identified in Australia (Graves and Stenos, 2009). World War II soldiers training in the bush of Queensland presented with clinical symptoms similar to murine typhus; fever and vesicular rash covering much of their body (Andrews, Bonnin and Williams, 1946), and the disease was called Queensland tick typhus (QTT). Because *R. australis* is the only currently known *Rickettsia* species to cause a spotted fever illness in an established C57BL/6 mouse model (Feng, Wen and Walker, 1993) (Walker, Olano and Feng, 2001), I

have chosen to utilize *Rickettsia australis* as the model organism of spotted fever illness in my studies.

1.1.2 Virulence factors and pathogenesis of rickettsiae. Two surface-exposed proteins, outer membrane protein A (OmpA) and OmpB, are found on SFG rickettsiae and are known molecules aiding in cell adhesion and invasion (Chan, Riley and Martinez, 2010). OmpB has been shown to bind specifically to Ku70, a DNA-dependent protein kinase, mediating *R. conorii* invasion of nonphagocytic mammalian cells (Martinez, Seveau, Veiga et al., 2005). Additionally, work from the Martinez group has shown that OmpB mediates Ku70-dependent invasion via clathrin- and caveolin-dependent endocytic events, thus facilitating rickettsial invasion (Chan, Cardwell, Mermanas et al., 2009). The process of rickettsial internalization occurs within minutes after contact with host cells, and rickettsiae use hemolysin C and phospholipase D to quickly escape phagosomal membranes, gaining access to the host cytosol (Whitworth, Popov, Yu et al., 2005).

As reviewed by Walker and Ismail (2005), many of the clinical manifestations of SFG rickettsial diseases are attributed to disseminated infection of the vascular endothelium ultimately leading to cerebral edema and non-cardiogenic pulmonary edema. The most obvious pathophysiological effects of rickettsial infection of the endothelium lead to increased vascular permeability, systemic inflammation mediated by proinflammatory cytokines, release of vasoactive mediators promoting coagulation, and sustained leukocyte/endothelium interaction (Walker and Ismail, 2008).

The infection of endothelium with a variety of rickettsial species causes increased secretion of IL-1α, IL-6, IL-8 and MCP-1 (Sporn and Marder, 1996) (Clifton, Rydkina, Huyck et al., 2005) (Bechelli, Smalley, Milhano et al., 2015). IL-6 and IL-8 are specifically correlated with the development of vasculitis

induced by rickettsial infection (Mansueto, Vitale, Cascio et al., 2012). In a paper published in 2015, I show that severe rickettsiosis caused by the Israeli spotted fever strain of *R. conorii* (ISF) is associated with endothelial pro-inflammatory cytokine production (IL-6 and IL-8), while less pathogenic rickettsial infection (*R. massiliae*) of endothelial cells correlates with increased levels of MCP-1 (Bechelli, Smalley, Milhano et al., 2015).

1.1.3 Host control of rickettsiae. Upon activation of Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain (NOD)-like receptors, TLRs transduce signals primarily through the adaptor molecule, MyD88 (Medzhitov, Preston-Hurlburt, Kopp, et al., 1998). The activation of MyD88 leads to the activation of transcription factor nuclear factor κB (NF-κB) and the induction of pro-inflammatory cytokines (Kawai, Adachi, Ogawa et al., 1999) (Schnare, Barton, Holt, et al., 2001). Work from Dr. Sahni's group has shown that rickettsiae activate NF-κB in endothelial cells leading to the production of IL-8 and MCP-1 (Clifton, Rydkina, Freeman et al., 2005). Work from Dr. Walker's group has shown that TLRs are involved in host responses to rickettsial infection. C3H/HeJ mice that are naturally defective in TLR4 signaling succumb to a normally sublethal inoculum of *R. conorii* (Jordan, Woods, Olano, et al., 2008). Additionally, the causative agent of rickettsialpox, *R. akari*, activates macrophages utilizing TLR2 and TLR4 (Quevedo-Diaz, Song, Xiong, et al., 2010).

In my most recently published paper, I show that MyD88$^{-/-}$ mice are more susceptible to infection with *R. conorii* or *R. australis* compared to wild type (WT) mice and that MyD88 deficiency leads to lower levels of secretion of IFN-γ, IL-12, IL-6 and G-CSF. Furthermore, mice lacking MyD88 have significantly fewer inflammatory infiltrates of macrophages and neutrophils in infected tissues

compared to WT mice and an inability to fully activate dendritic cells. We also demonstrated that the secretion of IL-1β by *Rickettsia*-infected BMDCs and in the serum of infected mice are significantly reduced in MyD88$^{-/-}$ mice suggesting that IL-1β is MyD88-dependent *in vitro* and *in vivo* (Bechelli, Smalley, Zhao, et al., 2016). Taken together, we showed that MyD88 signaling mediates instructive signals in DCs, the secretion of IL-1β (likely through NF-κB activation), and the type 1 immune cytokine response critical for the protective inflammatory response against rickettsial infection.

As mentioned, a T_H1-type response is critical to fight rickettsial infection *in vivo*. C3H/HeJ mice are susceptible to infection and succumb to fatal disease because they have a reduced T_H1-type response and a higher percentage of T-regulatory cells in their peripheral lymph nodes. It's believed that this suppresses the proinflammatory responses by increasing IL-10 or transforming growth factor-β (TGF-β), to hamper the adaptive immune response essentially by decreasing the number of effector cells. In WT mice resistant to infection, there is an increase in T_H1- and T_H17-polarized $CD4^+$ and $CD8^+$ T lymphocytes which function as antirickettsial effector cells (Jordan, Woods, Feng, et al., 2007) (Jordan, Woods, Olano, et al., 2008). Additionally, the WT mice have significantly higher serum levels of IFN-γ compared to C3H/HeJ mice, which is important for the induction of nitric oxide (NO) in infected ECs (Jordan, Woods, Soong, et al., 2009), a known rickettsiacidal mechanism (Feng and Walker, 2000).

Fang et al. (2009) have shown using a C3H/HeN *R. conorii* model of MSF that a sublethal inoculum induced protective immunity, and all mice recovered. Their work showed that T cells from lethally infected mice (3×10^5 PFU) produced low levels of IL-2 and IFN-γ, a higher level of IL-10 and suppressed CD4+ T-cell proliferation in response to *R. conorii*. Their study ultimately shows that CD4+ CD25+ T cells produced during spotted fever rickettsiosis are T_H1-

cell-related adaptive T-regulatory cells that are responsible for suppressing the systemic immune response, possibly through IL-10 (Fang, Ismail, Shelite, et al., 2009).

Natural killer (NK) cells are key initiators of the innate immune system due to their ability to eliminate of a variety of pathogens by secreting IFN-γ and perforin/granzyme (Korbel, Finney and Riley, 2004) (Shekhar and Yang, 2015). Work by Fang et al. (2012), has shown that bacterial clearance early during infection of resistant WT animals is associated with elevated IFN-γ produced by CD8 T cells as well as heightened activation and increased NK cell cytotoxicity. They show that NK cell deficiency impairs host resistance to *R. conorii* and promotes the progression of severe pathology in both susceptible and resistant mouse strains, highlighting the critical role of NK cells for rickettsial clearance (Fang, Ismail and Walker, 2012).

In acute human cases of Mediterranean spotted fever (MSF), serum levels of TNF-α, IFN-γ, IL-6 and IL-10 are elevated compared to healthy controls. Additionally, there are reduced circulating $CD4^+$ (helper/inducer T cells), $CD4^+/CD45RO^+$ (memory T cells), as well as $CD4^+/CD45^+$ (naive cells) while there is a significant expansion of monocytes (CD14+/HLA-DR+) (Cillari, Milano, D'Agostino, et al. 1996). The role of IL-10 in human *R. conorii* infection is not clearly defined; both IL-10 and IL-6 can disrupt the critical T_H1-type cytokine response critical to fight rickettsial infections. The thought is that IL-10 may be elevated to minimize tissue injury mediated by the T_H1 cytokine response (Mansueto, Vitale, Cascio et al., 2012).

As discussed previously, IFN-γ is critical for survival during rickettsial infections by activating rickettsiacidal mechanisms. CD4+ and CD8+ T cells, in addition to NK cells, are both plentiful sources of IFN-γ. However, CD8+ T cells, along with rickettsial antigens on class I major histocompatibility complex

(MHC-I), contribute to protective immunity against rickettsiae (Walker, Olano and Feng, 2001). Interestingly, removal of CD4+ T cells has no effect on the course of infection.

In summary, severe rickettsial infections are characterized immunologically as diseases that have transient immune dysregulation, stimulate a type 1 immune response and suppression of CD4+ T cells. The innate immune system including macrophages, DCs and NK cells are important for clearance of the pathogen, and become infected themselves throughout later stages of the illness. There is an acute phase reponse involving the promotion of inflammatory events including the secretion of IL-1α, IL-1β, IL-6, IL-8, IFN-γ, MCP-1, complement proteins, C-reactive protein (CRP), and fibrinogen. Endothelial cell activation induces platelet activation, CD40L secretion, and ultimately leads to the leakiness of the vessels promoting edema and fatal outcomes during severe illness when not controlled by the innate and adaptive immune responses (Mansueto, Vitale, Cascio, et al., 2012).

1.1.4 Tick vectors, ecology and rickettsial transmission. Arthropod vector and vertebrate host distribution correlates with the presence of diseases transmitted by the vector (Estrada-Peña and Jongejan, 1999). An example of this vector/host relationship can be observed in QTT and the distribution of the vector *Ixodes holcyclus, I. tasmani* and *I. cornuatus* in Australia (Parola, Paddock, Socolovschi et al., 2013). The distribution of ticks harboring *R. australis* range from the Torres Strait islands to Wilson's Promontory (Pinn and Sowden, 1998) (Unsworth, Stenos, Faa, et al., 2007). Not surprisingly, QTT cases have been identified occurring down the east coast of Australia, from the tip of the continent and Torres Strait Island to the southeastern corner in Victoria.

However, since nearly all cases are diagnosed serologically and *R. australis* and *R. honei* (causative agent of Flinders Island spotted fever) crossreact, it is difficult to know which species is causing the infection, or the true distribution of the agents (Unsworth, Stenos, Graves, et al., 2007). Queensland tick typhus is considered mild, although serious illness and death have been reported (Graves and Stenos, 2009). Most cases of QTT occur during the winter and spring (Fenner, 1946); the prevalence of nymphal and adult *I. holocyclus* ticks peaks during the similar time periods (Barker and Walker, 2014).

As SFG rickettsiae are transmitted primarily via a tick vector, the role of tick saliva in the dissemination and transmission of rickettsial agents is of considerable interest. The brown dog tick, *Rhipicephalus sanguin*eus, is recognized as the principal vector of Mediterranean spotted fever (Peter, Burgdorfer, Aeschlimann et al., 1984). It is widely believed that rickettsiae are maintained transovarially and transstadially in the tick vectors.

Levin et al. (2009), have shown that *R. conorii* (Malish) was not successfully maintained in ticks from both North American and Mediterranean colonies, and was lost within one generation. They propose that vertebrate reservoirs likely facilitate the persistence of *R. conorii* strains, and that co-feeding transmission of *R. conorii* between ticks may be an important amplification mechanism (Levin, Killmaster, Eremeeva et al., 2009). Interestingly, the same group later showed that dogs are competent reservoirs for *R. conorii* and that tick-infected dogs remained a source of acquisition of rickettsiae by ticks for at least a month postinfection (Levin, Killmaster and Zemtsova, 2012). Examining the effects of *R. rickettsii* on *Dermacentor variabilis*, Schumacher et al. (2015) found that infection caused a slight decrease in fecundity in sympatric vector ticks, but overall there were no obvious deleterious effects observed (Schumacher, Snellgrove, and Levin, 2015).

Work from our laboratory has shown that intradermal inoculation of *R. conorii* into C3H/HeJ mice in the presence of saliva from *Rhipicephalus sanguineus* inhibits the proinflammatory effects of IL-1β and NF-ϰB (Milhano, Saito, Bechelli et al., 2015). Additionally, work from the Macaluso group has shown that intradermal inoculation of *R. parkeri* in the presence of feeding ticks resulted in significantly increased levels of rickettsiae when compared with the group of mice that received intradermal inoculation of *R. parkeri* without tick feeding (Grasperge, Morgan, Paddock et al., 2014).

In a prospective study of human boutonneuse fever cases caused by *R. conorii*, Walker et al. (1988) examined the *taches noires* from patients with a documented diagnosis of boutonneuse fever. *R. conorii* was detected in 14 of 17 samples studied (Walker, Occhino, Tringali et al., 1988). Furthermore, spotted fever group rickettsiae (presumably *R. conorii*), have been observed in the *tache noire*, specifically in the lining of the vessel walls in the reticular dermis; however, the exact cell type first infected during tick feeding remains elusive for rickettsial infections but is an area of considerable interest in the community (Mansueto, Tringali, Di Leo, et al., 1984).

1.1.5 Animal models of spotted fever rickettsiosis. Studies of the immune response to SFG rickettsiae and the pathogenesis of these diseases have been limited by the lack of appropriate animal models. Many early studies relied on the intraperitoneal inoculation of rickettsiae into guinea pigs or inbred mouse strains leading primarily to the infection of cells of the peritoneal lining, or macrophages instead of endothelial cells (Eisemann, Nypaver and Osterman, 1984). Additionally, the lack of reagents for many animal models including guinea pigs and cotton rats compounds the difficulties in studying agents like *R. rickettsii in vivo*. Additionally, many patients that present with SFG infections

are immune competent; thus immunocompromised, newborn, weanling or vitamin-deficient animals do not provide ideal models. Our laboratory and others have developed murine models of SFG rickettsial infections that better mimic the various human pathologies observed during mild and severe forms of the disease.

R. parkeri is a member of the SFG rickettsiae causing American boutonneuse fever (Grasperge, Reif, Morgan et al., 2012) or Tidewater spotted fever in humans (Wright, Sonenshine, Gaff et. al, 2015). First reported as a human pathogen in 2004, *R. parkeri* is believed to be transmitted primarily via *Amblyomma maculatum* and *A. americanum* to a lesser extent, and has been isolated from ticks throughout the southern United States (Wright, Sonenshine, Gaff et. al, 2015) and South America (Lado, Costa, Verdes, et al., 2015). Grasperge et al., (2012) described a murine model of American boutonneuse fever using C3H/HeJ mice that exhibit facial edema, marked splenomegaly and greater concentrations of rickettsial DNA in heart, lung, liver, and spleen compared to A/J, BALB/c, and C3H/HeN mice strains. However, this model demonstrates that only 25% of intradermally inoculated mice developed eschar-like lesions, and only 50% of inoculated mice presented with necrotic lesions at the initial injection site, a prominent clinical sign of *R. parkeri* rickettsiosis in humans. Additionally, the C3H/HeJ model of *R. parkeri* rickettsiosis does not result in fatal disease, but may be useful in understanding the immunology of a non-fatal SFG rickettsiosis (Grasperge, Reif, Morgan et al., 2012). Additional work from this group has provided preliminary data toward an immunocompetent rhesus macaque model using intradermal inoculations of *R. parkeri* during *A. maculatum* feeding. Interestingly, eschars formed at 100% of the inoculation sites, and all four infected macaques developed inflammatory serum cytokine profiles, increased C-reactive protein concentrations, elevated T_H1 (interferon-γ, interleukin-15) and

acute phase inflammatory cytokines (interleukin-6) post-inoculation (Banajee, Embers, Langohr, et al., 2015).

R. akari, the causative agent of rickettsialpox, is uniformly lethal for Mai:(S) and BALB/c mice (Sammons, Kenyon, Hickman, et al., 1977). *R. rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), lacks a suitable animal model for understanding the pathogenesis of disease. The pine vole, *Microtus pinetorum,* demonstrated signs of acute disease with a 45% mortality rate following intraperitoneal infection with 3×10^6 pfu/animal. While the results show that the pine vole is a valuable experimental model for studying infection with *R. rickettsii*, the immunological reagents are almost nonexistent, making research studies difficult. While several models of *R. rickettsii* infection exist, the best model mimicking human disease uses the guinea pig (Price, 1953). However, like issues relating to the other animal models of RMSF, there are few immunological reagents for use in the guinea pig, hindering our understanding of the immune response against this pathogen. Additionally, the cost of housing many animals can be cost prohibitive, as well as the lack of genetic tools for guinea pigs (Padilla-Carlin, McMurray and Hickey, 2008).

Work by Walker et al. (1994) has shown that C3H/HeN mice inoculated intravenously with 2.25×10^5 *R. conorii* (Malish 7 strain) showed disseminated endothelial infection on day 1, became ill with progressive increase in rickettsiae on day 4, and expired on days 5 or 6 due to vascular injury-based meningoencephalitis and interstitial pneumonia. Mice inoculated with 2.25×10^3 pfu became ill on day 5 and recovered by day 10. The C3H/HeN mouse strain provides the best model to date for examining rickettsial disease with endothelial infection and injury, rickettsial clearance by T_H1 mediated immune events, regeneration of endothelium, and repair of the vascular lesions (Walker, Popov, Wen et al., 1994).

The C3H/HeN mouse model of Mediterranean spotted fever shows delayed CD4(+) T-cell activation and suppressed T_H1/T_H2 cell development compared to resistant C57BL/6 mice (Fang, Ismail, Soong et al., 2007). Fang et al. (2009) also demonstrated that CD4(+) CD25(+) T cells generated in the C3H/HeN model are T_H1-cell-related adaptive T-regulatory cells that contribute to suppressing the systemic immune response and leading to a fatal outcome (Fang, Ismail, Shelite, et al., 2009).

The *R. australis* BALB/c mouse model of SFG rickettsiae infection demonstrates a disseminated intracellular endothelial infection that causes lesions in the liver, lung and testis of animals that mimic pathologies observed during human Mediterranean and Rocky Mountain spotted fever (Feng, Wen and Walker, 1993). In this study, mice were injected intravenously with 1 X 10^5 and 2 X 10^6 pfu/mouse, and mice initially showed signs of illness on day 3 post infection as indicated by ruffled fur. The 2 X 10^6 dose was uniformly lethal on day 6 post infection due to overwhelming rickettsial proliferation including infection of the pulmonary interstitium, splenic red pulp, testicular interstitium and hepatic sinusoids, as well as renal and cardiac interstitial capillaries.

Endothelial cells were shown to be a primary target by immunohistology and electron microscopy from day 3-post infection, as well as macrophages and other extravascular cells. As in human cases of SFG infections, the serum levels of key cytokines including IL-1, IL-6, TNF and IFN are increased significantly during the infection, though their kinetics vary throughout the course of illness. Importantly, this model has been the best to characterize the *in vivo* expression of rickettsial invasiveness in an experimental animal model; "this *R. australis* mouse model includes entry of rickettsiae into and growth within target cells, rickettsial escape from these cells and spread to other target cells, and injury to the infected cells and the blood vessels that they comprise" (Feng, Wen and

Walker, 1993). Most importantly, *R. australis* also establishes a dose-dependent fatal infection model in C57BL/6 (B6) mice (Walker, Olano and Feng, 2001), which are the murine background on which most of the gene knockout mice have been developed. Therefore, the mouse model of *R. australis* infection in B6 mice provides a very useful tool for mechanistically studying both immunity to and pathogenesis of rickettsial diseases.

1.1.6 Autophagy and inflammatory regulation in rickettsial infections. Rickettsiae are the first microbes that were described to induce autophagy (Rikihisa, 1984). In 1997, Walker et al. reported that *R. conorii* appeared to be engulfed in autophagosomes and were subsequently destroyed in structures resembling autolysosomes in mouse endothelial cells treated with IFN-γ and TNF-α (Walker, Popov, Crocquet-Valdes, et al., 1997). However, it was undetermined whether rickettsiae induce autophagy without an activation stimulus and whether autophagy can serve as a mechanism of host defense against rickettsial infection in other immune cells, such as macrophages.

Another obligatory intracellular pathogen, *Anaplasma phagocytophilum*, is the causative agent of human granulocytic anaplasmosis. Niu et al. (2008), have shown that stimulating autophagy by treating cells with rapamycin enhances bacterial replication, while the inhibition of autophagy by 3-methyladenine (3-MA) decreases bacterial burden. They further showed that *A. phagocytophilum* not only subverts autophagic killing, but establishes itself in an autophagosome-like compartment to facilitate its growth (Niu, Yamaguchi and Rikihisa, 2008).

Similarly, *Coxiella burnetii*, the agent of Q fever, has enhanced bacterial load and increased replicative vacuole size when autophagy is induced by amino acid deprivation, suggesting the autophagic pathway provides a favorable niche for bacterial growth and survival (Gutierrez, Vázquez, Munafó, et al., 2005).

Orientia tsutsugamushi, the causative agent of scrub typhus, activates autophagy early during infection, 0.5 to 1 hour post infection (up to 4 hours in macrophages), but the intracellular growth of bacteria is unaffected by autophagy as determined by using autophagy-deficient $Atg3^{-/-}$ cells and 3-MA treatment. Additionally, it has been suggested that *O. tsutsugamushi* not only evades autophagic killing, but actively blocks autophagic microbicidal defense mechanisms using unidentified bacterial effector proteins (Ko, Choi, Ha et al., 2013).

Pathophysiological studies of rickettsial infection have focused primarily on endothelial cells, as most rickettsiae show a strong tropism for the vascular endothelium. Of particular interest are vascular inflammation, and the release of vasoactive mediators promoting pro-inflammatory cytokine production. *Rickettsi*a-infected endothelial cells have long been reported to produce several proinflammatory cytokines and chemokines including IL-6, IL-8 and MCP-1 (Bechelli, Smalley, Milhano et al., 2015), as well as the activation of nuclear transcription factor-kappa B (NF-kB) and p38 mitogen-activated protein (MAP) kinase, expression of heme oxygenase 1 (HO-1) and cyclooxygenase 2 (COX-2), and secretion of chemokines and prostaglandins (Rydkina, Turpin and Sahni, 2010). In response to inflammatory stimuli, endothelial cells can be activated to gain new functions such as displaying surface adhesion molecules and the secretion of chemokines leading to the recruitment and activation of circulating leucocytes (Pober, 1988). However, the role of innate immune cells including dendritic cells, macrophages and natural killer cells in modulating the inflammatory profile during rickettsial infection has been less well characterized.

Macrophages and NK cells and are involved in the innate immune response against *Rickettsia* (Walker, Valbuena and Olano, 2003). The role of dendritic cells has been less explored in regards to rickettsial induced infection; however, Fang et al. (2007), have shown that *R. conorii*-infected BMDCs derived

from resistant B6 mice produce higher levels of IL-12p40 than C3H mice, which are susceptible to *R. conorii* infection but produce little IL-12p70, the biologically active form of IL-12, a prominent T_H1 cytokine (Fang, Ismail, Soong et al., 2007). Interestingly, lethal rickettsial infection is associated with lower levels of IFN-γ but higher levels of IL-10 in the spleen compared to sublethal infection, suggesting that lethal infection results in local and systemic suppression of the type 1 T-cell immune response, which was associated with enhanced production of IL-10. Ultimately, Fang et al., (2009) showed that CD4(+) CD25(+) T cells produced during infection with *R. conorii* contribute to suppressing the systemic immune response, possibly by a mechanism involving IL-10 (Fang, Ismail, Shelite et al., 2009).

Understanding the role of autophagy during rickettsial infection has been an intriguing idea for several years but has not been studied in unstimulated cells until now. Additionally, the function of macrophages during rickettsial infection is not well characterized. The role of *Rickettsia*-induced inflammatory pathology has been well-studied and characterized in human and animal models. However, the regulation of inflammation by autophagy during rickettsial infection has never been examined, and experiments to address these key questions on autophagy will be elucidated in chapters 3 and 4 of this dissertation.

1.2 Autophagy and autophagy as a mechanism of host defense

1.2.1 Introduction. The word "autophagy" comes from the Greek language, and means self-eating. Autophagy is an evolutionarily conserved intracellular catabolic pathway that plays a key role in maintaining intracellular homeostasis. Autophagy is essentially a bulk degradation process involved in the clearance of damaged proteins and organelles, protein aggregates and foreign materials. During starvation conditions, autophagy functions to recycle intracellular energy

resources in response to nutrient or amino acid depletion (Chang, Su, Zhang et al., 2015). Additionally, autophagy removes cytotoxic proteins and damaged organelles under stressful conditions (Kroemer, Marino and Levine, 2010). Recent research has shown that autophagy has additional ancillary roles, including the regulation of immune signaling via inflammation, and the clearance of pathogens.

Three major types of autophagy have been described, including macroautophagy, microautophagy, and chaperone-mediated autophagy. The best characterized, and the focus of this dissertation is macroautophagy. A specific form of macroautophagy is termed xenophagy and functions in the removal or degradation of intracellular pathogens (Gomes and Dikic, 2014). Hereafter, xenophagy, a subset of macroautophagy, will be referred to as autophagy and will be the focus of this dissertation.

This review will focus on canonical autophagy signaling (starvation/rapamycin model), which is comprised of AMPK activation or mTOR inhibition resulting in ULK1 (Unc-51-like kinase-1) activation (Kim, Kundu, Viollet, et al., 2011) (**Illustration 1**). Activated ULK1 then phosphorylates Beclin 1 and activates the kinase VPS34, which causes a complex of ULK1 and Beclin 1–VPS34 to localize to an open, double membrane structure known as the phagophore.

Autophagy induced by nutrient deprivation or rapamycin treatment leads to the response of more than 35 autophagy-related genes (Atg). Mammalian ULK1 is the yeast homologue of Atg1. The Atg12-Atg5-Atg16 complex of proteins initiates phagophore elongation and membrane binding, and this protein complex (Atg12-Atg5-Atg16) is essential for effective promotion of Atg8 lipidation and is more frequently known as LC3 in mammalian systems (Walczak and Martens, 2013). The phagophore enlarges and engulfs cytoplasmic material

while forming a double membrane vacuole termed the autophagosome, a distinguishing feature of autophagy visualized by electron microscopy. Microtubule-associated protein 1 light chain 3 (LC3) is normally found in the cytoplasm of cells not undergoing autophagy and is traditionally known as LC3-I. Upon activation, LC3-I is cleaved, lipidated with phosphatidylethanolamine (hereafter known as LC3-II), and inserted into the autophagic membrane upon autophagy initiation (Kabeya, Mizushima, Ueno et al., 2000). Autophagosomes targeted for degradation bind with polyubiquitinated adaptor proteins including SQSTM1 (also known as p62), OPTINEURIN, or NDP52, which then bind to both LC3-II and ubiquitinated molecules present on the autophagosome membrane. SQSTM1 contains an LIR (LC3-interacting region) that is made up of an acidic cluster and hydrophobic residues that interact with multiple sites on LC3 allowing for autophagic degradation of SQSTM1 (Katsuragi, Ichimura and Komatsu, 2015). Additionally, SQSTM1 facilitates the degradation of ubiquitinated cargos through their interaction with ubiquitin tagged proteins on host cell proteins and viral or bacterial proteins (Komatsu, Kageyama and Ichimura, 2012). Because the SQSTM1 protein and SQSTM1 bound polyubiquitinated proteins are degraded in autolysosomes, the quantification of SQSTM1 levels by Western blot is often used as a readout of autophagic degradation, or the complete process of autophagy (Klionsky, Fabio, Abeliovich, et al., 2012). Autophagy is responsible for the degradation of SQSTM1, and thus the inhibition of autophagy is usually accompanied by massive accumulation of SQSTM1. Thus, the correlation between increases in LC3-II and decreases in SQSTM1 are used throughout this dissertation as a measurement of autophagy.

LC3-II is present both inside and outside of autophagosomes, and the amount of LC3-II is associated with the degree of autophagosome formation (Kabeya, Mizushima, Ueno et al., 2000). However, the LC3-II localized to the

outer membrane is re-cleaved by Atg4B after autophagosome formation and recycling. In contrast, LC3-II located on the inner membrane is degraded along with cytoplasmic proteins engulfed by the phagophore by lysosomal proteases (Katsuragi, Ichimura and Komatsu, 2015). Therefore, Atg8/LC3 is the most widely monitored autophagy-related protein and is often measured by monitoring the conversion of LC3-I to LC3-II by Western blotting. To measure autophagy flux, the accumulation of LC3-II can be determined by preventing the autophagosomes from fusing with the lysosome using the inhibitors bafilomycin A_1, chloroquine, or the cysteine protease inhibitor E-64d. The significant consideration of this approach is the difference in the amount of LC3-II in the presence and absence of inhibitors, used to assess the conversion of LC3-II throughout autophagy; if autophagy flux is occurring, the concentration of LC3-II increases in the presence of the inhibitor (Klionsky, Fabio, Abeliovich, et al., 2012).

1.2.2 Mechanisms and regulation of autophagy. Mammalian target of rapamycin (mTOR) is a conserved protein kinase that is evolutionarily conserved and has diverse functions throughout eukaryotes (Wataya-Kaneda, 2015). The deregulation of mTOR has been associated with several human diseases due to its involvement in autophagy, cell growth and senescence, cell viability and immune regulation (Wataya-Kaneda, 2015).

The mammalian target of rapamycin (mTOR) is a large protein of approximately 300 kDa that functions as a serine/threonine kinase and is a member of the phosphatidylinositol-3-kinase (PI3K) -related protein kinase family. mTOR can be found within two distinct protein complexes, target of

rapamycin complex 1 (mTORC1) and mTORC2, which are conserved from yeast to humans (Yerlikaya, Meusburger, Kumari, et al., 2015). Both mTORC1 and mTORC2 are multiprotein complexes composed of mTOR as its central protein as well as Tel2-interacting protein 1 and telomere maintenance 2 (Tel2). However, mTORC1 has regulatory-associated protein of mTOR (Raptor), the DEP domain-containing mTOR-interacting protein (Deptor), mammalian lethal with SEC13 protein 8/G-protein β-subunit-like protein (mLST8/GβL), and protein-rich Akt substrate of 40-kDa (PRAS40). mTORC2 is composed of mTOR, rapamycin-insensitive companion of mTOR (RICTOR), mLST8 and mammalian stress-activated protein kinase-interacting protein 1 (mSin1) (Wataya-Kaneda, 2015). Because mTORC1 is a master regulator of autophagy (Castets and Ruegg, 2013), and mTORC2 is minimally targeted by rapamycin (due to Rictor), this study will focus on mTORC1.

mTORC1 is found in the cytoplasm and can be activated by low levels of cellular amino acids, depletion of growth factors, stress responses, and low ATP levels among other signals. mTORC1 positively regulates protein, ribosome, nucleic acid and lipid synthesis, transcription and nutrient uptake (Perl, 2015). mTORC1 phosphorylates S6 ribosomal kinase (S6K) leading to increased protein synthesis and 4E binding protein 1 (4E-BP1) to promote translation (Dann and Thomas, 2006). Specially, mTORC1 phosphorylates S6K at threonine 389, activating S6K to phosphorylate ribosomal protein S6 (S6RP), a component of the 40S ribosomal subunit. Simultaneously, mTORC1 phosphorylates the translation

inhibitor 4E-BP1, causing the liberation of a key initiation factor of eukaryotic translation initiation, factor eIF4E (Huo, Iadevaia and Proud, 2011).

Growth factors and cytokines can stimulate mTORC1 by activation of the PI3K pathway that in turn activates Akt by phosphorylating threonine 308. Activated Akt then phosphorylates TSC2 (Tuberous sclerosis complex 2), at serine 939, serine 981 and threonine 1462, which aids in the binding of TCS2 to the cytosolic anchoring 14-3-3 protein. This protein interaction disrupts the TSC1-TSC2 complex thus activating mTORC1 (Wataya-Kaneda, 2015). Additionally, the MAP/ERK pathway activates mTORC1 by inhibiting the TSC1-TSC2 pathway, and the Wnt pathway inhibits glycogen synthase kinase 3β, which ultimately leads to the inhibition of TSC1-TSC2 pathway and subsequent activation of mTORC1. Interestingly, the cytokine TNF-α induces mTOR activation via Iκ kinase β, which causes the disassociation of the TSC complex, thus keeping mTORC1 in the inactive state (Salminen, Hyttinen, Kauppinen et al., 2012).

mTORC1 regulates autophagy by directly interacting with the Ulk1 complex in a nutrient-dependent manner. The association between Ulk1 and Raptor causes a multi-site Ulk1-dependent Raptor phosphorylation that directly inhibits mTORC1 activity (Jung, Seo, Otto et al., 2011) or interferes with Raptor-substrate interactions leading to a reduction in phosphorylation of downstream targets (Dunlop, Hunt, Acosta-Jaquez et al., 2011).

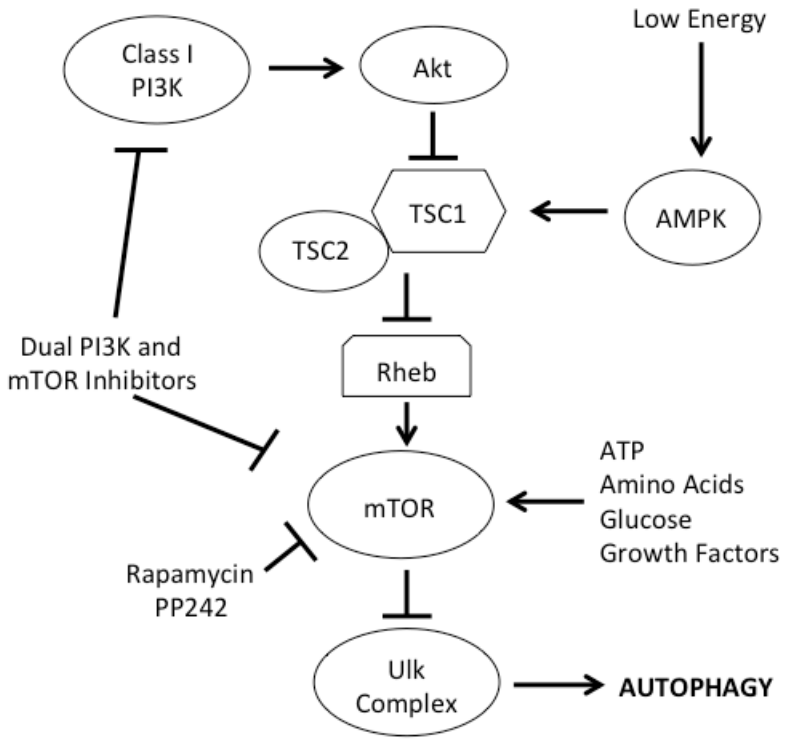

Illustration 1. Autophagy is negatively regulated by mTOR. The mTOR protein complex (mTORC1) is inhibited by rapamycin and PP242, and are used as autophagy inducers. Upstream of mTOR, growth factors suppress the GTPase activity of the TSC complex, leading to full activation of GTP–Rheb, which is the activator of the mTOR–raptor complex. Alternatively, nutrient or energy depletion (amino acids or ATP) inhibits mTOR through the activation of the TSC complex by the activation of the AMP kinase, thereby directly phosphorylating TSC2. It is the ULK complex (comprising ULK1/2–Atg13–FIP200–Atg101) that is ultimately responsible for initiating autophagy.

However, starvation is the classical means of inducing autophagy and remains the best-characterized mechanism of autophagy induction to this date. Phosphorylation sites in Ulk1/2 are dephosphorylated during starvation leading to Ulk1/2 autophosphorylation and the phosphorylation of Atg13 and FIP200 (Alers, Löffler, Paasch et al., 2011). Additionally, Ulk1 phosphorylates

autophagy/beclin-1 regulator-1 (AMBRA1), which leads to the PI3K complex being released, which then translocates to the endoplasmic reticulum (ER) where autophagosomes initiation occurs (Di Bartolomeo, Corazzari, Nazio, et al., 2010). AMBRA1 enhances the interaction with Beclin-1 and its target lipid kinase VPS34, leading to autophagosome nucleation (Fimia, Stoykova, Romagnoli et al., 2007). After the separation of mTORC1 from unc-51–like kinase 1/autophagy-related gene 1 (ATG1), ATG1 phosphorylates beclin-1 and binds to membranes to start autophagosome formation (Nazio, Strappazzon, Antonioli, et al., 2013). Decreases in glucose and amino acid levels lead to the induction of autophagy due to the reduced mTORC1 activity. When amino acids are plentiful, mTORC1 is targeted to the lysosomal membrane and activated by Rheb to inhibit autophagy (Carroll, Maetzel, Maddocks, et al., 2016).

1.2.3 Autophagy in antimicrobial immunity. In the context of infectious diseases, autophagy can act as a mechanism of host defense, a function of the innate immune system termed xenophagy as discussed previously. Autophagy induction and the innate immune system are linked via the TRIF-dependent, myeloid differentiation factor 88 (MyD88)-independent TLR4 signaling pathway (Xu, Jagannath, Liu, et al., 2007). Additionally, TLR3 and TLR7 have been shown to stimulate autophagy in murine macrophages in a MyD88-dependent manner (Delgado, Elmaoued, Davis et al., 2008). Engagement with TLRs activates NF-κB to induce the production of several proinflammatory cytokines including inflammasome-associated pro-IL-1β and pro-IL-18. Interestingly, NLRs are inflammasome components present in the cytoplasm that have been

shown to negatively regulate autophagy (See section 1.3 for a more detailed description of inflammasomes).

Salmonella enterica subsp. *enterica* serovar Typhimurium (*S.* Typhimurium), is a well studied bacterial pathogen targeted by autophagy, which is essential for controlling the infection in both *Caenorhabditis elegans* and *Dictyostelium discoideum* model systems (Jai, Thomas, Akbar et al., 2009). *Salmonella* Typhimurium invades epithelial cells by using its two type III secretion systems and resides in the Salmonella-containing vacuole (SCV) (Brumell, Steele-Mortimer and Finlay, 1999). However, a small portion of bacteria escape the SCV membrane early after infection and enter the cytosol where they are targeted by the autophagy system, which protects the cytosol from bacterial infection (Birmingham, Smith, Bakowski, et al., 2006). While ubiquitylated proteins are known to be involved in targeting the bacteria to autophagosomes (Zheng, Shahnazari, Brech et al., 2009), the ubiquitylated host proteins and bacterial effectors involved are currently unknown.

Mycobacterium tuberculosis is another bacterium targeted by autophagy inside of damaged vacuoles of macrophages. *Mycobacterium tuberculosis* blocks phagosome maturation and replicates in the phagosome. Approximately 30% of bacteria are targeted by LC3 and ATG12 at 4 hours post-infection. This binding interaction occurs by the recognition of bacterial DNA by the STING-dependent cytosolic pathway to tag the bacteria with ubiquitin, ultimately delivering the pathogen to autophagosomes using SQSTM1 and the DNA-responsive kinase TBK1 (Watson, Manzanillo and Cox, 2012). The ubiquitin ligase parkin has been shown to be involved in the ubiquitin-mediated autophagy of *M. tuberculosis* (Manzanillo, Ayres, Watson et al., 2013).

Group A Streptococcus (GAS) is a cytosolic bacterium that is selectively targeted by autophagy. GAS uses the toxin streptolysin O to escape from

endosomes to the cytoplasm where they become encased in LC3 proteins, GAS-containing autophagosome-like vacuoles (GcAVs), and are eventually destroyed through autophagy (Nakagawa, Amano, Mizushima et al., 2004). Interestingly, streptolysin O is required for autophagy to recognize the bacteria, evidenced by streptolysin O mutants escaping the LC3-positive autophagic structures and having enhanced survival over wild-type bacteria (Sakurai, Maruyama, Funao et al., 2010).

However, autophagy is not always detrimental to the invading pathogen. *Coxiella burnetii*, the etiologic agent of Q fever, is an obligate intracellular bacterium similar to rickettsiae. The bacterium enters the host cell via classical phagocytosis and replicates in acidic vacuoles that resemble lysosomes and not only survives, but actively replicates inside of these vacuoles with autophagosomal features (Beron, Gutierrez, Rabinovitch et al., 2002). While autophagy is not up-regulated by *Coxiella* infection, the autophagosomal features present include LC3 richly present on and inside of replicating *Coxiella*-containing vacuoles, which are likely recruited from existing autophagosomes (Moffatt, Newton and Newton, 2015). Additionally, when ATG5 is silenced, *C. burnetii* bacterial burden is not altered suggesting that canonical autophagy is not required for infection.

1.2.4 Evasion of autophagy. Autophagy functions in the cytoplasm to sequester and remove adverse cytosolic contents including organelles, protein aggregates and certain pathogens. However, many pathogens including both bacteria and viruses reside in the cytoplasm making their evasion of autophagy, remodeling of autophagic compartments, or manipulation of the autophagic machinery critical for their survival. The ability to manipulate the host cell's

autophagic processes to aid pathogen survival is critical for our understanding of pathogenesis because it functions as a virulence mechanism.

Francisella tularensis is the causative agent of the tularemia, also known as rabbit fever. *F. tularensis* bacteria evade autophagy in macrophages to ensure survival and proliferation (Chong, Wehrly, Child et al., 2012). The surface polysaccharide of *Francisella tularensis*, O-antigen, has been shown to provide a protective role against autophagy as the O-antigen deletion mutants were ubiquitinated, and colocalized with SQSTM1 and LC3 prior to cytosolic clearance (Case, Chong, Wehrly et al., 2014). Interestingly, ATG5 is not required for *F. tularensis* intracellular growth; however, ATG5-independent autophagy causes the degradation of cellular components which results in the generation of nutrients that *F. tularensis* utilizes for bacterial replication (Steele, Brunton, Ziehr, et al., 2013)

Burkolderia pseudomallei is the causative agent of melioidosis, a severe disease causing a mortality rate of approximately 40% in Southeast Asia and Northern Australia (Wiersinga, Currie and Peacock, 2012). Interestingly, *B. pseudomallei* has been shown to escape LC3-asociated phagocytosis (LAP), and evade autophagy; however, the exact mechanisms leading to evasion are still under investigation. There is some support to suggest that *B. pseudomallei* blocks the initiation of autophagosome formation by using the bacterial protein BopA (Devenish and Lai, 2014). Interestingly, BopA is a homologue of *Shigella* IcsB (Stevens, Haque, Atkins et al., 2004), which is an effector that has been shown to be responsible for autophagy evasion by preventing IcsA (the *Shigella* autophagy inducing protein) from associating with ATG5 (Ogawa, Yoshimori, Suzuki et al., 2005).

Staphylococcus aureus is a Gram-positive bacterium that typically causes acute food-borne illnesses, skin infections, meningitis and septic and toxic shock.

Following internalization, *S. aureus* becomes enclosed inside endosomal vacuoles, where it quickly escapes and enters the cytosol thus avoiding the phagolysosome pathway (Kubica Guzik, Koziel et al., 2008). However, some bacteria persist in a phagophore structure, and bacteria actually replicate within LAMP-1 and LC3-positive endosomal vacuoles, although the exact mechanism has yet to be fully identified (Schnaith, Kashkar, Leggio et al., 2007). During autophagy inhibition via wortmannin treatment or cells deficient in ATG5, intracellular replication of *S. aureus* is severely inhibited. These data suggest that autophagic components provide an advantageous niche to facilitate *S. aureus* replication.

1.2.5 Bacteria exploit autophagy for survival. While autophagy is typically thought of as a means of host defense, it can facilitate the replication of bacterial pathogens. *Brucella* is a group of Gram-negative facultative intracellular pathogens of veterinary importance. However, many species infect humans as incidental hosts, and are classified as category B pathogens with potential for bioterrorism (de Figueiredo, Ficht, Rice-Ficht, et al., 2015). Inside phagocytic cells, *Brucella* reside in a *Brucella*-containing vacuole (BCV), a nutrient lacking microenvironment called the brucellosome (Kohler, Foulongne, Ouahrani-Bettache, et al., 2002). Similar to rickettsiae, *Brucella* inhibits apoptosis and prevents DC maturation (Billard, Dornand and Gross, 2007). BCVs gradually acquire autophagy proteins ULK1, Beclin 1, Atg14L and PI3-kinase activity but are independent of Atg5, Atg7, Atg16L1 and LC3-II (Starr, Child, Wehrly, et al., 2012). Starr et al. (2012), shows that BCVs with autophagic features are essential for bactieral replication, cell-to-cell spread, subverting host clearance and promoting infection (Starr, Child, Wehrly, et al., 2012).

Coxiella burnetii is an additional bacterium that subverts autophagy but replicates inside of a vacuole, using the process as a means of promoting its own replication and survival (Campoy and Colombo, 2009). LC3 is quickly recruited to the *Coxiella*-containing vacuole. However, there is a delay of fusion with the bacteria-containing vacuole and lysosome that enables the bacteria to replicate. When rapamycin is added to induce autophagy, the number of infected cells increases, the size of the vacuole grows significantly, and the bacterial burden increases significantly compared to untreated cells highlighting the advantage of autophagy for *Coxiella* replication (Mostowy and Cossart, 2012). Additionally, *Yersinia pseudotuberculosis* bacterial burden increases in the presence of rapamycin, while 3-methyladenine (3-MA) treatment to prevent autophagy allows the vacuole to become acidified, killing the bacteria inside (Moreau, Lacas-Gervais, Fujita, et al., 2010).

Staphylococcus aureus, the causative agent of several human infections, induces an autophagic response that facilitates bacterial replication (Mestre, Fader, Sola et al., 2010). As described by Schnaith et al. (2007), *S. aureus* inhibits the fusion of phagosomes with lysosomes and then permeabilizes phagosomes using secreted toxins, damaging the phagosome. The damaged phagosomes containing *S. aureus* become substrates for the autophagic process allowing the bacteria to eventually escape into the host cell cytoplasm where replication can occur. Interestingly, rapamycin treatment results in significantly elevated bacterial burden compared to untreated cells, and the inhibition of autophagy using wortmann significantly reduces bacterial replication (Schnaith, Kashkar, Leggio, et al., 2007).

1.3 Inflammation and IL-1β/IL-18

1.3.1 Introduction. The innate immune system is an evolutionarily conserved system responsible for targeting pathogenic microbes early during the infection. The pattern-recognition receptors (PRRs) expressed primarily on monocytes, macrophages, neutrophils, and dendritic cells (DCs) recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Host-derived endogenous molecules released by damaged and dead cells after trauma or infection can activate PRRs including the TLRs. PRRs are generally classified as TLRs, C-type lectins (CTLs), and galectins and recently, the inflammasome (Shao, Xu, Han et al., 2015).

1.3.2 Inflammasome-mediated production of IL-1β/IL-18. Inflammasomes are cytoplasmic signaling complexes that detect pathogenic microorganisms and cellular stressors that activate the highly pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. Inflammasomes consist of a protein complex consisting of an inflammasome sensor molecule, the adaptor protein ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD) and caspase 1. Sensor molecules contain a NOD-like receptor (NLR) such as NLRP1 (NOD-, LRR- and pyrin domain-containing 1), NLRP6, NLRP7, NLRP12 or NLRC4 with the best-characterized being NLRP3 (Latz, Xiao, and Stutz, 2013). Using its CARD (caspase activation and recruitment domain), ASC facilitates the self binding of pro-caspase 1 monomers that initiate caspase 1 self- cleavage and the formation of the active heterotetrameric caspase-1 protein (Karasawa, Kawashima, Usui et al., 2015). Activated caspase-1 then cleaves several host proteins including pro-IL-1β and pro-IL-18 rendering the biologically active proinflammatory cytokines IL-1β and IL-18 (Gu, Kuida, Tsutsui et al., 1997) (Illustration 2).

Interestingly, IL-1β is a "leaderless" protein meaning that it lacks the N-terminal secretory signal sequence typically used to target secreted proteins to the endoplasmic reticulum and their translocation into the lumen (Schatz and Dobberstein, 1996). There are 6 major mechanisms for the secretion of IL-1β; 1) active secretion through an unknown mechanism utilizing an ER/golgi-independent pathway, 2) secretion via secretory lysosomes, 3) microvesicle shedding from the plasma membrane, 4) secretion via exosomes, 5) pyroptotic cell death and 6) secretory autophagy (Piccioli and Rubartelli, 2013). Interestingly, pyroptosis is an inflammatory lytic, programmed cell death pathway initiated by either caspase-1 or caspase-11 that may be involved in rickettsia-induced cell death of endothelial cells (Bechelli, Smalley, Milhano et al., 2015).

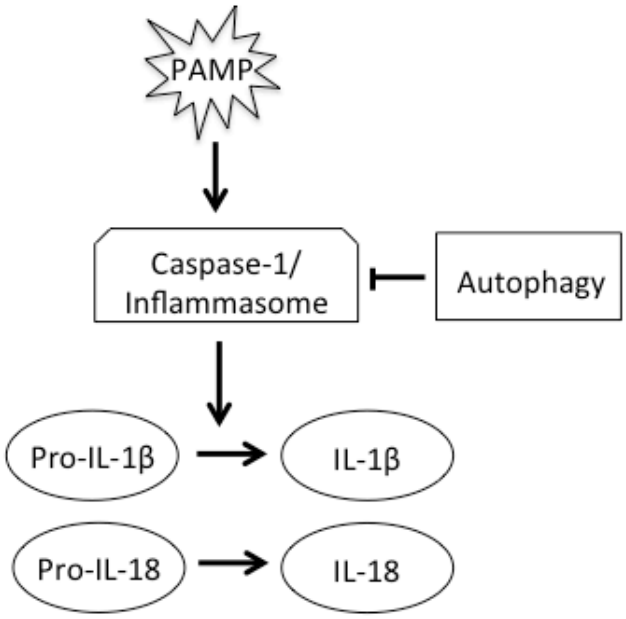

Illustration 2. Autophagy regulates inflammasome-mediated production of proinflammatory cytokines. This process is mediated by inflammasome activation, which activates caspase-1, leading to the processing of pro IL-1β/IL-18 into the biologically active forms. Autophagosomes are unable to degrade excess inflammasome components leading to unregulated inflammation in autophagy deficient cells.

1.3.3 Autophagy-regulated inflammation. It has been suggested that autophagy acts as a regulatory mechanism for the control of inappropriate or dangerous inflammatory immune responses (Harris, 2013). As described by Harris et al. (2011), upon stimulation with TLR3 or TLR4 ligands, autophagy inhibition leads to the increased release of IL-1β, IL-18 and IL-1α. Moreover, autophagy induction by rapamycin inhibits IL-1β release in response to LPS. Interestingly, the processing and secretion of IL-1β during the inhibition of autophagy is NLRP3- and TRIF-dependent. Upon TLR4 stimulation, but in the absence of inflammasome activation, pro-IL-1β is degraded by autophagosomes. These data suggest that autophagy contributes to the regulation of the NLRP3 inflammasome (Harris, Hartman, Roche, et al., 2011).

Work by Shi et al. (2012), further expanded the knowledge of inflammasome regulation by autophagy using 3-MA and rapamycin to augment cellular autophagy in murine macrophages. Their work has shown that activation of the NLRP3 and AIM2 inflammasomes induces autophagy. They show that 3-MA (autophagy inhibitor) increases inflammasome activation, while autophagy induction by rapamycin or starvation reduces inflammasome activation. More importantly, AIM2, NLRP3, and ASC inflammasome constituents co-localize with LC3 and LAMP-1 suggesting that inflammasomes are destroyed within the autophagosomes (Shi, Shenderov, Huang et al., 2012). Their data suggest that

autophagy reduces extraneous inflammation by the removal of activated inflammasomes.

Several cytokines are known to regulate autophagy including IL-10, IL-4, IL-13, IL-23, IFN-γ, TNF-α, IL-1α and IL-1β (Harris, 2013). These data further suggest that autophagy may function in a negative feedback loop to control the secretion of inflammatory cytokines. Walker et al. (1997) have shown that murine endothelial cells infected with *R. conorii* are present in autophagosomal structures when cells are stimulated with IFN-γ and TNF-α. Their data show that endothelial cells stimulated with IFN-γ and TNF-α induce nitric oxide-dependent rickettsicidal effects and also suggest that cytokine-stimulated endothelial cells kill rickettsiae via an autophagic mechanism (Walker, Popov, Crocquet-Valdes, et al., 1997).

1.4 Research objectives

1.4.1 Introduction. My ***long-term goal*** is to facilitate vaccine development and new therapeutic strategies by targeting autophagy and to have a better understanding of the regulatory mechanisms of autophagy as well as how autophagy interacts with the host immune response. The ***objective*** of this proposal is to determine the role of autophagy in host defense against rickettsiae and to determine the mechanism of autophagy and inflammation regulation during rickettsial infections. My ***central hypothesis*** underlying this proposal is that rickettsiae modulate the autophagy pathway to promote a favorable environment for rickettsial survival. Specifically, I hypothesize that rickettsiae activate mTORC1 to inhibit autophagy initiation and that autophagy functions as a key factor involved in the host cell's proinflammatory innate immune responses by degrading the inflammasome complex to prevent excessive inflammation in

response to infection. Understanding the role of autophagy during rickettsial infection is critical, not only for enhancing our knowledge of rickettsial biology, but also for augmenting current treatment strategies and future vaccine candidates for fatal rickettsial diseases.

1.4.2 Objective 1. Determine the role of autophagy during *R. australis* infection of murine macrophages. The *working hypothesis* is that *R. australis* induces autophagy in murine macrophages and human endothelial cells without an activation stimulus, and autophagy serves as a means of host defense against rickettsiae. The *rationale* is that Walker et al. (1997) reported that by electron microscopy *R. conorii* appeared to be engulfed in autophagosomes and destroyed in structures resembling autolysosomes in mouse endothelial cells treated with IFN-γ and TNF-α. In my studies, autophagy was measured by Western blotting of LC3 and SQSTM1 levels, electron microscopy and confocal microscopy of LC3 puncta formation. I inhibited autophagy using 3-MA and LY294002 and assessed the genetic inhibition of autophagy using BMMs from *Atg5*$^{flox/flox}$ Lyz-*Cre* mice and evaluated autophagy. Additionally, I evaluated the role of autophagy in human endothelial cells using western blotting, determination of bacterial burden and electron microscopy.

1.4.3 Objective 2. Determine the mechanisms involved in *R. australis* evasion of autophagy. The *working hypothesis* is that *R. australis* utilizes mTORC1 activation as a counter-strategy to evade autophagy within host macrophages. The *rationale* is that during infection with *R. australis*, the level of autophagy flux decreased to an undetectable level at 24 hours post infection (p.i.), the same time rickettsiae have been shown to induce the activation of mTORC1, a potent negative regulator of autophagy (Thomasson et al., 2013). I measured mTORC1

activation in macrophages infected with *R. australis* using Western blotting. Additionally, I measured autophagy induction and bacterial burden in the presence of the mTORC1 inhibitor rapamycin to assess the effects of mTORC1 inhibition on bacterial replication. Finally, I examined the ability for *R. australis* to infect human monocyte-derived macrophages by qPCR, light microscopy and confocal microscopy.

1.4.4 Objective 3. Identify the mechanisms by which autophagy regulates the inflammatory response during rickettsial infection. The *working hypothesis* is that autophagy negatively regulates the inflammatory response including caspase-1-dependent IL-1β secretion during rickettsial infection. The *rationale* for this aim is that IL-1β is an inflammatory cytokine associated with the inflammasome, and IL-1β-driven inflammation plays a critical role in antimicrobial immunity. Inflammasome activation has been reported due to the inability of autophagy-deficient cells to clear damaged mitochondria induced by the production of excessive reactive oxygen species and excessive pro-IL-1β (Nakahira et al., 2010). Negative regulation of inflammasome by autophagy has been reported previously (Chen and Sun, 2013) (Nakahira et al., 2010). I infected $Atg5^{flox/flox}$ Lyz-*Cre* (autophagy-deficient), and $Atg5^{flox/flox}$ (control) mice and BMMs with *R. australis* and measured IL-1β and IL-18 by ELISA, and other inflammatory cytokines using Bio-Plex analysis. Additionally, I measured bacterial burden in autophagy-competent and -deficient cells/animals, measured pathological foci in the organs of infected animals, and determined the cellular influx/efflux of CD3+ T cells and macrophages in infected animals at 4 days p.i..

Chapter 2

Materials and Methods

2.1 Rickettsiae and mice

For *in vitro* infection, *R. australis* (Cutlack strain) was cultivated in Vero cells and purified by either Renografin density gradient centrifugation or using a Renografin "cushion" as previously described (Hanson, Wisseman, Waddell et al., 1981) (Fang, Ismail, Shelite, et al., 2009) (Ammerman, Beier-Sexton and Azad, 2008). The concentration of rickettsiae propagated in cell culture was determined by plaque assay after purification as described in previous studies (Fang, Ismail, Shelite, et al., 2009). The rickettsial stock was stored at -80°C until use, and all the experiments described in this study were performed in a certified biosafety level 3 (BSL3) laboratory at UTMB. *Atg*5 $^{flox/flox}$ Lyz-*Cre* (autophagy deficient), and *Atg*5 $^{flox/flox}$ (control) mice were kindly provided by Dr. Noboru Mizushima at The University of Tokyo and Dr. Herbert Virgin IV at Washington University School of Medicine in St. Louis (Zhao, Thackray, Miller et al., 2007) (Hara, Nakamura, Matsui et al., 2006) (Zhao, Fux, Goodwin et al., 2008). Bone marrow from *Atg*16L1 $^{flox/flox}$ (control), *Atg*16L1 $^{flox/flox}$ Lyz-*Cre* (autophagy deficient) mice were kindly provided by Dr. Seungmin Hwang at the University of Chicago in Chicago, Illinois. For *in vivo* experiments, mice were maintained and manipulated in an animal biosafety level-3 (ABSL3) facility at UTMB and inoculated intravenously (i.v.) through the tail vein with *R. australis* at the doses indicated. *R. australis* used in animal studies was grown in embryonated chicken yolk sac egg culture as described previously (Fang, Ismail and Walker, 2012). Animals were monitored daily for signs of illness and sacrificed at times indicated. The UTMB Animal Care and Use Committee approved all experiments

and procedures, and experiments in mice were performed according to the guidelines of the Guide for the Care and Use of Laboratory Animals.

2.2 Generation of bone marrow-derived macrophages

Generation of primary bone marrow-derived macrophages (BMMs) from 6-8 week old female wild type (WT) B6 mice, *Atg*5 $^{flox/flox}$ mice and *Atg*5 $^{flox/flox}$ Lyz-*Cre* mice, *Atg*16L1 $^{flox/flox}$ mice and *Atg*16L1 $^{flox/flox}$ Lyz-*Cre* mice, was performed as previously described (Zhao, Thackray, Miller et al., 2007) (Hara, Nakamura, Matsui et al., 2006) (Zhao, Fux, Goodwin et al., 2008) (Zhang, Goncalves and Mosser, 2008). Briefly, after femurs and tibias were dissected, bone marrows were flushed, and cells were cultivated in low-endotoxin DMEM containing 10% (v/v) fetal bovine serum (FBS; Hyclone, Utah, SV30160) supplemented with either 20% supernatant from L929 cell culture or recombinant M-CSF (PeproTech, NJ, 315-02) at 37℃ in 5% CO_2. On day 6 of culture, cells were harvested and characterized by flow cytometric analysis after staining with anti-F4/80 and CD11b antibodies. Approximately 90% of these cells were F4/80 (+) and CD11b (+). These cells were plated in 24-well plates at a density of 1×10^6 cells/well in RPMI 1640 containing 10% fetal bovine serum and used for experiments within 24 hrs.

2.3 Macrophage *in vitro* infections

Primary mouse BMMs and RAW264.7 cells were infected with *R. australis* at a multiplicity of infection (MOI) of 2:1 or 5:1. To synchronize bacterial internalization, rickettsiae were centrifuged onto the cells at 560 × g for 5 min. Cells were continuously incubated at 37°C in 5% CO_2. At 1 h, 3 h and 24 h p.i., cells were collected and washed for further experiments. Uninfected macrophages served as negative controls.

2.4 Immunofluorescence microscopy of autophagy elements

For immunofluorescence detection of LC3 puncta and localization of LC3 and rickettsiae, cells were seeded on glass coverslips in 12-well plates one day before infection. At the indicated time points, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton-X in PBS for 20 min and blocked with 3% BSA in PBS for 30 min. Samples were incubated with rabbit polyclonal antibodies directed against *R. australis*, goat anti-mouse LC3 (Santa Cruz, CA, sc-16755), rat monoclonal antibody against lysosome- associated membrane protein-2 (LAMP-2, Santa Cruz, CA, sc-19991) followed by appropriate secondary antibodies including Alexa Fluor 488-conjugated chicken anti-goat IgG, Alexa Fluor 647 conjugated donkey anti-rabbit IgG, Alexa Fluor 568- conjugated goat anti-rat IgG antibodies (Life Technologies, NY, A21467, A31573, A11077). The anti-LC3 Ab preferentially labels autophagosome-associated LC3-II (Deuretzbacher, Czymmeck, Reimer et al., 2009). Autolysosomes were labeled as LC3 (+) and LAMP-2 (+). Nuclei were stained with DAPI in ProLong® Gold Antifade Mountant (Life Technology, NY, P-36931). Coverslips were sealed with nail polish and visualized by confocal microscopy (Olympus Fluoview 1000) using FV10-ASW software (Olympus, PA). The percentage of cells containing LC3 (+) puncta, number of LC3 puncta per 10 cells, percentage of rickettsiae co-localized with LC3 (+) puncta and percentage of rickettsiae colocalized with autolysosomes were calculated in more than 300 cells or 300 bacteria from at least 12 randomly selected images using Image J software as previously described (Potze, Mullauer, Colak et al., 2014).

2.5 Transmission electron microscopy

For examination of infected BMMs by electron microscopy, BMMs were harvested at indicated time points p.i. and immersed in Ito's fixative (2.5%

formaldehyde, 0.1% glutaraldehyde, 0.03% $CaCl_2$, and 0.03% trinitrophenol in 0.05 M cacodylate buffer, pH 7.3) at room temperature for 1 h and then overnight at 4°C. After washing, samples were processed further as described previously (Fang, Ismail, Soong, et al., 2007). Ultrathin sections were cut on a Leica EMUC7 ultramicrotome (Leica Microsysytems, Inc., Bannockburn, IL) and examined in a Phillips 201 transmission electron microscope (Phillips Electron Optics, Eindhoven, The Netherlands) at 60 kV.

2.6 Western immunoblotting of components of autophagy activity and mTOR signaling

For assessment of the cellular LC3 levels and the conversion of naïve LC3-I to lipidated LC3-II, cells were lysed with RIPA lysis buffer (EMD Millipore, MA, 20-188) supplemented with protease inhibitors (Roche, IN). The soluble part of cell lysates was isolated by centrifugation and separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and probed with a rabbit polyclonal Ab directed against LC3B (Cell Signaling Technology, MA, 4108). Immunoreactive bands were visualized using an appropriate secondary Ab and ECL detection reagents (Thermo scientific, Pierce, IL, 32106). Equal protein loading of the gels was controlled by detecting ACTB with mouse mAb (Sigma, MO, A1978) in the cellular lysates. The detection of SQSTM1 is indicative of the autophagic process as described previously (Zeng and Carlin, 2013). SQSTM1-specific immunoblotting was performed as described above using antibodies directed against SQSTM1 (Cell Signaling Technology, MA, 5114). The amounts of LC3-II and SQSTM1 relative to ACTB and the ratio of LC3-II to SQSTM1 were calculated after densitometry using Image J software (Colasanti, Vomero, Alessandri et al., 2014). Antibodies directed against phospho-mTOR (Ser2448) (D9C2) XP® Rabbit mAb and phospho-p70 S6 Kinase (Thr389) (1A5) Mouse

mAb (Cell Signaling Technology, MA) were used for the analysis of the mTOR signaling pathway.

2.7 Quantification of bacterial loads by quantitative real-time PCR

To determine the number of intracellular rickettsiae following in vitro infection, *R. australis*-infected cells were collected at indicated time points after checking the viability of infected cells. Extracellular rickettsiae were eliminated after cells were washed with PBS three times. DNA was extracted from these cells using QIAGEN DNA extraction kit (Valencia, CA, 69506) as described previously (Fang, Ismail, Soong et al., 2007). Quantitative real-time PCR was performed using the iCycler from Bio-Rad (Hercules, CA). Rickettsial loads were determined by real-time PCR with primers and Taqman probes for the *Rickettsia*-specific citrate synthase (CS) gene (*gltA*) and mouse *Gapdh* as described in our previous studies (*Gapdh* forward, CAACTACATGGTCTACATGTTC; *Gapdh* reverse, CTCGCTCCTGGAAGATG; *Gapdh* probe, CGGCACAGTCAAGGCCGAGAATGGGAAGC; *gltA* forward, GAGAGAAAATTATATCCAAATGTTGAT; *gltA* reverse, AGGGTCTTCGTGCATTTCTT; *gltA* probe, CATTGTGCCATCCAGCCTACGGT) (Xin, Shelite, Gong et al., 2012). The *gltA* probe was labeled with FAM and Black Hole Quencher 1, and the probe for *Gapdh* was labeled with FAM and Black Hole Quencher 2 (Biosearch Technologies, CA). Two-step cycle parameters (95°C and 60°C) were used. The results were normalized to *Gapdh* or ng of genomic DNA in the same sample and expressed as CS gene copy number per 1000 *Gapdh* or per ng of genomic DNA.

2.8 Endothelial cell culture and infection

Normal pooled human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD), and cultured in EGMTM-2 medium supplemented with components of HUVEC BulletKit™. Cells were cultured without the addition of antibiotics or antifungal drugs and maintained in culture according to the manufacturer's instructions. Briefly, HUVECs were cultured on porcine gelatin-coated plates (0.1%) at 37°C with 5% CO_2 until 90-95% confluent. The human microvascular endothelial cell line HMEC-1 (Ades, Candal, Swerlick et al., 1992) was cultured in MCDB 131 medium (Gibco, Grand Island, NY) supplemented with L-glutamine (10 mmol/L; Gibco), mouse epidermal growth factor (10 ng/mL; BD Bioscience, San Jose, CA), hydrocortisone (1 μg/mL; Sigma Aldrich, St. Louis, MO), and 10% heat-inactivated fetal bovine serum. Endothelial cells were infected with *R. australis* at MOI of 6:1. At 1 h, 3 h, and 24 h p.i., cell lysates were collected for assessment of autophagy induction by the expression levels of LC3 and SQSTM1 as described above.

2.9 Isolation and differentiation of human monocyte-derived macrophages

Deidentified peripheral blood was obtained from healthy donors and provided by the UTMB blood bank. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood using Ficoll density centrifugation, and the light-density layer was removed, washed, and resuspended in phosphate-buffered saline (PBS). CD14+ cells were isolated using Miltenyi Biotec MiniMACS magnetic bead cell separation columns (Auburn, CA, USA). PBMCs (10^7) were resuspended in PBS with EDTA/BSA and incubated with 100 μl of monoclonal microbead-conjugated anti-CD14 antibody (Miltenyi Biotec) for 30 min at 4°C. Thereafter, the cells were washed and passed through a

100 μm nylon mesh and separated in a column exposed to the magnetic field of the MACS device. The column was washed three times with sorting buffer (500 μl aliquots) and removed from the separator as directed by the manufacturer. Cells were cultured in RPMI + 10% FBS and 50ng/mL M-CSF at 10^6 cells/ml of medium in 5% CO_2 at 37°C. Medium was replenished after 3 days using fresh RPMI + 10% FBS and 50 ng/mL M-CSF before harvesting and use on day 6. Cells were stained using CD11b and CD68 and used for experiments only if > 85% of cells were double positive for both CD11b and CD68.

2.10 Inhibition of autophagy and mTOR signaling

To study the effect of autophagy on bacterial replication, cells were incubated with medium containing 3-MA or LY294002 (Sigma, St. Louis, MO, M9281 and L9908, respectively). The culture medium in each well was replaced with fresh medium daily to avoid starvation. The cell number was counted, morphology was observed by microscopy, and cell viability was determined with the trypan blue dye exclusion method (Altman, Randers and Rao, 1993). The inhibitory effect of 3-MA and LY294002 on autophagy was examined by immunoblotting with antibodies against LC3 and SQSTM1 using 2 mM 3-MA and 10 μM LY294002 for experiments. RAW 267.4 cells and HUVECs were pre-treated with these autophagy inhibitors for 4 h before infection. To exclude the possibility that autophagy significantly regulates the phagocytosis of rickettsiae, bacterial replication was monitored in RAW267.4 cells treated with 3-MA either starting at 4 h before infection or 4 h after infection. After different time intervals of infection, cells were washed with PBS to remove extracellular bacteria, and intracellular bacterial replication was monitored using real-time PCR as described above. Rapamycin is the pharmacologic gold standard for inhibiting mTOR by associating with FK-506 binding protein 12 to selectively inhibit mTORC1 (Yu

and Toral-Barza, 2012). To inhibit mTORC1 signaling, cells were treated with 50 ng/mL of rapamycin (Sigma-Aldrich, St. Louis MO) for 4 hours prior to infection with rickettsiae. Medium containing fresh 3-MA, LY294002 or rapamycin was added daily to prevent starvation-induced autophagy and to maintain pharmacological activity.

2.11 Measurement of cytokines by ELISA

Supernatants from *Rickettsia*-infected cells and uninfected controls were filter-sterilized and stored as aliquots at -80°C. The concentration of IL-18 and IL-1β present in the supernatant was measured by ELISA using the IL-18 Platinum ELISA kit and the Ready-Set-Go IL-1β ELISA kit (eBioscience, San Diego, CA), following the manufacturer's instructions. The detection limits for the ELISA cytokine concentrations were 19.0 pg/mL for IL-18 and 8.0 pg/mL for IL-1β. Samples were assayed in duplicate and are presented as the average of at least 3 independent experiments. Absorbance values were obtained using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA), and the concentrations were calculated from values obtained within the linear range of the standard curve.

2.12 Bio-plex assay for cytokine/chemokine analysis

Cell culture supernatants and sera were processed according to the manufacturer's instructions and then analyzed using a Bio-plex 200 system (Bio-Rad, Hercules, CA). Briefly, the samples were filter sterilized and subsequently centrifuged for 10 minutes at 450 × g at 4°C to remove debris. The resulting supernatants were collected and aliquoted into 96-well plates and processed for use on the Bio-plex system. The cytokines were coupled to cytokine-specific multi-plex beads (Bio-Rad) in the Bio-Plex mouse cytokine 23-Plex immunoassay following the

manufacturer's instructions. Using the pre-designed assay, the kit measured the concentrations of cytokines/chemokines including interleukin (IL)-1α/β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40, p70), IL-13, IL-17, eotaxin, IFN-γ, KC (CXCL1), monocyte chemoattractant protein (MCP)-1 (CCL2), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)1α/β (CCL3/CCL4), tumor necrosis factor (TNF)-α and RANTES (CCL5). The plates were read at 450 nm, and the absorbances were transformed to pg/ml using calibration curves prepared with cytokine standards included in the kit.

2.13 Live/Dead staining and flow cytometry of BMMs

Primary BMMs were washed with PBS, and the cells were centrifuged at 330 × g for 10 min at 4°C. The pellet was washed with chilled PBS, and cells were stained using fixable LIVE/DEAD Near-IR Dead Cell Stain Kit (Life Technologies, Grand Island, NY) in accordance with the manufacturer's protocol. Cells were washed with PBS, fixed with 4% paraformaldehyde and stored at 4°C until subjected to fluorescence-activated cell sorting analysis. Flow cytometry was performed on 30,000 cells with a FACSCalibur laser cytometer (Becton-Dickinson, BD Biosciences, San Jose, CA). Data analysis on the entire ungated cell population was performed using FlowJo software.

2.14 Histopathological and immunohistochemical analysis

Formalin-fixed, hematoxylin and eosin (H&E)-stained uninfected and infected tissue sections from *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5*$^{flox/flox}$ animals were assessed by a pathologist by both low- (X 10) and high-magnification (X 40) microscopy. Images were taken using an Olympus BX41 microscope (Olympus America Inc., Center Valley, PA). The frequency and size of inflammatory infiltrates in the liver

were measured by Image J as described previously (Tang, Berman, Swanson et al., 2010). Antigen unmasking was performed by treatment with sodium citrate buffer at pH 6, and tissue sections were stained for neutrophils (Abcam, clone NIMP-R14), T cells (CD3+, clone F7.2.38, Dako, Carpinteria, CA) and macrophages (Santa Cruz, clone sc-71088). Biotinylated secondary antibodies (Vector Labs) were diluted in Dako antibody diluent (Dako, #S3022). Streptavidin/alkaline phosphatase tertiary antibodies were diluted in Dako diluent; 3,3'-diaminobenzidine (DAB) was used as the chromogen and hematoxylin as the counterstain. All washes were completed using TBS Tween-20 and the sections were dehydrated before glass coverslips were mounted with permount mounting medium. Slides were photographed with an Olympus DP71 camera (Olympus, Center Valley, PA, USA) attached to an Olympus Ix71 Inverted Microscope (Olympus, Tokyo, Japan) utilizing × 20 objectives. Slides were analyzed using ImmunoRatio (Institute of Biomedical Technology, University of Tampere).

2.15 Statistical analysis

For comparison of multiple experimental groups, the one-way analysis of variance (ANOVA) with Bonferroni's procedure was used. Two-group comparison was conducted using either Student t-test or Welch's t-test depending on whether the variance between two groups was significantly different. When two factors were included in the comparison, two-way ANOVA with Bonferroni post-test was used. All the statistical analyses were performed using GraphPad Prism software version 5.01. P values of 0.05 or less were the threshold for statistical significance.

Chapter 3

Autophagy Defends against Rickettsial Replication in RAW Macrophages but not Endothelial Cells

Introduction

Rickettsiae are Gram-negative, obligately intracellular bacteria that cause potentially life-threatening diseases, with fatality rates as high as 40% if not treated promptly with appropriate antibiotics (Angerami, Resende, Feltrin, et al., 2006) (Walker, 2007). Due to the high infectivity and severe illness after inhalation, *Rickettsia prowazekii* and *R. rickettsii* are considered as potential biological weapons (Azad, 2007). Although rickettsiae effectively invade macrophages, dendritic cells, vascular smooth muscle cells and hepatocytes, microvascular endothelial cells are the major targets of rickettsial infection (Sahni and Rydkina, 2009). A key feature in the pathogenesis of rickettsioses is that *Rickettsia* quickly escapes phagosomal vacuoles and replicates within the cytosol before phagolysosomal fusion occurs (Walker, 2007). Phospholipase D (PLD) and TlyC are potential effector proteins that mediate rickettsial escape from vacuoles (Balraj, Renesto and Raoult, 2009) (Whitworth, Popov, Yu et al., 2005). Very little is known about how rickettsiae interact with immune killing mechanisms in the cytosol. Rickettsiae are the first microbes that were described to induce autophagy (Rikihisa, 1984). As early as 1997, we reported that by electron microscopy *R. conorii* appeared to be engulfed in autophagosomes and destroyed in structures resembling autolysosomes in mouse endothelial cells treated with IFN-γ and TNF-α (Walker, Popov, Crocquet-Valdes et al., 1997). However, it remains undetermined whether rickettsiae induce autophagy without an activation stimulus in endothelial cells and other innate immune cells, such as

macrophages, and whether autophagy can serve as a mechanism of host defense against rickettsial infection in these host cells.

Autophagy is an intracellular, bulk degradation process in which a portion of a cytoplasmic component of the cell is engulfed in double-membrane structures known as autophagosomes and subsequently degraded upon fusion with lysosomes (Mizushima, Noda, Yoshimori et al., 1998) (Levine and Klionsky, 2004). Although autophagy was discovered many decades ago, only recently was a large body of information on the biological process and function of autophagy obtained, which led to better understanding of the role of autophagy in human health and diseases. Autophagosome formation requires 16 autophagy-related (*Atg*) genes including *Atg5*, which comprise the first ubiquitin-like conjugation system (Mizushima, Yoshimori and Ohsumi, 2011). Another ubiquitin-like conjugation system, microtubule-associated protein light chain 3 (LC3-Atg8), is also required in autophagosomal elongation (Choi, Ryter and Levine, 2013). The conversion of a cytosolic truncated form of LC3 (LC3-I) to its autophagosomal membrane-associated, phosphatidylethanolamine-conjugated form (LC3-II), visible as discrete puncta by immunofluorescent analysis, indicates autophagosome formation (Ravikumar, Sarkar, Davies et al., 2010). During infection, autophagy either assists the immune response by degrading intracellular pathogens or is beneficial to bacterial growth (Levine, Mizushima and Virgin, 2011) (Niu, Yamaguchi and Rikihisa, 2008). Several medically important human pathogens are degraded by autophagy, including bacteria (e.g., *Shigella flexneri, Salmonella enterica, Listeria monocytogenes,* and *Francisella tularensis*), viruses such as herpes simplex virus type 1 and chikungunya virus, and parasites such as *Toxoplasma gondii* (Choi, Ryter and Levine, 2013).

Rickettsia australis (*R. australis*) is the etiologic agent of Queensland tick typhus. Although Queensland tick typhus has been described as a mild illness,

severe cases have recently been reported (McBride, Hanson, Miller et al., 2007). *R. australis* establishes a dose-dependent fatal infection model in C57BL/6 (B6) mice (Feng, Wen and Walker 1993), which are the murine background on which most of the gene knockout mice have been developed. Therefore, the mouse model of *R. australis* infection in B6 mice provides a very useful tool for mechanistically studying both immunity to and pathogenesis of rickettsial diseases. In this study, I investigated the kinetic induction of autophagy by *R. australis* in mouse macrophages using a variety of approaches. My findings illustrated that autophagy serves as a mechanism of host clearance of intracellular rickettsiae in mouse RAW macrophages. In addition, autophagy plays a different role in endothelial cells from that in macrophages in infection with endothelium-targeting intracellular bacteria.

Results

R. australis induces autophagosome formation and autolysosome degradation at the early stage of infection in macrophages

To investigate whether *R. australis* infection induces the complete process of autophagy, namely autophagy flux (Mizushima, Yoshimori and Levine, 2010), I first examined the quantity of autophagosomes and the turnover of autophagosomes by immunoblotting and confocal immunofluorescence microscopic analysis throughout the time-course of infection. LC3 exists in two molecular forms; LC3-I (18 kD) is cytosolic, whereas LC3-II (16 kD) binds to autophagosomes (Tanida, Ueno and Kominami, 2008). The conversion of LC3-I to the lipidated LC3-II form is a hallmark of autophagy and indicates autophagosome formation (Klionsky, Abeliovich, Agostinis et al., 2008). The amount of LC3-II correlates directly with the number of autophagosomes. At 1 h p.i., *R. australis* induced an significantly increased LC3-II/β-actin (ACTB) ratio compared to uninfected controls (**Figs. 1A and B**), suggesting autophagosome accumulation in the cytosol. However, since LC3-II itself is degraded by autophagy, the increased number of autophagosomes may represent either induction of autophagy or blockade of any step downstream of autophagosome formation. To address this issue, I examined the levels of SQSTM1, which is an ubiquitin-binding protein that is specifically degraded by autolysosomes. SQSTM1 is considered a useful marker for autophagic vesicle turnover (Mostowy, Sancho-Shimizu, Hamon et al., 2011), and the level of SQSTM1 is inversely correlated with autophagy flux (Komatsu, Waguri, Koike et al., 2007). At 1 h p.i., *R. australis* induced a slightly decreased SQSTM1/ACTB ratio compared to uninfected controls (**Figs. 1A and B**), which excludes the possibility

that the increased levels of LC3-II resulted from the blockade of any step downstream of autophagosome formation.

Interestingly, as the infection progressed, the ratio of LC3-II/ACTB progressively decreased in association with insignificant changes in levels of SQSTM1, suggesting that autophagy flux induced by infection decreased over time (at 3 h and 24 h p.i.) (**Figs. 1A and B**).

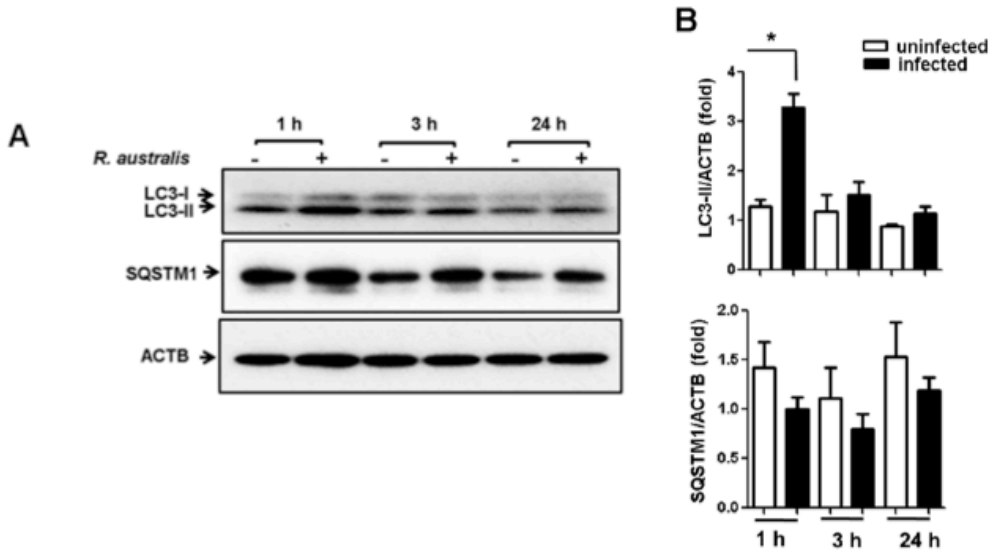

Figure 1 A and B. *R. australis* induced accumulation of LC3-II at the very early stage of infection. RAW264.7 cells were infected with *R. australis* at MOI of 5. Cells were collected at 1 h, 3 h and 24 h p.i.. Cell lysates were immunoblotted with antibodies directed against LC3, SQSTM1 and ACTB (A). The ratios of LC3-II/ACTB and SQSTM1/ACTB were analyzed by densitometry (B). Data shown are mean ± standard error (SE) of three independent experiments. *, $p<0.05$.

In line with these results, confocal immunofluorescence microscopic analysis also demonstrated LC3 puncta (green) in *R. australis*-infected macrophages (**Fig. 1C**). At 1 h p.i., 36.9 ± 9.9% of infected cells contained LC3 puncta, which progressively decreased to 11.1 ± 5.7% at 24 h p.i. (**Fig. 1D**). The number of LC3 puncta was significantly increased at 1 h p.i. compared to uninfected controls (**Fig. 1E**), suggesting that *R. australis* induced autophagosome formation and

turnover at 1 h p.i., but the level of autophagy progressively decreased as the infection progressed.

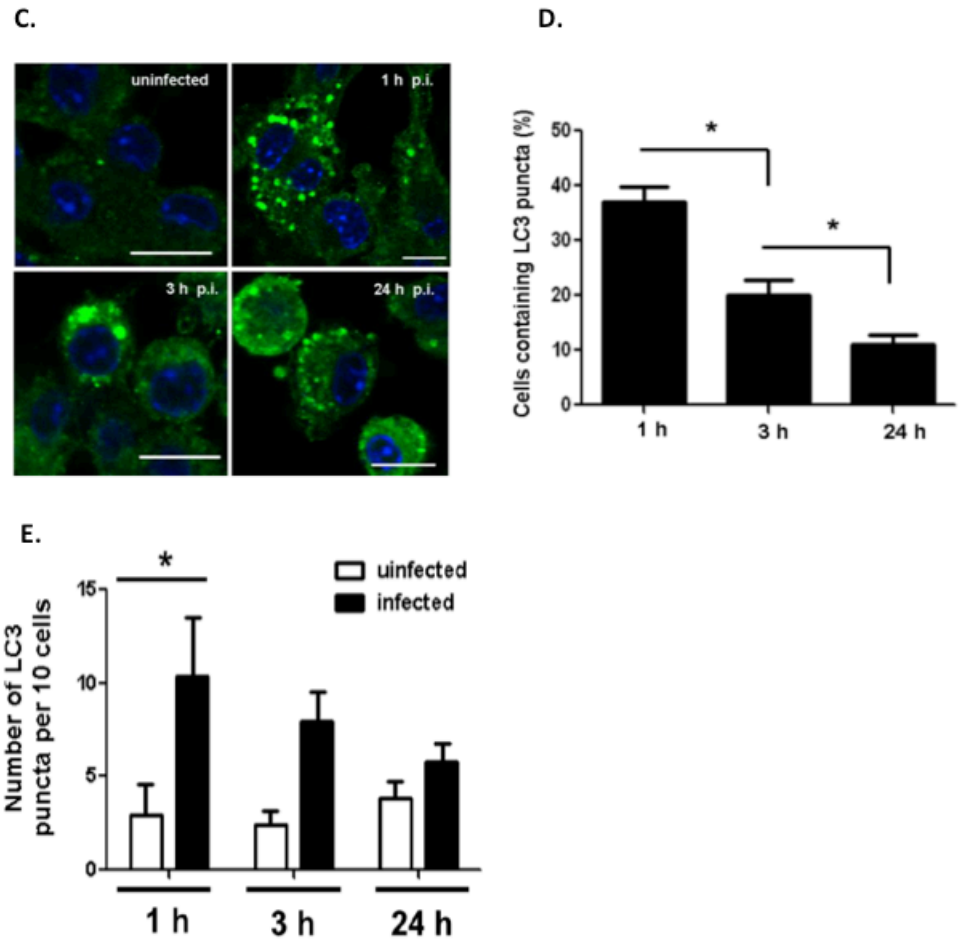

Figure 1 C, D and E. *R. australis* induced accumulation of LC3 puncta at the very early stage of infection. RAW264.7 cells were infected with *R. australis* at MOI of 5. Cells were collected at 1 h, 3 h and 24 h p.i.. Representative confocal microscopic images of infected macrophages. Each scale bar represents 10 µm. LC3 (+) puncta are depicted in green, DAPI (nuclei) in blue (C). The percentage of cells containing LC3 puncta (D) was determined and the number of LC3 puncta per 10 cells was counted (E). More than 300 cells from 12 randomly selected images were counted at each time point. Data shown are mean ± standard error (SE) of three independent experiments.

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Ultrastructural analysis of autophagy induction by rickettsiae

By transmission electron microscopy, the most traditional method to study mammalian autophagy, an autophagosome is defined as a double-membrane enclosed vacuole containing undigested cytoplasmic contents (Mizushima, Yoshimori and Levine 2010). At the ultrastructural level, *R. australis*-infected macrophages contained individual intact rickettsiae or groups of rickettsiae in vacuoles surrounded by double membranes (indicated by arrows) at both 1 h (**Figs. 2A and B**) and 3 h p.i. (**Figs. 2C and D**), around at least at part of their circumference. Rickettsiae (indicated by arrowheads) were found inside these vacuoles either intact (**Figs. 2A and C**) or with signs of degeneration (**Figs. 2B and D**). Most autophagosomes had only fragments of inner membrane (arrows in **Figs. 2A, B and D**), suggesting that they were gradually transforming into autophagolysosomes where rickettsiae were degraded.

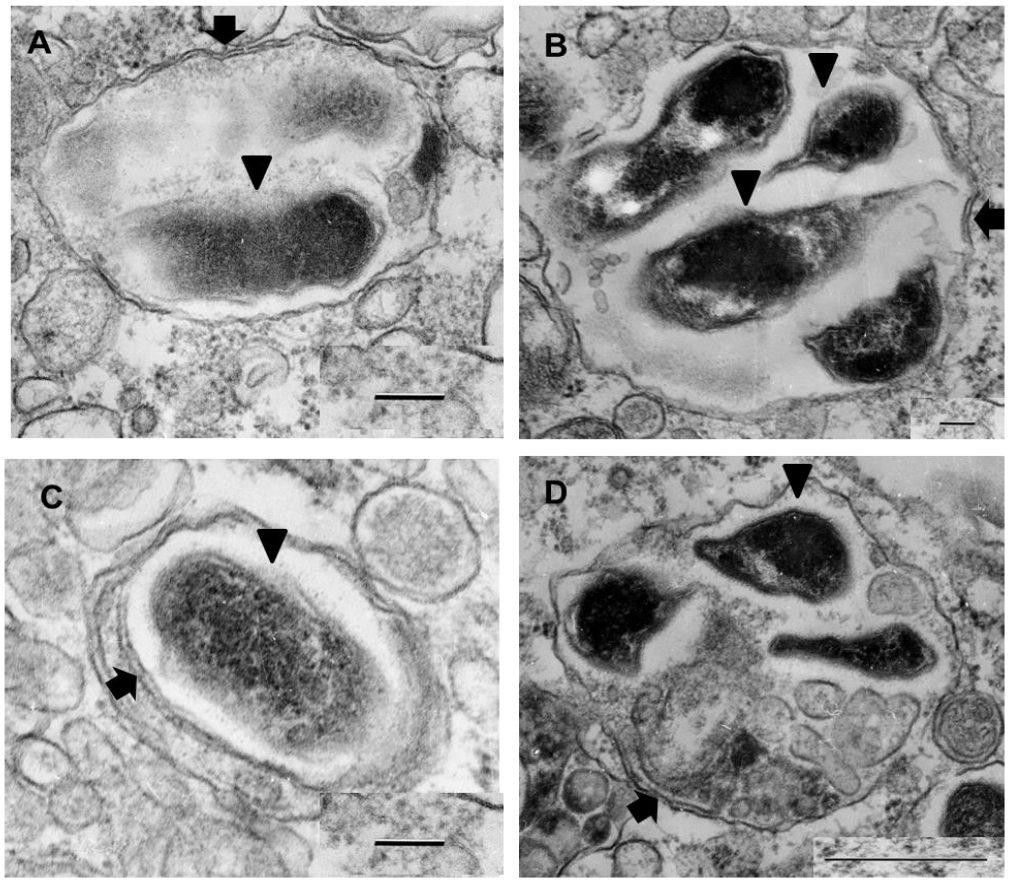


Figure 2. Transmission electron microscopy of *R. australis*-infected BMMs. Double-membrane-bound vacuoles containing rickettsiae (arrowheads) were found at 1 h (A and B) and 3 h p.i. (C and D). Arrows indicate double membranes either complete (C) or in fragments (A, B, D). Bars =100 nm in A, B and C and 500 nm in D. Data represent three independent experiments.

Autophagosomes and autolysosomes target *R. australis* in the cytosol

To determine whether autophagosomes target rickettsiae in macrophages and the number of bacteria captured by autophagosomes or autolysosomes, I examined the co-localization of autophagosomes or autolysosomes with *R. australis* at different time points of infection. The autophagosomes, which were

labeled by antibodies against LC3 (green), were colocalized with *R. australis* (red) in the cytosol (nucleus as blue) of infected macrophages as determined by confocal immunofluorescence microscopy (**Fig. 3A**). Quantitative analysis revealed 20.1 ± 8.1% of rickettsiae co-localized with autophagosomes at 1 h p.i. (**Fig. 3B**). The percentage of bacteria associated with autophagosomes decreased significantly as infection progressed. Only 9.4 ± 8.3% of *R. australis* colocalized with autophagosomes at 24 h p.i.. By confocal immunofluorescence microscopy, no significant difference was observed in the frequency of autophagosome-associated rickettsiae at 1 h p.i. compared to 3 h p.i.. These results suggest that a significant proportion of rickettsiae were trapped in autophagosomes at the early stage of infection. However, as infection progressed, the frequency of autophagosome-associated rickettsiae was significantly reduced.

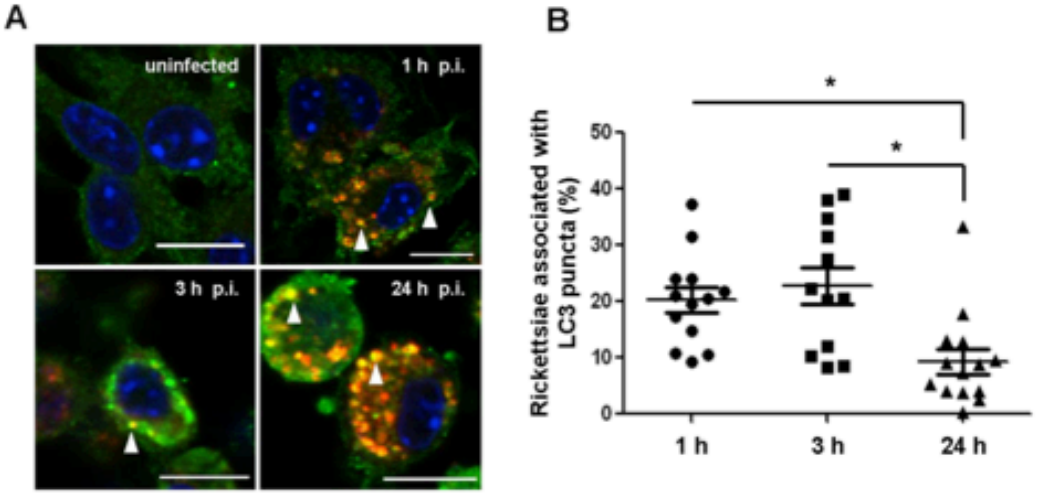


Figure 3 A and B. Intracellular *R. australis* is targeted by LC3-positive compartments in the cytosol. Co-localization of rickettsiae with autophagosomes (**A**). Representative confocal microscopic images of infected BMMs at different time points as indicated. Each scale bar represents 10 μm. LC3 puncta are depicted in green, nuclei (DAPI) in blue, and *R. australis* in red. Arrowheads indicate co-localization of rickettsiae with LC3 positive compartments (yellow) in the cytosol. Percentages of rickettsiae co-localized with LC3 were analyzed using MetaMorph software (**B**). More than 300 bacteria were analyzed at each time point. (*, $p<0.01$). Data represent two independent experiments.

Lysosome-associated membrane protein 2 (LAMP-2), a marker of lysosomes, was also acquired by rickettsiae-associated autophagosomes in macrophages throughout the infection (**Fig. 3C**). At 1 h, 3 h, and 24 h p.i., *R. australis* (red) co-localized with autolysosomes, which expressed both markers of autophagosomes (LC3, green) and lysosomes (LAMP-2, blue). These results showed that intracellular *R. australis* was truly trapped in autophagosomes, which were then delivered to lysosomes for degradation, suggesting that a portion of rickettsiae can be potentially cleared by the autophagic machinery.

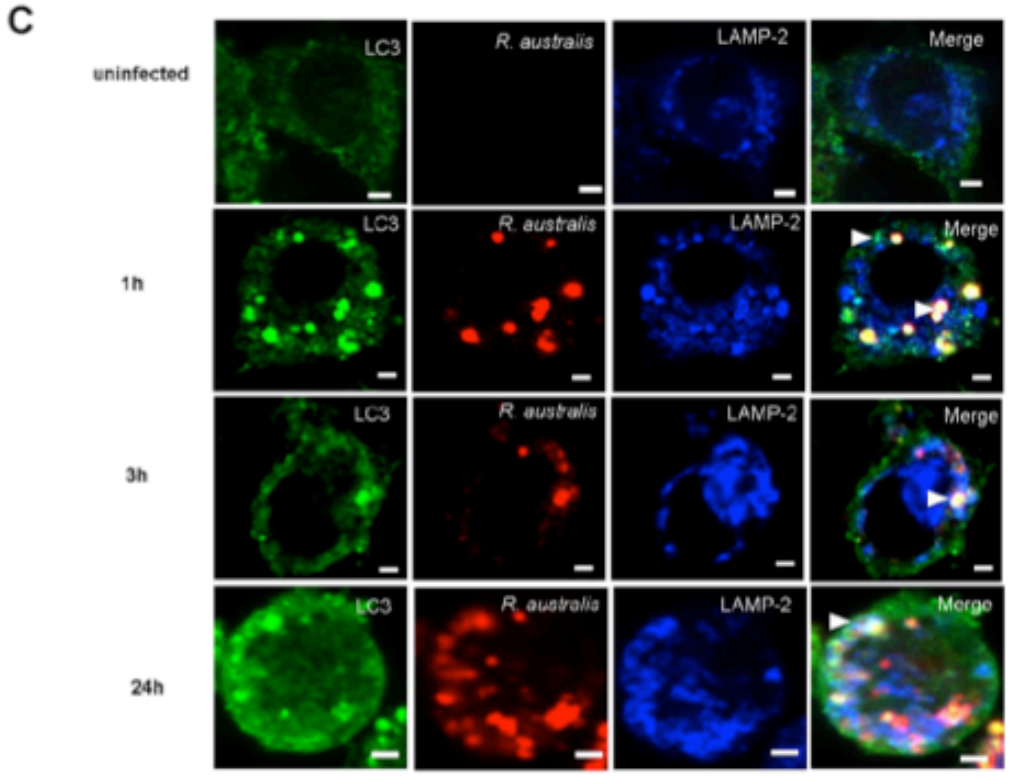


Figure 3C. Intracellular *R. australis* bacteria are targeted by autolysosomes in the cytosol. Infected macrophages were stained with antibodies directed against LC3 (green), *R. australis* (red), and LAMP-2 (blue). Bar, 2 µm. Arrowheads indicate co-localization of rickettsiae with autolysosomes. Data represent two independent experiments.

Inhibition of autophagy by 3-MA and LY294002 enhances rickettsial survival in murine RAW macrophages

Since we observed autophagosome formation, fusion of autophagosomes with lysosomes, and trapping of rickettsiae in autophagosomes and autolysosomes, I further determined whether autophagy contributed to intracellular bacterial killing in macrophages. 3-MA, a widely used inhibitor of autophagy inhibits the activity of class III phosphatidylinositol 3-kinase (Seglen and Gordon, 1982 and Lindmo and Stenmark, 2006). I first examined the viability of macrophages in response to infection and 3-MA treatment. Previous studies have demonstrated that rickettsiae induce negligible cytotoxicity in mouse peritoneal macrophages and the macrophage-like cell line, P388D1 (Radulovic, Price, Beier et al., 2002). In line with these previous studies, I did not find significantly reduced viability of RAW 264.7 cells after infection with *R. australis* within a limited time period of infection (data not shown) (Radulovic, Price, Beier et al., 2002). At a concentration of 2 mM, 3-MA was not toxic to RAW264.7 cells as morphologic and viability changes were not detected in these cells. I also confirmed that autophagy was inhibited in these cells by the treatment as assessed by levels of LC3-II and SQSTM1 using immunoblotting (data not shown) (Niu, Yamaguchi, Rikihisa, 2008). When these cells were pretreated with 3-MA and then infected with *R. australis*, the number of rickettsiae was significantly increased compared to cells without 3-MA treatment at 48 h p.i., but not at 24 h p.i. (**Fig. 4A**). I found very similar results when cells were first infected with *R. australis* and then treated with 3-MA (**Fig. 4B**). These results excluded the possibility that 3-MA treatment promoted rickettsial replication via increasing phagocytosis of bacteria.

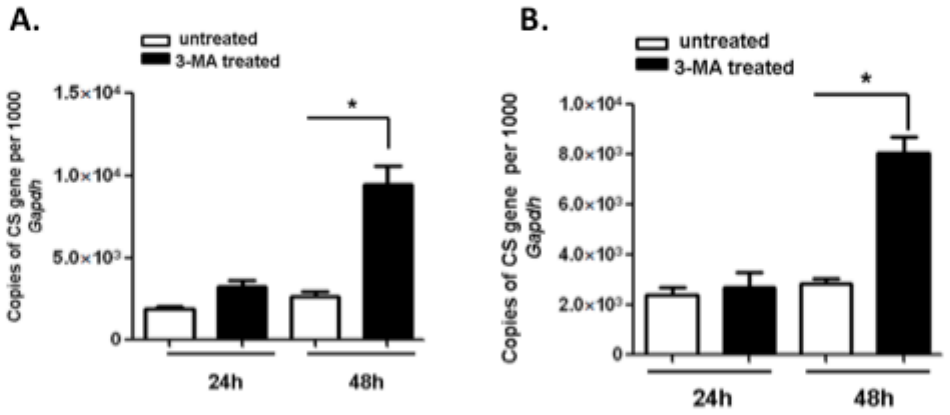


Figure 4 A and B. Inhibition of autophagy by 3-MA promotes rickettsial replication in murine RAW macrophages. RAW264.7 cells were infected with *R. australis* at an MOI of 2. The numbers of intracellular bacteria in adherent cells at indicated time points were quantified by real-time PCR after washing with PBS. Number of citrate synthase (CS) gene copies per 1000 *Gapdh* represents the quantity of rickettsiae. RAW macrophages were either pretreated with 3-MA (2 mM) for 4 hours and then infected with *R. australis* (A), or first infected with *R. australis* for 4 hours and then treated with 3-MA (2 mM) (B). Data shown are mean ± SE of two to five independent experiments with more than four wells in each experiment. *, $p<0.05$.

Although 3-MA is a well-known inhibitor of autophagy, it was recently reported to induce autophagy in some cell types (Wu, Tan, Shui, et al., 2010) (Lin, Kuo, Wang, et al., 2012). To confirm my results, I employed another inhibitor of phosphatidylinositol 3-kinase, LY294002 (Vlahos, Matter, Hui, et al., 2994). In line with what we have found using 3-MA treatment, the number of rickettsiae in cells treated with LY294002 was significantly greater than that in untreated controls at both 48 h and 72 h p.i. (**Fig. 4C**). Thus, these results strongly suggest that autophagic killing is one of the rickettsicidal mechanisms in mouse RAW macrophages.

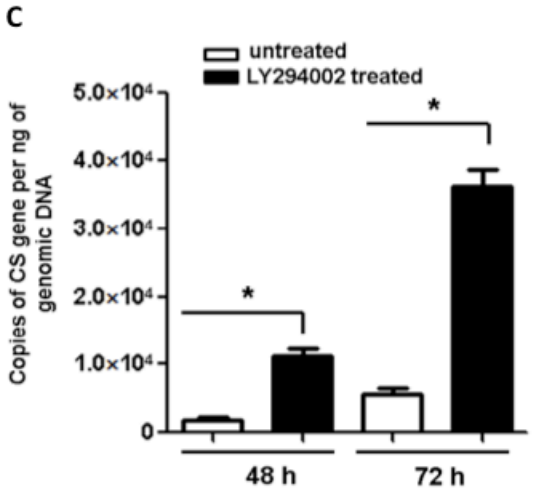


Figure 4 C. Inhibition of autophagy by LY294002 promotes rickettsial replication in murine RAW macrophages. RAW264.7 cells were infected with *R. australis* at an MOI of 2. The numbers of intracellular bacteria in adherent cells at indicated time points were quantified by real-time PCR after washing with PBS. Number of citrate synthase (CS) gene copies per ng of genomic DNA represents the quantity of rickettsiae. Rickettsial replication was monitored in cells pre-treated with LY294002 (10 μM) for 48 and 72 h (**C**). Data shown are mean ± SE of three independent experiments with more than four wells in each experiment. *, $p<0.05$.

Autophagy induction by rickettsiae in mouse macrophages is *Atg5*-dependent

To investigate the mechanisms involved in induction of autophagy by rickettsial infection in macrophages, I investigated autophagy flux in rickettsiae-infected bone marrow-derived macrophages (BMMs) from mice in which an autophagy gene, *Atg5*, was inactivated in macrophages/granulocytes. *Atg5* was inactivated in myelomonocytic cells by breeding of *Atg5* $^{flox/flox}$ mice with mice that express the *Cre* recombinase from the endogenous lysozyme M locus (Lyz-*Cre*) (Hara, Nakamura, Matsui, et al, 2006). In line with the results described above, *R. australis* induced a significantly increased LC3-II/ACTB ratio not accompanied by significantly increased SQSTM1/ACTB ratio at 1 h p.i. in *Atg5*-competent macrophages (*Atg5*$^{flox/flox}$) compared to their uninfected controls (**Figs.**

5A and B), suggesting the induction of autophagy flux. In contrast, *R. australis*-induced autophagy flux was completely abrogated in infected *Atg5*-deficient macrophages (*Atg5* $^{flox/flox}$ Lyz-*Cre*) as evidenced by insignificant changes in LC3-II/ACTB and SQSTM1/ACTB upon infection compared to uninfected cells at 1 h p.i.. These results suggest that that induction of autophagy by rickettsiae in mouse macrophages was dependent on *Atg5*.

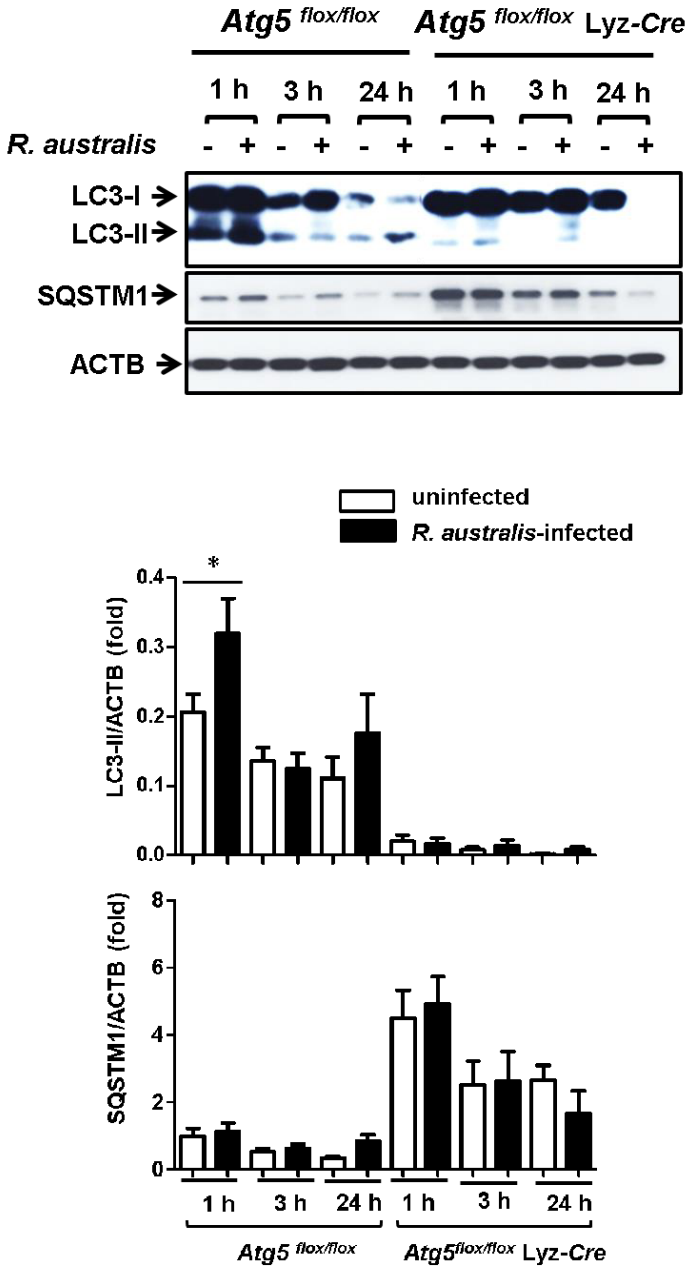


Figure 5. Induction of autophagy by *R. australis* is *Atg5*-dependent. BMMs were isolated from *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice, and then infected with *R. australis* at an MOI of 5. Induction of autophagy by rickettsiae in these infected macrophages was evaluated by expression levels of LC3-II, SQSTM1 and ACTB at different time points (A). The ratios of LC3-II/ACTB and SQSTM1/ACTB were analyzed by densitometry (B). Data shown are mean ± SE of three independent experiments. *, $p<0.05$.

Interactions of *R. australis* with autophagy in endothelial cells

To reveal the interactions of *R. australis* with autophagy system in the primary target cells of rickettsiae, endothelial cells, I kinetically determined the levels of LC3-II and SQSTM1 in human umbilical vein endothelial cells (HUVECs) upon infection at a multiplicity of infection (MOI) of 6 based on the plaque forming units of rickettsial stock. In contrast to the results described above, no significant changes in the amount of LC3-II in endothelial cells at 1 h p.i. were observed. *R. australis* induced a slightly increased level of LC3-II in infected HUVECs at 24 h p.i., compared to uninfected controls (**Figs. 6A and B**). However, the expression levels of SQSTM1 was also slightly increased at 24 h p.i.. Although the levels of LC3-II/SQSTM1 were slightly increased at 24 h p.i. compared to uninfected controls **(Figs. 6A and B)**, it remains elusive whether autophagy flux was actively induced upon infection.

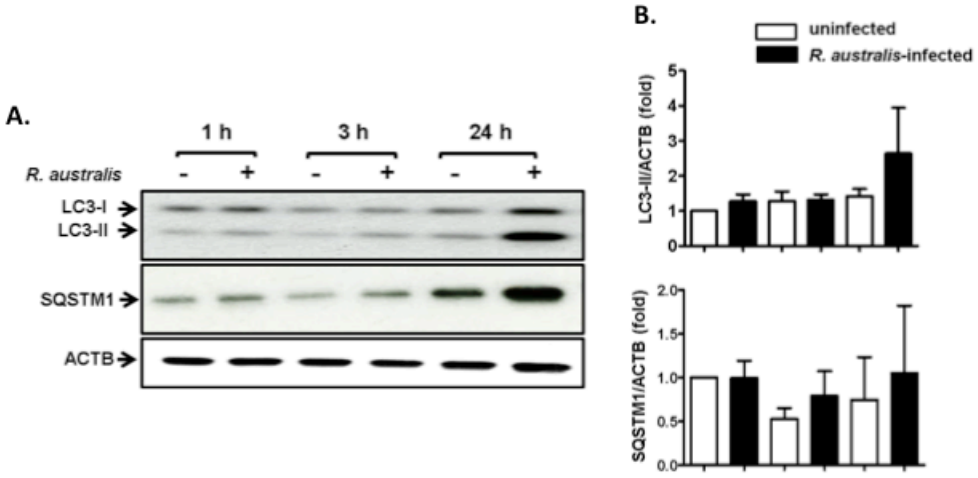


Figure 6 A and B. Interactions of *R. australis* with autophagy in human endothelial cells. (A) HUVECs were infected with *R. australis* at an MOI of 6, and autophagy induction was evaluated at different time intervals by Western blotting. (B) The levels of LC3-II/ACTB and SQSTM1/ACTB were evaluated by densitometry analysis. Data shown are mean ± SE of six independent experiments.

To further investigate the role of autophagy in endothelial cells, HUVECs were treated with 3-MA. As shown in **Fig. 6C**, 3-MA treatment significantly decreased the number of rickettsiae in human endothelial cells at 48 h p.i.

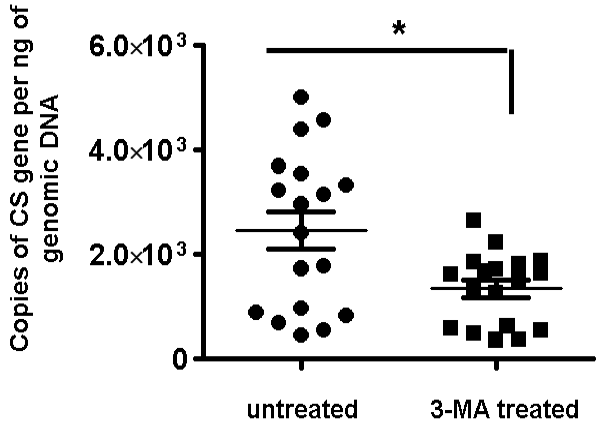


Figure 6 C. Interactions of *R. australis* with autophagy in human endothelial cells. Bacterial replication in the presence of 3-MA was monitored at 48 and 72 h p.i. by quantitative real-time PCR. Data shown are mean ± SE of two independent experiments with samples from 18 wells. *, $p<0.05$.

Transmission electron microscopy of *R. australis* infected HMEC-1 cells failed to demonstrate any characteristics of autophagy induction at 1 h, 3 h, 24, h or 48 h p.i. (**Figs. 7A, B, C and D**). Additionally, no autophagy was detected in HUVECs infected with *R. australis* (data not shown). These results suggest that the induction mechanisms and the immunological role of autophagy in endothelial cells are distinct from those in macrophages during rickettsial infection.

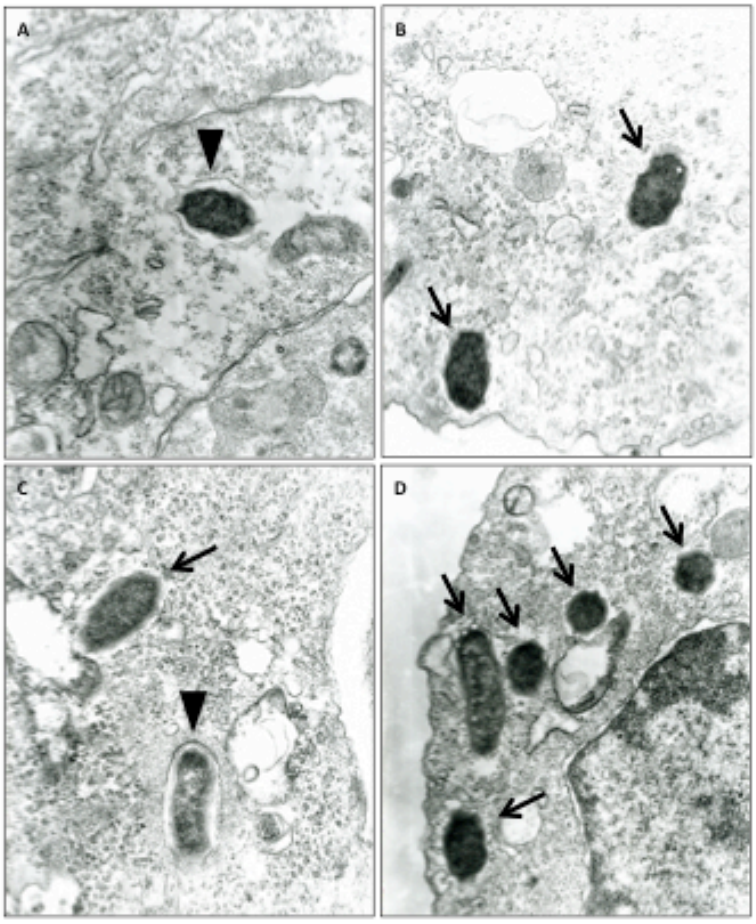


Figure 7. Transmission electron microscopy of *R. australis*-infected HMEC-1 cells. Single-membrane-bound vacuoles containing rickettsiae (arrowheads) and free cytosolic rickettsiae (arrows) were found at 1 h (A) 3 h p.i. (B), 24 h p.i. (C) and 48 h p.i. (D) indicating a lack of autophagy observed in HMEC-1 cells at the time points examined. Data represent two independent experiments.

DISCUSSION

These studies, for the first time, demonstrated that the obligately intracellular rickettsiae induce autophagy flux in mouse macrophages without an addition of exogenous immunological stimuli *in vitro*. More importantly, autophagy machinery only contributes to host clearance of *R. australis* in mouse RAW macrophages but not in human primary endothelial cells. Autophagy is a conserved membrane-traffic pathway in eukaryotic cells that sequesters cytoplasmic components, including invading pathogens, and delivers them to lysosomes. Recent research has highlighted the critical aspects of the intracellular surveillance systems mediated by autophagy (Schmid and Münz, 2005) and (Huang and Brumell, 2014).

The interactions of bacteria with autophagy have been investigated extensively in recent decades. Autophagy can serve as a mechanism of host innate immunity, targeting intracellular bacteria such as *Group A Streptococcus* (GAS) and *Mycobacterium tuberculosis* to restrict bacterial growth (Nakagawa, Amano, Mizushima, et al., 2004) and (Gutierrez, Master, Singh, et al., 2004). In contrast, some bacteria have developed strategies to escape this defense system or even hijack the autophagy machinery to promote their intracellular growth such as *Yersinia enterocolitica* and *Anaplasma phagocytophilum* (Deuretzbacher, Czymmeck, Reimer, et al., 2009) (Birmingham, Canadien, Gouin et al., 2007).

With analysis of several hallmarks of autophagy, we found that rickettsial invasion into the cytosol of macrophages induced Atg5-dependent formation of autophagosomes, which was followed by fusion of autophagosomes with lysosomes to form autolysosomes at 1 h p.i.. Furthermore, a subset (around 20%) of intracellular *R. australis* is targeted by the autophagy system. Will this early stage of autophagic targeting of the minority of rickettsiae cause a difference in

host control of the bacteria? Our findings demonstrated that autophagic degradation significantly limits the intracellular rickettsial growth in murine RAW macrophages.

Like other intracellular bacteria such as GAS and *Listeria monocytogenes*, *R. australis* induces autophagic flux in macrophages without external stimulation (Nakagawa, Amano, Mizushima et al., 2004 and Birmingham, Canadien, Gouin et al., 2007). However, the kinetics of autophagy induction by *R. australis* is distinct from other intracellular bacteria including GAS, *A. phagocytophilum* and *Coxiella burnetii*. GAS begin to co-localize with GFP-LC3 at 3 h p.i. (Nakagawa, Amano, Mizushima et al., 2004). *Coxiella burnetii* inclusions accumulate LC3 at 5 min p.i. (Romano PS, Gutierrez MG, Berón et al., 2007). *A. phagocytophilum*, a Gram-negative obligately intracellular bacterium in the order Rickettsiales (Dumler and Bakken, 1998), colocalizes with LC3 and Beclin 1 at 32 and 48 h p.i., respectively (Dumler and Bakken, 1998). Our results demonstrated that *R. australis* induced autophagy flux at as early as 1 h p.i. in mouse macrophages, which then progressively decreased and was evaded by rickettsiae. Thus, induction of autophagy by *R. australis* is a rapid, transient and dynamic response. In addition, *R. australis* induced autophagy in both mouse macrophage cell line (RAW 246.7) and primary macrophages (BMM), which followed very similar kinetics (**Figs. 1A and 5A**).

Macrophages are professional phagocytes and serve as an important effector of host defense against intracellular bacterial infection. However, the role of macrophages in the pathobiology of rickettsial diseases is poorly defined. Our study for the first time points out that macrophages eliminate and control intracellular rickettsiae via degradative autophagy. Autophagy induced by rickettsiae in mouse macrophages contributes to controlling bacteria documented as follows: i) LC3 puncta and accumulation of LC3-II both reached the peak at

the same time when bacteria were captured by autophagsosomes; ii) rickettsiae were found in the autolysosomes; iii) pharmacological inhibition of autophagy greatly promoted bacterial survival and replication in RAW macrophages. The interactions of autophagy with rickettsiae in macrophages are distinct from those of two other intracellular bacteria genetically close to rickettsiae, *A. phagocytophilum* and *Orientia tsutsugamushi* (Niu, Yamaguchi and Rikihisa) (Niu, Xiong, Yamamoto, et al., 2012) (Ko, Choi, Ha et al., 2013). Although *A. phagocytophilum* actively induces autophagy, the autophagosome-like compartment is hijacked by bacteria to facilitate its own growth (Niu, Yamaguchi and Rikihisa) (Niu, Xiong, Yamamoto, et al., 2012). *O. tsutsugamushi* actively escapes from autophagic recognition so that inhibition or activation of autophagy does not affect its intracellular growth (Ko, Choi, Ha et al., 2013). Although the main type of cells used in the studies of *A. phagocytophilum* and *O. tsutsugamushi* are not mouse RAW macrophages, from which most of our results were derived, our findings at least demonstrated a role of autophagy in rickettsial infection that is distinct from other two closely related intracellular bacteria.

To demonstrate the role of autophagy in controlling intracellular rickettsiae in macrophages, we employed different approaches to avoid false positive results. First, it has been reported recently that autophagy regulates phagocytosis in macrophages during infection with *Mycobacterium tuberculosis* (Bonilla, Bhattacharya, Sha, et al., 2013). Autophagy-deficient macrophages exhibit greater mycobacterial uptake. The data in Fig. 4 showed similar findings regarding bacterial replication in macrophages with 3-MA treatment before and after infection. These results exclude the possibility that the enhanced level of rickettsial replication in 3-MA treated cells is due to an increased number of rickettsiae entering the cell resulting from 3-MA treatment. Secondly, it has been reported that induction of autophagy is accompanied by apoptosis (Mariño, Niso-

Santano, Baehrecke, et al., 2014). To exclude this possibility, we examined the viability of macrophages throughout the infection with and without 3-MA and LY29400 treatment. We did not observe significant cell death induced by the treatment or infection with *R. autralis* based upon examination by trypan blue staining, at least until 48 h p.i.. Thirdly, we detected a significantly greater number of rickettsiae in 3-MA treated macrophages starting at 48 h p.i., but not at 24 h p.i., compared to untreated cells. This result of detecting no rickettsial growth at 24 h p.i. is probably due to the detection limits of quantitative real-time PCR, the growth kinetics of rickettsiae, or the timing of when rickettsiae escape autophagy in macrophages. Fourth, the difference in bacterial growth between 3-MA treated and untreated samples increased over time until 96 h p.i.. It is worth mentioning that a significant portion of infected-macrophages were detached from the culture plate after 72 h p.i.. This potential issue should not bias our results since we only quantified the intracellular rickettsiae in the adherent macrophages. It would be worthwhile to investigate any potential pathological effect on macrophages by rickettsiae at the late stage of infection in the future.

Our findings suggest that *R. australis* induces autophagy at the early stage of infection via an *Atg5*-dependent mechanism in mouse macrophages. However, it remains unknown if and how *Rickettsia* escapes and/or inhibits autophagy at the late stage of infection. At 24 h p.i., most of the bacteria (90%) were not inside autophagosomes. Fewer rickettsiae were co-localized with autolysosomes compared to autophagosomes (data not shown). This observation suggests that: i) autophagy flux was blocked possibly due to inhibition of the maturation of autophagosomes into autolysosomes by an unknown mechanism or ii) rickettsiae express strategies to escape autophagosomes. Future studies are required to further reveal whether rickettsiae escape and/or suppress autophagy. A very recent study reported that the actin cytoskeleton participates in the initiation of

autophagsosomes (Aguilera, Beron and Colombo, 2012) (Reggiori, Monastyrska, Shintani, et al., 2005). Therefore, it is possible that rickettsiae regulate autophagy via mechanisms involving actin, potentially by rickettsial proteins such as RickA and Sca2 that stimulate actin polymerization (Reed, Lamason, Risca et al., 2014).

Another significant finding in our study is that the interactions of autophagy with rickettsiae in endothelial cells are distinct from those in macrophages. First, *R. australis* induced significant autophagy flux in macrophages at 1 h p.i. but not in endothelial cells (**Figs. 1 and 6**). Although we found a slight, but not significant, increase in LC3-II at 24 h p.i. in endothelial cells, it still remains elusive whether it is due to the blockage of fusion of autophagosome with lysosome or accumulation of autophagosomes. This will be explained by monitoring the amount of LC3-II after using the inhibitors of maturation of autolysosomes such as chloroquine (Mizushima, Yoshimori and Levine, 2010). Considering the fact that no gold standard method is available for monitoring autophagy activity, the interactions of autophagy with rickettsiae in endothelial cells will be better characterized by a combination of several approaches including the amount of LC3 puncta and the localization of rickettsiae with autophagosomes/autolysosomes as described previously (Mizushima, Yoshimori and Levine, 2010). Secondly, inhibition of autophagy by 3-MA did not significantly change rickettsial replication in endothelial cells but promoted bacterial expansion in macrophages (**Figs. 4 and 6C**). These results suggest that autophagy serves as a mechanism of host defense in RAW macrophages but may be beneficial for bacterial replication in endothelial cells during rickettsial infection.

The differential role of autophagy in rickettsial infection in macrophages and endothelial cells is likely due to the intrinsic physiological properties and immune function of each type of cells in infections, which potentially highlights

the unique pathogenesis of rickettsial diseases and endothelial tropism of rickettsiae. In addition, our previous studies provide the first evidence that rickettsiae are engulfed in an autophagosome-like compartment and potentially destroyed in mouse brain endothelial cells after stimulation with IFN-γ and TNF-α (Walker, Popov, Crocquet-Valdes, et al., 1997). The cell types used and experimental conditions in these studies may explain the different conclusions from our current studies. Thus, further investigations on interactions of autophagy with rickettsiae in human and mouse primary macrophages and microvascular endothelial cells *in vitro* and *in vivo* would be greatly insightful for development of novel therapeutics by manipulating the autophagy system.

Our study raises questions that require further investigation in the future. What are the bacterial effectors involved in induction of autophagy, blocking fusion of autophagosomes with lysosomes, preventing their killing by autophagy machinery, or masking themselves to avoid autophagy recognition? Are these mechanisms cell-type specific? What are the host factors that mediate autophagic killing of rickettsiae in phagocytic cells? What is the role of autophagy in host immunity against rickettsial infection *in vivo*? A detailed elucidation of these questions will not only increase our knowledge of rickettsial biology and pathogenesis of rickettsioses, but also will be beneficial for control of other infectious diseases.

Chapter 4

***Rickettsia australis* induces mTOR activation to facilitate bacterial replication *in vitro*.**

Introduction:

The mammalian target of rapamycin (mTOR) is an atypical serine-theonine protein kinase that affects several aspects of cellular functions including metabolism, aging, growth, apoptosis, and autophagy (Weichhart and Säemann, 2009). Toll-like receptor activation, cytokines and low concentrations of amino acids will activate mTOR leading to the phosphorylation of S6 kinase, an established marker of mTORC1 activation (Hong, Mannan and Inoki, 2012). Additionally, mTOR is a master regulator of autophagy, and it is well documented that stimulation of mTORC1 reduces autophagy (Katholnig, Linke, Pham et al., 2013). In addition to S6 kinase, mTOR phosphorylates AMBRA1 (activating molecule in BECN1 (beclin-1)-regulated autophagy protein 1), preventing serine/threonine-protein kinase ULK1/ATG1 from starting the process of autophagosome formation (Nazio, Strappazzon, Antonioli, et al., 2013).

Recently, Owen et al. (2014) have shown that focal adhesion kinase (FAK) is recruited to the surface of *Salmonella*-containing vacuoles, which leads to amplified signaling through the Akt-mTOR axis and inhibition of the autophagic response. However, when Akt/mTOR signaling is attenuated by removing FAK, autophagy is enhanced, resulting in reduced bacterial survival. Although there are studies of virus-mTOR interactions, the study of bacterial/mTOR interactions is severely limited, and non-existent with respect to

obligately intracellular bacterial pathogens. Preliminary work from the Sahni lab has shown that endothelial cells infected with *R. prowazeki, R. conorii*, or *R. rickettsii* induce mTORC1 activation as demonstrated by immunoblotting of phosphorylated S6 kinase (P70S6K) threonine 412/serine 424, as well as phosphorylated mTOR on serine 2448. Additionally, they have shown both indirect as well as direct evidence that rickettsiae from the spotted fever and typhus groups activate mTORC1 in infected endothelial cells. My work described in chapter 3 shows that autophagy flux induced by rickettsiae progressively decreases from 1 to 3 hours p.i., and is undetectable at 24 hours. These data suggest that *R. australis* may actively induce evasion from autophagic killing in BMMs, possibly through modulation of the mTOR pathway.

Rapamycin was first isolated from *Streptomyces hygroscopicus* taken from a soil sample on Easter Island (Vézina, Kudelski and Sehgal, 1975). Rapamycin forms a complex with FK506 binding protein 1A 12kDa (FKBP12), which inhibits mTOR's kinase activity by blocking the substrate recruitment domain and restricting access to the protein's active site (Waldner, Fantus, Solari, et al., 2016). Rapamycin is only functional against the mTORC1 protein complex, while the ATP-competitive inhibitor PP242 effectively inhibits both mTORC1 and mTORC2 (Feldman, Apsel, Uotila, et al., 2009). Interestingly, PP242 is a more potent inhibitor of mTORC1 than rapamycin and inhibits rapamycin-insensitive cap-dependent translation, making PP242 an effective pharmacological inhibitor of mTOR activity (Feldman, Apsel, Uotila, et al.,

2009). Both rapamycin and PP242 have been used to induce autophagy through the inhibition of mTOR (Tong, Chen, Zhang, et al., 2015).

Several pathogens induce mTOR activation including *Campylobacter jejuni* (Sun, Threadgill and Jobin, 2012), various mycobacteria species (Zullo and Lee, 2012), human cytomegalovirus and Simian virus 40 (Brunton, Steele, Ziehr, et al., 2013) and the toxin produced by *Pasteurella multocida* (Kloos, Chakraborty, Lindner, et al., 2015). It has been hypothesized that the activation of mTOR may benefit pathogens by stimulating fatty acid and lipid synthesis, or by inhibiting autophagy (Brunton, Steele, Ziehr, et al., 2013).

Given the key role of mTOR in regulating the autophagy response, I sought to examine the role of mTOR during rickettsial infection. In this study I investigated the kinetics and activation status of mTOR during infection of macrophages with *R. australis* as a potential evasion mechanism against host defense. Additionally, I examined the consequences of mTOR inhibition on bacterial replication. My findings illustrate that *R. australis* induces the activation of mTOR in murine macrophages and show that mTOR inhibition suppresses bacterial replication in murine macrophages. Finally I show that *R. australis* survives inside human macrophages and establishes an active infection in this cell type often overlooked during rickettsial infections.

Results:

***R. australis* induced activation of mTORC1**

To investigate the impact of *R. australis* on mTORC1 activation, BMMs were infected with *R. australis* at an MOI of 5 for 1, 3 and 24 hours post infection. I examined the direct phosphorylation of mTOR on serine 2448 and the indirect activation of mTORC1 by measuring the phosphorylation of P70S6 kinase (threonine 389) using Western blotting. The phosphorylation of these proteins is indicative of mTORC1 activation and is seen when BMMs are infected with *R. australis* after 1 h p.i. until 24 hours p.i. (**Figs. 8A, B and C**). mTOR is significantly activated at 1 h p.i, peaks at 3 h p.i and remains statistically increased at 24 h p.i as indicated by the densitometry analysis (**Fig. 8B**). Additionally, the phosphorylation of P70S6K is not significantly increased at 1 h p.i (**Fig. 8C**), but is highly increased compared to ACTB loading controls at 3 and 24 h p.i. (**Fig. 8C**). Interestingly, as mTORC1 activation peaks at 3 h p.i., the level of autophagy decreases (**Figs. 1 A, B, C, D and E**), suggesting that *R. australis* may utilize mTORC1 activation as a counter-strategy to evade autophagy within host macrophages.

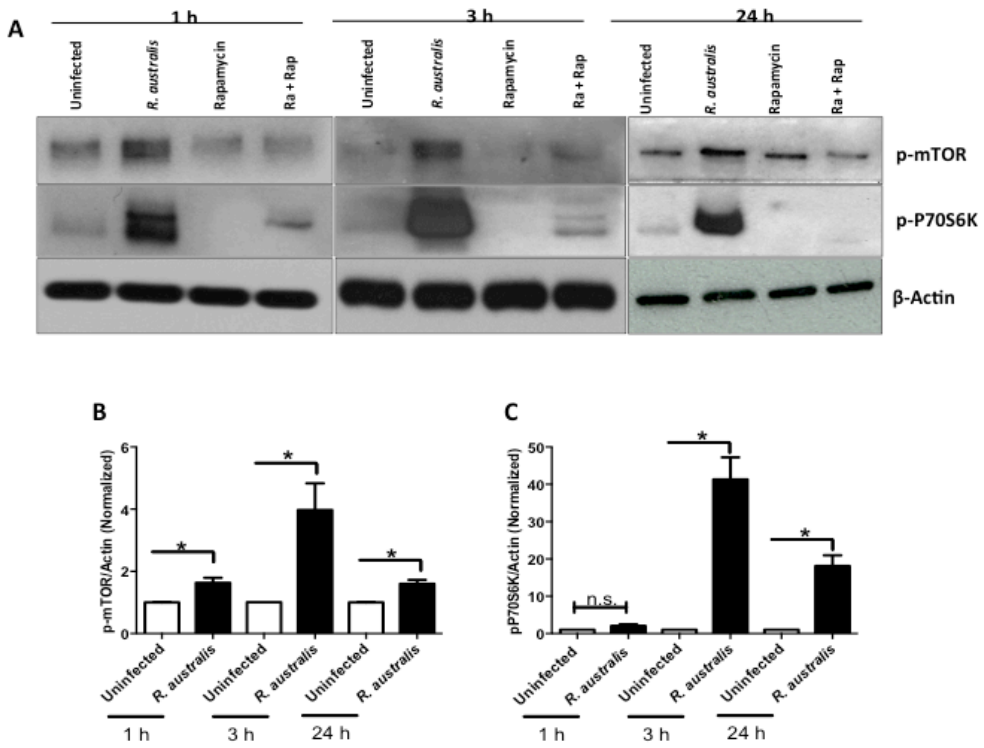


Figure 8. *R. australis* induced activation of mTOR signaling pathway in murine BMMs. Primary murine BMMs were infected with *R. australis* at MOI of 5. Cells were collected at 1 h, 3 h and 24 h p.i., and cell lysates were immunoblotted with antibodies directed against phospho-mTOR and phospho-p70S6K and ACTB (A). The activation of phospho-mTOR and (C) phospho-p70S6K were analyzed by densitometry using ACTB as a normalization control (B).

R. australis induced mTOR-dependent autophagy in murine BMMs

To investigate whether mTOR is a potential negative regulator of autophagy during *R. australis* infection in BMMs, I utilized pharmacologic inhibition of mTOR using rapamycin to evaluate the effects on host cell

autophagy. At 1 h p.i., *R. australis* induced a significant increase in the LC3-II/ACTB ratio compared to uninfected controls, and a slight, but not significant, increase in SQSTM1/ACTB, indicating that autophagosomes were forming early during infection. Interestingly, treatment with a low dose of rapamycin did not induce significant autophagy, as there was only a minimal increase in LC3-II/ACTB. However, the dose used (50 nM), was sufficient to inhibit mTORC1 activation as shown in figure 8A. When comparing LC3-II or SQSTM1 levels in infected cells to infected cells treated with rapamycin, there were no significant differences at any time points tested (**Figs. 9A and B**).

I next evaluated how inhibition of mTOR activity with rapamycin would impact *Rickettsia*-induced autophagy by examining the levels of LC3-II in infected cells with or without rapamycin. I treated cells with rapamycin and infected cells with *R. australis* for 1 hour. Cells were stained with antibody specific for LC3-II (green) and DAPI (blue), and the total fluorescence was calculated using Image J as a measure of LC3-II. As expected, *R. australis* induced a significantly increased level of LC3-II compared to uninfected cells. Additionally, rapamycin induced a significant amount of staining, indicating that autophagosomes were present in the cytosol of treated cells. Interestingly, infected cells treated with rapamycin had LC3-II fluorescence intensities that were similar to those of infected cells alone; no synergistic or additive effects were observed when mTOR was inhibited during infection with rickettsiae (**Fig. 9 C**). These data suggest that autophagy is likely initiated in an mTOR-dependent mechanism.

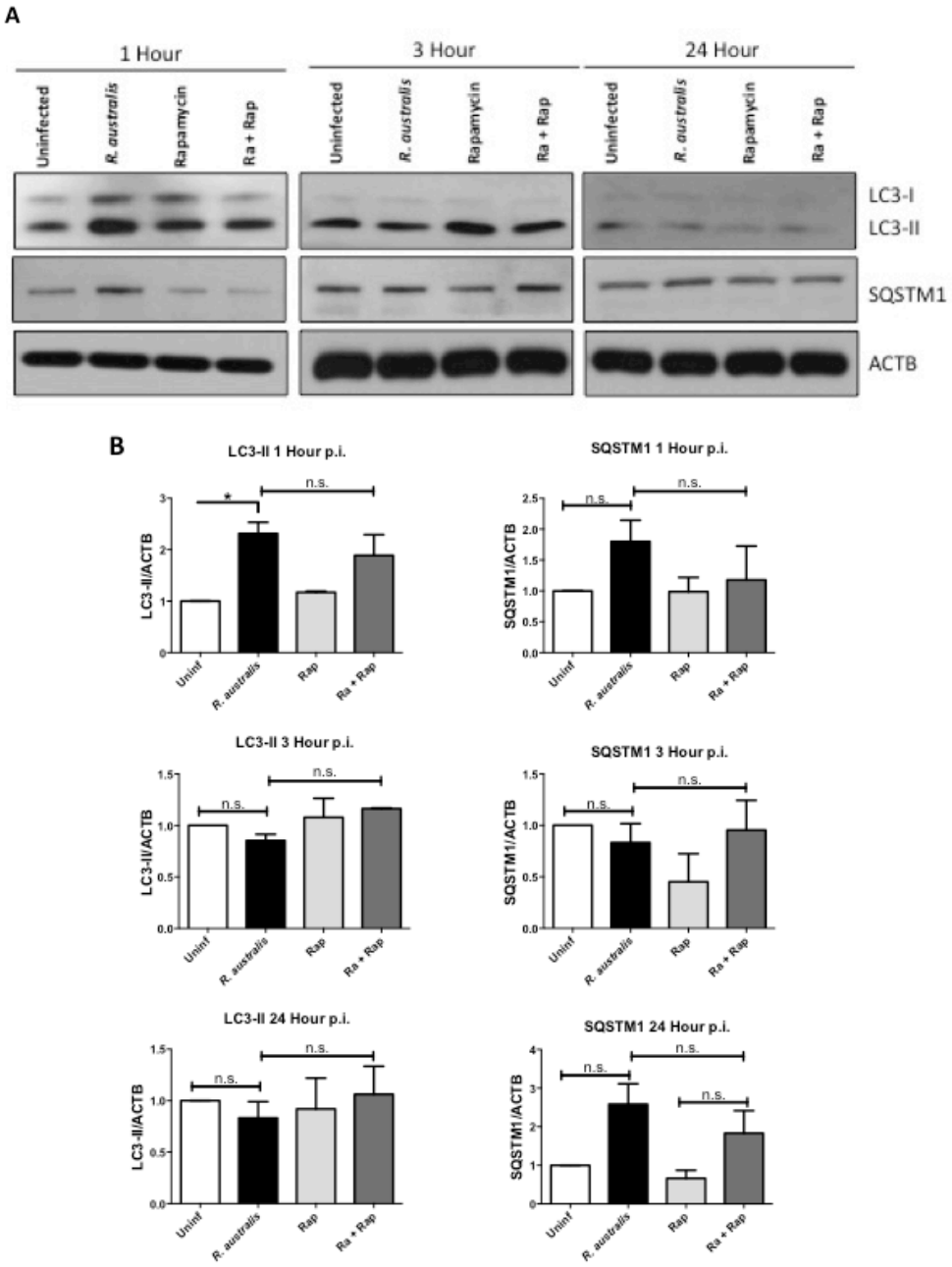


Figure 9 A and B. *R. australis* induced activation of autophagy during mTOR inhibition in murine BMMs. Primary murine BMMs were infected with *R. australis* at MOI of 5. Cells were treated with 50 nM rapamycin as indicated 4 hours prior to infection. Cells were collected at 1 h, 3 h and 24 h p.i. and cell lysates were immunoblotted with antibodies directed against LC3, SQSTM1 and ACTB (**A**). The ratios of LC3-II/ACTB and SQSTM1/ACTB were analyzed by densitometry for each time point (**B**). Data shown are mean ± SE of three independent experiments. *, $p<0.05$.

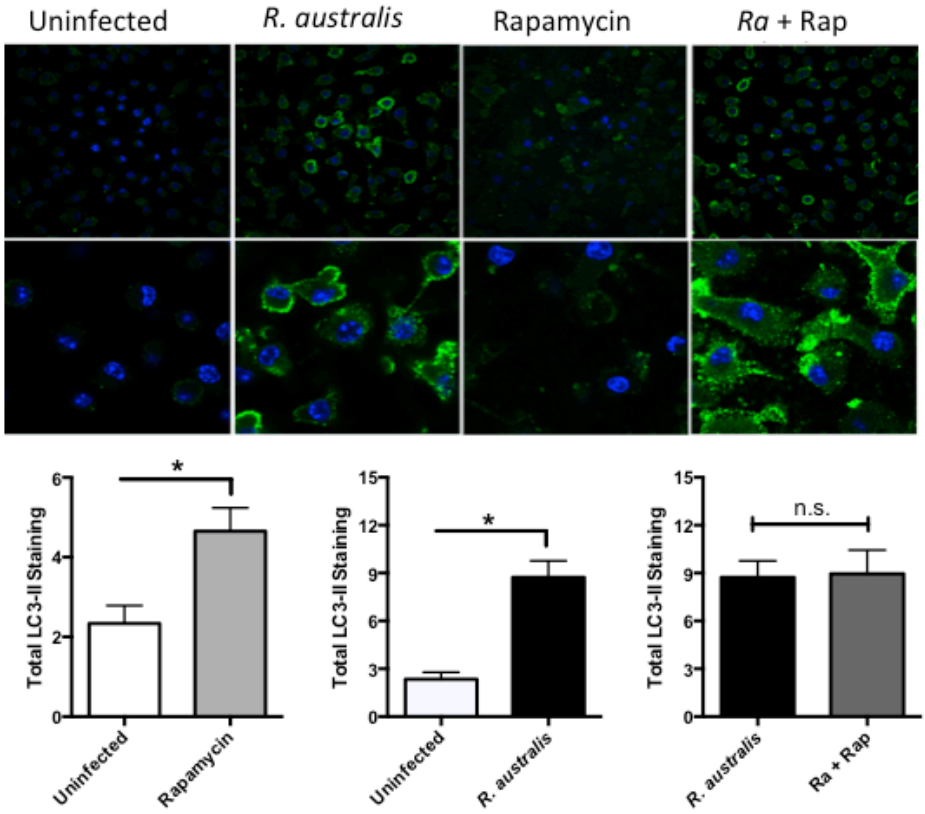
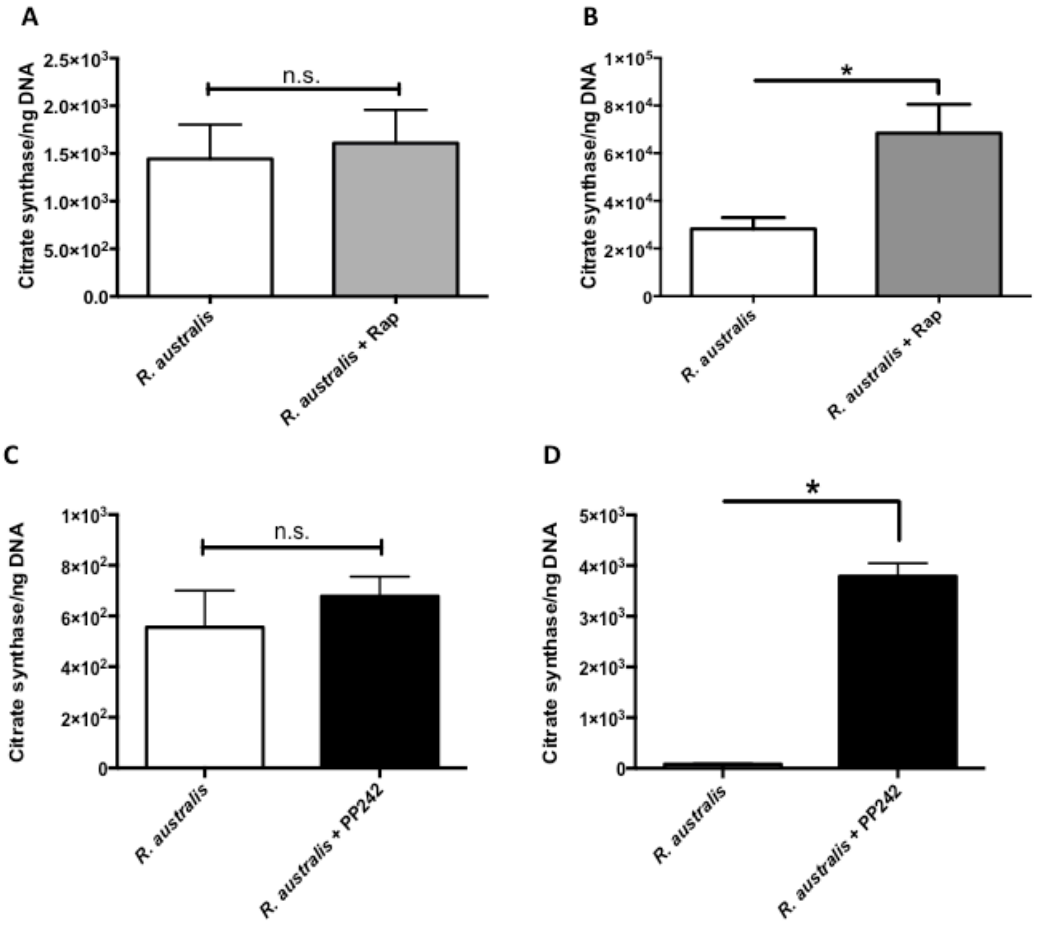


Figure 9C. *R. australis* induced LC3-II in BMMs is mTOR-independent. Primary murine BMMs were infected with *R. australis* at MOI of 5. Cells were grown on glass coverslips and collected at 1 h p.i.. Cells were stained using an LC3-II specific antibody (green), the nuclei were stained with DAPI (blue), and slides were visualized using confocal microscopy. Figures shown are representative confocal microscopic mages of infected macrophages. Total LC3-II staining was quantified using Image J software. Microscopy data represent two independent experiments. Data shown are mean ± SE. *, $p<0.05$.

mTOR inhibition promoted bacterial replication *in vitro*

To assess the consequences of mTOR inhibition on *R. australis* elimination in bone marrow derived macrophages, I determined the bacterial burden in cells treated with the pharmacological inhibitor of mTORC1,

rapamycin. As illustrated in figure 10 A, the treatment of BMMs with rapamycin has no effect on bacterial entry into host cells as determined by the insignificant differences in bacterial burden at 30 minutes p.i. (**Fig. 10A**); however, treatment with rapamycin significantly increases the bacterial replication at 48 h p.i. (**Fig. 10B**). Unlike rapamycin, PP242 inhibits both mTORC1 and mTORC2 by targeting the ATP domain of mTOR and is a more effective mTORC1 inhibitor than rapamycin. Like rapamycin, PP242 has no effect on the ability of murine macrophages to take up rickettsiae at 30 minutes p.i. (**Fig. 10C**). However, at 48 h p.i., cells treated with PP242 had a significantly increased bacterial burden compared to untreated cells (**Fig. 10D**).



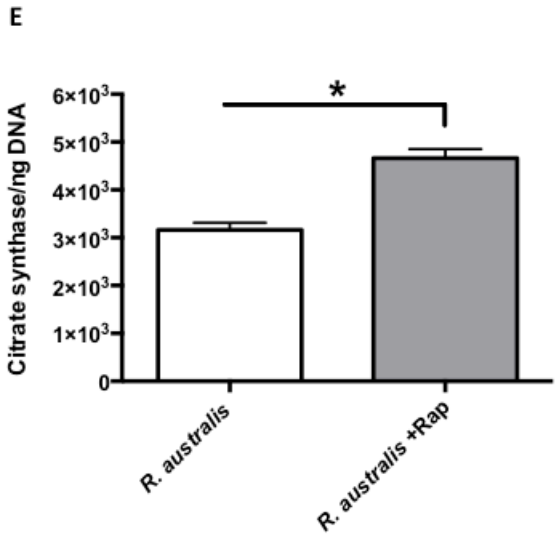


Figure 10. The inhibition of mTOR promotes significant bacterial replication in murine macrophages. BMMs were infected with *R. australis* at an MOI of 2. The burden of intracellular bacteria in adherent cells at indicated time points was quantified by real-time PCR after washing with PBS. The number of citrate synthase (CS) gene copies per ng of genomic DNA represents the quantity of rickettsiae. BMMs were pretreated with rapamycin (50 nM) for 4 hours and then infected with *R. australis* for 30 minutes (A), and 48 h (B) before collecting DNA. Rickettsial replication was also monitored in cells pre-treated with PP242 (1.0 μM) for 30 minutes (C) and 48 h (D). Bacterial burden was also examined in RAW264.7 cells pretreated with rapamycin (50 nM) and infected at an MOI of 2 for 48 h (**E**). Data shown are mean ± SE of at least three independent experiments. *, $p<0.05$.

Additionally, the murine macrophage cell line RAW264.7 treated with rapamycin had significantly increased rickettsial burden compared to untreated cells 48 h p.i. (**Fig. 10E**). These data suggest that mTOR activation, or other downstream effects of mTOR activation, may be a host defense mechanism against rickettsiae infection. Rapamycin and PP242 induce autophagy by

inhibiting mTOR activation, suggesting that the induction of autophagy or the inhibition of mTOR activation facilitates bacterial replication in murine macrophages.

R. australis established an active infection in human monocyte derived macrophages

Because human macrophages are generally believed to be a minor cell target during rickettsial infections (Walker, 2007), the relevance of using macrophages in these studies could be questioned. Therefore, to establish that *R. australis* infects human macrophages *in vitro*, I infected human monocyte-derived macrophages with *R. australis* for 3, 24 and 48 hours and visualized bacteria by microscopy and determined bacterial burden using qPCR. *R. australis* infected and persisted in human macrophages for at least 48 hours as shown by the presence of bacteria in the cytoplasm of cells 48 h p.i.. Rickettsiae were clearly visible as short purple-stained bacilli in the cytoplasm of macrophages visualized using Diff-Quik and light microscopy at 48 hours, while uninfected cells showed no infection (**Fig. 11A**).

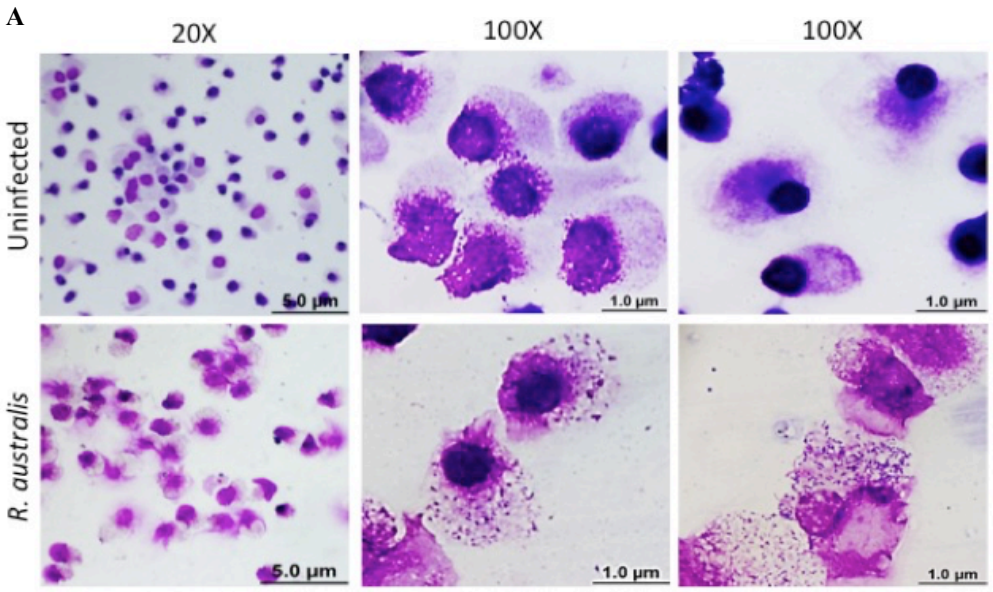


Figure 11 A. *R. australis* established an active infection in primary human monocyte-derived macrophages. Primary human CD14+ monocytes were differentiated into macrophages using M-CSF for 7 days and were infected with *R. australis* at MOI of 5. Cells were grown in glass chamber slides and collected at 48 h p.i.. Cells were visualized using Diff-Quik stain and visualized at 20X and 100X magnification (A). Data shown represents two independent experiments.

To further characterize the infection of human macrophages, I infected cells grown on glass chamber slides with *R. australis* for 48 hours and stained the cells using antibodies against CD68 and rickettsiae as well as DAPI to visualize DNA during confocal microscopy. With CD68 as a cytoplasmic marker of macrophages/monocytes (green), confocal microscopy confirmed that cells are in the monocyte/macrophage lineage as well as delineated the cytoplasmic area. Rickettsiae-specific antibodies labeled with Alexa Fluor 647 (red) showed bacteria present in the cytoplasm at 48 hours p.i., (**Fig. 11B**).

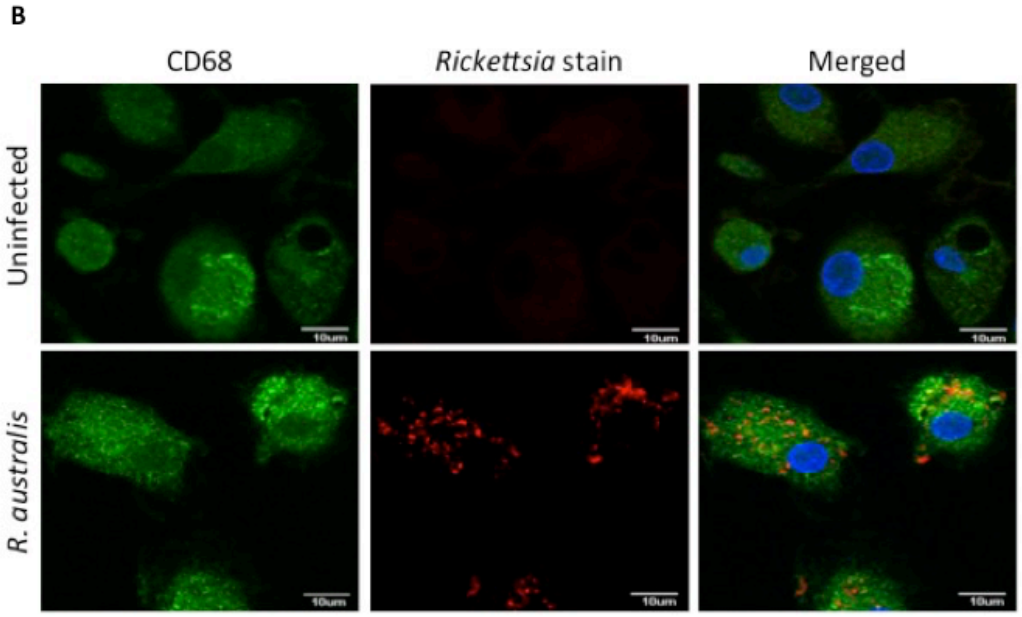


Figure 11 B. *R. australis* established an active infection in primary human monocyte-derived macrophages. Primary human CD14+ monocytes were differentiated into macrophages using M-CSF for 7 days and were infected with *R. australis* at MOI of 5. Cells were grown in glass chamber slides and collected at 48 h p.i.. Representative confocal microscopic images of infected macrophages at 48 h p.i... The macrophage marker CD68 is depicted in green, nuclei (DAPI) in blue, and *R. australis* in red. More than 100 cells were examined under each condition (B). Data shown represents two independent experiments.

I confirmed pathogen infection by performing qPCR, using primers specific for rickettsial citrate synthase. While I did not observe the presence of *R. australis* in uninfected cells, macrophages infected with *R. australis* were positive for the citrate synthase gene at 3, 24 and 48 h p.i. (**Fig. 12**). Quantitative qPCR provided convincing evidence that *R. australis* survives inside human monocyte-derived macrophages. Using a time point series, I detected small numbers of bacteria at 3 h p.i., followed by a statistically significantly increased load at 24 and 48 hours compared to the early time point. These data provide convincing evidence that *R. australis* induces an active infection in human macrophages.

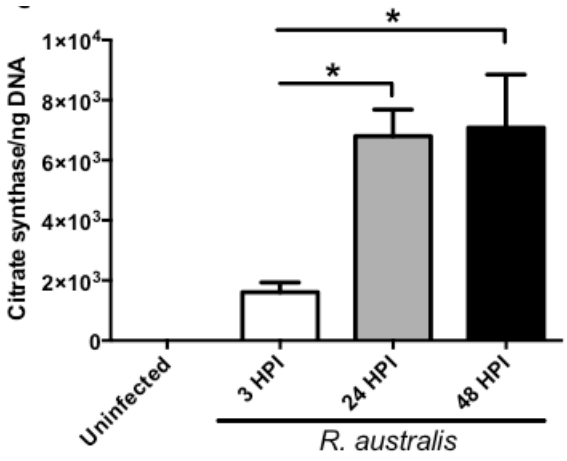


Figure 12. *R. australis* established an active infection in primary human monocyte-derived macrophages established using qPCR. Primary human CD14+ monocytes were differentiated into macrophages using M-CSF for 7 days and were infected with *R. australis* at MOI of 2. The numbers of intracellular bacteria in adherent cells at indicated time points were quantified by real-time PCR after washing with PBS. Number of citrate synthase (CS) gene copies per ng of genomic DNA represents the quantity of rickettsiae. Bacterial burden was assessed using 3 different donor samples. Data shown are mean ± SE of three independent experiments. *, $p<0.05$.

Discussion

Although the available evidence shows that *R. australis* induces autophagy early during infection of murine macrophages but that it is significantly reduced by hour 3 (Chapter 3), the mechanisms leading to inhibition of autophagy and the role of mTOR activation during rickettsial infection are not understood. In this study I demonstrated that *R. australis* induced mTOR activation as soon as 1 hour p.i., and maintained an activated status through 24 hours p.i.. My results also suggest that inhibition of mTOR promoted bacterial replication in murine macrophages as evidenced by greater rickettsial burden in cells treated with rapamycin or PP242 at 48 hours p.i.. My results also showed that *R. australis* established an infection in human monocyte-derived macrophages as seen by increased bacterial burden in HMDMs from 3 to 48 hours p.i. as well as by direct visualization of rickettsiae inside cells by Diff-Quik staining and confocal microscopy.

Modulation of the mTOR signaling cascade plays distinct roles in the response against several human pathogens. Hepatitis C virus (HCV) has been show to activate mTOR leading to enhanced cell survival by blocking apoptosis. The protein responsible has been identified as the viral nonstructural protein 5A (NS5A), which impairs the binding of FKBP38, a member of the FK506-binding proteins (Peng, Liang, Tong, et al., 2010). Human cytomegalovirus (HCMV) has also been shown to induce mTOR activation and attempts to maintain this activation throughout infection even during strong inhibitory stress responses (Kudchodkar, Yu, Maguire, et al., 2004) (Kudchodkar, Yu, Maguire, et al., 2006).

HIV-1 prevents autophagy in human DCs by activating mTOR as a strategy to evade immune-mediated control (Blanchet, Moris, Nikolic, et al., 2010)

While well studied in viral infections, mTOR activation by bacterial pathogens is less characterized and contains few examples. *Campylobacter jejuni* is a leading cause of enteritis throughout the world. In mice infected with *C. jejuni*, rapamycin decreased *C. jejuni*-induced phosphorylation of p70S6 kinase. More importantly, the authors showed that *C. jejuni* induces mTOR activation to evade host-mediated killing via the autophagy response and ultimately induces colitis through mTOR activation (Sun, Threadgill and Jobin, 2012). Additionally, the *Pasteurella multocida* toxin has been shown to activate mTOR signaling in both fibroblasts (Oubrahim, Wong, Wilson, et al., 2013) and macrophages (Kloos, Chakraborty, Lindner, et al., 2015). Zullo and Lee reported that several species of *Mycobacterium* activate mTOR signaling and induce autophagy; however, they show that induction of autophagy in macrophages is mTOR-independent (Zullo and Lee, 2012).

Similar to other studies, my results indicate that *R. australis* induces mTOR activation in murine macrophages early after infection (by 1 hour), that peaks at 3 hours p.i., and remains significantly increased over uninfected controls at least until 24 hours p.i. (**Figs. 8A, B and C**). Interestingly, while mTOR is phosphorylated at 1 hour p.i., there is not a significant increase in phosphorylated P70S6K, indicating that while mTOR is activated, the ability of phosphorylated mTOR to activate the downstream protein P70S6K is incomplete or potentially stalled. It is possible that activated mTOR observed 1 hour p.i. is not actually

biologically significant because activated P70S6K is not significantly increased until 3 hours p.i. (**Fig. 8C**).

Rickettsial growth is relatively slow compared to other bacteria with doubling times of approximately every 8 hours. Because rickettsiae are obligately intracellular pathogens, they must maintain a favorable environment for long periods of time. Work by Clifton et al., (1998) shows that inhibition of apoptosis is essential for endothelial cell survival during *R. rickettsii* infection (Clifton, Gross, Sahni, et al., 1998), and my previous work has shown that *R. rickettsii* protects host endothelial cells from staurosporine-induced cell death, (Bechelli, Rydkina, Colonne, et al., 2009). Interestingly, mTOR activation has been linked to promoting survival of several types of cancer cells (Huang and Fingar, 2014), and may serve as a means of promoting host cell survival during infection.

During infection, the increased transcription and translation of NF-κB-dependent genes and other genes, increase host metabolism, and activation of innate immune responses such as inflammasome will induce cellular stress. This stress response is an evolutionarily advantageous mechanism that evolved to control the stress and facilitate cell survival. Under a normal stress response, the cell would limit mTORC1 activation to reduce extraneous transcription/translation, saving energy and essential resources needed for cell survival. However, during infection with an obligately intracellular pathogen, the inhibition of transcription/translation would have adverse consequences for rickettsiae. It is an attractive hypothesis that the constitutive activation of mTOR in cells infected with rickettsiae may serve as a mechanism for bacterial growth

by promoting transcription/translation. Additionally, the activation of mTORC1-dependent translation has been shown to predispose mouse macrophages toward producing an anti-inflammatory cytokine profile, a strategy that would be beneficial to rickettsiae (Ivanov and Roy, 2013). It is also possible that the increase in mTOR signaling during rickettsial infection is due to the increase in cytokine synthesis and other effector functions that consume nutrients when macrophages become infected with rickettsiae.

Recent research has shown that infection with *S. flexneri* or *S.* Typhimurium elicits a potent starvation response due to the depletion of amino acids that ultimately leads to the inhibition of mTOR signaling and thus the induction of autophagy (Tattoli, Sorbara, Vuckovic, et al., 2012). It has been suggested that xenophagy originates from a primitive metabolic stress response, bolstering the association between metabolism and immune defense (Lapaquette, Guzzo, Bretillon et al., 2015). Specifically, mTORC1 activation promotes gene transcription involved in glycolysis, the pentose phosphate pathway, and de novo lipogenesis, factors that would be useful for obligate intracellular pathogens (Düvel, Yecies, Menon, et al., 2010). Rickettsiae provide a unique tool to study the induction of mTOR and autophagy as well as the interactions between mTOR and inflammation.

Rickettsiae show a strong tropism for vascular endothelium, particularly small and medium sized vessels (Valbuena and Walker, 2009). However, *R. akari* preferentially infects monocytes/macrophages in eschars and *R. typhi* survives in murine macrophages (Beaman and Wisseman, 1976) (Radulovic,

Price, Beier, et al., 2002). Interestingly, Riley et al., (2016), recently showed that *R. conorii* transformed with the fluorescent plasmid pRam18dRGA[AmTrCh] not only infects endothelial cells during in vivo infection, but also is found within circulating leukocytes, including macrophages (Riley, Fish, Garza et al., 2016). The animal model of Mediterranean spotted fever using C3H/HeN mice inoculated intravenously with *R. conorii* (Malish 7) also demonstrates that rickettsiae infect cells other than vascular endothelium including hepatocytes and macrophages (Walker, Popov, Wen, et al., 1994). Additionally, during fatal human infections, *R. conorii* has been described in macrophages (Walker and Gear, 1985).

While there are significant data showing that rickettsiae infect macrophages *in vitro* and *in vivo*, I have shown for the first time that *R. australis* actively replicates in primary human monocyte-derived macrophages (**Fig. 12**). My data show that at 3 hours post infection, rickettsiae are taken up by macrophages and remain inside cells for 24 and 48 hours post infection as seen directly by light and confocal microscopy. However, using quantitative PCR, I showed that bacterial numbers are significantly increased following infection for 24 hours suggesting that bacteria are dividing within human macrophages as opposed to simply infecting cells.

Overall, my research into mTOR signaling during *Rickettsia* infection provided more questions than answers. However, the study did reveal that *R. australis* induced mTORC1 activation in macrophages and showed that the inhibition of mTOR promoted rickettsial replication. These data suggest that

autophagy may promote bacterial replication; or mTOR inhibition specifically enhances rickettsial replication. Additionally, I showed for the first time that human macrophages are an additional cell type facilitating rickettsial replication *in vitro*. Moreover, several questions remain unanswered regarding the exact role of mTOR during rickettsial disease including the role of mTOR activation on regulating inflammation, the role of mTORC2 during rickettsial infection and the molecular mechanism of mTOR activation. While mTOR-deficient animals are embryonically lethal, mTOR, Raptor and Rictor conditional knockouts are available, which would greatly enhance our understanding of mTOR-mediated control of rickettsiae. Taken together, these findings suggest that mTOR-mediated signaling pathways could serve as potential targets for therapeutic interventions during this intracellular bacterial infection.

Chapter 5

Autophagy regulates inflammation induced by rickettsiae *in vitro* and *in vivo*

Introduction:

Although autophagy has long been considered a cornerstone of intracellular surveillance and host defense, the role of autophagy in regulating inflammation is a more recent development. Autophagy has been shown to regulate the inflammasome, a large multi-protein complex consisting of nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) and the protease caspase-1 (Schroder et al., 2010). The inflammasome functions as a key cytosolic surveillance mechanism and a regulator of the host inflammatory response through controlling caspase-1-mediated secretion of IL-1 family cytokines (IL-1β and IL-18) and pyroptosis (Saitoh et al., 2008).

IL-1β-driven inflammation plays a pivotal role in antimicrobial immunity, and its secretion requires synthesis of pro-IL-1β and cleavage of pro-IL-1β into biologically functional IL-1β by activated caspase-1 following assembly of the inflammasome. Autophagy was first observed to suppress inflammasome activation when IL-1β and IL-18 were elevated in an Atg16L1 deficient mouse model of Crohn's Disease (Saitoh, Fujita, Jang, et al., 2008). Additionally, numerous studies show that autophagy negatively regulates inflammasome activation and the role of basal autophagy in controlling excess inflammation (Nakahira, Haspel, Rathinam et al., 2011) (Zhou, Yazdi, Menu et al., 2011). In

addition to inflammasome, autophagy can affect PRR-mediated type I IFN signaling by amplifying TLR signaling by delivering cytoplasmic PAMPs to TLRs (Lee, Lund, Ramanathan, et al., 2007). Furthermore, there are examples of autophagy directly inhibiting the formation of, or suppressing the activation of, pro-inflammatory protein complexes. Major factors in autophagy-regulated inflammation are attributed to the removal of damaged mitochondria leading to increased mitophagy (Lupfer, Thomas, Anand, et al., 2013) and aggregated inflammasome components (Shi, Shenderov, Huang, et al., 2012).

Previous studies using mouse models of rickettsial disease have identified that IFN-γ and cytotoxic CD8 T cells are critical for host clearance of rickettsiae *in vivo* (Walker, 2007). Additionally, TNF-α is critical for controlling rickettsial infection by inducing the synthesis of nitric oxide to inhibit the intracellular growth of *R. conorii* in mouse cells (Feng and Walker, 1993). Furthermore, I investigated the involvement of MyD88 in rickettsial induced inflammation and showed that IFN-γ, IL-6, IL-12 and IL-1β are significantly reduced in MyD88$^{-/-}$ animals, and that MyD88 signaling mediates a type 1 immune cytokine profile essential for the protective inflammatory response during rickettsial infection (Bechelli, Smalley, Zhao, et al., 2016). Importantly, elevated concentrations of serum IFN-γ, TNF-α, and IL-6 are also reported in human cases of mild and severe rickettsioses (Cillari, Milano, D'Agostino, et al., 1996). Whether autophagy regulates the production of these inflammasome-independent cytokines in macrophages infected with rickettsiae was unknown.

To the best of our knowledge, my study is the first project to identify the critical role of autophagy in macrophages in infections with rickettsiae both *in vitro* and *in vivo*. It has been proposed that autophagy evolved as one of the antimicrobial defense systems in eukaryotic cells (Deretic, Saitoh and Akira, 2013). The regulation of inflammasome-derived IL-1 family cytokines has been established in bacterial model systems (Ilyas, Zhao, Liu, et al., 2016) (Zhou, Zhao, Yue, et al., 2015). In the studies presented in this chapter, I focused on the interactions of inflammation and autophagy in mouse macrophages in *in vivo* and *in vitro* infection with rickettsiae. I hypothesized that autophagy negatively regulates the inflammatory response including caspase-1-dependent IL-1β secretion during rickettsial infection. My results will enhance greatly our understanding of the interplay between autophagy and rickettsiae, and how the autophagic process regulates inflammation during infection with an obligately intracellular bacterium.

Results:

R. australis* induces *Atg5*-dependent and –independent cytokines and chemokines *in vitro* and *in vivo

To investigate the role of autophagy in rickettsial induced inflammation in macrophages *in vitro*, we determined the production of cytokines and chemokines in the supernatant of *R. australis*-infected *Atg5* $^{flox/flox}$ and *Atg5* $^{flox/flox}$ Lyz-*Cre* BMMs (**Fig. 13**). At 24 hours p.i., the levels of IL-1β, IL-1α, IL-6 and TNF-α were significantly increased in *Atg5* $^{flox/flox}$ Lyz-*Cre* BMMs compared to those in *Atg5* $^{flox/flox}$ BMMs. Levels of IL-18, MIP-1α, KC, MCP-1, MIP-1β, RANTES, IL-12p40, G-CSF, IL-10, and IL-12p70 were not significantly altered in *Atg5* $^{flox/flox}$ vs. *Atg5* $^{flox/flox}$ Lyz-*Cre* BMMs, suggesting that these cytokines/chemokines were produced in an *Atg5*-independent manner (**Fig. 13**).

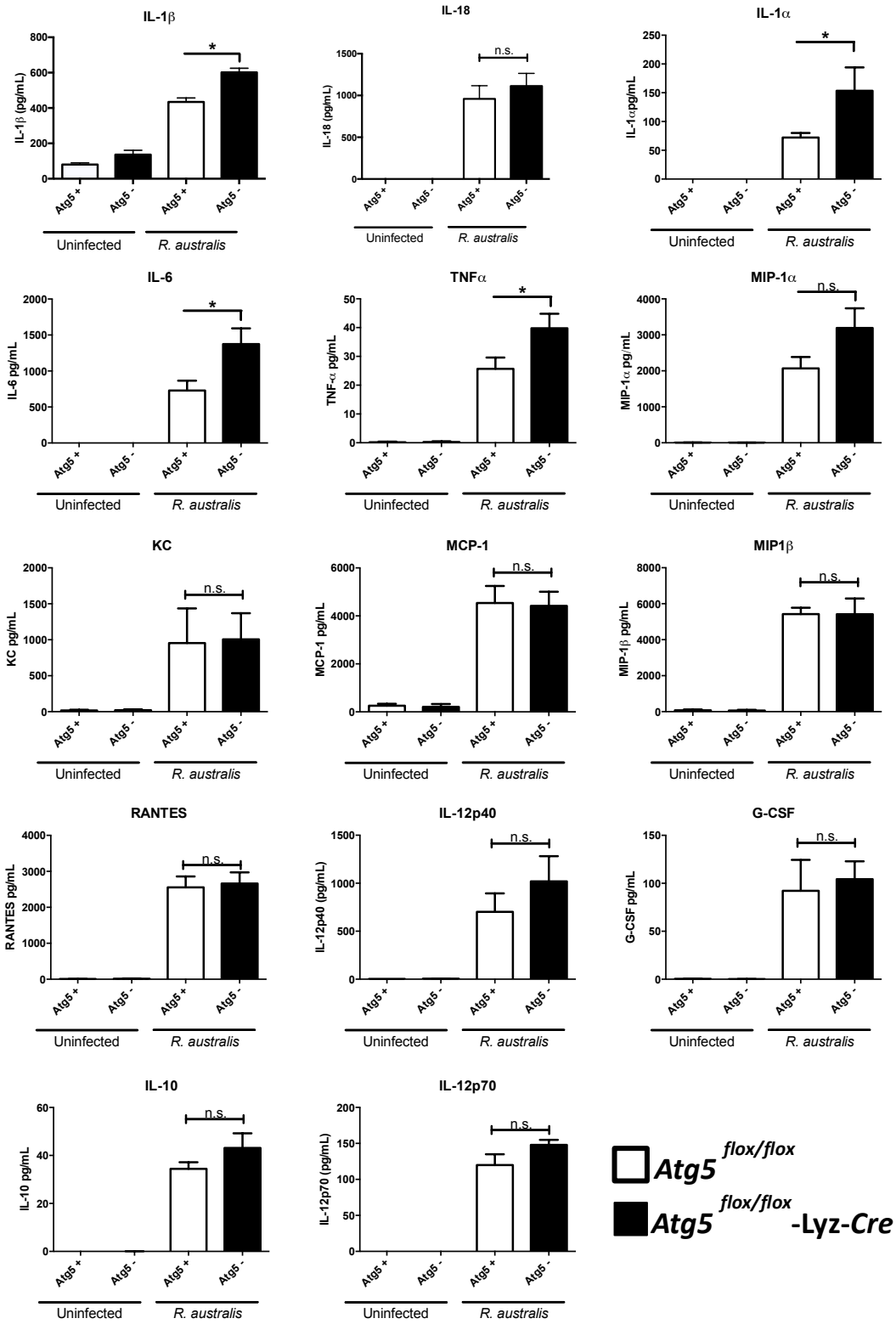

IL-1β
IL-1β (pg/mL)
800
600
400
200
0
*
Atg5 +
Atg5 -
Atg5 +
Atg5 -
Uninfected
R. australis
IL-18
IL-18 (pg/mL)
1500
1000
500
0
n.s.
Uninfected
R. australis
IL-1α
IL-1α pg/mL
250
200
150
100
50
0
*
Uninfected
R. australis
IL-6
IL-6 pg/mL
2000
1500
1000
500
0
*
Uninfected
R. australis
TNFα
TNF-α pg/mL
50
40
30
20
10
0
*
Uninfected
R. australis
MIP-1α
MIP-1α pg/mL
4000
3000
2000
1000
0
n.s.
Uninfected
R. australis
KC
KC pg/mL
2000
1500
1000
500
0
n.s.
Uninfected
R. australis
MCP-1
MCP-1 pg/mL
6000
4000
2000
0
n.s.
Uninfected
R. australis
MIP1β
MIP-1β pg/mL
8000
6000
4000
2000
0
n.s.
Uninfected
R. australis
RANTES
RANTES pg/mL
4000
3000
2000
1000
0
n.s.
Uninfected
R. australis
IL-12p40
IL-12p40 (pg/mL)
1500
1000
500
0
n.s.
Uninfected
R. australis
G-CSF
G-CSF pg/mL
150
100
50
0
n.s.
Uninfected
R. australis
IL-10
IL-10 pg/mL
60
40
20
0
n.s.
Uninfected
R. australis
IL-12p70
IL-12p70 (pg/mL)
200
150
100
50
0
n.s.
Uninfected
R. australis
Atg5 flox/flox
Atg5 flox/flox -Lyz-Cre

Figure 13. *In vitro* production of cytokines/chemokines in infected *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ BMMs**.** Cells were infected with *R. australis* and supernatant harvested 24 h p.i.. Supernatant levels of cytokines/chemokines including IFN-γ, TNF-α, IL-10, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, G-CSF, MCP-1, GM-CSF, eotaxin, MIP-1α, MIP-1β and RANTES were assessed by Bioplex assay. Cytokines that were not produced or were below the limit of detection were not shown. Results are means ± SE of data from three independent experiments. * Significant difference in *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ BMMs (p<0.05).

To determine the mechanisms by which autophagy benefits rickettsial replication in macrophages *in vivo*, we measured the systemic inflammatory response in *Atg5* $^{flox/flox}$ and *Atg5* $^{flox/flox}$ Lyz-*Cre* mice infected with *R. australis*. On day 4 p.i., the production levels of cytokines and chemokines in the serum of *R. australis*-infected mice were measured by Bio-plex assay. The levels of most of the cytokines and chemokines in the serum of infected *Atg5* $^{flox/flox}$ Lyz-*Cre* mice were not significantly changed compare to *Atg5* $^{flox/flox}$ mice, including IL-1β, IL-18, IL-1α, IL-6 and TNF-α, MIP-1α, KC, MCP-1, MIP-1β, RANTES, IL-12p40, IL-10, and IL-12p70. Interestingly, the level of G-CSF was significantly decreased in *Atg5* $^{flox/flox}$ Lyz-*Cre* mice vs. *Atg5* $^{flox/flox}$ mice (**Fig. 14 A**).

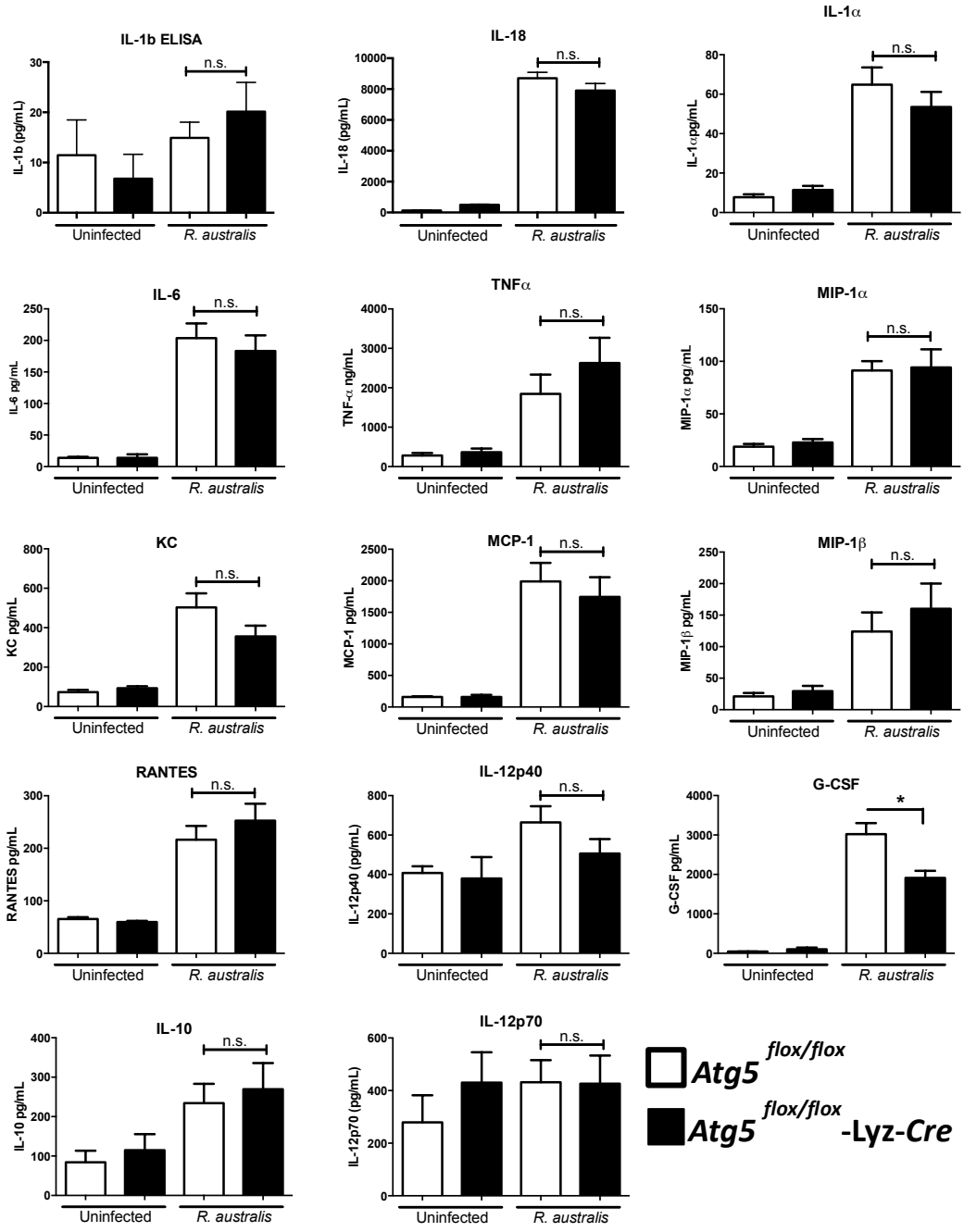

IL-1b ELISA
IL-1b (pg/mL)
n.s.
Uninfected
R. australis
IL-18
IL-18 (pg/mL)
n.s.
Uninfected
R. australis
IL-1α
IL-1α pg/mL
n.s.
Uninfected
R. australis
IL-6
IL-6 pg/mL
n.s.
Uninfected
R. australis
TNFα
TNF-α ng/mL
n.s.
Uninfected
R. australis
MIP-1α
MIP-1α pg/mL
n.s.
Uninfected
R. australis
KC
KC pg/mL
n.s.
Uninfected
R. australis
MCP-1
MCP-1 pg/mL
n.s.
Uninfected
R. australis
MIP-1β
MIP-1β pg/mL
n.s.
Uninfected
R. australis
RANTES
RANTES pg/mL
n.s.
Uninfected
R. australis
IL-12p40
IL-12p40 (pg/mL)
n.s.
Uninfected
R. australis
G-CSF
G-CSF pg/mL
*
Uninfected
R. australis
IL-10
IL-10 pg/mL
n.s.
Uninfected
R. australis
IL-12p70
IL-12p70 (pg/mL)
n.s.
Uninfected
R. australis
Atg5flox/flox
Atg5flox/flox-Lyz-Cre

Figure 14 A. Systemic production of cytokines/chemokines in infected *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice**.** Mice were infected with *R. australis* i.v. and then sacrificed on day 4 p.i.. Serum levels of cytokines/chemokines including IFN-γ, TNF-α, IL-10, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, G-CSF, MCP-1, GM-CSF, eotaxin, MIP-1α, MIP-1β and RANTES were assessed by Bioplex assay. Cytokines that were not produced or were below the limit of detection were not shown. Results are means ± SE of data from three independent experiments containing 4-6 mice per group. * Significant difference in *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice (p<0.05).

Interestingly, serum levels of IFN-γ in *Atg5* $^{flox/flox}$ Lyz-*Cre* animals were significantly reduced compared to *Atg5* $^{flox/flox}$ animals (Fig 14 B). I found two chemokines significantly reduced in *Atg5* $^{flox/flox}$ Lyz-*Cre* vs. *Atg5* $^{flox/flox}$ mice *in vivo,* while four cytokines significantly increased in *Atg5* $^{flox/flox}$ Lyz-*Cre* vs. *Atg5* $^{flox/flox}$ mice *in vitro* in response to rickettsial infections. No cytokine/chemokines were significantly increased in *Atg5* $^{flox/flox}$ Lyz-*Cre* vs. *Atg5* $^{flox/flox}$ mice *in vivo,* or reduced in *Atg5* $^{flox/flox}$ Lyz-*Cre* vs. *Atg5* $^{flox/flox}$ mice *in vitro*. These results suggest that *Atg5*-dependent autophagy in macrophages suppressed inflammation *in vitro*, and promoted systemic production of G-CSF and IFN-γ *in vivo*. The increased systemic levels of G-CSF and IFN-γ *in vivo* are likely attributed to increased bacterial burden and/or from other cell types such as endothelial cells and/or dendritic cells, which have been shown to play significant roles during rickettsial infection.

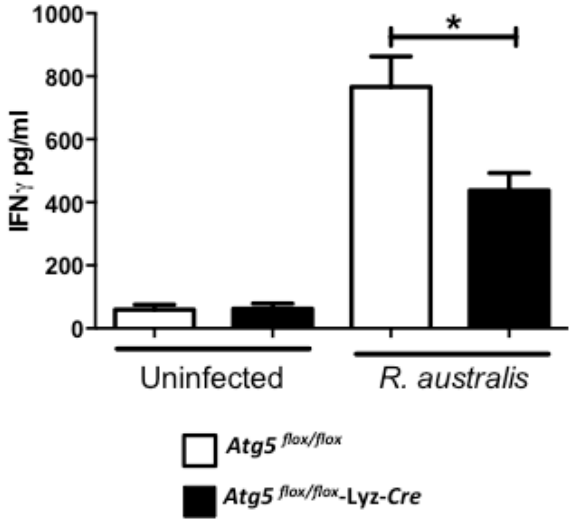


Figure 14 B. Systemic production of IFN-γ in infected *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice. Results are means ± SE of data from three independent experiments containing 4-6 mice per group. * Significant difference in *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice (p<0.05).

Autophagy promotes *R. australis* replication *in vitro*

Previous studies have shown that autophagy is critical for the elimination of mycobacteria in macrophages (Jagannath, Lindsey, Dhandayuthapani et la., 2009) (Watson, Manzanillo and Cox, 2012). Additionally, my data have shown that the inhibition of autophagy by 3-MA promoted a significantly greater bacterial burden compared to untreated controls in RAW 264.7 cells (**Figs. 5A and B**). To assess the role of autophagy in bacterial replication in primary BMMs, I isolated and infected BMMs from *Atg5* $^{flox/flox}$ vs. *Atg5* $^{flox/flox}$ Lyz-*Cre*

mice with *R. australis*. The bacterial burden in these cells was determined at 30 minutes p.i. and 48 hours p.i.. Previous reports using *Atg7*-deficient macrophages have shown that these cells have enhanced mycobacterial uptake (Bonilla, Bhattacharya, Sha et al., 2013). *Atg5*-deficiency did not alter the ability of the cells to uptake rickettsiae as evidenced by similar bacterial burdens at 30 minutes p.i. (Data not shown). However, *Atg5* $^{flox/flox}$ Lyz-*Cre* BMMs are not as permissive for rickettsial replication as evidenced by significantly lower bacterial burden in *Atg5* $^{flox/flox}$ Lyz-*Cre* macrophages compared to *Atg5* $^{flox/flox}$ cells (**Fig. 15A)**. To exclude the possibility that rickettsial replication was enhanced by ATG5 protein itself, but not *Atg5*-dependent autophagy, I infected BMMs of *Atg16L1* $^{flox/flox}$ Lyz-*Cre* and *Atg16L1* $^{flox/flox}$ mice and determined the bacterial burden using quantitative RT-PCR. Again, there was a significant reduction in the rickettsial burden in *Atg16L1* $^{flox/flox}$ Lyz-*Cre* cells compared to *Atg16L1* $^{flox/flox}$ controls (**Fig. 15B**). These data suggest that autophagy promotes rickettsial replication possibly providing an intracellular niche within which *R. australis* multiply and/or by suppressing the inflammatory response of macrophages that has a significant effect on rickettsial killing.

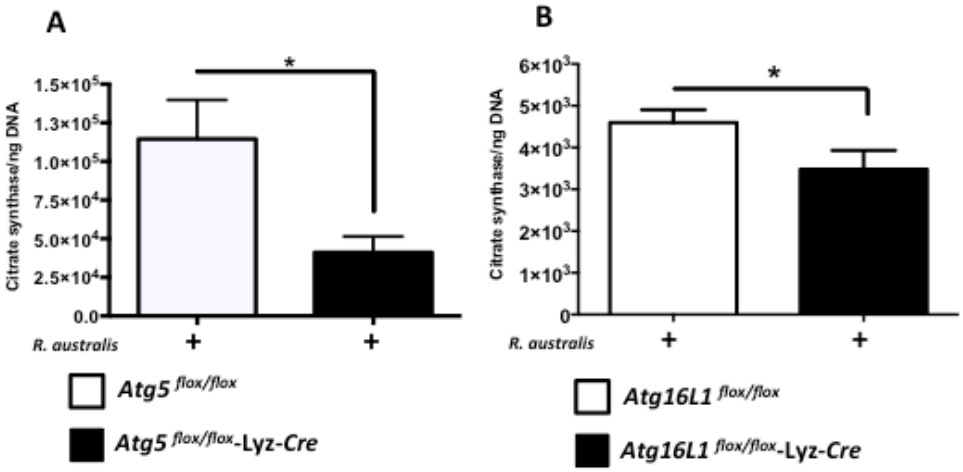
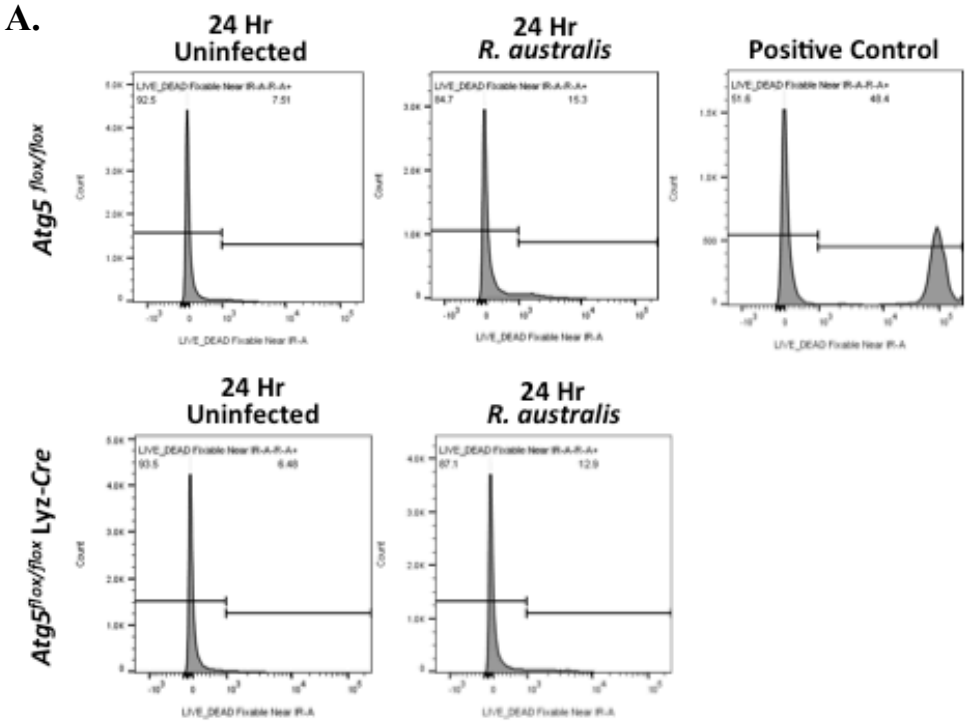


Figure 15. Autophagy promotes bacterial replication *in vitro* in BMMs. BMMs were infected with *R. australis* at an MOI of 2 and DNA was extracted at 48 h p.i.. The numbers of intracellular bacteria in adherent cells at indicated time points were quantified by real-time PCR after washing with PBS. The number of citrate synthase (CS) gene copies per ng of genomic DNA represents the quantity of rickettsiae. Rickettsial replication was monitored in *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ *x* BMMs (**A**) and in *Atg16L1* $^{flox/flox}$ Lyz-*Cre* and *Atg16L1* $^{flox/flox}$ BMMs (**B**). Data shown are mean ± SE of three independent experiments (**A**), 1 experiment using two different mice of each genotype with more than four wells in each experiment (**B**). *, $p<0.05$.

To exclude the possibility that cell death contributes to the reduced bacterial burden in *Atg5* $^{flox/flox}$ Lyz-*Cre* BMMs, I measured the percentage of dead cells by flow cytometry at 24 and 48 hours p.i. (**Figs. 16A and B**). There is minimal difference in cell death between uninfected *Atg5* $^{flox/flox}$ and *Atg5* $^{flox/flox}$

Lyz-*Cre* cells at 24 hours p.i. (7.51% and 6.48% respectively). *R. australis* induced 15.3% and 12.9% cell death in *Atg5* $^{flox/flox}$ and *Atg5* $^{flox/flox}$ Lyz-*Cre* BMMs 24 hours p.i. (**Fig. 16A**). Similarly, uninfected *Atg5* $^{flox/flox}$ and *Atg5* $^{flox/flox}$ Lyz-*Cre* BMMs at 48 hours show minimal cell death, 6.58% and 5.11% respectively. However, *Atg5* $^{flox/flox}$ Lyz-*Cre* BMMs have double the amount of cell death (12.9%), compared to *Atg5* $^{flox/flox}$ BMMs (6.18%) following 48 hours of infection with *R. australis* (**Fig. 16B**). The positive control consisted of equal portions of boiled and fresh BMMs (48% cell death) (**Figs. 16A and B**). These data suggest that cell death induced by rickettsiae is autophagy-independent and likely does not contribute to the reduced bacterial burden in autophagy-deficient cells.



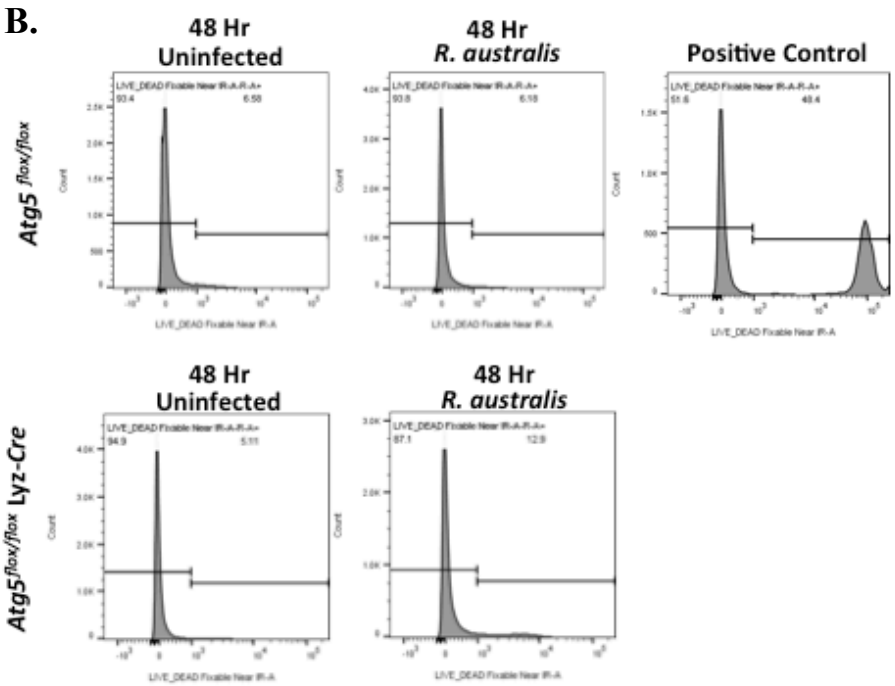


Figure 16. Autophagy-independent macrophage viability. BMMs were infected with *R. australis* at an MOI of 2 and incubated at 37°C for 24 (A) and 48 (B) h p.i.. Cells were stained on ice with fixable Live/Dead Near-IR viability stain for 30 minutes according to manufacturer's instructions. The cells were fixed with 1% paraformaldehyde prior to analysis by a FACSCalibur flow cytometer and the data were analyzed using FlowJo software. Data shown are raw counts from one experiment.

Autophagy and inflammatory infiltrations in rickettsial infection *in vivo*

Histologic analysis of the H&E stained liver sections of infected animals showed lobular foci that were randomly distributed throughout the tissue and consisted of cellular infiltration involving mainly macrophages, but also lymphocytes and neutrophils (**Figs. 17 A and B**). The lung and spleen sections showed no obvious pathological features in infected *Atg5* $^{flox/flox}$ vs. *Atg5* $^{flox/flox}$ Lyz-*Cre* mice (Data not shown). Interestingly, the frequency of pathological foci was significantly reduced in *Atg5* $^{flox/flox}$ Lyz-*Cre* livers compared to *Atg5* $^{flox/flox}$ controls (**Fig. 17 C**), and the inflammatory foci in the livers of *Atg5* $^{flox/flox}$ Lyz-*Cre* mice were significantly greater in size compared to *Atg5* $^{flox/flox}$ mice on day 4 p. i. with *R. australis* (**Fig. 17D**).

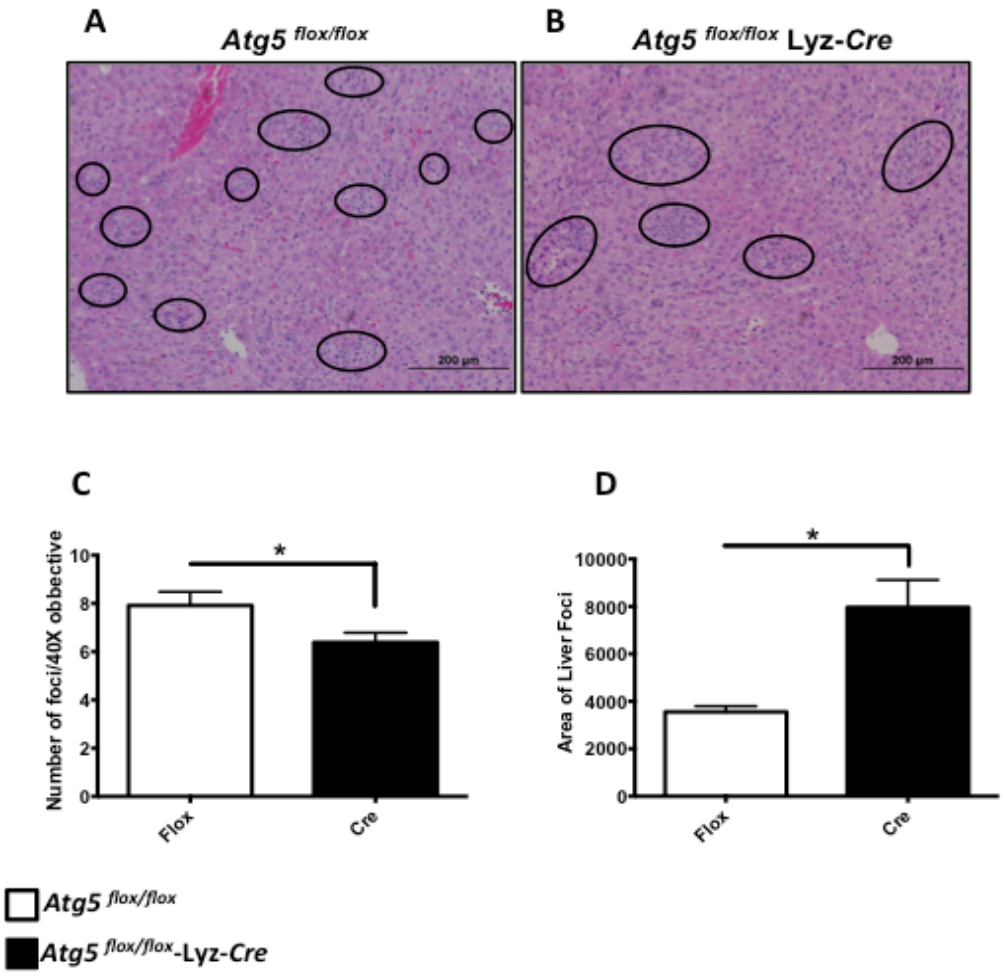


Figure 17. *Atg5*-dependent liver pathology during *R. australis* infection. *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice were infected i.v. with *R. australis* (3×10^5 PFUs per mouse). On day 4 p.i., mice were sacrificed and tissues were isolated and analyzed. Histopathogical staining of liver from infected mice under 20 X magnification (**A** and **B**). Foci of inflammatory infiltration are circled in black. The frequency (**C**) and size (**D**) of inflammatory lesions were analyzed using Image J (magnification 20 X). These results are representative of three independent experiments with at least 4 mice per group. *, $p<0.05$ using unpaired *t* test.

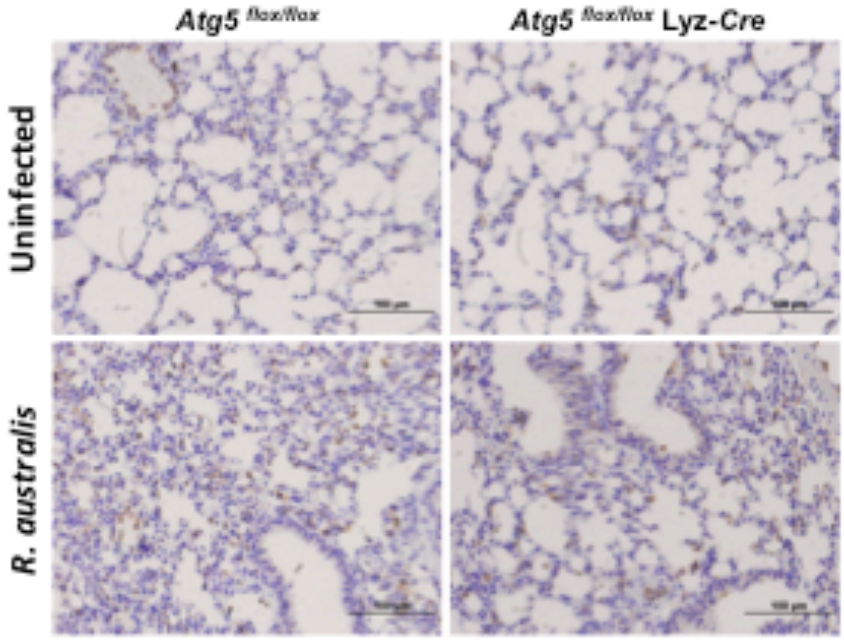
To further characterize the inflammatory response regulated by ATG5 *in vivo* through detecting inflammatory infiltrations, I determined the type and frequency of infiltrated inflammatory cells in the liver, spleen and lung on day 4 p.i. by immunohistochemical staining and quantitative analysis. Macrophages and T cells were stained with individual specific antibodies (F4/80 and CD3 antibodies, respectively) and analyzed using Immunoratio (Tuominen, Ruotoistenmäki, Viitanen, et al., 2010).

As shown in figure 18 A, the *in vivo* levels of CD3 positive cells in the lungs were unchanged in uninfected *Atg5* $^{flox/flox}$ Lyz-*Cre* mice compared to *Atg5* $^{flox/flox}$ animals (**Fig. 18A**). However, there was a significant influx of T cells into the lungs of infected *Atg5* $^{flox/flox}$ mice compared to uninfected animals. This influx of cells was not seen in *Atg5* $^{flox/flox}$ Lyz-*Cre* mice, suggesting *Atg5*-dependent migration of T cells in the lung. On day 4 p.i., there was a significant increase in CD3 positive cells in the livers of *Atg5* $^{flox/flox}$ animals which is not observed in *Atg5* $^{flox/flox}$ Lyz-*Cre* mice (**Fig. 18B**), suggesting an *Atg5*-dependent CD3 T cell recruitment to the liver 4 days post infection with *R. australis*. These data suggest that a robust T cell recruitment or migration to infection sites was initiated in animals with autophagy-competent-macrophages, resulting from either higher levels of rickettsial burden, or macrophage-specific autophagy.

Surprisingly, *Atg5* $^{flox/flox}$ Lyz-*Cre* animals had an elevated basal level of macrophages in the lungs compared to *Atg5* $^{flox/flox}$ animals; however, there was no significant difference in macrophage levels between *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ animals when mice were infected with *R. australis* (**Fig. 18D**).

Interestingly, the basal level of macrophages in the livers of uninfected animals showed a significant decrease in *Atg5* $^{flox/flox}$ Lyz-*Cre* animals compared to *Atg5* $^{flox/flox}$ animals and when infected, there was no significant difference in numbers of macrophages between these two genotypes. Furthermore, the level of macrophages in the liver of *Atg5* $^{flox/flox}$ mice significantly declined when infected with *R. australis*, suggesting macrophage emigration out of the liver upon infection (**Fig. 18E**). Similarly to macrophages in the lungs, uninfected *Atg5* $^{flox/flox}$ Lyz-*Cre* animals had significantly higher levels of macrophages in the spleen compared to *Atg5* $^{flox/flox}$ mice. Additionally, *Atg5* $^{flox/flox}$ mice infected with *R. australis* demonstrate macrophage infiltration on day 4 p.i.; however, infected *Atg5* $^{flox/flox}$ Lyz-*Cre* animals have significantly decreased macrophage levels in the spleen, suggesting macrophage efflux out of this organ (**Fig. 18F**).

Figure 18 a


Atg5 flox/flox
Atg5 flox/flox Lyz-Cre
Uninfected
R. australis

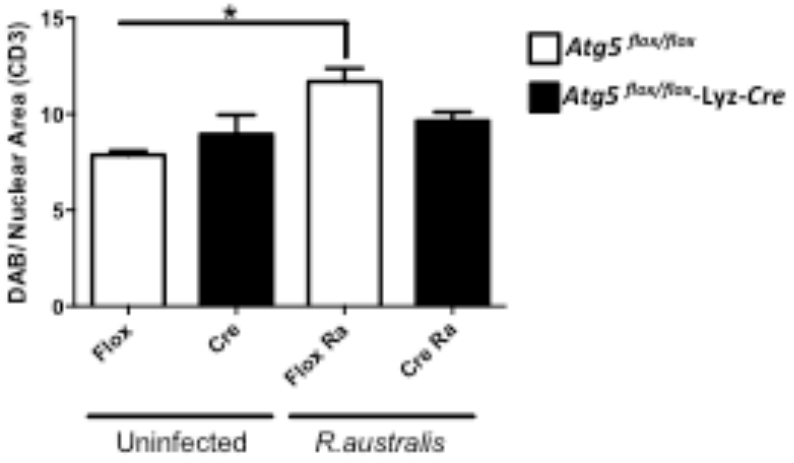

DAB/ Nuclear Area (CD3)
15
10
5
0
*
Flox
Cre
Flox Ra
Cre Ra
Uninfected
R.australis
Atg5 flox/flox
Atg5 flox/flox-Lyz-Cre

Figure 18 b

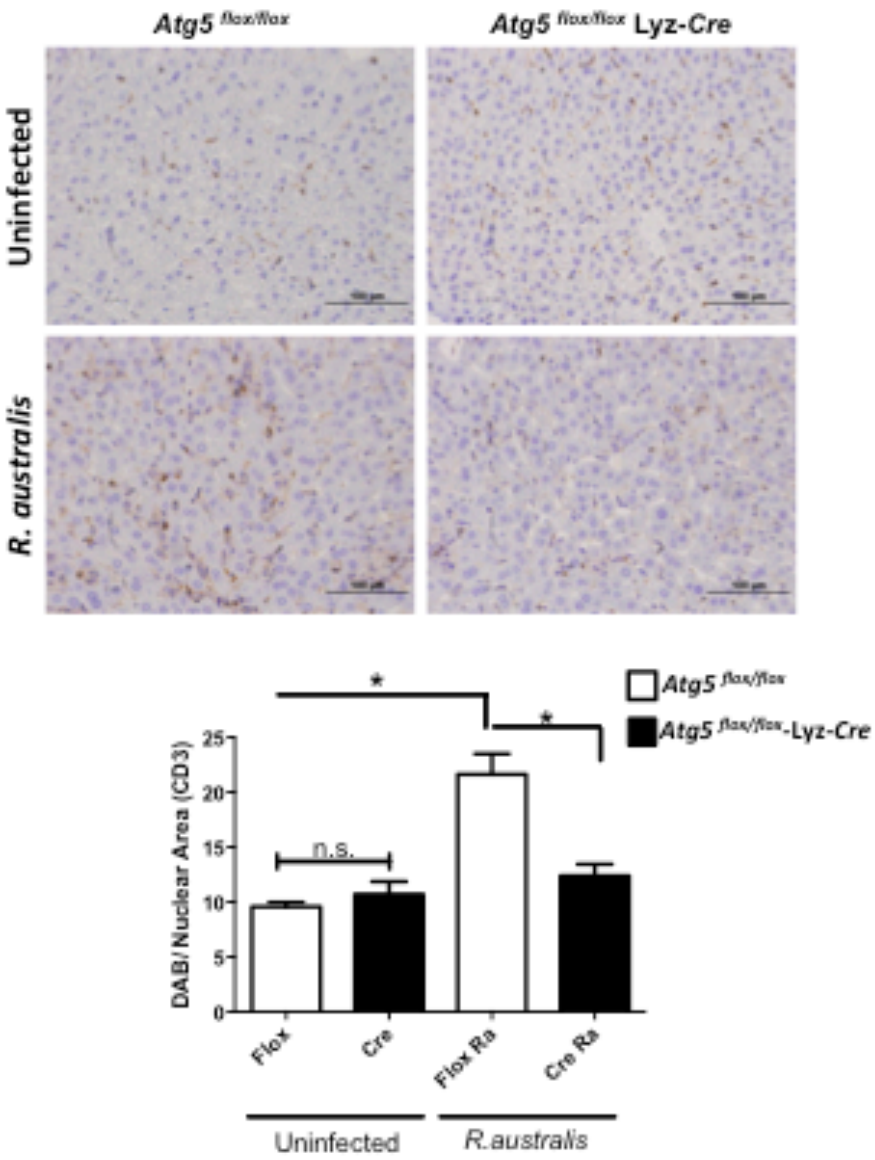
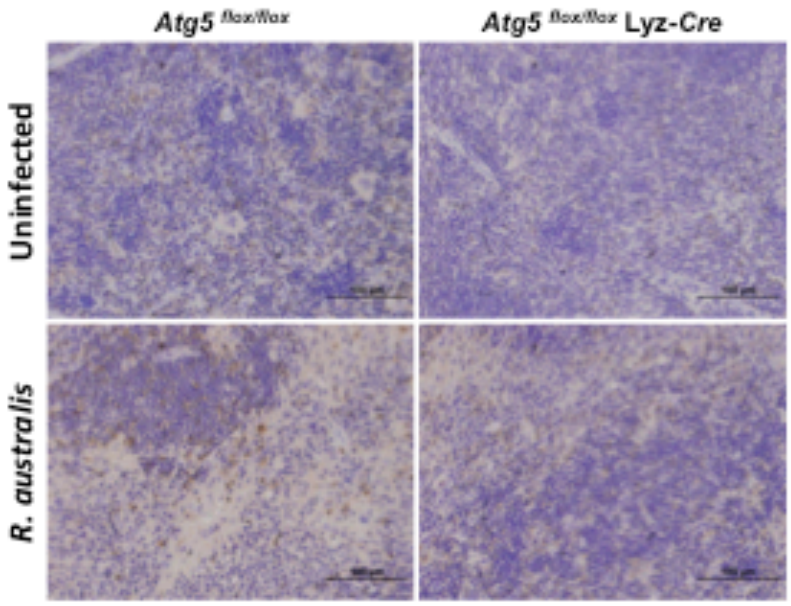

Atg5 flox/flox
Atg5 flox/flox Lyz-Cre
Uninfected
R. australis
Atg5 flox/flox
Atg5 flox/flox-Lyz-Cre
DAB/ Nuclear Area (CD3)
25
20
15
10
5
0
n.s.
*
*
Flox
Cre
Flox Ra
Cre Ra
Uninfected
R.australis

Figure 18 c


Atg5 flox/flox
Atg5 flox/flox Lyz-Cre
Uninfected
R. australis

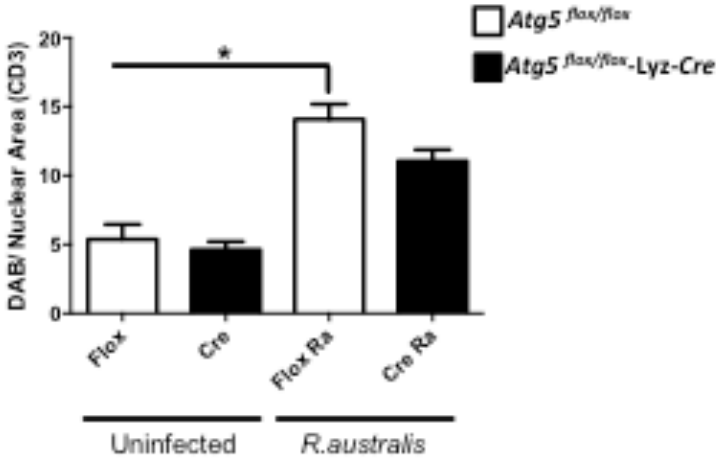
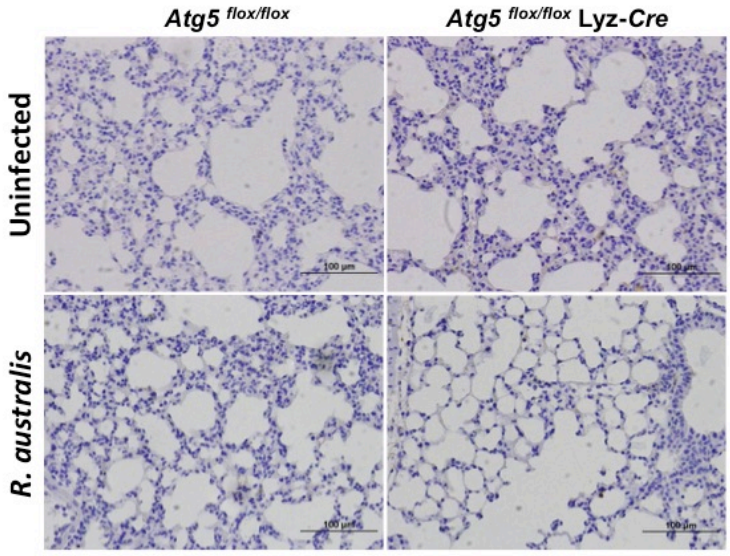

Atg5 flox/flox
Atg5 flox/flox-Lyz-Cre
DAB/ Nuclear Area (CD3)
20
15
10
5
0
*
Flox
Cre
Flox Ra
Cre Ra
Uninfected
R.australis

Figure 18 d


Atg5 flox/flox
Atg5 flox/flox Lyz-Cre
Uninfected
R. australis

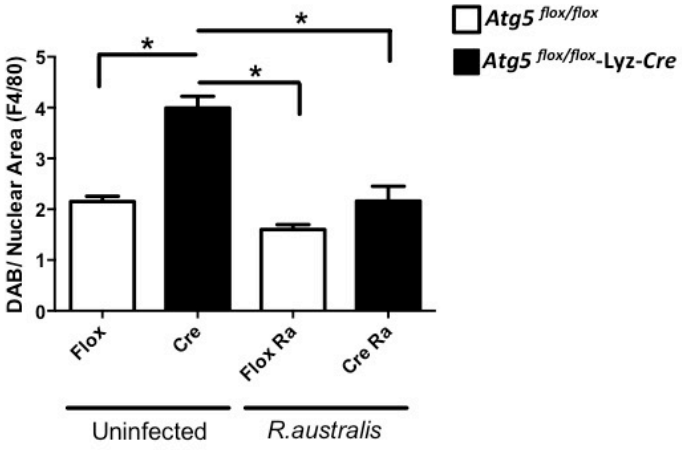

Atg5 flox/flox
Atg5 flox/flox-Lyz-Cre
DAB/ Nuclear Area (F4/80)
0
1
2
3
4
5
*
*
*
Flox
Cre
Flox Ra
Cre Ra
Uninfected
R.australis

Figure 18 e

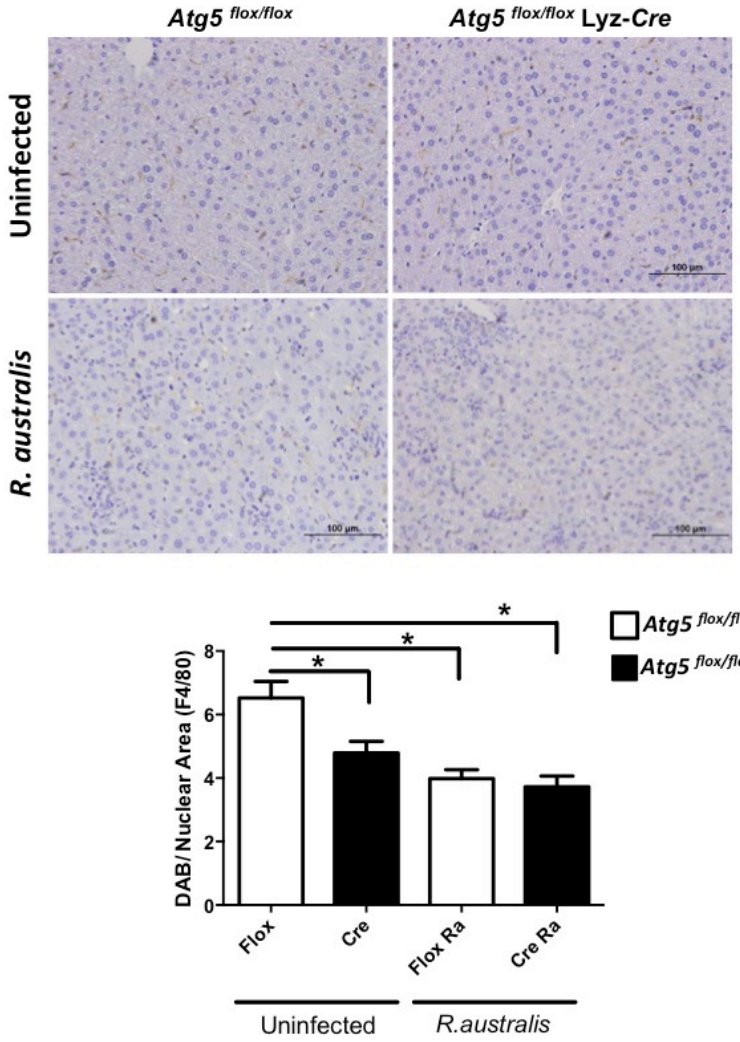

Atg5 flox/flox
Atg5 flox/flox Lyz-Cre
Uninfected
R. australis
100 µm
Atg5 flox/flox
Atg5 flox/flox-Lyz-Cre
DAB/ Nuclear Area (F4/80)
0
2
4
6
8
*
*
*
Flox
Cre
Flox Ra
Cre Ra
Uninfected
R.australis

Figure 18 f

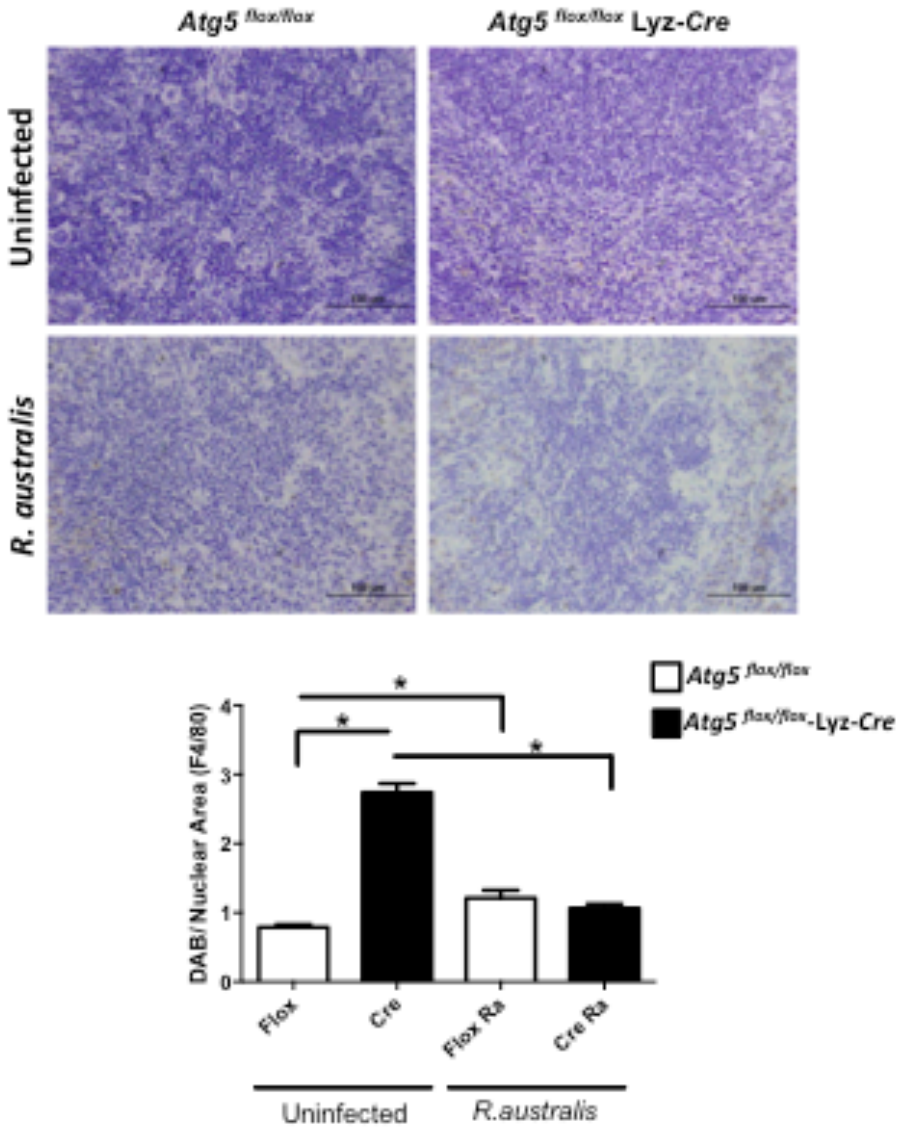

Atg5 flox/flox
Atg5 flox/flox Lyz-Cre
Uninfected
R. australis
Atg5 flox/flox
Atg5 flox/flox-Lyz-Cre
DAB/ Nuclear Area (F4/80)
0
1
2
3
4
*
*
*
Flox
Cre
Flox Ra
Cre Ra
Uninfected
R.australis

Figure 18. *Atg5*-dependent and -independent inflammatory cellular accumulation *in vivo.* Infected tissues were stained with antibody specific against CD3 by immunohistochemistry for detection of T cells in lung (**A**), liver (**B**) and spleen (**C**) on day 4 p.i.. Infected tissues were stained with antibody specific against F4/80 by immunohistochemistry for detection of macrophages in lung (**D**), liver (**E**) and spleen (**F**) on day 4 p.i.. Frequencies of inflammatory cells in different tissues were compared using ImmunoRatio by selecting 8 random microscopic fields from slides of immunohistochemical staining for each comparison. These results are representative of three independent experiments. *, $p<0.05$ using unpaired *t* test.

Atg5 $^{flox/flox}$ ***Lyz-Cre*** **mice are more permissive to *R. australis* replication *in vivo***

To assess the role of ATG5 deficiency on bacterial replication *in vivo*, *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice were infected with a sublethal dose of *R. australis* and sacrificed on day 4 p.i.. The bacterial burden in the liver (**Fig. 19 A**), spleen (**Fig. 19 B**) and lungs (**Fig. 19 C**) was significantly decreased in *Atg5* $^{flox/flox}$ Lyz-*Cre* versus *Atg5* $^{flox/flox}$ mice. *R. australis* levels in the heart were not significantly changed *Atg5* $^{flox/flox}$ Lyz-*Cre* versus *Atg* $^{flox/flox}$ mice (**Fig. 19 D**).

Figure 19

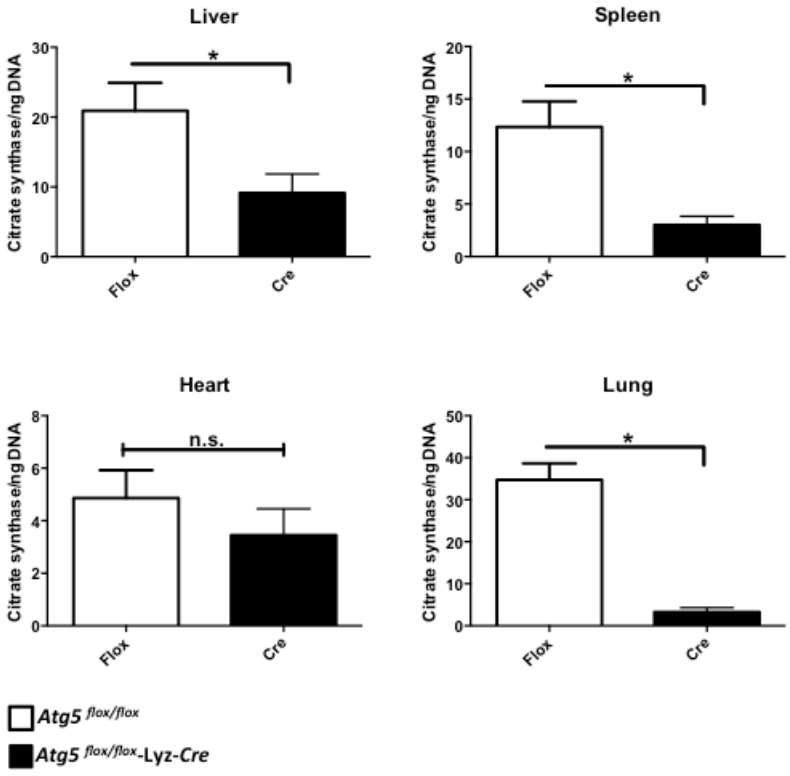


Figure 19. ATG5 facilitates bacterial replication *in vivo*. *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice were infected i.v. with *R. australis* (3×10^5 PFUs per mouse). On day 4 p.i., mice were sacrificed and tissues were isolated. DNA was extracted from these infected tissues, and the numbers of bacteria were quantified by real-time PCR. Number of citrate synthase (CS) gene copies per ng of genomic DNA represents the quantity of rickettsiae. Rickettsial replication was monitored in the livers of *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice, spleen, heart, and lungs. Data shown is representative of three independent experiments contains at least 3 individual mice of each genotype. *, $p<0.05$.

Discussion:

This study investigated the contributions of autophagy to host immunity by examining the inflammatory response to rickettsial infection. My current results demonstrated that autophagy inhibited the production of several cytokines by BMMs during *R. australis* infection *in vitro* while autophagy also promoted the systemic production of G-CSF and IFN-γ *in vivo*. Importantly, my study showed that autophagy promoted rickettsial replication both *in vitro* and *in vivo*, which suggests that autophagy in macrophages serves as one of the mechanisms involved in pathogenesis of rickettsioses.

Several studies have examined the regulation of cytokines by autophagy, particularly the IL-1 family of cytokines. IL-1, IL-2, IL-6, TNF-α, IFN-γ and TGF-β are known to induce autophagy, IL-4, IL-10 and IL-13 inhibit autophagy, and autophagy can regulate the production of IL-1, IL-18, TNF-α, and Type I IFN (Harris, 2011). However, this is the first study to examine the role of autophagy in the regulation of inflammation with an obligate intracellular bacterium. I hypothesized that autophagy would negatively regulate the rickettsiae-induced inflammatory response in macrophages *in vitro* including caspase-1-dependent IL-1β secretion. Additionally, I have examined the importance of ATG5 in rickettsial infection; a novel area of study in the *Rickettsia* field. This study is the first to examine the critical role of phagocyte-specific autophagy in infection with rickettsiae *in vivo*; however, the underlying molecular mechanisms by which rickettsiae trigger the inflammatory response remain unclear.

IL-6 has been previously identified as an autophagy-regulated cytokine (Luo, Wong, Chan, et al., 2015). Interestingly, autophagy-deficient human lung epithelial cells and mouse lung tissues show reduced influenza-induced proinflammatory responses including IL-1β, TNF-α, IL-6, MCP-1, and RANTES, both *in vitro* and *in vivo* (Pan, Zhang, Luo et al., 2014). Additionally, IL-6 has been shown to induce autophagy and induce an antiapoptotic effect in macrophages, an important feature for rickettsial survival (Roca, Varsos, Sud, et al., 2009). Our results demonstrate autophagy-dependent regulation of IL-1β, IL-1-α, IL-6 IFN-γ and TNF-α, and several autophagy-independent cytokines including IL-18 in murine macrophages infected with *R. australis in vitro* (**Fig. 13**). While IL-18 requires caspase-1 and inflammasome activation like IL-1β, it is unclear at this time how autophagy regulates IL-18 (Harris, 2011).

Notably, my data showed that autophagy promoted rickettsiae replication in murine macrophages as demonstrated by enhanced bacterial replication in rapamycin- or PP242-treated cells, decreased bacterial burden in *Atg16L1* $^{flox/flox}$ Lyz-*Cre* BMMs compared to *Atg16L1* $^{flox/flox}$ BMMs and the significantly lower bacterial replication in *Atg5* $^{flox/flox}$ Lyz-*Cre* mice/BMMs compared to *Atg5* $^{flox/flox}$ mice/BMMs. Niu et al. have shown (2008) that the stimulation of autophagy by rapamycin enhances *Anaplasma phagocytophilum* infection, and this obligately intracellular pathogen replicates in early autophagosome-like compartments decorated with LC3 and Beclin 1 (Niu, Yamaguchi, Rikihisa et al., 2008). Work from the same group also shows that *A. phagocytophilum* induces autophagy by using *Anaplasma* translocated substrate-1 which binds to beclin-1 to initiate

autophagy. They propose that autophagosomes traffic and fuse with *A. phagocytophilum* inclusion bodies, which then deliver degraded proteins into the inclusions serving as essential nutrients for bacterial growth (Niu and Rikihisa, 2013). In light of our findings regarding inflammation, I hypothesize that a likely reason for rickettsial induction of autophagy early during infection is to delay and dampen the inflammatory immune response to facilitate rickettsial infection.

IL-18 deficient mice are extremely susceptible to *Burkholderia pseudomallei* infection, which is due to the inefficient T_H1 response required to clear the infection (Ceballos-Olvera, Sahoo, Miller et al., 2011). Mice unable to secrete IL-18 or IL-1β during *Francisella tularensis* infection show significantly increased bacterial burdens and mortality compared to wild-type mice (Mariathasan, Weiss, Dixit, et al., 2005). Additionally, IL-1β and IL-18 are essential for inflammation-based control during *Shigella flexneri* infection (Sansonetti, Phalipon, Arondel, et al., 2000).

Interestingly, IL-1β has a negative effect during *B. pseudomallei* infection, which was shown by the administration of IL-1R antagonists that protected mice from a lethal dose of *B. pseudomallei* (Ceballos-Olvera, Sahoo, Miller et al., 2011). Studies using *Pseudomonas aeruginosa* also show IL-1R antagonist improves host defense against this pathogen (Schultz, Rijneveld, Florquin et al., 2002). We see that ASC-deficient mice that are unable to produce IL-18 or IL-1β succumb to a sublethal dose of *R. australis* suggesting that IL-18 and/or IL-1β may have a positive role in the clearance of rickettsiae *in vivo* (unpublished data). Because IL-18 is known for promoting a potent T_H1 response through the

induction of IFN-γ (Novick, Kim, Kaplanski et al., 2013), it is an attractive hypothesis that the elevated level of IL-18 *in vivo* plays a critical role during rickettsial disease.

Because the *Atg5* $^{flox/flox}$ Lyz-*Cre* mice are autophagy-deficient only in the granulocytes lineage including macrophages and neutrophils, it was not surprising that on day 4 p.i., only minimal changes were observed in cytokine secretion between *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice. This observation is likely due to other cell types that contain ATG5 including fibroblasts, endothelial cells and dendritic cells responding to infection (**Fig. 14A**). I found significantly reduced levels of G-CSF and IFN-γ in *Atg5* $^{flox/flox}$ Lyz-*Cre* mice compared to *Atg5* $^{flox/flox}$ mice. Bacterial infections induce a systemic hematopoietic response called "emergency granulopoiesis" to enhance neutrophil production (Boettcher, Gerosa, Radpour et al., 2014). G-CSF is the major regulator of neutrophilic granulocytes, and infections with a broad range of agents (including *R. australis*) induce a considerable increase in serum G-CSF in both humans and mice (Panopoulos and Watowich, 2008). G-CSF production is mainly from endothelial cells as well as macrophages, and is induced by IL-1β, TNFα and LPS (Demetri and Griffin, 1991), and is regulated by NF-κB (Dunn, Coles, Lang, et al., 1994). While Guo et al. (2015), show that G-CSF promotes autophagy in neural tissue (Guo, Liu, Zhang, et al., 2015), I am the first to show that G-CSF may be regulated by autophagy during *in vivo* infection with *R. australis*. An alternative hypothesis for the decreased G-CSF production is that the lower *Rickettsia* burden in *Atg5*

flox/flox Lyz-*Cre* mice translates to fewer infected endothelial cells, which stimulate a lower G-CSF response compared to *Atg5* *flox/flox* animals.

Work from Franzke et al. (2003) shows that G-CSF stimulation of T cells induces the up-regulation of GATA-3, the master transcription factor for T_H2 immune responses (Franzke, Pio, Lauber, et al., 2003). After *in vivo* G-CSF stimulation, T cells have reduced cytotoxic activity and proliferative response upon *in vitro* stimulation (Reyes, García-Castro, Esquivel et al., 1999). An additional mechanism accounting for the reduced bacterial burden in *Atg5* *flox/flox* Lyz-*Cre* mice could be due to an enhanced T_H1 cell response owing to the significantly lower levels of G-CSF compared to *Atg5* *flox/flox* mice. Further work will be required to determine the mechanisms involved in G-CSF regulation and the implications for G-CSF during rickettsial infection.

An effective T_H1-type cytokine response (TNF-α and IFN-γ) is critical for controlling rickettsial infection (Mansueto, Vitale, Cascio, et al., 2012). Elevated IFN-γ production is essential for inducing NO production that has rickettsiacidal effects (Li, Jerrells, Spitalny, et al., 1987) (Feng, Popov and Walker, 1994). IFN-γ facilitates the formation and maturation of autophagosomes in murine macrophages (Matsuzawa, Kim, Shenoy, et al., 2012), and autophagy regulates the production of IL-1 family cytokines including Type I IFN, important for polarizing T_H1 cytokine responses (Harris, 2011). Additionally, IL-18 has been shown to promote T_H1 cell development, which is critical for rickettsial clearance (O'Garra and Arai, 2000). Macrophages are not a major producer of IFN-γ, and our *in vitro* data confirm this account (data not shown). However, the serum of

Atg5 $^{flox/flox}$ Lyz-*Cre* mice had significantly lower levels of IFN-γ compared to *Atg5* $^{flox/flox}$ mice on day 4 p.i. (**Fig. 14B**). Interestingly, a lower bacterial burden was found in the tissues of *Atg5* $^{flox/flox}$ Lyz-*Cre* mice (**Fig. 19**); the low level of IFN-γ in *Atg5* $^{flox/flox}$ Lyz-*Cre* mice compared to *Atg5* $^{flox/flox}$ animals may be due to the lower number of bacteria in the tissues, or the lack of production mechanisms mediated by autophagy in macrophages.

Besides its role in autophagy initiation, ATG5 has several other functions in various cell types. The ATG12-ATG5 protein complex associates with the retinoic acid-inducible gene I (RIG-I) and interferon-beta promoter stimulator 1 (IPS-1) proteins that are necessary for recognition of RNA viruses and for the upregulation of type I interferons (Takeshita, Kobiyama, Miyawaki, et al., 2008). This group showed that the Atg12-Atg5 protein complex negatively regulates the type I IFN pathway and actually promotes viral replication. Furthermore, the ATG12-ATG5 complex is required for IFN-γ-mediated host defense against murine norovirus replication (Hwang, Maloney, Bruinsma, et al., 2012). Interestingly, work from Colonne et al. (2011), demonstrated the critical role of IFN-β in controlling *R. conorii* replication in endothelial cells (Colonne, Eremeeva and Sahni, 2011) (Colonne, Sahni and Sahni, 2013). While my studies did not examine the type I IFN response, it is possible that the decreased bacterial burden in Atg5-deficient cells is due to a increased level of type I IFN.

In vivo pathology data showed that *Atg5* $^{flox/flox}$ Lyz-*Cre* mice had significantly fewer pathological foci that were larger in size in the liver compared to *Atg5* $^{flox/flox}$ mice (**Figs. 17 A, B, C and D**). I hypothesize that *Atg5* $^{flox/flox}$ Lyz-

Cre mice are better able to control the infections based on fewer foci and the greater area of cellular infiltration. Additionally, I showed that in the livers of *Atg5* $^{flox/flox}$ mice, CD3+ T cells significantly increase in response to *R. australis*; however, there is no increase in the CD3+ T cell numbers in *Atg5* $^{flox/flox}$ Lyz-*Cre* mice. While I did not observe any pathology in the lungs or spleen of infected animals, CD3+ T cells in *Atg5* $^{flox/flox}$ Lyz-*Cre* mice failed to increase significantly during infection as observed in *Atg5* $^{flox/flox}$ animals (**Figs. 18A, B and C**). Interestingly, macrophages in the lung and spleen of *Atg5* $^{flox/flox}$ Lyz-*Cre* mice are significantly higher than in *Atg5* $^{flox/flox}$ mice, possibly a compensatory mechanism related to the lack of autophagy in this cell type (**Figs. 18D and F**). Surprisingly, the macrophage number in the lungs and spleen decreased significantly after infection, likely a result of migration to the lymphatic system as only minimal dead cells were observed in the histopathology. Staining intensity was normalized using the advanced threshold function available within the ImmunoRatio software program.

When I measured the bacterial burden in the tissues of infected animals, *Atg5* $^{flox/flox}$ Lyz-*Cre* mice had a significantly lower bacterial burden in liver, lungs and spleen compared to *Atg5* $^{flox/flox}$ mice. There are several possibilities to account for the reduced bacterial burden in *Atg5* $^{flox/flox}$ Lyz-*Cre* mice and BMMs; 1) autophagosomes protect rickettsiae from immune surveillance or killing mechanisms in host cytosol, 2) autophagy suppresses inflammatory cytokine responses that have rickettsiacidal effect such as TNF-α and IL-β, 3) autophagy inhibits other intracellular bacterial killing mechanisms such as ROS and nitric

oxide (Tai, Sasai, Lee, et al., 2009) (Olano, 2005). Additionally, ATG5 has been shown to be involved in the regulation of cell death; however, my data showed that there is no significant difference in cell death between *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ BMMs infected with *R. australis* (**Fig. 16**), suggesting that reduced bacterial burden in autophagy-deficient cells is not due to host cell death.

Several questions remain unanswered including the role of G-CSF during rickettsial infection, the mechanism of autophagy enhanced rickettsial replication, the role of IL-1β and IL-18 during rickettsial infection and the pathological consequences of autophagy deficiency during *R. australis* infection. However, my results have shown that autophagy promotes bacterial replication both *in vivo* and *in vitro* and demonstrates the role of autophagy in regulating rickettsial-triggered inflammatory responses. Our data suggest that manipulating the autophagic pathway may be a novel strategy to treat a severe rickettsial infection.

Conclusions and Future Directions

In my dissertation, I have studied the induction of autophagy by rickettsiae as well as the role of autophagy in the pathogenesis of rickettsioses *in vitro* and *in vivo*. I found that autophagy was induced in murine BMMs upon infection with *R. australis* and bacteria are specifically targeted by autophagosomes. Considering that a number of intracellular bacteria have been reported to induce autophagy (Niu, Yamaguchi and Rikihisa, 2008)(Choi, Cheong, Ha, et al., 2013)(Berón, Gutierrez, Rabinovitch, et al., 2002), it was not surprising that *R. australis* induced autophagy in unstimulated host macrophages. However, rickettsial induced autophagy significantly decreased at 3 hours post infection and is almost completely ablated by 24 hours p.i. (**Fig 1**).

The mechanisms by which bacterial pathogens induce and evade autophagy are diverse. Bacteria can induce autophagy using effector molecules, or toxins in the case of *Staphylococcus aureus* (Mestre and Colombo, 2012), while others cause direct damage to cellular components leading to initiation of the autophagic response (Birmingham, Smith, Bakowski, et al., 2006). Because rickettsiae are unable to make all of the amino acids necessary for their survival, and they require host ATP early during infection, they are obligately intracellular pathogens (Andersson, Zomorodipour, Andersson, et al., 1998). Cellular decreases in ATP and amino acids are classic signals to initiate autophagy. Thus, it is possible that lack of ATP and/or essential amino acids may be responsible for the early autophagic response observed in murine BMMs at 1 hour p.i.. Interestingly, I was unable to detect autophagy induction by rickettsial infection in

human endothelial cells, suggesting that autophagy induction by rickettsiae seems to be either cell type-specific or host-specific. Interestingly, using BMMs from *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ mice, I showed that *Rickettsia*-induced autophagy is *Atg5*-dependent. More importantly, deletion of autophagy gene *Atg5* in *Atg5* $^{flox/flox}$ Lyz-*Cre* BMMs significantly reduced bacterial replication compared to *Atg5* $^{flox/flox}$ littermates. Furthermore, rickettsial loads in liver, lung and spleens of autophagy-deficient *Atg5* $^{flox/flox}$ Lyz-*Cre* animals were significantly less compared to *Atg5* $^{flox/flox}$ littermates, suggesting that autophagy in macrophages promoted rickettsial replication *in vitro* and *in vivo*. Additionally, pharmacological modulators of autophagy including rapamycin and 3-MA confirmed that autophagy promotes bacterial replication in murine macrophages. Future work will be needed to fully understand the molecular mechanisms of autophagy induction and how rickettsiae exploit the autophagic pathway to promote their survival.

R. australis-infected BMMs of *Atg5* $^{flox/flox}$ Lyz-*Cre* mice secreted significantly higher levels of several proinflammatory cytokines including IL-1α, IL-6 and TNF-α and the inflammasome-dependent IL-1β when infected with rickettsiae, suggesting that autophagy suppresses the induction of these inflammatory cytokines. These inflammatory cytokines such as TNF-α activate macrophages to kill intracellular bacteria (Walker, Popov, Crocquet-Valdes, et al., 1997). Thus, bacteria exploit autophagy to promote their own replication possibly through inhibiting anti-microbial inflammatory response. Additionally, I showed for the first time that rickettsiae not only infect, but also replicate in human

macrophages *in vitro*. Interestingly, I did not find significant rickettsial replication in mouse macrophages, suggesting that macrophages potentially play an important role in the pathogenesis of human rickettsial infections. Furthermore, I demonstrated that rickettsiae did not induce autophagy in human endothelial cells at time points up to 48 hours post infection. Our studies suggest that rickettsiae induce autophagy at the early stage of infection in mouse macrophages to escape the bactericidal effect possibly mediated by inflammatory cytokines including IL-1β and TNF-α. These data also suggest that rickettsiae interact with the autophagy system in cell-type specific mechanisms.

To investigate the regulation of autophagy induced by rickettsiae, I examined the mammalian target of rapamycin (mTOR), a master regulator of autophagy, during infection of BMMs with *R. australis*. Rickettsiae induced mTOR phosphorylation and activation of the downstream protein P70S6K at 3h p.i., a measurement of mTORC1 activation. Furthermore, I showed that treatment with mTOR inhibitors, rapamycin and PP242, significantly promoted rickettsial replication in BMMs compared to untreated controls.

Future studies of mTOR during rickettsial infection are necessary to fully understand the regulation of inflammation in macrophages, and the immune system in general. mTOR and signal transducer and activator of transcription (STAT) signaling pathways are both key regulators in the development, survival, and function of $CD4^+$ T helper cells, $CD4^+$ T regulatory cells, $CD8^+$ T cells, DCs, and macrophages (Saleiro and Platanias, 2015). STAT proteins function as sensors for several cytokines, including interferons and interleukins. There is also

growing evidence demonstrating the crosstalk between mTOR and STAT pathways in the regulation of the immune response (Saleiro and Platanias, 2015).

My studies have not examined mTORC2 activation during rickettsial infection. However, mTORC2 is known to be essential for transcriptional regulation of IFN-stimulated genes (ISGs) (Kaur, Sassano, Majchrzak-Kita, 2012). As discussed in chapter 5, the Type 1 IFN response is important for polarizing the T_H1 cytokine response (O'Shea and Visconti, 2000) that is critical for successful clearance of rickettsiae. Interestingly, my data show that treatment of cells with PP242, an mTORC1 and mTORC2 inhibitor, increases bacterial burden in BMMs. While I have attributed the increase in bacterial burden to an induction of autophagy, the role of mTORC2 in this system has not been evaluated. Future studies examining the role of mTORC2 activation would not only be a novel area of study in *Rickettsia* infection, but in understanding the elusive roles of mTORC2 during host infection.

Interestingly, my data described in chapter 3 using RAW macrophages show that treatment with 3-MA, a known autophagy inhibitor, induces significantly higher levels of bacteria at 48 hours p.i. (**Fig 4**). These data seemingly contradict data shown in chapter 5 where I conclude that autophagy promotes bacterial replication *in vivo* and *in vitro* (**Figs 15 and 19,** respectively). 3-MA has been described to inhibit autophagosome formation by interfering with class III PI3K nonspecific effects including inhibition of class I PI3K, mTOR, and suppression of protein degradation. However, in a paper by Lin et al. (2012), 3-

MA acts as an autophagy inducer in RAW264.7 macrophages and functions as an inhibitor of class I PI3K to inhibit Akt signaling (Lin, Kuo, Wang, et al., 2012).

A second possibility to explain why autophagy acts as a mechanism of host defense in RAW macrophages, but not in primary BMMs, could be due to the crosstalk of autophagy with inflammasome activation. When RAW macrophages are infected with *R. australis*, we are unable to detect active caspase-1 and the subsequent production of IL-1β or IL-18 (unpublished observation). In the absence of increased anti-microbial inflammatory cytokines, autophagy may serve as a pathway utilized by the host to eliminate rickettsiae in macrophages. While rickettsiae induce inflammasome activation in primary BMMs (unpublished data), the role of IL-1β and/or IL-18 during rickettsial infection is currently unknown.

Studies examining the role of 3-MA in our experimental system should be performed to exclude the possibility that this drug is inhibiting class I PI3K; and what affects rickettsiae have on the PI3K signaling pathways. The increased levels of inflammatory cytokines, particularly IL-1β, in autophagy-deficient BMMs may play a role in rickettsial killing. Experiments examining the exogenous addition of IL-1β and/or IL-18 would be useful to assess rickettsiacidal mechanisms of these two cytokines. Alternatively, the use of caspase-1-deficient BMMs and caspase-$1^{-/-}$ mice would help to further explain my observations in *Atg5* $^{flox/flox}$ Lyz-*Cre* and *Atg5* $^{flox/flox}$ cells.

Taken together, my data show that rickettsiae benefit from the autophagic response in murine macrophages, and activate mTOR shortly after infection.

Autophagy induced by rickettsiae in BMMs is negatively regulated by an mTOR-dependent mechanism and contributes to the regulation of the innate immune response by modulating IL-1β and IFN-γ *in vivo*, and several cytokines *in vitro* including IL-1α, IL-6, IL-1β and TNF-α. The contribution of my research proposed here is highly significant because it is the first step toward understanding the mechanisms by which rickettsiae exploit autophagy to promote the pathogenesis of rickettsial diseases. Once such strategies become available, there is the promise that we can overcome the pathogen's ability to evade killing, while enhancing the ability to kill bacteria via modulation of the inflammatory cytokine response.

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Vita

Jeremy Bechelli was born on March 5, 1980 in Olean, NY. He is the son of Deborah Bechelli and Michael Bechelli. Jeremy attended Portville Central School from 1986 until graduation in 1998. Jeremy attended the State University of New York College at Brockport where he completed his B.A. in biology from 1998 until 2002. After working at the University of Rochester for several years, Jeremy completed his M.S. in microbiology and immunology under the direction of Dr. Sanjeev Sahni and graduated in 2009. Jeremy continued work at the University of Rochester on leukemia biology while working as an adjunct teaching faculty member at Monroe community college until August 2012. Jeremy started his Ph.D. in experimental pathology in August 2012 at the University of Texas Medical Branch in Galveston TX under the direction of Drs. David H. Walker and Rong Fang. Jeremy successfully defended his dissertation on March 30, 2016.

Permanent address: 289 West Bend Drive, League City TX, 77573

This dissertation was typed by Jeremy Bechelli.