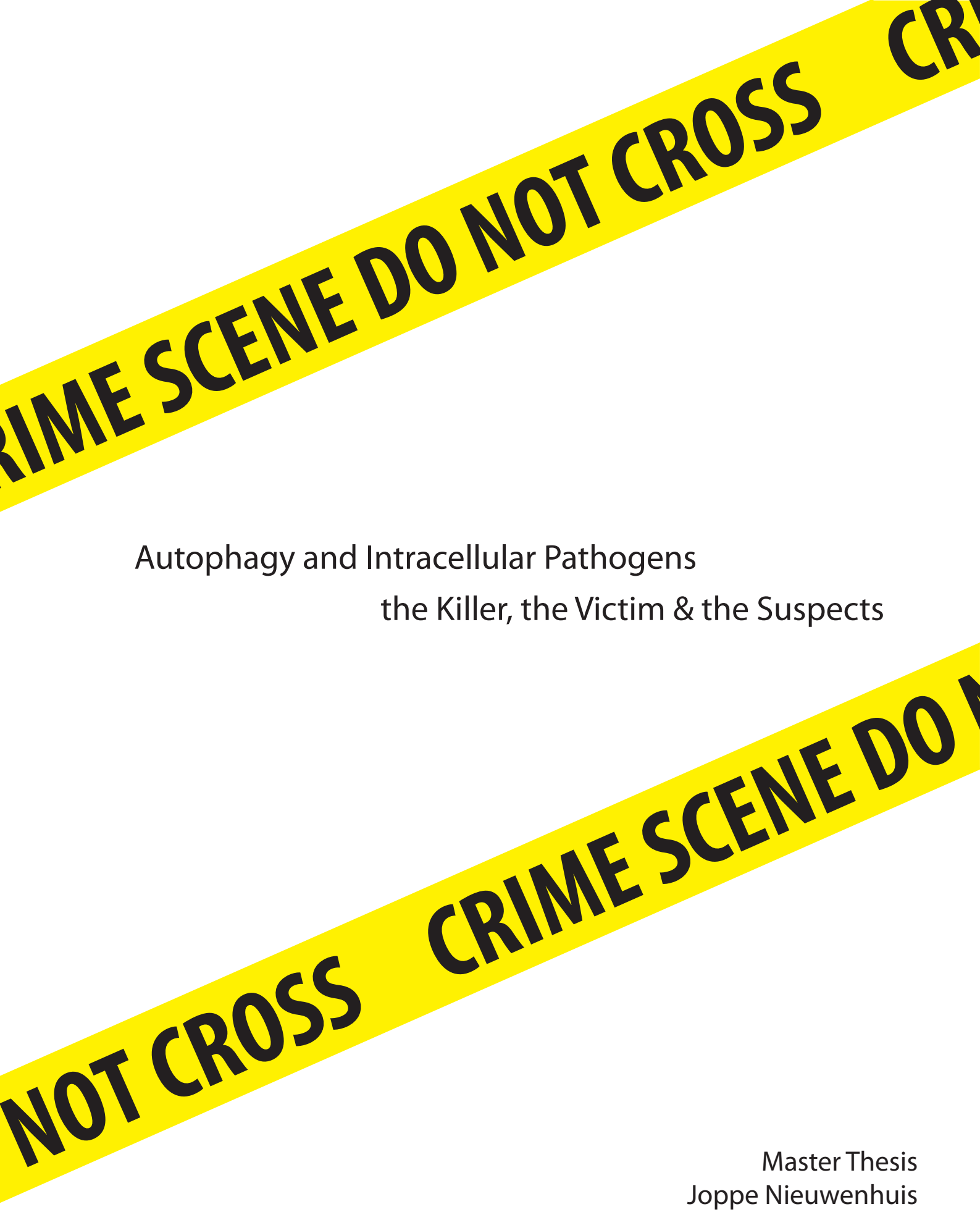

Autophagy and Intracellular Pathogens
the Killer, the Victim & the Suspects

Master Thesis
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Cancer Genomics and Developmental Biology

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ABSTRACT

Until recently, autophagy was viewed as a process primarily involved in controlling intracellular biomass as well as protecting cells against toxic protein aggregates and the accumulation of damaged organelles. Recent research, however, has revealed a central role of autophagy in the innate immune system by mediating the degradation of intracellular pathogens. Various viruses and bacteria, including *Streptococcus pyogenus,* are subjected to this elimination, whereas others have developed strategies to overcome autophagy or even subvert it for their own advantage like *Coxiella burnetii*. Molecular links between pathogen receptors and the initiation of autophagy have been identified, which increased the insights into autophagy regulation during infection. Research to pathogens suspected to interact with the autophagic pathway such as *Chlamydia trachomatis* have shed light into the autophagy-independent function of the autophagosome protein marker LC3. The aim of this thesis is to review the intracellular life cycle of few bacteria to illustrate the different types of interactions that have evolved between bacteria and autophagy.

Abbreviations

3-MA	3-Methyladenine
Atg	Autophagy-related gene
Atg14L	Autophagy gene 14 like
BAF	Bafilomycin A1
Bcl-2	B-Cell lymphoma-2
BH3	Bcl-2 homology 3
C. trachomatis	*Chlamydia trachomatis*
C. Burnetii	*Coxiella burnetii*
CD46	Cluster of Differentiation 46
CFU	Colony Forming Units
CR3	Complement Receptor 3
DC	Dendritic Cell
DN	Dominant-Negative
EB	Elementary Body
ER	Endoplasmatic Reticulum
ECM	Extracellular matrix
F-actin	Filamentous actin
FIP200	FAK-family interacting protein 200
GAS	Group A *Streptococcus*
GcAV	GAS containing LC3-positive autophagosome-like vacuoles
GOPC	Golgi associated PDZ and coiled-coil motif containing
HIV-1	Human Immunodeficiency Virus-1
IL	Interleukin
LAMP	Lysosomal Membrane Protein
LC3	microtubule-associated protein 1 Light Chain 3
LC3-I	microtubule-associated protein 1 LC3, C-terminally cleaved
LC3-II	microtubule-associated protein 1 LC3, cleaved and conjugated to PE
LPS	Lipopolysaccharide
MDC	Monodansylcadaverine
MHC-II	Major-Histocompatibility Complex-II
MHV	Mouse hepatitis virus
mTOR	mammalian Target Of Rapamycin
PAMP	Pathogen Associated Molecular Pattern
PAS	Phagopore Assembly Site
PE	Phosphatidylethanolamine
PI3K	Phosphatidylinositol-3-Kinase
PRR	Pattern Recognition Receptor
PV	Parasituphorous Vacuole
RB	Reticulate Body
RGD-motif	Arginyl-Glycin-Aspartic Acid-motif
RIG-1	Retinoic acid-inducible gene-1
RV	Replicative Vacuole
SLO	Streptolysin O
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-α
TOR	Target of Rapamycin
ULK1	Unc-51-like-kinase 1
UVRAG	Ultraviolet irradiation resistant-associated gene
VAMP8	Vesicle Associated Membrane Protein 8
VMP1	Vacuolar membrane protein 1
Vps	Vacuolar protein sorting
Vti1b	Vesicular transport through interaction with t-SNAREs homolog 1b

1. INTRODUCTION TO AUTOPHAGY

1.1 Autophagy

Until recently, autophagy was primarily viewed as a catabolic process protecting cells for obsolete and potentially harmful mitochondria and toxic aggregates formed by misfolded proteins (Xie and Klionsky, 2007). Various forms of autophagy have been described and macroautophagy is the best understood. In addition to macroautophagy, other forms of autophagy have been identified: Chaperone-mediated autophagy (reviewed by Lie, 2011), microautophagy (Mijaljica, 2011) and piecemeal microautophagy of the nucleus (Kvam, 2007). Macroautophagy is the catabolic cellular process by which excess or damaged organelles or misfolded proteins are sequestered into double-membrane vesicles, the autophagosomes, and delivered in the lysosome for degradation. The products obtained by degradation are transported into the cytoplasm to be used to generate new cellular structures or as a source of energy (Salminen, 2009). Macroautophagy also enables cells to overcome periods of nutrient deprivation by generating an internal pool of nutrients (Xie and Klionsky, 2007). Additionally to these cellular housekeeping and survival functions, autophagy can be triggered by mitochondrial damage, endoplasmatic reticulum (ER) stress or by cytokines (Harris, 2011).

More recently, it has been shown that autophagy is a key player in host cell defence against invading pathogens through a selective type of autophagy that has been named xenophagy in which intracellular pathogens are specifically incorporated in autophagosomes and degraded (Levine, 2005). This makes autophagy part of the arsenal of weapons of our immune system.

1.2 The formation of autophagosomes by the core autophagy machinery

The formation of autophagosomes can be divided in morphologically distinct steps (Deretic and Levine, 2009; Xie and Klionsky, 2007). The initiation of autophagy is characterized by the formation of the phagopore assembly site or pre-autophagosomal structure (PAS) (Deretic and Levine, 2009). The PAS consists of membranous structures as well as a wide variety of autophagy regulating proteins. At the PAS, a small cistern called the phagopore or isolation membrane is initially formed. Expansion of the phagopore by the acquisition of extra lipids leads to the fusion of the two growing extremities and the formation of a spherical double-membrane vesicle: the autophagosome. Subsequently, autophagosomes fuse with endosomal structures to form amphisomes and eventually with lysosomes to form the autolysosome. Through this latter event the inner vesicle as well as the cargo of the autophagosome are liberate into the interior of lysosome and degraded by resident hydrolases (figure 1) (reviewed by Tanida, 2011).

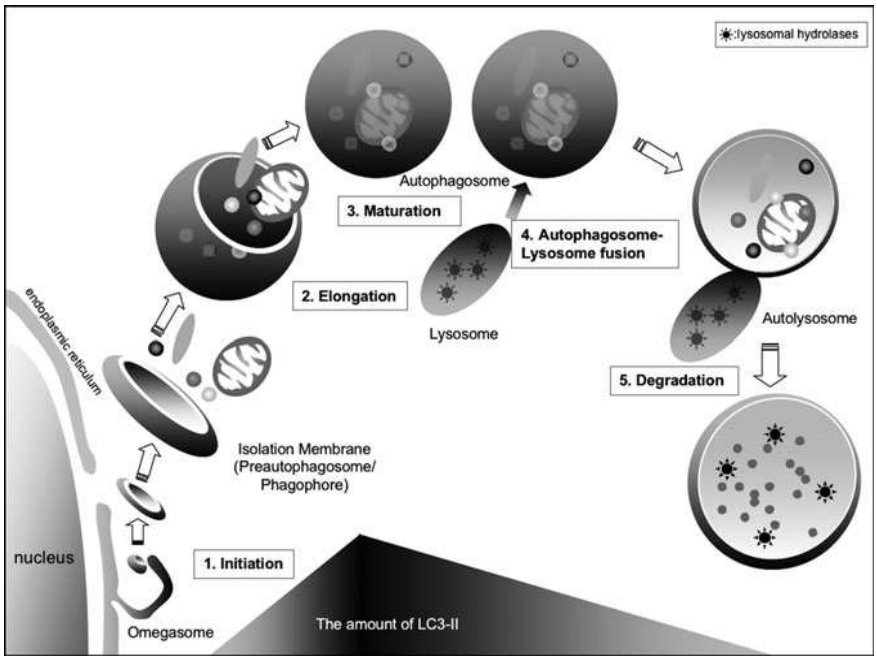

Figure 1. Multistep formation of autophagosomes. The PAS, a site defined as the location where all Atg proteins associate upon autophagy induction, appears to be the precursor structure of the omegasome. This latter is derived from the ER. It remains unclear whether the omegasome and phagopore are the same structure. Nevertheless, elongation and the fusion of the extremities of these compartments lead to the formation of autophagosomes which finally fuses with the lysosome to degrade their cargo.

(Tanida, 2011).

Regulation of autophagy has been studied extensively in yeast and over 35 autophagy-related genes (*ATG*) have been identified (Xie and Klionsky, 2011), many of which possess orthologues in mammals, indicating a strong evolutionary conservation of the autophagy machinery (Diaz-Troya, 2008). A subset of Atg proteins is indispensable in the onset of autophagy in all organisms and under all conditions, these proteins are referred to as the 'core autophagy machinery' (Yang and Klionsky, 2010). Four clusters of proteins can be distinguished: the Unc51-like kinase (ULK) complexes, the ubiquitin-like protein conjugation systems, the phosphatidylinositol 3-kinase (PI3K) complex and a set of transmembrane proteins (Yang and Klionsky, 2010).

The ULK complexes are the direct link between signalling cascades and initiation of autophagy in response to nutrients and growth factors. Both in yeast as in mammals, autophagy is controlled by the (mammalian) Target of Rapamycin (mTOR). Signal transduction pathways sensing nutrient availability converge to mTOR (reviewed by Wullschleger, 2006), which activity is directly linked to the inhibition of autophagy through the control of the activity of Ulk1 and Ulk2, two kinases both homologs of the yeast protein Atg1 (Yang and Klionsky, 2010). During nutrient deprivation mTORC1, a complex containing mTOR, becomes inactive and autophagy is activated (Diaz-Troya, 2008). The exact mechanism by which mTOR controls autophagosome formation remains to be fully elucidated, but mTORC dependent phosphorylation of both ULK1/2 as well as the mammalian homolog of Atg13 (mAtg13), a binding partner of ULK proteins, plays a key role in autophagy regulation (Yang and Klionsky, 2010). In nutrient-rich conditions mTORC1 is associated with ULK1/ULK2, mAtg13 and the scaffold protein FAK-family interacting protein 200 (FIP200). In this complex, mTORC1 phosphorylates ULK1/2 as well as mAtg13, which is also phosphorylated by ULK1/2, keeping the autophagy inducing complex inactive. When nutrient deprivation occurs, mTORC1 dissociates from this complex. This results

in a reduction of the mTOR-dependent phosphorylation on ULK1/2 and mAtg13 that activates ULK1/2, which then is able to phosphorylate FIP200, triggering the PAS and autophagosome formation (Xie and Klionsky, 2007; Yang and Klionsky, 2010).

The elongation of the phagopore is dependent on two ubiquitin-like proteins, Atg12 and the microtubule-associated protein light chain 3 (LC3, the mammalian homolog of atg8), the activity of both being regulated by different conjugation systems. Atg12 is conjugated through the E1- and E2-like enzymes Atg7 and Atg10 to Atg5, which in turn form a complex together with Atg16L (Yang and Klionsky, 2010). During elongation, the Atg12-Atg5-Atg16L complex is found on the outer membrane of the expanding phagopore and, when the maturation of the autophagosome proceeds, the amount of Atg12-Atg5-Atg16L decreases (Xie and Klionsky, 2007). LC3 is first cleaved (generating LC3-I) before being conjugated to phosphatidylethanolamine (LC3-II) through the action of Atg7 and the E2-like enzyme Atg3. The lipidated form of LC3 is initially found on both the inner as well as the outer membrane of the phagopore, but is removed from the outer membrane once an autophagosome is formed by the proteolytic cleavage carried out by Atg4 (Yang and Klionsky, 2010).

Two transmembrane proteins are part of the autophagy machinery: Mammalian Atg9 (mAtg9) and vacuole membrane protein 1 (VMP1). mAtg9 is located in the *trans*-Golgi network as well as in late endosomes. Upon nutrient deprivation it is directed to the PAS in an ULK1 and Vps34-dependent manner (Yang and Klionsky, 2010). The precise function of mAtg9 still remains elusive but it has been hypothesized that mAtg9 delivers at least part of the lipids necessary to mediate the phagopore elongation (Yang and Klionsky, 2010). VMP1, which has no yeast homolog, is a potent autophagy inducer as overexpression of VMP1 leads to autophagosome formation, regardless to the nutritional state. Cells depleted of VMP1 are unable to induce mTORC-dependent autophagy. VMP1 is located at the plasma membrane but co-localizes with LC3 and Beclin1 after autophagy induction. It is postulated VMP1 recruits the factors forming the PI3K complex to the PAS (Yang and Klionsky, 2010).

In yeast, there is one PI3K complex involved in autophagosome formation, which comprises Vps34, Vps15, Atg6 and Atg14. Vps34 is the lipid kinase responsible for generating phosphatidylinositol 3-phosphate which is essential to direct specific Atg proteins to the PAS (Yang and Klionsky, 2010). In mammalian cells, two classes of PI3K complexes are crucial for the progression of autophagy. They both consist of Beclin1/Atg6, p150/Vps15, the human homolog of Vps34 (hVps34), plus either the Atg14-like protein (Atg14L, or Barkor) or the ultraviolet irradiation resistant-associated gene (UVRAG) (Xie and Klionsky, 2007; Yang and Klionsky, 2010). While the Atg14L-containing complex is crucial for the initiation of autophagy, the one that comprises UVRAG is important for the maturation of autophagosomes first into amphisomes and then into autolysosomes (Deretic and Levine, 2009). The activity of the PI3K complex is counteracted at least in part by the phosphatase Jumpy/MTMR14, indicating the

phosphatidylinositol phosphorylation status needs to be strictly regulated during autophagy (Deretic and Levine, 2009).

1.3 Autophagy in immunity

Various intracellular pathogens have been shown to be degraded by the autophagy machinery, e.g. Group A *Streptococcus* (GAS) (Nakagawa, 2004) and *Mycobacterium tuberculosis* (Gutierrez, 2004). However, there are also pathogens that subvert the autophagy machinery for their advantage in order to be able to replicate and propagate in host cells. Examples include *Coxiella burnetii* (Beron, 2002).

Intracellular pathogens are recognized by pattern recognition receptors (PRRs) (reviewed by Hansen, 2011). A link between autophagy and PRRs has been reported (Sanjuan, 2007; Xu, 2007; Yano and Kurata, 2011). These receptors can activate autophagy either directly, in a Beclin1-dependent way (Shi and Kehrl, 2010), or indirectly via secreted cytokines (Deretic and Levine, 2009). The PRRs found to directly triggering autophagy in response to intracellular invasions of pathogens are the Toll-like Receptors (TLRs) (Shin, 2010), the Nod-like Receptors (NLRs) (Suzuki and Nunez, 2008) and the RNA recognizing RIG-I-like receptors (RLRs) (Yoneyama, 2005; Hansen, 2011).

In addition to a role in host cell defence, autophagy also performs a function in the regulation of cytokine secretion (Harris, 2011), indicating that this pathway is a factor contributing to the decision of what type of immune reaction is established. Induction of autophagy in the presence of lipopolysaccharide (LPS), a molecule present on the surface of Gram-positive bacteria, leads to the inhibition of interleukin-1β (IL-1β) secretion in murine dendritic cells (DCs) (Harris, 2011). Similarly, the expression of Tumor Necrosis Factor-α (TNF-α) is regulated by autophagy activity in macrophages exposed to LPS (Harris, 2011).

Vice-versa, autophagy activation upon cytokine stimulation is seen in experiments in which cells were treated with Interferon-γ (IFN-γ) before exposure to *M. tuberculosis*. Cells untreated with this cytokine displayed a severe delay in autophagy-dependent degradation of the pathogen compared to cells subjected to IFN-γ (Gutierrez, 2004).

In addition to intracellular bacteria, autophagy provides a defence against viruses as well (Deretic and Levine, 2009). Among others, Human Immunodeficiency Virus-1 (HIV-1), Sindbis virus and Vesicular Stomatitis Virus are degraded by autophagy (reviewed by Kudchodkar and Levine, 2009). Picornavirales and Nidovirales, in contrast, are able to subvert the autophagic pathway for their own advantage (Cottam, 2011; Klein and Jackson, 2011).

Blanchet and co-workers have reported that HIV-1-infected DCs show LC3-positive autophagosome-like structures that contain degraded HIV-1. However, the number of these compartments decreases dramatically within a few hours post-infection, indicating that HIV-1 has developed an immune evasion strategy directed against autophagy-dependent degradation (Blanchet, 2010). Importantly, the HIV-1-infected cells display a decreased response to TLR

agonist treatment, suggesting that the TLR-induced autophagy could be impaired. Moreover, HIV-1 abrogates Major-Histocompatibility Complex-II (MHC-II) cross-presentation of HIV-1 antigens probably by using its Env protein, which activates mTOR signalling, thereby inhibiting basal autophagy (Joubert, 2009). The work of Joubert and co-workers shows that autophagy is inhibited in multiple manners by HIV-1, highlighting the importance of autophagy in host cell defence because pathogens develop complementary solutions to efficiently inhibit this pathway.

1.4 Aim of this literature study

In this thesis the interplay between autophagy the intracellular bacteria *Streptococcus pyogenes*, *Coxiella burnetii* and *Chlamydia trachomatis* is described. These bacteria represent three different examples of possible interactions between pathogens and autophagy. *S. pyogenes* is efficiently degraded by autophagy (Nakagawa, 2004), whereas *C. burnetii* requires autophagy to create a replication niche (Beron, 2002). The role of autophagy in *C. trachomatis* is not defined yet as conflicting data has been reported (Al-Younes, 2004; Pachikara, 2009). So, the precise function of autophagy for this pathogen remains elusive although autophagy appears not to be essential for *C. trachomatis* replication (Pachikara, 2009).

2. *STREPTOCOCCUS PYOGENES* IS EFFICIENTLY DEGRADED BY AUTOPHAGY

Streptococcus pyogenes, or Group A *Streptococcus* (GAS), is a Gram-positive extracellular bacterium (Amano, 2006). By colonizing the throat and skin, GAS becomes the etiological agent of a wide range of infections in humans, ranging from the easily curable pharyngitis to the life-threatening necrotizing fasciitis ('flesh eating disease') (Cole, 2011).

2.1 Cell invasion mechanism of *Streptococcus pyogenes*

Despite the extracellular living mode of GAS, it has been shown in various *in vitro* studies that this microbe is also able to invade epithelial cells (Greco, 1995; Molinari, 1997). This invasion enables GAS to escape from the extracellular weaponry of the host immune system and protects the pathogen from the action of antibiotics. This feature could also contribute to the spread of the infection into deeper tissues (Ozeri, 1998). Invasion of host cells is initially mediated by the binding of GAS through it surface protein F1 to fibronectin, a component of the extracellular matrix (ECM). Thereby, the pathogen is not only associated to the ECM, which enables it to proliferate, but it is also indirectly bound to the epithelial cells via integrins, which are bound to fibronectin (Caswell, 2008). Additionally, a direct interaction between GAS and various plasma membrane receptors, including CD46, is documented (Joubert, 2009; Meiffren, 2010). This (in-)direct interaction with the cell surface leads to intracellular uptake of the bacterium, which is mediated via clathrin- and dynamin-dependent endocytosis (Molinari, 1997; Logsdon, 2011) In contrast to some other intracellular pathogens, e.g. *Yersinia pseudotuberculosis* (Mills, 1998), GAS is initially not consumed in the lysosomes, the end stage of the endocytic pathway. To escape this route to degradation, GAS secretes the pore forming toxin streptolysin O (SLO), which enables it to escape from early endosomes and enter the cytosol (Sakurai, 2010).GAS knockout strains lacking SLO remain in the early endosomes up to 2 hours after infection before being degraded by the fusion of endosomes with lysosomes (Sakurai, 2010). In contrast, the wild-type GAS populations found in early endosomes decrease after 1 hour of infection. This is not due to their elimination, but rather their escape into the cytosol (Nakagawa, 2004).

2.2 Formation of GAS containing autophagosome-like vesicles

Once in the cytosol, GAS is rapidly captured in another vesicular structure as first described by Nakagawa and co-workers (Nakagawa, 2004). In particular, they reported that autophagy protects the host cells from cytosolic GAS and thereby defends the organism against the dissemination of the infection (Nakagawa, 2004). GAS escaped from the early endosomes is found in vacuolar structures, which have been named the GAS-containing LC3-positive autophagosome-like vacuoles (GcAVs). These membranes are positive for the autophagy marker protein LC3, but differ in size from the conventional autophagosomes. In starved cells, these

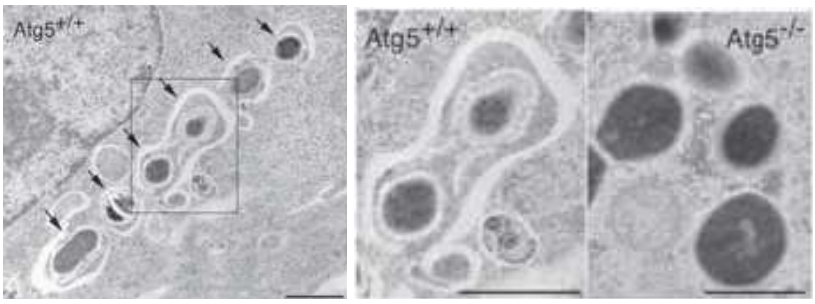

Figure 2. Ultrastructural differences between ATG5-deficient and competent cells after GAS infection.
Left and middle panel show EM image of GAS engulfed by autophagosomal-like membrane in $Atg5^{+/+}$ cells. Right panel shows $Atg5^{-/-}$ cells GAS is not found to be trapped in these membranes.

(Nakagawa, 2004). Bars represent 1 µm.

latter vesicles have a diameter of 0.5-1 µm while GcAVs have a diameter of up to 10 µm. The formation of these compartments depends at least partially, if not totally, on the activation of same molecular machinery that is responsible for autophagy progression, as no GcAVs are found in $Atg5^{-/-}$ cells, which are unable to generate autophagosomes (figure 2) (Nakagawa, 2004).

GAS sequestration into GcAVs appears to be an effective defense mechanism against the intracellular form of this bacterium as its sequestering into GcAVs leads to its killing (Nakagawa, 2004). By measuring the amount of colony forming units (CFU) of intracellular GAS the authors revealed that the number of CFU starts to decrease in wild-type cells at 4 hours post-infection, reflecting the GAS elimination, while this CFU decrease was blocked in *ATG5*-deficient cells. Accordingly to the fact of an involvement of autophagy, the intracellular killing of GAS in GcAVs occurs in lysosomes. In particular, GcAVs fuse with lysosomes and partially degraded GAS have been detected in the interior of these organelles by electron microscopy. This notion is supported by the finding GAS degradation is delayed in cells treated with lysosomal protease inhibitors, emphasizing the role of lysosomes in GAS killing (Nakagawa, 2004).

While Nakagawa and co-workers have highlighted the role of autophagy in the host cell defense against GAS, at the time it was totally elusive how cytosolic GAS escaping from early endosomes were detected by autophagy and how the selective process of GAS engulfment by the LC3-positive membrane was initiated. This finding was in contrast to canonical autophagy, which was thought to be mainly a non-selective process. This view has now changed and the work of Nakagawa and co-workers has been a milestone in this direction.

2.3 The pathogen receptor CD46 leads to the activation of autophagy

Joubert and co-workers have reported that CD46, a transmembrane protein that is expressed on all nucleated mammalian cells, is one of the molecular links between the sensing of various bacteria and the activation of autophagy (Joubert, 2009). It was already known that GAS is able to bind to keratinocytes by interacting with the pathogen receptor CD46 (Okada, 1995). However, the work of Joubert and co-workers showed that the activation of this receptor by agonistic antibodies leads to the formation of autophagosomes in an Atg5- and Atg7-dependent manner (Joubert, 2009).The increase of autophagosome number reflects an increase in the autophagy flux as the efficiency of autolysosome formation is not affected. So, the observed

phenotype is not caused by a fusion defect between autophagosomes and lysosomes (Joubert, 2009).

CD46 is expressed in two spliced forms, CD46-cyt1 and CD46-cyt2, which differ in the cytosolic tail of the receptor. A yeast two-hybrid assay-based screen has revealed that there is an interaction between CD46-Cyt1 with the scaffold protein Golgi associated PDZ and coiled-coil motif-containing (GOPC) but not between this protein and CD46-Cyt2 (Joubert, 2009). Importantly, it has been demonstrated that GOPC, via its PDZ domain, binds with Beclin1 in murine cells (Yue, 2002). Joubert and co-workers have detected the same interaction in human cells and determined that the coiled-coil domain of GOPC is essential for its binding to CD46-cyt1. These data suggests that GOPC is the scaffold protein that probably links the pathogen receptor CD46 and a well-defined positive regulator of autophagy, e.g. Beclin1. As a result, the CD46-cyt1/GOPC/Beclin1 pathway could be one of the ways to activate autophagy during infections (Joubert, 2009). This pathway would be a way to stimulate autophagy independently from the canonical signaling cascades, which are stimulated by nutrient deprivation. This is relevant because autophagy is needed as a defense mechanism against invading pathogens also in well-fed cells. This notion is highlighted by experiments where GOPC was knocked-down and a significant reduction of autophagosome formation was observed after stimulation of CD46, but not when autophagy was induced by starvation. In agreement with these results, infection with the emm49^{+} GAS strain, which is not able to bind CD46 (Darmstadt, 2000), displayed a severe delay in the degradation of the bacterium indicating that CD46 activation is necessary for efficient GAS elimination (Joubert, 2009).

The experiments of Joubert and co-workers make conceivable that the potentially invaded host cells are ready to eventually destroy the GAS escaping from the early endosomes (and possibly other cytosolic pathogens) through autophagy. In particular, this scenario evokes the detection of the pathogen by CD46 when it is still present in the extracellular environment and a consequent activation of autophagy before the GAS entry into the host cells. This would provide a mechanism to prepare the host cells for a possible pathogen invasion. Still, it remains to be determined how GAS is recognized by the forming autophagosome.

2.4 Rab7 in the initiation of GcAVs formation

To gain insight in the early development of GcAVs formation, the role of Rab7, a member of the small G protein family has been investigated because Rab proteins regulate a multitude of intracellular membrane trafficking steps, including formation and transportation of vesicles as well as their docking to target organelles (Sakurai, 2010; Seachrist and Ferguson, 2003; Yamaguchi, 2009). Rab5 plays a role in the formation of clathrin-coated vesicles (Seachrist and Ferguson, 2003) and is thereby associated with early endosomes. During the maturation process of endosomes Rab7 replaces Rab5 and therefore Rab7 is often used as a marker protein for late endosomes (Chavrier, 1990). Besides this, Rab7 is shown to be essential for the maturation of

autophagosomes as Rab7 knockdown results in a delay of autophagosome formation and overexpression of dominant-negative Rab7 (DN-Rab7) inhibits the formation of autolysosomes (Gutierrez, 2004; Jager, 2004).

Considering the role of Rab7 in endosomes and its role in autophagosome maturation, it has been proposed that this GTPase could play a similar role in the maturation of GcAVs (Yamaguchi, 2009). By closely following GcAVs formation for 2 hours post-infection using both electron and fluorescence microscopy, it has been shown that the number of GcAVs is high shortly after infection and decreases over time, while the number of bacteria trapped in the GcAVs increases over time (Yamaguchi, 2009). These observations suggest that the early GcAVs fuse with other GcAVs to create large vacuolar structures, which contain a high number of GAS (figure 3). Quantification of the various types of GcAVs supports this hypothesis. To test whether Rab7 played a role in this homotypic fusion of GcAVs, DN-Rab7 mutant was overexpressed in cells infected with GAS. Surprisingly, no GcAVs were detected thus suggesting Rab7 is essential in the earliest steps of GcAVs formation (Yamaguchi, 2009).

By further analyzing the early events leading to GcAVs formation, a precursor structure of the GcAVs was identified using correlative fluorescence- and electron-microscopy (Yamaguchi, 2009). This structure resembles to the isolation membrane and it is positive for both Atg5 and LC3. Remarkably, in addition to Atg5 and LC3, endogenous Rab7 was also found to be present in these structures, something not reported for the early steps of the biogenesis of the canonical autophagosomes (Yamaguchi, 2009). Once more, this observation emphasizes the difference in the regulation of the GcAVs and classical autophagosome formation.

To determine whether GAS sequestered into GcAVs is from either the cytosolic or early endosomal pools (Levine, 2005), electron- and immune-fluorescence microscopy analyses were performed to closely analyze GAS-containing membranous structures in infected cells. GAS was only found to be trapped in the single membrane Rab5-positive early endosomes, or as

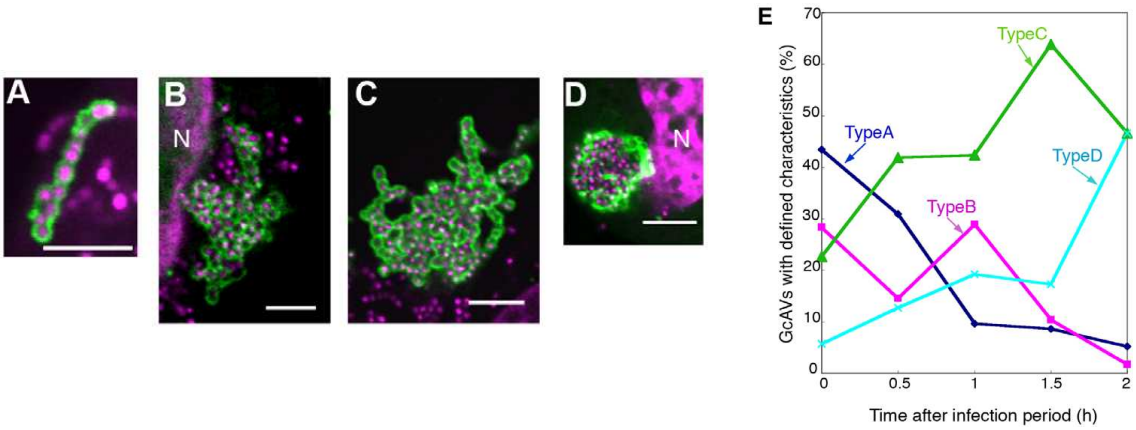

Figure 3. GcAVs increase in size during time. Cells expressing GFP-LC3 were transfected with GAS for 1 hour and fixed at various post-infection times. Different sizes and shapes of GcAVs were detected (A-D) and quantified at each time point (E). Green: GFP-LC3, Magenta: DAPI, reflecting GAS DNA or nuclear (N) areas.

(Yamaguchi, 2009).

mentioned above in the Atg5, LC3 and Rab7 positive membranes. These observation suggested that only cytosolic GAS is enwrapped by GcAVs (Sakurai, 2010).

2.5 SNARE proteins Vti1b and VAMP8 facilitate the interaction between GcAVs and lysosomes

The soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are well known mediators in vesicle trafficking. These proteins are important players in the docking and hetero- and homotypic fusion of various vesicular membranes. SNAREs are subdivided in v-SNAREs when in the vesicles and t-SNAREs when located in the target organelle (reviewed by Jena, 2011). Various SNAREs have been tested to determine which one of them is present in the GcAVs (Furuta, 2010). Among others, the t-SNARE VAMP8 and the v-SNARE Vti1b were found to be localized in the limiting membrane of GcAVs and their knockdown resulted in the inhibition of fusion between GcAVs and lysosomes, while the number of GcAVs after infection was not affected (Furuta, 2010). These results indicated that these SNAREs are essential for the fusion between GcAVs and lysosomes but not for the GcAVs biogenesis. Previous reports have shown that the interaction between the Vti1b and the VAMP8 occurs after fusion of Vti1b-containing endosomes with VAMP8-positive lysosomes (Wade, 2001). As a result, the appearance of both proteins on the same membranous structure can only be possible after fusion of the GcAVs with lysosomes.

Closer inspection of the localization of Vti1b and VAMP8 showed that during the earliest steps of GcAVs formation, Vt1b is present on the GcAVs but VAMP8 puncta almost exclusively co-localize with the lysosomal marker protein LAMP1 (Furuta, 2010). Over time, however, the number of VAMP8 puncta co-localizing with GcAVs increases while the number of Vti1b-positive GcAVs stays constant proving that indeed there is fusion between GcAVs and lysosomes (Furuta, 2010).

In their report, Furuta and co-workers claimed to be the first to describe a role for SNARE proteins in mammalian autophagy. Comparable experiments performed under starvation conditions showed that the same set of SNAREs is also involved in the fusion of canonical autophagosomes with lysosomes (Furuta, 2010). Consequently, while there are substantial differences, the maturation of autophagosomes and GcAVs have some mechanistic analogies.

2.6 Future challenges in the research about the role of autophagy in the defense against GAS

The cell invasion mechanism of GAS has been established (see above). The bacterium enters the cell via the endocytic pathway. By expressing and secreting the pore forming cytolysin SLO, GAS is able to escape from the Rab5-positive early endosomes to momentanely prevent its degradation in the lysosome and access the cytosol. Once in the cytosol, however, GAS is rapidly engulfed by an autophagosome-like membrane positive for Atg5, LC3 and Rab7. This process is triggered at least in part by the binding of extracellular GAS to CD46, which in turn triggers

autophagy and thereby primes the host cell for an efficient immune response. Over time, GcAVs fuse to form larger GcAVs, from which the GAS are not able to escape. GcAVs fuse eventually with lysosomes, an event mediated by Vti1b, VAMP8 and possibly Rab7. Once in the lysosome, GAS are destroyed by lysosomal hydrolases.

The importance of the molecular machinery of autophagy in the defense against invading GAS is thus well described. Although at least one signaling pathway regulating the link between the onset of autophagy in response to bacterial invasion has been identified, the exact mechanism of activation of the autophagy machinery after host cell invasion remains elusive. A remarkable difference between GcAVs and autophagosome formation is the selective aspect of GcAVs, where GAS are specifically enwrapped into this compartment. This is underlined by the observation that all the Atg5/LC3/Rab7-positive membranes are adjacent to invading bacteria, making it unlikely that these nascent autophagosomes are formed before the entering of GAS in the cytosol. Probably, a yet not identified factor is able to detect the presence of GAS in the cytosol and can function as an initiator for the membrane development in the vicinity of the bacterium. Such factors have been found in directing autophagy to the elimination of *Salmonella typhimurium* (Cemma, 2011). This pathogen is heavily ubiquitinated upon entering the host cell (Birmingham, 2006) and subsequently gets enveloped by a single membrane, forming a Salmonella-containing vesicle, before being targeted by an autophagosome (Kageyama, 2011). The targeting of these vesicles to the site of autophagosome formation is mediated by two adaptor molecules, p62 and Nuclear Dot Protein 52 (NDP52), which are able to bind both LC3 as well as ubiquitin (Cemma, 2011). Interestingly, the membrane of these vesicles appear to be ruptured (Birmingham, 2006), theoretically allowing NDP52 and p62 to mediate autophagosome formation adjacent to SCVs (Cemma, 2011; Kageyama, 2011).

Another remarkable observation is that GAS efficiently escape from the early endosomes by secreting SLO, but are unable to escape from the GcAVs possibly by using the same molecule. It is conceivable that this is caused by a difference in lipid composition of the two membranes. SLO is a cholesterol-dependent pore-forming factor. Therefore it is conceivable that the GcAVs do not contain any or sufficient cholesterol. Interestingly, *Shigella flexneri* escapes from autophagosomes in a cholesterol dependent manner (Kayath, 2010). Additionally, a difference in pH between early endosomes and GcAVs could be the cause of this difference. Analysis of both lipid composition and pH in maturing GcAVs is needed to test these hypotheses.

Yamaguchi and co-workers proposed that Rab7 functions in the homotypic fusion of GcAVs. With their experiments, they discovered that Rab7 is essential for the formation of GcAVs, making it impossible to test their original hypothesis by overexpressing a DN-Rab7 mutant. Future research is needed to unveil whether Rab7 indeed functions as hypothesized. A possible way to test this would be to transfect wild-type cells with a plasmid carrying an inducible DN-Rab7. Thus after infection with GAS and formation of the GcAVs, one could induce the expression of DN-Rab7. The fact that the DN-Rab7 mutant competes with endogenous Rab7

on the GcAVs membrane would provide a way to test the need for Rab7 activity in homotypic fusion of GcAVs.

3. *COXIELLA BURNETII* REPLICATION DEPENDS ON AUTOPHAGY

The Gram-negative obligate intracellular bacterium *Coxiella burnetii* is the agent of the zoonosis Q fever, causing flu-like symptoms and in severe cases, pneumonia and hepatitis (Ghigo, 2009). Most patients overcome this disease but for certain risk groups, e.g. patients with valvulopathy or pregnant women, Q fever can lead to death. The initial target of the bacteria are the alveolar macrophages but the infection can spread to a wide variety of tissues (Heinzen, 1999). Importantly, *C. burnetii* infections are persistent and its DNA can be detected in host cells months after the acute infection (Ghigo, 2009).

3.1 *C. burnetii* invasion of the host cell

The virulence of *C. burnetii* is different from strain to strain. This is probably caused by the expression of different variants of the surface LPS (Maurin and Raoult, 1999). *C. burnetii* phase I forms, expressing the smooth, full-length form of LPS are considered to be highly virulent, whereas phase II forms, expressing a truncated, rough variant of LPS are poorly virulent (Ghigo, 2009; Maurin and Raoult, 1999). Interestingly, the phase II *C. burnetii* strain Nine Mile Crazy expresses an intermediate form of LPS that enables the pathogen to be efficiently taken-up by various cell types (Beron, 2002; Ghigo, 2009).

The phase I pathogen infects phagocytic myeloid cells, whereas phase II organisms are eliminated in the extracellular environment (Ghigo, 2009). Physical interaction of the pathogen with the target cell is necessary to establish internalization (Ghigo, 2009). Phase I *C. burnetii* binds the integrins αvβ3 and complement receptor 3 (CR3) present on the surface of myeloid cells leading to phagocytic uptake. The factor on *C. burnetii* that is responsible for this interaction has not been elucidated yet, but multiple receptors capable of binding the Arginyl-Glycin-Aspartic Acid (RGD)-motif of αvβ3 have been identified in the *C. burnetii* genome (Capo, 1999; Seshadri, 2003).

Contact of phase I form of *C. burnetii* with the target cell leads to major cytoskeleton modifications, in particular formation of filamentous actin (F-actin) filaments resulting in the generation of membrane protrusion and cell polarization (Meconi, 1998). Importantly, phase II *C. burnetii* fails to induce these effects (Meconi, 1998). Uptake by microfilament-dependent parasite-directed endocytosis is probably passive as killed pathogens are internalized in equal rates as the living *C. burnetii* (Heizen, 1999). After internalization via the classical phagocytic pathway (Romano, 2007), the bacterium ends in the parasitophorous vacuoles (PVs), which matures with the same characteristics as endosomes (Romano, 2007) to become large secondary lysosomes that are positive for Rab7, LAMP1 as well as other lysosomal marker proteins (Heinzen, 1999; Baca, 1993; Beron, 2002). These compartments have been designated as the Coxiella Replicative Vacuoles (CRVs) (Beron, 2002). Acidification of the CRVs takes place until a

pH as low as 4.8 is established, which is essential for replication of the pathogen that is resistant to the harsh environment created by the lysosomal hydrolases and toxins present in this compartment (Heinzen, 1999).

C. burnetii reproduction depends on the transformation from the infecting small cell variant (SCV) into a replicating large cell variant (LCV). The SCV is metabolically dormant and has a low replication rate, but is resistant to the harsh environment of the phagolysosomes. The differentiation of the SCV to the LCV is believed to be triggered by the lowering of the pH and the presence of nutrients in the CRVs (Gutierrez, 2005; Heinzen, 1999).

3.2 The role of autophagy in *C. burnetii* life cycle

In a study aimed to characterize the PVs and to examine a possible role for autophagy in the formation of this organelle, Beron and co-workers reported that PVs are positive for both LC3 and the autophagosome specific marker monodansylcadaverine (MDC), and that MDC-positive vacuoles are found in the vicinity of PVs (Beron, 2002). Note that later research revealed that MDC is not as specific for autophagosomes as initially was thought, rather it stains acidic compartments such as late autophagosomes and lysosomes (Bampton, 2005; Klionsky, 2008). In addition, treating infected cells with the PI3K inhibitor 3-Methyladenine (3-MA) inhibits, but not totally prevents, the formation of PVs (Beron, 2002) indicating that autophagy is a major player in PVs formation.

In a subsequent work, the same investigators showed that serum-starved cells infected with *C. burnetii* appear to form more CRVs than cells cultured under nutrient rich conditions (Gutierrez, 2005). This difference is due to an increased rate in CRVs formation and not

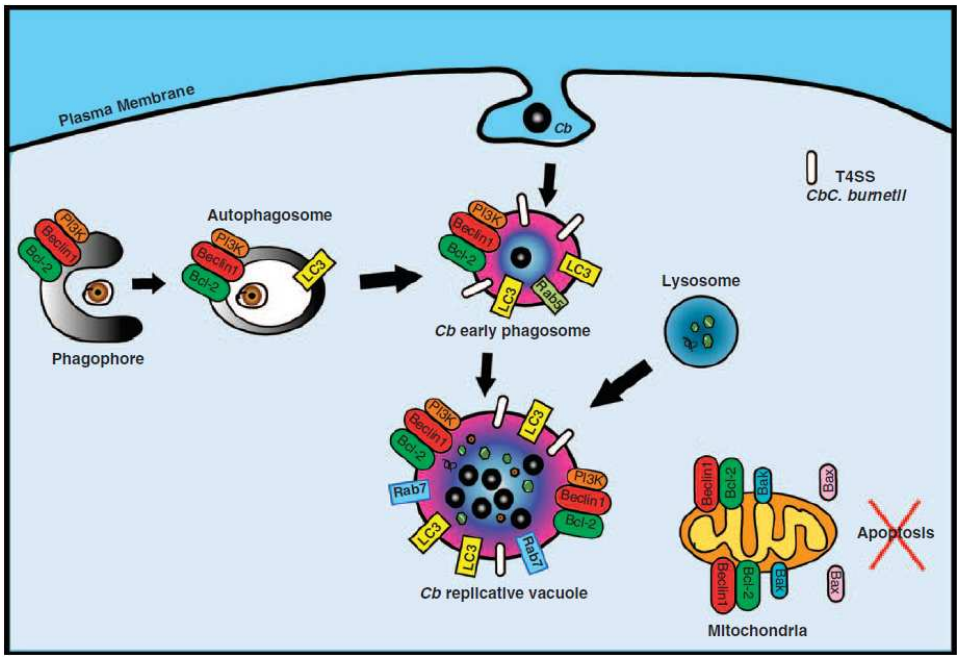

Figure 4. *C. burnetii* intracellular life cycle. After internalization *C. burnetii* recruits autophagic proteins such as LC3, Rab7 and Beclin-1 and thereby forms the CRVs.
Probably via its T4SS *Coxiella* secretes effectors that interfere with autophagy progression and inhibits apoptosis, while fusion with lysosomes is severely delayed it eventually takes place. In the CRVs SCVs mature to LCVs.

Adapted from Vazquez et al. 2009

phagocytosis, because the number of internalized bacteria did not change in the two experimental conditions (Gutierrez, 2005)

By investigating the initial steps of *C. burnetii* infection, it was found that Rab5, Rab7 and LC3 are present on the PVs in early phases of *C. burnetii* cell entry (Romano, 2007). The amount of Rab5, however, diminishes over time while Rab7 remains associated to the *C. burnetii*-containing compartments (Romano, 2007). LC3 is detected on the PVs membrane 40 minutes post-infection and it is actively directed to the membrane by the pathogen, as treatment of the cells with chloramphenicol to specifically inhibit prokaryotic protein synthesis, blocks the recruitment of LC3 and phagosomes fail to mature into PVs (Howe, 2003; Romano, 2007). Autophagy appears to play a role in the regulation of the fusion of lysosomes to the PVs as well (Romano, 2007). Chloramphenicol treated *C. burnetii* display an inability to recruit lysosomal marker proteins to the CRVs, including cathepsin D (Romano, 2007; Vazquez and Colombo, 2010).

These experiments indicate that *Coxiella*, while sequestered from the cytosol, influences autophagy by using its own proteins in order to regulate the maturation process of the vacuoles in which it resides. The bacterial factors responsible for these interferences are not been identified yet. The genome of *Coxiella* encodes for orthologues of type IV secretion system (T4SS) proteins known as the Dot/Icm system in *Legionella pneumophilia* (Gutierrez, 2005; Romano, 2007; Seshadri, 2003; Zamboni, 2003). These genes are expressed early after infection (Morgan, 2010) and the secretion system appears to be an essential factor in the manipulation of lysosomes.

3.3 Beclin1 as a link between autophagy and apoptosis during *C. burnetii* infection

C. burnetii infection can be persistent and therefore it requires the survival of the infected host cells. To obtain this, the pathogen actively inhibits the induction of apoptosis (Voth, 2009, Luhrmann, 2007). Autophagy has been linked to apoptosis through Beclin1 (reviewed by Kang, 2011). Beclin1 was initially discovered as the binding partner of the well-defined inhibitor of apoptosis B-cell lymphoma (Bcl-2), which interacts with the Bcl-2 Homology 3 (BH3) domain of Beclin1 (Liang, 1998). The Beclin1-Bcl-2 association inhibits autophagy because it interferes with the formation of the Beclin1-hVps34 complex (Pattingre, 2005). The anti-apoptotic function of Bcl-2, however, is not affected by this interaction (Kang, 2011; Vazquez and Colombo, 2010). Nevertheless, Bcl-2 regulates autophagy activity as overexpression of a Beclin1 point mutant not able to bind Bcl-2 (Beclin1-BD) in nutrient rich conditions leads to an extensive increase of autophagy and cells die of autophagic cell death (Pattingre, 2005).

In a work designed to elucidate the role of Beclin1 in both autophagy and apoptosis during *C. burnetii* infection, cells overexpressing Beclin1 were infected with *C. burnetii.* This led to an increase in both the CRVs size and the number of infected cells (Vazquez and Colombo,

2010). Knockdown of Beclin1 led to a decrease in CRVs, both in number as in size, underling that Beclin1 is essential to promote of the formation of CRVs (Vazquez and Colombo, 2010).

The observed effect of Beclin1 on CRVs formation is dependent on the interaction of Beclin1 with Bcl-2 because overexpression of Beclin1-BD fails to show the beneficial effects on CRVs formation (Vazquez, 2010). Further analysis of the role of Beclin1 in CRVs formation showed that this protein, together with hVsp34, is associated with the CRVs. Although a maximum of these proteins was found to be at the CRVs membrane 6 hours post-infection, these factors were detected by immune-fluorescence at this site also after 48 hours (Vazquez and Colombo, 2010).

Beclin1 localization on the membrane of CRVs depends on the kinase activity of hVps34 (Vazquez and Colombo, 2010). Surprisingly, the autophagy-inhibiting pool of Bcl-2 was also detected on the CRVs surface (Vazquez and Colombo, 2010). Overexpression of Bcl-2 thus resulted in an increase in the CRVs number but these compartments had a smaller diameter. These two differences, however, were lost at 48 hours post-infection. Overexpressed Bcl-2 probably disassembles the Beclin1-hVps34 complex, thereby decreasing the autophagy flux resulting in changes in CRVs formation mechanisms early in infection (Vazquez and Colombo, 2010)

The inhibition of apoptosis by *C. burnetii* appears to be dependent on the interaction of Beclin1 and Bcl-2. Overexpression of Beclin1-BD in cells infected with *C. burnetii* led to an increase of fragmented nuclei, a phenotype typical of apoptosis. Moreover, the simultaneous knockdown of Beclin1 and overexpressing of Bcl-2 in cells infected with *C. burnetii* increased apoptosis (Vazquez and Colombo, 2010). Consequently, the interplay between Beclin1 and Bcl-2 after *C. burnetii* infection is essential for the establishment of proper CRVs as well as the inhibition of apoptosis to generate a persistent infection (figure 4) (Vazquez and Colombo, 2010).

3.4 Future challenges in the research about the role of autophagy in *C. burnetii* replicative cycle

While only few reports have been published about the role of autophagy in *Coxiella* biology, it is clear that this bacterium manipulates autophagy to create a niche necessary for the development of the SCV form into LCV and probably exploits Beclin1 to induce a persistent infection (figure 4).

It has been postulated that nutrients, together with a decrease in pH, are the trigger for *Coxiella* transformation from SCV into LCV (Gutierrez and Colombo, 2005). It is currently unclear how *C. burnetii* obtains nutrients when present in the CRVs, in which it resides for up to 48 hours (Heinzen, 1999). The presence of MDC positive vacuoles adjacent to the CRVs, as reported by Berón and co-workers (Beron, 2002), suggests that autophagosomes could deliver nutrients to the CRVs. However, it is unclear whether autophagosomes fuse with the endocytic membranes

containing *C. burnetii*, or whether Atg proteins are recruited to the CRVs membrane and thereby CRVs acquire the autolysosome-like characteristics. Fusion of autophagosomes with bacteria-containing compartment to deliver nutrients has been reported for *Leishmania Mexicana* (Schaible, 1999).

It is very likely that *Coxiella burnetii* uses its T4SS to deliver bacterial effectors into the host cell cytosol. However, to date no secreted proteins have been identified that could be the candidates for activation of autophagy. In addition, it is unknown which factors in the autophagic pathway are the targets of the bacterial effectors. Identification of these virulence factors could lead to new insights into the regulation of autophagy.

Vazquez and co-workers reported the localization of Bcl-2 on CRVs, and thus they have been the first to describe the presence of Bcl-2 on a pathogen-containing compartment (Vazquez and Colombo, 2010). This is a surprising observation because Bcl-2 inhibits autophagy, which is actively induced by *Coxiella* as well as apoptosis when bound to Bak on the mitochondrial membrane. The role of Bcl-2 on autophagy has been extensively described (Kang, 2011) but the function of Bcl-2 on CRVs has not been elucidated yet. It has been postulated that Bcl-2 dampens the activation of *Coxiella*-induced autophagy (Vazquez and Colombo, 2010). It has not been tested, however, whether the recruitment of Bcl-2 onto the CRVs is induced by *Coxiella*. Further research should reveal whether Bcl-2 is also present on vacuoles containing other bacteria or parasites than *Coxiella*.

4. THE PUTATIVE ROLE FOR AUTOPHAGY IN *CHLAMYDIA TRACHOMATIS* INFECTION

Chlamydiae are obligate intracellular Gram-negative bacteria that infect a wide variety of mammalian cells (Hammerschlag, 2002). The best described member of this genus of bacteria is *Chlamydiae trachomatis*, which is the most prevalent cause of sexual transmitted diseases and leads to persistent infection such as urogenital infections and trachoma (Al-Younes, 2004; Corsaro and Greub, 2006).

4.1 The pathogenic cycle of *C. trachomatis*

Chlamydiae have a biphasic developmental cycle characterized by two morphologically distinct forms, the Elementary Bodies (EBs) and the Reticulate Bodies (RBs) (figure 5) (Hammerschlag, 2002). *C. trachomatis* infects organisms by entering mucosal epithelial cells. Interaction between the infectious EBs, a spore-like particle that is metabolically inactive, and the host cell is based on electrostatic binding of the bacteria to the surface of the host cell (reviewed by Hammerschlag, 2002). The EBs are internalized by a poorly understood process that is independent from microtubules (Hammerschlag, 2002). Internalized EBs are detected in inclusions, which are vacuoles negative for endosomal marker proteins such as Rab5 and Rab7 (Rzomp, 2003), underlining that the internalization of this pathogen is not based on classical endocytosis. These inclusions do not fuse with lysosomes and do not contain acidifying factors, thus being a non-acidic niche for *Chlamydiae* development and replication (Hammerschlag, 2002). Lipids necessary to enlarge the inclusions are derived from the Golgi apparatus, which is fragmentized by proteins expressed by the EBs (Heuer, 2009) and secreted into the cytosol via *Chlamydiae*'s Type 3 Secretion System (Subtil, 2000).

In the inclusions, EBs differentiate into RBs. The latter either undergo binary fission or remain silent leading to persistent infections (Hammerschlag, 2002). Almost all RBs re-

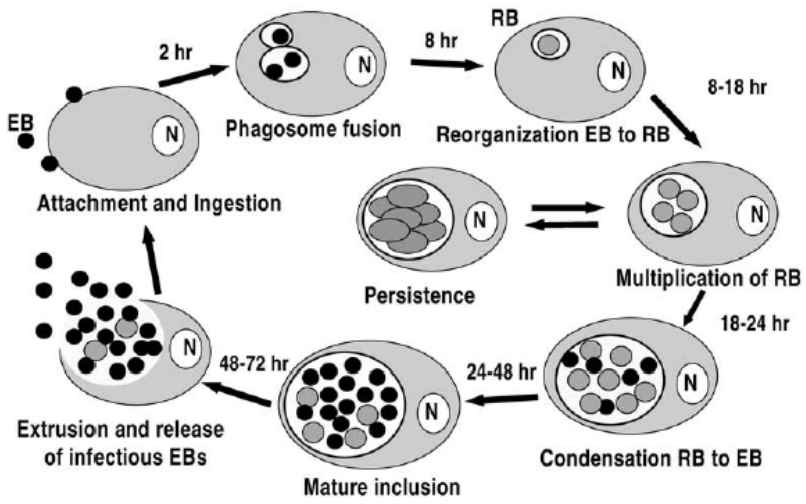

Figure 5. The replicative cycle of *C. trachomatis*. After infection, *C. trachomatis* concentrates into the inclusions. In these vesicles the differentiation from EBs to RBs takes place. RBs actively replicate and eventually almost all of them re-differentiate into EBs, which leave the cell by cytolysis or extrusion, spreading the infection.
EB, elementary body; RB, reticulate body; N, nucleus of host cell

(Hammerschlag, 2002)

differentiate into EBs and up to 1,000 of these EBs can be present in a single vacuole and they are eventually released by cytolysis or extrusion to spread infection (Hammerschlag, 2002).

C. trachomatis resides for up to 72 hours in the inclusions (figure 5). How nutrients are obtained is poorly understood. Particularly, the demand of nutrients increases in presence of the metabolically active RBs (Hammerschlag, 2002). Some bacteria, e.g. *Toxoplasma gondii* create a molecular tube in the parasitophorous vesicle to allow free diffusion of molecules of a size up to 1,900 Da to obtain nutrients (Schwab, 1994). The membrane of the *Chlamydia*-containing inclusion does not possess such a transporter and does not allow free passage of molecules larger than 520 Da. Thus theoretically, amino acids and small oligopeptides could pass the membrane freely but this has not been observed to date (Heinzen and Hackstadt, 1997). An postulated way to deliver nutrients to internalized *C. trachomatis* is through the fusion of the inclusions with early endosomes (Al-Younes, 2004).

4.2 The putative role of autophagy in *C. trachomatis* infection

Research into the role of autophagy in the intracellular life of *C. trachomatis* was performed principally to investigate whether this pathway plays a role in delivering nutrients to the *Chlamydia*-containing vesicles (Al-Younes, 2004). Al-Younes and co-workers were the first to report that autophagy is necessary for the normal development of these inclusions (Al-Younes, 2004). The inhibition of autophagy by 3-MA previous to infection resulted in a complete inhibition of the formation of inclusions (figure 6) (Al-Younes, 2004). When autophagy was inhibited after infection, inclusion growth was arrested resulting in smaller vesicles (Al-Younes, 2004). Comparable results were obtained when autophagy was inhibited by addition of the essential amino acids phenylalanine, methionine, isoleucine and leucine (figure 6) (Al-Younes, 2004). In response to infection, LC3 co-localizes with LAMP1 indicating that autophagy is active and contributes to normal inclusions formation (Pachikara, 2009). By which mechanism autophagy promotes *C. trachomatis* infection is unclear. Fusion between autophagosomes and inclusions has not been observed and LC3 is not found to be internalized by or associated to the inclusion membrane, but is present juxtaposed to it (Al-Younes, 2004). However, LC3 localization after *C. trachomatis* infection remains a matter of intense debate as

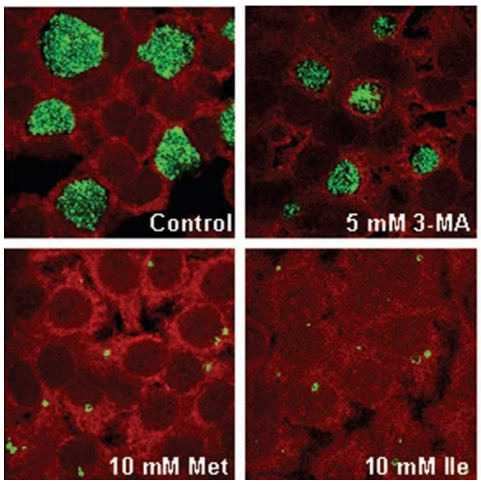

Figure 6. Inhibition of autophagy by 3-MA or excess of essential amino acids leads to the abrogation of inclusion growth.
Cells were treated for 30 min with 3-MA, methionine or isoleucine before and during infection. Cells were finally fixed 44 hours post-infection before being analyzed by confocal microscopy.
Green: *C. trachomatis.* Red: host cells

(Al-Younes, 2004)

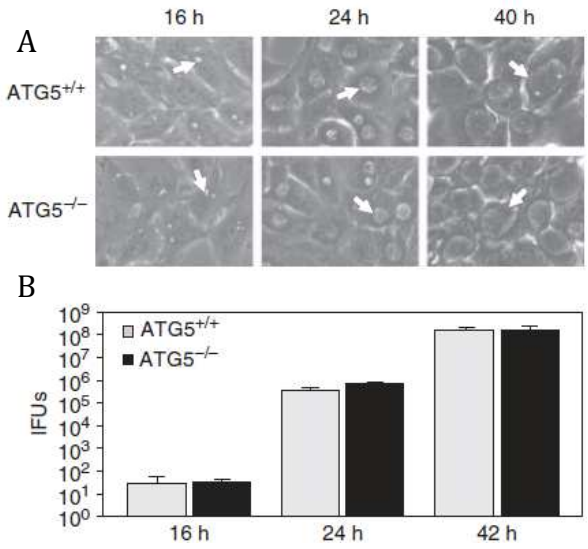

Figure 7. *C. trachomatis* inclusions are indistinguishable in morphology and quantity between autophagy competent- and autophagy deficient-cells.
Atg5$^{+/+}$ and *Atg5*$^{-/-}$ mouse embryonic fibroblasts were infected with *C. trachomatis* for 16, 24 and 40 hours.
A. No morphological differences are observed in the inclusions when analyzed by phase-contrast microscopy. White arrows point to the inclusions.
B. At various post-infection times, the same number of EBs are produced in both cell lines.

(Pachikara, 2009)

Pachikara and co-workers were not able to observe LC3 co-localization with *Chlamydia* inclusions (Pachikara, 2009).

Analysis of the levels of p62, a protein directed to autophagosomes and degraded into autolysosomes, has showed that in infected cells autophagy is not completed as p62 levels remain constant in these cells (Yasir, 2011). To test whether autophagy is essential for *C. trachomatis* infection, *Atg5*$^{-/-}$ cells were infected (Pachikara, 2009). Surprisingly, the same number of EBs are generated in these cells as in the control *Atg5*$^{+/+}$ cells. The inclusions observed in the autophagy-deficient cells are indistinguishable from inclusions observed in wild-type cells (figure 7) (Pachikara, 2009). Similar experiments with *Atg7*$^{-/-}$ cells led to the same results emphasizing that the autophagy machinery is not essential for *C. trachomatis* infection (Ouellette, 2011).

To further analyse the autophagy activity during the *C. trachomatis* infection, the ratio of overexpressed LC3-II/LC3-I was determined (Pachikara, 2009). These experiments showed that at 24 hours post-infection there is a significant elevation of LC3-II levels, which also appeared to co-localize with LAMP1. UV- and heat-treated EBs fail to induce this LC3 increase (Pachikara, 2009), showing that LC3 lipidation is actively induced by *C. trachomatis* (Pachikara, 2009). As *ATG5* and *ATG7* are dispensable for *C. trachomatis* infection, it was postulated that LC3 is processed in an autophagy-independent manner (Pachikara, 2009).

However, it appears that LC3 associates with the inclusions in a microtubule-dependent way, something also emphasized by the fact that the microtubule associated proteins 1A (MAP1A) and MAP1B are co-recruited to the inclusions (Al-Younes, 2011). MAP1A and MAP1B are two well described binding partners of LC3 (Mann and Hammarback, 1994). LC3 and MAP1A knockdowns have revealed that these proteins are highly important for *C. trachomatis* infection as the infectivity of this bacterium was reduced to 25% and 50%, respectively (Al-Younes, 2011). Although the exact function of LC3 and MAP1A in *C. trachomatis* life cycle is not known, it has

been hypothesized that these two proteins mediate the attraction of exosomes to the bacterial inclusions to deliver nutrients to *Chlamydiae* (Al-Younes, 2011).

4.3 Discussion about the role of autophagy and LC3 in *C. trachomatis* infected cells

The most recent research has challenged the concept that autophagy has an essential role for the intracellular life cycle of *Chlamydia trachomatis* (Ouellette, 2011; Yasir, 2011). However, the report by Al-Younes and co-workers shows that the inhibition of autophagy by 3-MA and an excess of amino acids abrogates normal inclusions formation (Al-Younes, 2004). Besides hVps34, other PI3Ks are inhibited by distribution of 3-MA and amino acids are intermediates of numerous signal transduction pathways. In addition, hVps34 is not only involved in autophagy but it also generates phosphatidylinositol-3-phosphate on endosomal membranes. This makes conceivable that other pathways are affected by the treatments with these chemicals and this could impair the growth of *C. trachomatis* (Al-Younes, 2004). The microtubule network organization and dynamics is, at least partially, dependent on the generation of phosphatidylinositol (3,4,5)-trisphosphate via the LL5β and the PKB/Akt pathways, both regulated by PI3K activity (Paranavitane, 2003).

Pachikara and co-workers reported co-localization of LC3 with LAMP1, indicating an increase in autophagy as a response to *C. trachomatis* infection (Pachikara, 2009). Perhaps the presence of inclusions in the cytoplasm or EBs secreted proteins are recognized by TLRs or other PPRs, which in turn initiate autophagy, that later during infection is inhibited by *C. trachomatis*. Another explanation is that the observed autophagy is induced by starvation as a result from the decrease in cytosolic amino acids, if these are metabolized by *C. trachomatis.*

As described above, normal amounts of *Chlamydia* EBs are formed as in autophagy-deficient cells (Ouellette, 2011; Pachikara, 2009). As no fusion between autophagosomes and inclusions has been reported, it is highly unlikely that autophagy directly delivers nutrients to the inclusions. As the inclusion membrane possibly allows amino acids to pass it would be favourable for *Chlamydiae* to increase the cytosolic levels of amino acids. This could be obtained by increasing the autophagy flux in infected cells. However, by analyzing p62 levels in infected cells, it appears that *C. trachomatis* infection does not stimulate the completion of autophagy and therefore it is possible that this bacterium block the last steps of this pathway (Yasir, 2011). Ouellette and co-workers showed that nutrients, at least partially, are retrieved from the lysosome and *Chlamydia* growth is severely inhibited by Bafilomycin A1, a potent inhibitor of the lysosomal H^+-ATPase (Ouellette, 2011).

The observation that LC3 has an autophagy-independent role in *C. trachomatis* infection is probably not the explanation for the increase in LC3-II/LC3-I ratio after infection. The experiments of Al-Younes and co-workers show in particular that it is most likely the non-lipidated form of LC3 performs the propagation of *C. trachomatis* (Al-Younes, 2011). Further research is needed to determine the exact function of LC3 during *C. trachomatis* infection.

Reggiori and co-workers reported an autophagy-independent function of LC3-I in coronavirus infected cells (Reggiori, 2010). The mouse hepatitis virus (MHV), a coronavirus, induces the formation of double-membrane vesicles that are derived from EDEMosomes, ER-derived structures that normally deliver their cargo to endosomal vesicles for disposal. These EDEMosomes are coated with LC3-I, which appears to be essential for viral replication as knockdown of LC3 protects cells from viral replication. *ATG7* does not play a role in MHV infection as highly similar replication efficiency is observed in *Atg7*$^{-/-}$ cells compared to wild-type cells (Reggiori, 2010). Indicating that the autophagosomal machinery is dispensable but LC3 processing is essential for MHV replication. Thus, a possible scenario could be that *C. trachomatis* also hijacks EDEMosomes to supply its EBS with nutrients.

The function of LC3 obviously goes beyond its function in autophagy. Further research is needed to completely elucidate the cellular processes using LC3 and it modified forms independently from the autophagy core machinery.

5. CONCLUDING REMARKS

This thesis has been designed to give an overview about various possible scenarios between autophagy and intracellular pathogen. Autophagy is part of the innate immune system in the killing of *Streptococcus pyogenes* but is subverted by *Coxiella burnetii* to contribute to the replication of this bacterium. Research to both *Chlamydia trachomatis* as well as to coronaviruses, in contrast, provided new insights into the function of LC3.

Although relatively well understood, some questions remain for the interaction between autophagy and GAS. Research to both recognition and specificity of autophagy directed against GAS will probably provide us with new insights in the regulation of selective autophagy. The same learning opportunities are there for the elucidation of how *Coxiella* subverts autophagy as these insights could lead to new therapeutic opportunities to inhibit autophagy, which in some cases could be applicable to cancer treatment (Hanahan and Weinberg, 2011).

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