Targeting of autophagy by *Yersinia enterocolitica* in epithelial cells

Dissertation zur Erlangung des Doktorgrades an der Fakultät für Mathematik, Informatik und Naturwissenschaften Fachbereich Biologie, der Universität Hamburg

vorgelegt von

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Hamburg 2015

Tag der Disputation: 17. Juli 2015

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ABBREVIATIONS

General abbreviations

AA	Amino acid
Amp	Ampicillin
AMPK	AMP-activated protein kinase
APS	Ammonium persulfate
Atg	Autophagy-related gene
CatD	Cathepsin D
CCV	*Coxiella*-containing vacuole
CFU	Colony forming unit
Chl	Chloroquine
Chlor	Chloramphenicol
CLEM	Correlative light and electron microscopy
CREB	cAMP response element-binding protein
DAPI	4',6'-diamidino-2-phenylindole
ddH_2O	Doubled-distilled water
DFCP1	Double FYVE-containing protein 1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
EGFP	Enhanced GFP
EGTA	Ethylenglycoltetraacetate
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
FAE	Follicle-associated epithelium
FBS	Fetal bovine serum

FFA	Free fatty acid
FIP200	FAK family kinase interacting protein of 200 kDa
Gal8	Galectin-8
Gal8IR	Galectin-8 interacting region
GAP	GTPase-activating protein
GFP	Green fluorescent protein
HPI	High-pathogenicity island
IF	Immunofluorescence
Ig	Immunoglobulin
IL-1β	Interleukin-1β
IL-18	Interleukin-18
Inv	Invasin
KO	knockout
LAMP-1	Lysosome-associated membrane protein 1
LAP	LC3-associated phagocytosis
LB	Luria-Bertani
LC3	Light chain protein 3
LCV	*Legionella*-containing vacuole
LDH	Lactate dehydrogenase
LIR	LC3-interacting region
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
mIC_{cl2}	mouse intestinal cell clone 2
MLN	Mesenteric lymph node
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
Nal	Nalidixic acid
NBR1	Neighbour of Brca1 gene
NDP52	Nuclear dot protein 52
NFκB	Nuclear factor-κ-light-chain-enhancer of activated B cells

NLR	(NOD)-like receptor
OD	Optical density
OPTN	Optineurin
p62	Nucleoporin p62
p70S6K	Ribosomal S6 kinase 1
PAMP	Pathogen-associated pattern
PAS	Phagophore assembly site
PBS	Phosphate-buffered saline
PBST	PBS Tween
PE	Phosphatidylethanolamine
PEI	Polyethylenimine
PI3K	Phosphoinositide 3-kinase
PP	Peyer's patch
PRR	Pattern recognition receptor
PRK2	Protein kinase C-like 2
PtdIns3P	Phosphatidylinositol-3-phosphate
PtdIns(4,5)P_2	Phosphatidylinositol-4,5-biphosphate
PTPase	Protein tyrosine phosphatase
pYV	Plasmid of *Yersinia* virulence
Rapa	Rapamycin
RFP	Red fluorescent protein
RLR	(RIG-I)-like receptors
RNA	Ribonucleic acid
RSK1	Ribosomal S6 protein kinase 1
SCV	*Salmonella*-containing vacuole
SDS	Sodium dodecyl sulphate
SDS-Page	SDS-polyacrylamide gel electrophoresis
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
Starv	Starvation
Syc	Specific Yop chaperone
TEM	Transmission electron microscopy

tfLC3	Tandem fluorescent LC3
TLR	Toll-like receptor
TRIF	TIR domain-containing adaptor inducing INF-β
TTSS	Type three secretion system
Ub	Ubiquitin
UBA	Ubiquitin associated domain
ULK1	UNC51-like Ser/Thr kinase
VAMP	Vesicle associated membrane protein
v-ATPase	Vacuolar proton ATPase
WB	Western-Blot
WIPI	WD repeat domain phosphoinositide-interacting 1
WT	Wild-type
YadA	*Yersinia* adhesin A
YCV	*Yersinia*-containing vacuole
YFP	Yellow fluorescent protein
YplA	*Yersinia* lipoprotein A
Yop	*Yersinia* outer protein
Ysc	*Yersinia* secretion protein
Yst	*Yersinia* stable toxin
ZnF	Zinc-finger domain

Abbreviations for microorganisms

E. coli	*Escherichia coli*
WAC	Virulence plasmid-cured derivative of wild-type Y. enterocolitica strain WA-314, serotype O:8
S. Typhimurium	*Salmonella enterica* subsp. *enterica* serovar Typhimurium
Y. enterocolitica	*Yersinia enterocolitica*
Y. pestis	*Yersinia pestis*
Y. pseudotuberculosis	*Yersinia pseudotuberculosis*

Units

Å	Angstrom
°C	Degree Celsius
Cm	Centimetre
g	Grams
h	Hour
kDa	KiloDalton
kV	KiloVolt
L	Litre
M	Mol
Min	Minute
mL	Millilitre
rpm	Revolutions per minute
sec	Second
µF	MicroFarad
nm	Nanometre
µm	Micrometre
µL	Microlitre

ABSTRACT

Autophagy is a cellular pathway that delivers cytoplasmic proteins, lipids and organelles to lysosomes for degradation. It occurs at basal levels to maintain cellular homeostasis and is upregulated in response to various stress conditions. Moreover, autophagy has also emerged as an innate immune response that selectively targets intracellular bacteria in the cytosol or within damaged vacuoles in a selective process, called xenophagy, in order to restrict bacterial growth. Importantly, some bacteria have evolved strategies to combat autophagy or to exploit the autophagy machinery to promote intracellular growth. Until now, little is known about the role of autophagy in *Y. enterocolitica* infection of epithelial cells. This work provides evidence that, after invading epithelial cells, autophagic processes are triggered which may influence the lifestyle of *Y. enterocolitica.* We have shown that *Yersinia*-induced autophagy is mediated by the *Yersinia* surface protein invasin, which requires viable bacteria. Once within the epithelial cells, a population of *Y. enterocolitica* resides within autophagosome-like vacuoles that display markers of autophagosomes (LC3) and late endosomes (LAMP-1 but no v-ATPase or cathepsin D). Notably, the ability of these vacuoles to fuse with lysosomes and the concomitant acidification are blocked. Transmission electron microscopy studies revealed that these vacuoles consist in their ultrastructure mainly of double or multiple membranes around the bacteria, characteristic of xenophagy. Furthermore, manipulation of the autophagic responses by using a knockout embryonic mouse fibroblast line deficient in autophagy ($Atg5^{-/-}$ MEFs) suggested that the bacteria may employ the autophagy machinery to create a niche that could support intracellular bacterial survival and replication. This may eventually assist spread of the bacteria from the infected cells. It appears that the *Yersinia*-containing vacuoles (YCVs) are targeted by ubiquitin, and the autophagy receptors p62 and NDP52 to promote autophagy. Finally, using wild-type mice and mice deficient in intestinal epithelial cell autophagy, pilot experiments were performed to investigate whether autophagy is hijacked *in vivo* by *Y. enterocolitica* to promote the dissemination of the bacteria from the intestinal mucosa to deeper tissues. However,

in these experiments we did not observe a significant difference in the dissemination of the bacteria in autophagy deficient versus competent mice, which suggests a more complex picture of epithelial autophagy in the pathogenesis of intestinal yersiniosis. In total, our study shows that *Y. enterocolitica* may manipulate the normal autophagy defense machinery of epithelial cells to promote survival, replication and spread of the bacteria from the infected cells. Whether this is beneficial for the pathogen *in vivo* remains to be specified.

1 INTRODUCTION

1.1 Y*ersinia enterocolitica*

1.1.1 Background

Bacteria of the genus *Yersinia* can cause diseases that range from enteritis to bubonic plague. The initial characterization of this genus was performed in 1894 by Alexandre Emile John Yersin, who identified *Yersinia pestis* (formerly *Pasteurella pestis*) as the causal agent of bubonic plague (Bottone 1997). However, the first description of human isolates belonging to *Yersinia enterocolitica* was made in 1939 by Schleifstein and Coleman as "an unidentified microorganism resembling *Bacterium lignieri* and *Pasteurella pseudotuberculosis*". Later, Frederiksen proposed the name *Yersinia enterocolitica* in 1964 (Cover & Aber 1989).

Members of the genus *Yersinia* are non-spore forming, Gram-negative or Gram-variable, rod-shaped bacteria of 1-3 µm in length. All species, with the exception of *Y. pestis*, are motile at 22-30 °C but lack this ability when grown at 37 °C. Yersiniae grow both under aerobic and anaerobic culture conditions between 0-45 °C, but their optimum growth occurs in the temperature range of 25-28 °C. Like other *Enterobacteriaceae*, yersiniae ferment glucose and are oxidase-negative and, most isolates reduce nitrates (Cover & Aber 1989; Fàbrega & Vila 2012).

Among the 17 species that belong to the genus *Yersinia* (Koornhof et al. 1999), only *Y. pestis, Y. pseudotuberculosis* and some strains of *Y. enterocolitica* are pathogenic for humans or certain warm-blooded animals, whereas the other species are of environmental origin (Murray et al, 1999). All pathogenic species carry a virulence plasmid (pYV) and share a common tropism for lymphoid tissue and the ability to resist the innate immunity of the host (Wren, 2003). Their main strategy seems to consist of: (1) avoiding lysis by complement; (2) avoiding phagocytosis by polymorphonuclear leukocytes and macrophages; and (3) forming extracellular microcolonies in the infected tissues (Cornelis 1994). The enteropathogenic yersiniae, *Y. enterocolitica* and

Y. pseudotuberculosis (**Fig. 1.1**), are fecal-oral pathogens that cause a range of invasive gastrointestinal diseases that are called yersiniosis. The occasional dissemination into blood, liver and spleen gives rise to enteric fever. *Y. pestis* is the etiological agent of bubonic plague and alternately grows in fleas or mammalian hosts (Straley & Perry 1995).

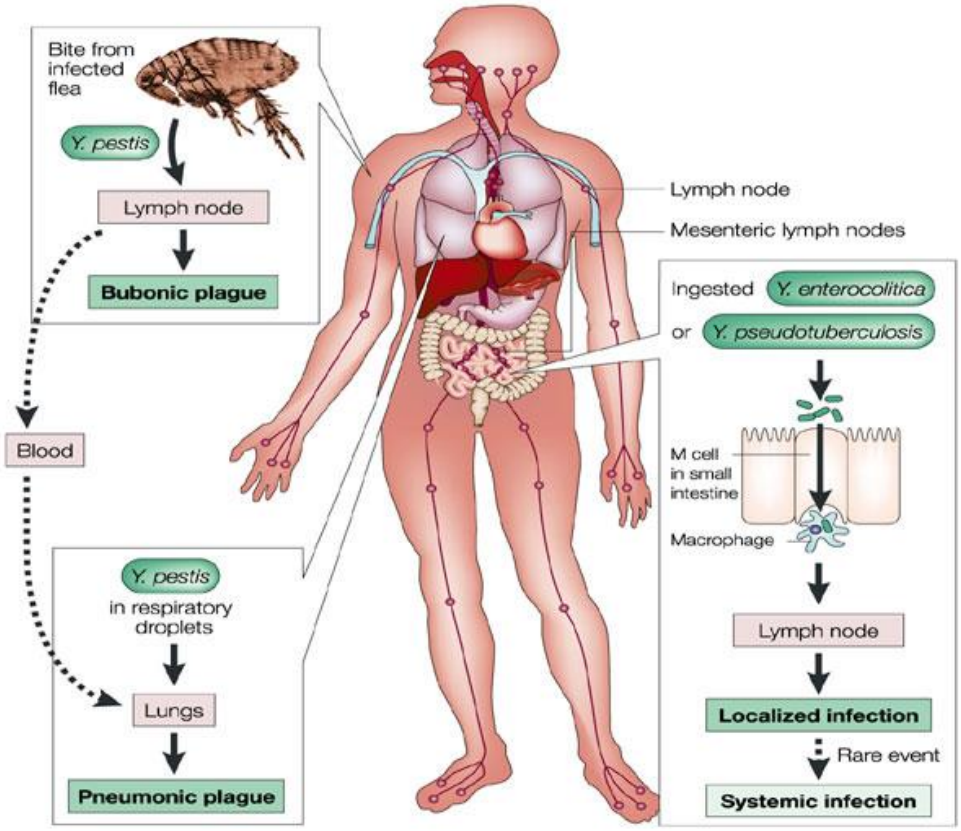


Fig. 1.1: Lifestyles and pathogenesis of the human pathogenic *Yersinia* species.
The enteropathogenic *Yersinia* species *Y. enterocolitica* and *Y. pseudotuberculosis* are transmitted by contaminated food or water. They are ingested and enter the lymphatic system, mainly, through the small intestine. The main reservoirs of *Y. pestis* are rodents. Transmission of the bacteria to humans occurs through the bite of an infected flea resulting in bubonic plague. Pneumonic plague is developed when *Y. pestis* reaches the lung and is transmitted via respiratory droplets (from Wren, 2003).

Y. enterocolitica is an heterogeneous group of strains that are mainly distinguished by biochemical testing (biogroups) and serotyping according to O antigens (lipopolysaccharide) (Cover & Aber 1989). There are 6 biogroups (1A, 1B and 2-5) and 57 serogroups (Wauters et al. 1987; Aleksić & Bockemühl 1990). Strains that belong to serogroup O:3 (biogroup 4), O:5,27 (biogroups 2 and 3), O:8 (biogroup 1B) and O:9 (biogroup 2) are frequently isolated from human samples. In many European

countries, O:3 and O:9 are the most important serogroups, whereas serogroup O:8 is mainly isolated in the United States (Bottone, 1997).

1.1.2 Clinical manifestations and pathogenesis

Y. enterocolitica is one of the most common causes of gastrointestinal disease in the moderate and subtropical climes of the world. The clinical manifestations of the infection depend on the age and physical condition of the patient, presence of any underlying medical disorder and the bioserotype of *Y. enterocolitica* (Fàbrega & Vila 2012). Yersiniosis may appear as enteritis, terminal ileitis or mesenteric lymphadenitis with watery or sometimes bloody diarrhoea. Sepsis is a rare complication of infection, except in immunocompromised patients or patients with states of iron overload (e.g. haemolytic anemia) (Bottone 1997). Due to the fact that an analogous disease state occurs in mice in response to oral infection, a murine model was developed to study the mode of transmission and the pathogenicity of *Y. enterocolitica* as a paradigm of bacterial pathogenesis (Carter 1975).

The primary event of *Y. enterocolitica* pathogenesis is colonization of the intestinal tract, particularly the terminal ileum and proximal colon (Bottone 1997). There, yersiniae attach and penetrate the mucus barrier overlying the mucosal epithelial cells, and eventually adhere to intestinal cells. The attachment and invasion of the cells is mediated by a number of bacterial adhesins including invasin and YadA (Isberg 1996). Bacteria preferentially bind to and penetrate M cells of the follicle-associated epithelium (FAE) of the Peyer's patches (PPs) and, once internalized, they are transported across the epithelial barrier by transcytosis and released from the basolateral side of the M cell (**Fig. 1.2**) (Westphal et al. 2008; Schulte et al. 2000; Autenrieth & Firsching 1996). In the following early step of infection, macrophages phagocytose the bacteria. Internalized bacteria replicate inside those phagocytes and are transported within migrating macrophages to mesenteric lymph nodes (MLN), causing inflammatory responses (Fàbrega & Vila 2012). Furthermore, phagocytes that take up bacteria can disseminate via the bloodstream to the liver and spleen. Once located in PPs, MLNs, spleen or liver, *Y. enterocolitica* replicates extracellularly within micro-abscesses (Autenrieth et al. 1996; Wuorela et al. 1999). Inside these lesions

bacteria appear to be resistant to phagocytosis by macrophages and neutrophils (Oellerich et al. 2007; Trülzsch et al. 2007). This is possible due to intracellular injection of *Yersinia* outer proteins (Yops) mediated by a type three secretion system (TTSS) that paralyses phagocytes of the innate immune system (Heesemann et al. 2006).

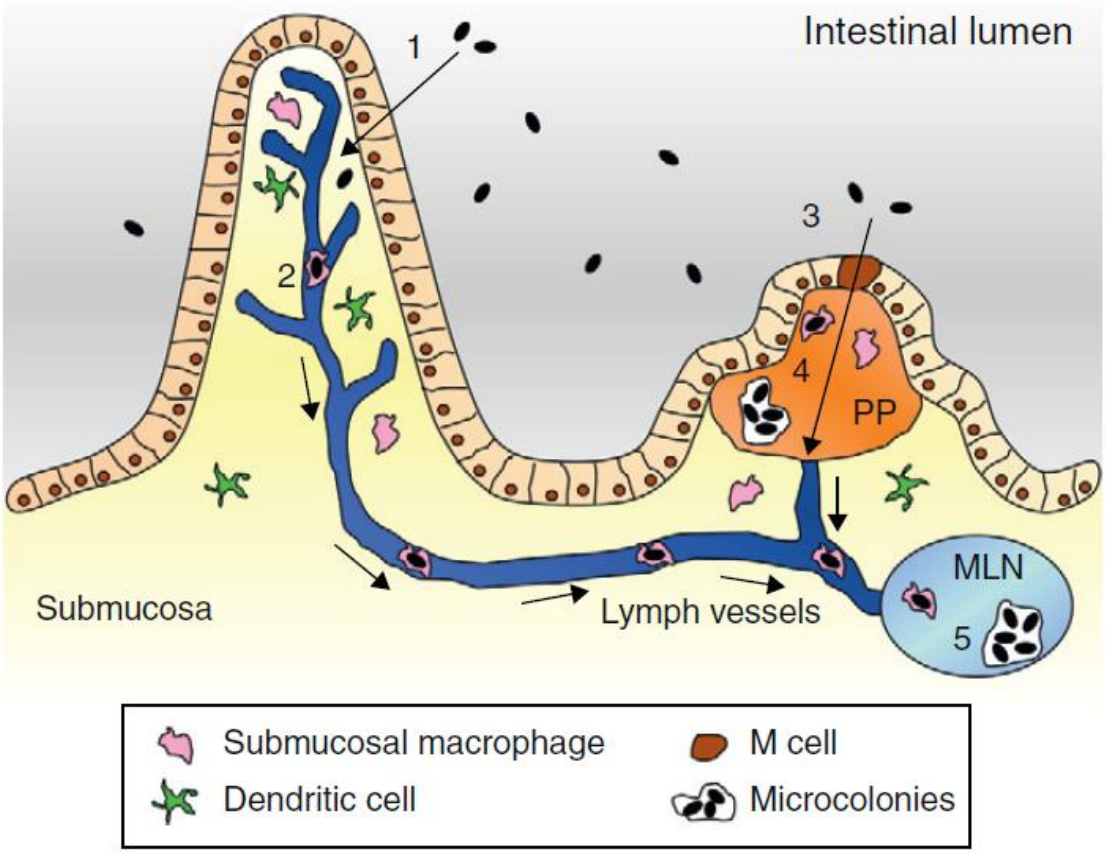


Fig. 1.2: Pathogenesis of *Y. enterocolitica*.
(**1**) Yersiniae traverse the intestinal epithelium via epithelial cells to the submucosa. (**2**) Submucosal macrophages phagocytose the pathogen and enter into the lymphatic system thereby reaching the MLN. (**3**) Alternatively, bacteria can be internalized by M cells. (**4**) Once in the PP yersiniae form microcolonies and replicate. (**5**) Eventually, bacteria are located in the MLN, form microcolonies and replicate. MLN, mesenteric lymph nodes; PP, Peyer's Patch (from Fàbrega & Vila, 2012)

1.1.3 Virulence factors encoded by the chromosome

Pathogenesis of *Yersinia* has multifactorial origins: it requires both chromosomal and plasmid-encoded genes (Portnoy & Falkow 1981) that act cooperatively to mediate invasion and to enable the bacteria to survive inside the host organism (Portnoy & Falkow 1981). Virulence gene expression depends on environmental signals. All

pathogenic *Y. enterocolitica* strains show a temperature-regulated adaptation process which allows the transition from the environment to the adverse conditions inside the mammalian host. The regulation of the virulence genes plays a key role in the successful adaptation to the host at 37 °C (Bottone 1997). In addition to temperature, either the presence or absence of calcium in the cellular environment or culture medium can be an important regulator of the expression of virulence genes (Cornelis 1994).

The chromosomal virulence factors that are strongly expressed at low temperatures (25-28 °C), are the adhesion protein invasin, the heat-stable enterotoxin Yst, proteins related to scavenge iron, and the flagellin-encoding genes *fleABC*. These factors are important for bacterial survival in the conditions that are encountered during the early stages of infection and their expression may persist during the passage of the yersiniae to the terminal ileum (Straley & Perry 1995).

1.1.3.1 Invasin

When growing at low temperatures and at the very beginning of the stationary phase of growth, the outer membrane protein called invasin is heavily expressed. These particular conditions correspond to those found when *Y. enterocolitica* and *Y. pseudotuberculosis* are ingested by contaminated food or water (Pepe & Miller 1993; Grassl et al. 2003). Invasin is present on the surface of pathogenic strains and it is essential for the initial step of invasion. This is because this protein is the major bacterial factor that initiates the adhesion, uptake and translocation of the bacteria through the intestinal epithelial cells due to its strong interaction with various members of the β1-integrin receptor family located on those cells (**Fig. 1.3**) (Isberg & Falkow 1985; Isberg & Leong 1990; Pepe & Miller 1993). After the bacteria have crossed the epithelial layer, invasin is not longer required, since *in vivo* bacterial survival and replication take place extracellularly due to an arsenal of anti-phagocytic Yops (Section 1.1.4.2.3) that are injected into target cells to block the internalization of bacteria upon adhesion (Pizarro-Cerdá & Cossart 2006). It is worth mentioning that within the intestinal epithelium, M cells are rich on β1-integrins on their apical side, while enterocytes express integrins only at their baso-lateral membrane. For this

reason, enterocytes are not expected to be heavily invaded like M cells during oral infection (Isberg et al. 2000; Schulte et al. 2000).

β1-integrins that bind invasin are normally either receptors for extracellular matrix proteins or are involved in cell-cell interaction. Their cytoplasmic domain interacts with the cytoskeleton in focal complexes of adhesion plaques and transmit signals after substrate binding. In case of the infection by *Yersinia*, binding of invasin to β1-integrins induces internalization of the bacteria into epithelial cells by a "zipper" mechanism (Finlay & Cossart 1997). The "zipper" mechanism (**Fig. 1.3**) can be divided into three successive steps: (1) contact and adherence, (2) phagocytic cup formation, and (3) phagocytic cup closure and retraction. The first step is independent of the actin cytoskeleton and involves the invasin protein and the β1-integrin receptors. Invasin can oligomerize, leading to integrin clustering, which ensures an efficient downstream signaling. During the second step, the activation of β1 receptors by the ligand-receptor engagement induces an autophosphorylation of the tyrosine kinase FAK (Focal Adhesion Kinase), that allows the subsequent recruitment of Src kinases, followed by a mutual activation of both kinases (Grassl et al. 2003). FAK not only acts as a kinase but also as a molecular scaffold. It is a docking site for class IA phosphoinositide 3-kinase (PI3K) and factors like $p130^{cas}$, which transmit signals downstream of the kinase and regulate the activity of Rho-family GTPases. In turn, actin rearrangements are induced at the site of bacterial entry and plasmatic membrane extension occurs (Reis & Horn 2010). At the last step, the closure of the phagocytic cup occurs by cytoskeleton rearrangements and membrane traffic that depend on local concentration of phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P_2). PtdIns(4,5)P_2 acts as a scaffold for actin-remodelling proteins. The Rho GTPase Rac1 together with Arf6 may play a role in the recruitment and activation of phophoinositol-4-phosphate-5-kinase responsible for the local production of PtdIns(4,5)P_2. (Wong & Isberg 2003; Cossart & Sansonetti 2004; Pizarro-Cerdá et al. 2014). Furthermore, the activity of class IA PI3K is necessary for the recruitment of Rab5 and inositol 5-phosphatases, which later on cleave PtdIns(4,5)P_2 from the PtdIns(4,5)P_2-rich compartment surrounding invading *Yersinia*. This allows vacuolar fission from the plasma membrane into the cytosol of the host cell (Sarantis et al. 2012).

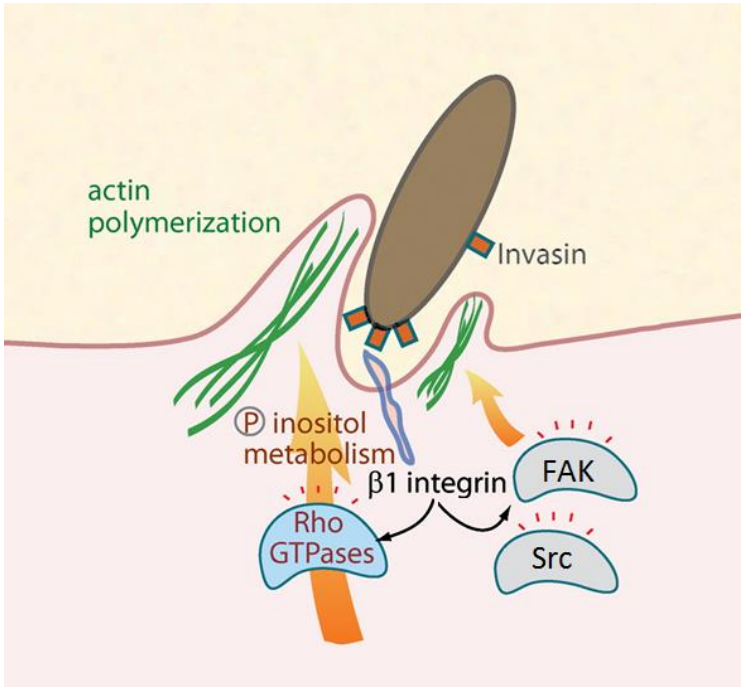


Fig. 1.3: Molecular invasion strategy of *Yersinia*.
Yersinia invasin interacts with β1-integrin receptors. That causes bacterial internalization following activation of FAK and Src, which are involved in actin cytoskeletal rearrangements. The Rho GTPase Rac1 also participates in this process, by modification of the phosphatidylinositol metabolism at the site of bacterial entry (modified from Pizzaro-Cerdá & Cossart, 2006).

1.1.3.2 Enterotoxin Yst

Y. enterocolitica produces a heat-stable chromosomally encoded enterotoxin (Pai & Mors 1978), known as Yst (for "*Yersinia* stable toxin"). This peptide resembles both the heat stable enterotoxin of *E. coli* and guanylin, an endogenous activator of intestinal guanylate cyclase (Cornelis 1994). It was speculated that the production of Yst is responsible for the diarrheal manifestation associated with yersiniosis (Delor & Cornelis 1992).

1.1.3.3 High-Pathogenicity Island (HPI)

This chromosomal region is only present in *Y. enterocolitica* biotype 1B (Carniel et al. 1996). Most of the genes located on this island are involved in the biosynthesis, transport and regulation of the siderophore yersiniabactin (Carniel 2001). Thus, the HPI may be regarded as an iron-capture island. The locus is organized into four

operons (*fyuA, irp2, ybtA* and *ybtP*) which possess a Fur-binding site and are negatively regulated by this repressor in the presence of iron (Carniel 1999; Carniel et al. 1996).

1.1.3.4 Flagella

Before *Y. enterocolitica* establishes intimate contact with the intestinal epithelium, flagella and motility play an important role in initiating the host invasion (Young et al. 2000). The flagellum is a heterooligomeric structure and its assembly involves the synthesis of a type three protein export apparatus-related structure that subsequently transports flagellin proteins from the cytoplasm to the outer surface of the cell, where oligomerization occurs (Young et al. 1999).

Motility is regulated by environmental and physiological conditions (D'Amato & Tomfohrde 1981; Kapatral et al. 1996) and the inactivation of the flagellar regulatory genes has been associated with decreased invasion comparable to that of strains in which *inv* has been inactivated (Young et al. 2000).

The flagellum is not only an organelle for cell motility, but it can also be used to secret extracellular virulence factors under certain conditions. One of the proteins exported by the flagellar secretion system is the phospholipase YplA, which may also contribute to *Yersinia* virulence (Young et al. 1999; Young & Young 2002; Minnich & Rohde 2007).

1.1.4 Virulence factors encoded by the virulence plasmid

Pathogenic strains of *Yersinia* have a 70 kb virulence plasmid, pYV (for "plasmid of *Yersinia* virulence" (Portnoy & Falkow 1981)). pYV is essential to allow extracellular survival and multiplication of the bacteria in the host lymphoid tissue after yersiniae have overcome the epithelial barrier. The plasmid harbours genes encoding for the *Yersinia* adhesin A (YadA) and the type three secretion system TTSS Ysc (Aepfelbacher et al. 2007; Cornelis et al. 1998).

Two different regulatory networks govern pYV gene expression. The first one responds to temperature and regulates all pYV encoded virulence functions, while the second one responds to calcium and only regulates the production of the Yops and of *Yersinia* lipoprotein A (YlpA) (Cornelis 1994; Gemski et al. 1980; Portnoy & Falkow 1981).

1.1.4.1 YadA

YadA is a surface-exposed protein that serves as a multi-functional virulence factor that, unlike invasin, is positively regulated at 37 °C (Dube 2009; El Tahir & Skurnik 2001).

This protein mediates adherence to epithelial cells and phagocytes (Heesemann & Grüter 1987). It also binds to extracellular matrix components (collagen fibers and fibronectin) which promotes indirect interaction to host cell integrin receptors (Tertti et al. 1992; Schulze-Koops et al. 1993) and it is required for persistence, survival and replication in PPs (Pepe et al. 1995). YadA is also responsible for the autoagglutination that occurs after growth in tissue culture medium at 37 °C (Skurnik et al. 1984; Balligand et al. 1985).

Additionally, YadA plays an important role in the defense against the nonspecific immune response, inhibiting the formation of the complement membrane attack complex at the bacterial surface. Furthermore, it impairs opsonization, which reduces phagocytosis and killing by polymorphonuclear leukocytes (Cornelis 1994; Dube 2009).

1.1.4.2 Ysc type three secretion system (TTSS)

Yersinia uses specialized secretion systems called TTSS to deliver virulence factors into the cytoplasm of target host cells (Cornelis 2002). The TTSS encoded by the pYV-plasmid is also known as Ysc (for “Yop secretion”) (Michiels et al. 1991) to distinguish it from two other TTSS encoded by the *Y. enterocolitica* chromosome, including the flagellar TTSS and the TTSS Ysa (Haller et al. 2000; Young et al. 1999). The TTSS Ysc (**Fig. 1.4**) comprises the Yop effector proteins, the injection apparatus itself (called “injectisome”) and the Yop translocators (needed to deliver the effectors across the eukaryotic plasma membrane). The injected Yops are exotoxins that mimic the action of host cell enzymes such as phosphatases, proteases, kinases and acetylases to modulate the cytoskeleton and immune signaling (Cornelis 2002; Viboud & Bliska 2005). This ultimately leads to inhibition of phagocytosis, proinflammatory cytokine production, and in the case of macrophages, the induction of apoptosis and the

prevention of pyroptosis, thus favouring the survival of the invading *Yersinia* (Cornelis 2002; Dube 2009).

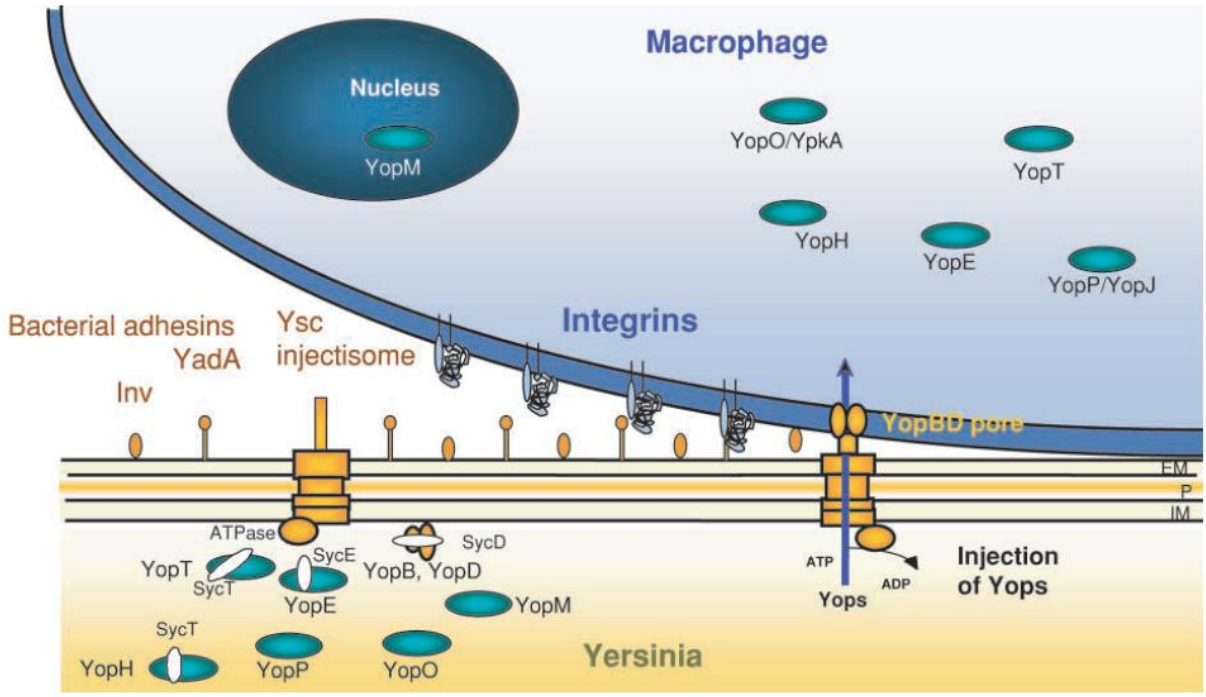


Fig. 1.4: Secretion of Yops by the Ysc injectisome and translocation across the target cell membrane. When *Yersinia* are exposed to 37°C in a rich environment, the Ysc injectisome is installed and a stock of Yop proteins is synthesized. During their intrabacterial stage, some Yops are capped with their specific Syc chaperone. Upon contact with a eukaryotic target cell, the adhesins YadA or invasin (Inv) interact with integrins and the bacterium docks at the host cell surface. Then, the secretion channel opens and Yops are exported. YopB and YopD form a pore in the target cell plasma membrane, and the effector Yops are translocated across this membrane into the eukaryotic cell cytosol. YopM migrates to the nucleus. EM, outer membrane; P, peptidoglycan; IM, inner membrane (from Cornelis, 2002).

1.1.4.2.1 Ysc injectisome

The Ysc injectisome functions as a protein pump, spanning the peptidoglycan layer and the two bacterial membranes topped by a stiff needle-like structure protruding outside the bacterium. The whole organelle comprises 27 Ysc proteins encoded by the *ysc* genes, which are distributed in four contiguous loci that were initially called virA, virB, virG and virC (for “virulence”) (Cornelis et al. 1986; Cornelis 2002).

The proteins YscC, YscD and YscJ form the rigid scaffold spanning the two bacterial membranes and the peptidoglycan (Diepold et al. 2011). The size of the pore within

the ring-shaped structure is ~50 Å (Koster et al. 1997). YscR, YscS, YscT, YscU and YscV are integral inner membrane proteins, which form the export channel in the internal membrane and are believed to recognize export substrates (Diepold et al. 2011). YscN is an essential part of the pump, which is localized at the cytosolic side and resembles the catalytic α and β subunits of the F_0F_1 proton translocase and related ATPases (Woestyn et al. 1994). Finally, the injectisome ends with a 60-80 nm-long and 6-7 nm-wide needle formed by the polymers of the YscF protein that is secreted by the Ysc apparatus itself (Hoiczyk & Blobel 2001). The length of the needle is determined by YscP (Journet et al. 2003).

1.1.4.2.2 Syc cytosolic chaperones

The secretion of some Yops requires the presence of small cytosolic chaperones called Syc proteins (for "specific Yop chaperone") (Woestyn et al. 1994; Cornelis et al. 1998): SycE (for YopE) (Wattiau & Cornelis 1993; Birtalan & Ghosh 2001), SycH (for YopH) (Phan et al. 2004), SycT (for YopT) (Büttner et al. 2005; Locher et al. 2005), SycN (for YopN) (Day & Plano 1998), SycD (for YopB and YopD) (Schmid et al. 2006; Büttner et al. 2008) and SycO (for YopO) (Letzelter et al. 2006). Sycs are small acidic proteins with little or no sequence similarity but with a common, putative COOH-terminal amphiphilic α-helix. They bind to their specific partner Yop, and in their absence Yop secretion is importantly reduced, if not abolished. They are usually encoded by genes located close to the genes of the respective Yops (Cornelis 2002).

1.1.4.2.3 *Yersinia* outer proteins (Yops)

Upon contact with cells, two translocator Yops (YopB and YopD) are inserted into the host plasma membrane and function to transport six effector Yops (YopH, YopO/YpkA, YopP/YopJ, YopE, YopM and YopT) via the TTSS into the cytosol of the host cells. Effector Yops counteract multiple signaling responses in the infected host cell that are initiated by phagocytic receptors, Toll-like receptors, translocator Yops, etc (Viboud & Bliska 2005).

YopH: It is a protein tyrosine phosphatase (PTPase), which antagonizes several signaling pathways associated with phagocytosis of the bacteria by host cells (Forsberg et al. 1994; Viboud & Bliska 2005). This effector is among the most powerful PTPase known: high expression of YopH alone is sufficient to block internalization of the bacteria (Persson et al. 1997). Substrates of YopH include $p130^{Cas}$, FAK, paxillin, Fyn-binding protein (Byb) and the scaffolding protein SKAP-HOM (Black & Bliska 1997; Hamid et al. 1999; Persson et al. 1997; Viboud & Bliska 2005), which regulate the interaction between the actin cytoskeleton and integrins in focal adhesions or similar structures. Cytoskeletal uptake structures formed upon contact of YadA or invasin with cellular integrins resemble focal adhesions/complexes and their disruption by YopH could explain its antiphagocytic activity (Aepfelbacher et al. 2007). Additional immunomodulatory effects of YopH include suppression of the oxidative burst of macrophages (Green et al. 1995), reduction of Ca^{2+} in neutrophils (Andersson et al. 1999), inhibition of T and B lymphocyte activation (Yao et al. 1999), blockage of monocyte chemoattractant protein 1 production by macrophages (Sauvonnet et al. 2002) and induction of apoptosis in T cells (Bruckner et al. 2005).

YopP (YopJ in *Y. pseudotuberculosis* and *Y. pestis*): It is an acetyltransferase that acts as a potent inhibitor of the NFκB signaling pathway of host cells, which is an important initiator of inflammation. As a result, YopP reduces the release of TNF-α by macrophages (Boland & Cornelis 1998) and of IL-8 by epithelial cells (Schesser et al. 1998) and endothelial cells (Denecker et al. 2002). YopP is also an inhibitor of the mitogen-activated protein kinase (MAPK) signaling pathways (Ruckdeschel et al. 1997; Orth et al. 1999) that abrogates phosphorylation of transcription factor CREB (cAMP response element-binding protein), which is involved in the proinflammatory immune response (Meijer et al. 2000). Last but not least, YopP can induce apoptosis only in macrophages but not in another cell types (Denecker et al. 2001).

YopM: It is an effector Yop that does not exert any enzymatic activity. This strongly acidic protein, that contains a varying number of a leucin-rich repeat motifs (Leung & Straley 1989), has a nuclear localization signal. Four YopM monomers stack together to form a hollow cylinder (Skrzypek et al. 1998). It serves as a scaffold that interacts with

two cytoplasmic kinases: protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1), which are implicated in the regulation of gene transcription and cell cycle progression (McDonald et al. 2003). YopM also binds caspase-1, which inhibits caspase-1 activity and blocks the formation of a functional inflammasome complex. The inflammasome is a key multiprotein platform that processes the proinflammatory cytokines pro-IL-1β and pro-IL-18 into their mature and active forms, leading to both the release of IL-1β and IL-18 and pyroptotic cell death, an inflammatory program of cell death directed by caspase-1 (LaRock & Cookson 2012).

YopE, **YopT** and **YopO** interact with Rho GTPases, that regulate diverse cellular functions, from dynamic regulation of the actin cytoskeleton to gene expression (Barbieri et al. 2002).

YopE: It is a GTPase-activating protein (GAP) that exerts GAP activity on the Rho GTPases RhoA, Rac-1 and Cdc42, switching them to the inactive state by accelerating GTP hydrolysis (Black & Bliska 2000; Von Pawel-Rammingen et al. 2000). Through its ability to deactivate Rho GTPases, YopE disrupts the actin cytoskeleton causing an effect called cytotoxicity that consists in cell rounding and detachment from the extracellular matrix (Goguen et al. 1986; Rosqvist et al. 1990; Rosqvist et al. 1991). By inactivation of Rho and Rac, YopE not only blocks phagocytosis (Grosdent et al. 2002), but also inhibits caspase-1-mediated maturation and release of IL-1β in macrophages (Schotte et al. 2004). In addition, this effector plays a role in the regulation of Yop translocation (Viboud & Bliska 2001).

YopO (YpkA in *Y. pseudotuberculosis* and *Y. pestis*): It is a serine/threonine kinase that becomes autophosphorylated upon contact with actin and that interacts with RhoA and Rac-1 (Galyov et al. 1993; Dukuzumuremyi et al. 2000; Juris et al. 2000). Additionally, it mimics a Rho-family nucleotide dissociation inhibitor (Prehna et al. 2006). Although its role on host actin cytoskeletal rearrangements is relevant in the context of phagocytosis inhibition, the potential host cell kinase target and the exact mode of action of YopO remain unknown (Wiley et al. 2006; Navarro et al. 2007).

YopT: It is cysteine protease (Shao et al. 2002) that cleaves the geranylgeranyl groups of RhoA, Rac-1 and Cdc-42, removing the GTPases from the membrane and inactivating them (Shao et al. 2003; Aepfelbacher et al. 2007). This leads in turn to the disruption of stress fibers, cell rounding and inhibition of phagocytosis (Iriarte & Cornelis 1998; Aepfelbacher et al. 2007).

1.2 Innate immune responses against pathogenic bacteria

The success of infection is a continuously evolving battle between the host and the infecting microbes. In order to resist and prevent infections by pathogenic microorganisms, vertebrates have developed an immune system, which consist of innate and adaptive immune responses (Akira et al. 2006). The innate immunity is an evolutionary ancient part of the host defense mechanisms and the first line of defense against pathogens (Janeway & Medzhitov 2002). It comprises the complement system (a series of proteolytic cascades), macrophages, polymorphonuclear leukocytes, natural killer cells, dendritic cells and mast cells. Alternatively, the adaptive immunity is involved in the elimination of pathogens in later phases of infection and in the establishment of immunological memory, and involves T and B lymphocytes (Akira et al. 2006).

The innate immunity relies on a limited number of germline-encoded receptors, called pattern recognition receptors (PRRs), which recognize conserved microbial pathogen components, called pathogen associated molecular patterns (PAMPs) (Janeway & Medzhitov 2002). Major PAMPs are microbial nucleic acids, including DNA (e.g. unmethylated CpG motifs), double-stranded RNA, single-stranded RNA, and 5‘-triphophate RNA, as well as lipoproteins, surface glycoproteins, and membrane components like lipopolysaccharide (LPS) (Tang et al. 2012). The main PRRs are the Toll-like receptors (TLRs), retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs), AIM2 like receptors (ALRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Mogensen 2009). After PAMP recognition, activated PRRs localized on the cell surface, the cytoplasm and/or in intracellular vesicles, signal the presence of microbial infection by activating different pathways (Janeway & Medzhitov 2002;

Tang et al. 2012). Signal transduction is mediated by adaptor molecules which may determine the specificity of the response (Mogensen 2009). In the case of TLRs, they recruit one or several adaptor molecules (like MyD88 or TIR domain-containing adaptor inducing INF-β (TRIF)) that allow the activation of important downstream signaling pathways, including NF-κB and MAPK cascades (Mogensen 2009). As result of activation of these signal transduction pathways transcription factors are induced that regulate the expression of genes involved in inflammation and antimicrobial responses (Janeway & Medzhitov 2002; Mogensen 2009). This may culminate in phagocytosis, elimination of the pathogen via autophagy, synthesis of inflammatory and chemotactic cytokines, apoptosis and lytic cell death or necrosis via pyroptosis (**Fig. 1.5**) (Tang et al. 2012; Baxt et al. 2013).

Even when the immune system has been shaped during evolution by interactions with infectious microorganisms, several infectious agents have developed mechanisms to evade or manipulate the immune responses in order to survive in their hosts (Janeway & Medzhitov 2002).

Many pathogens interfere with the induction of host protective inflammation. In response to infection, a proinflammatory transcriptional response is activated through the MAPK and NFκB pathways, leading to the synthesis of several cytokines and chemokines (Baxt et al. 2013). The release of cytokines and chemokines trigger the recruitment of phagocytic cells and other components of the immune response to the site of infection. To subvert these processes, pathogenic bacteria may have effector proteins that intercept the activation of those pathways in several ways (Janeway & Medzhitov 2002). For example, *Shigella* spp. possess an effector called IpaH9.8 that modulates NFκB activation and dampens the inflammatory response (Ashida et al. 2010). Furthermore, OspF is another secreted effector protein of *Shigella* that inactivates the MAPKs in an irreversible way, reducing the influx of inflammatory cells into infected tissue (Li et al. 2007). *Yersinia* spp. are also capable of subverting inflammation by the action of YopP on the NFκB and the MAPK signaling pathways (Section 1.1.4.2.3). YopP also induces apoptosis in macrophages by the abrogation of a potent anti-apoptotic signaling loop provided by NFκB pathway (Bhavsar et al. 2007). Moreover, yersiniae, by the action of YopM, block the activation of the

proinflammatory cytokines IL-1β and IL-18 as well as pyroptotic cell death (Section 1.1.4.2.3).

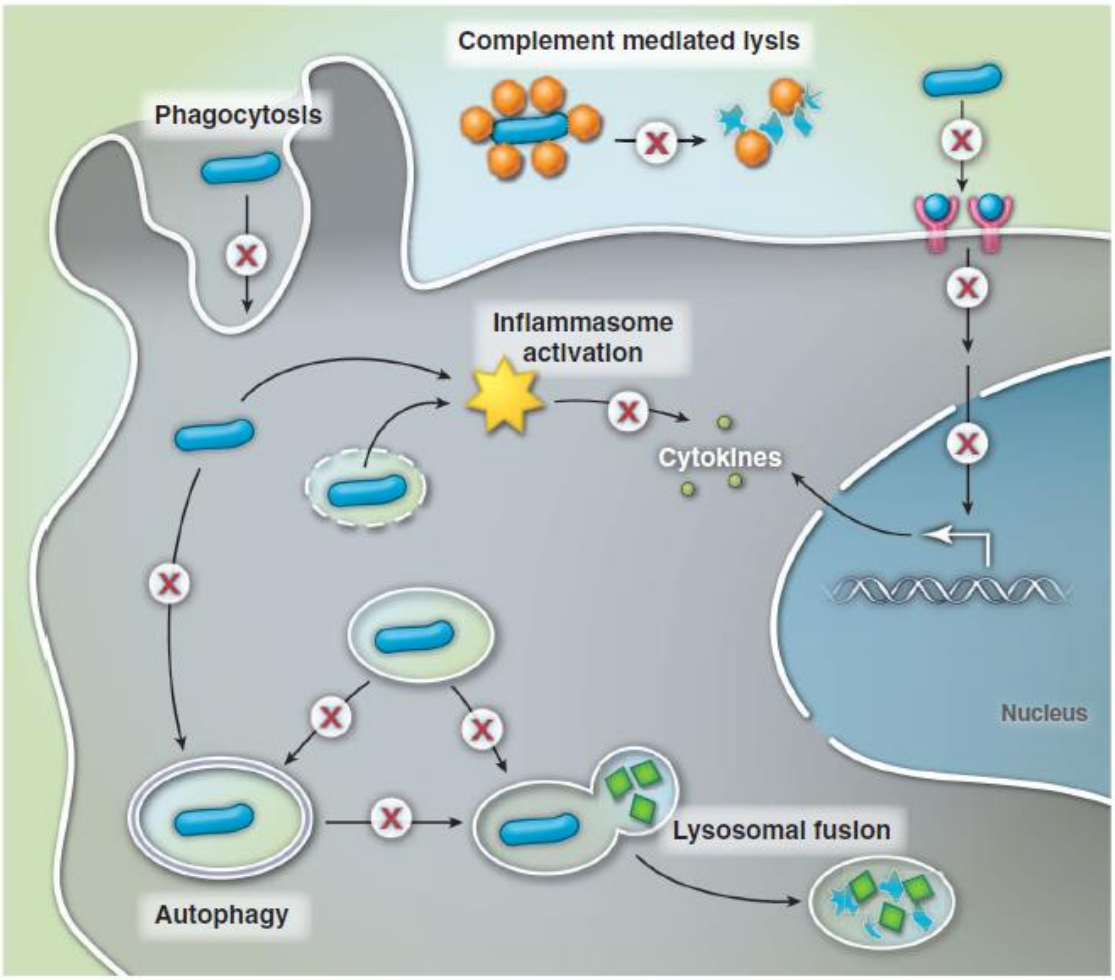


Fig. 1.5: Innate immune responses against bacteria.
Extracellular bacteria are susceptible to phagocytosis by phagocytes and to complement-mediated lysis. Intravacuolar and intracytoplasmic bacteria are subjected to the autophagy pathway. Detection of PAMPs by extracellular or intracellular receptors activates signaling cascades that mediate to a proinflammatory transcriptional response. Detection of bacterial PAMPs in the cytosol may trigger activation of inflammasomes to induce the proinflammatory cytokine IL-1β, which activate proinflammatory cytokines. Steps in these pathways known to be inhibited by bacteria are marked with a red X. Blue ellipses and circles represent bacteria; orange hexagons, complement; green diamonds, degradative enzymes within a lysosome; yellow star, inflammasome; pink "cups" on cell surface, Toll-like receptors (extracellular PRR); white arrow, proinflammatory cytokine transcriptional response (from Baxt et al., 2013).

In addition to the intervention with inflammatory processes, microbial pathogens may also manipulate other antimicrobial responses, such as phagocytosis and autophagy. Professional phagocytes internalize extracellular pathogens by phagocytosis. As a result of this process the microorganisms are included within a phagosome. This

organelle undergoes maturation by fusion with endosomes and lysosomes, to become an acidified, degradative compartment where most infectious agents are killed (Deretic 2008b). However, some pathogens can avoid phagocytosis or the subsequent fusion of bacteria-containing vacuoles with lysosomes. As mentioned above, *Yersinia* spp. are an example of pathogens that counteract phagocytosis by the action of Yops (YopH, YopE, YopO and YopT) on the host actin cytoskeleton. Another pathogen, *Neisseria meningitidis,* inactivates complement by recruiting host complement-inhibitory proteins. In this way, the bacterium inhibits complement-mediated phagocytosis and killing (Schneider et al. 2009). Some pathogens are also able to manipulate vesicle trafficking pathways by exploiting host guanosine triphosphatase (GTPase) signaling to avoid degradation. *S.* Typhimurium is a paradigm of a pathogen that traffics through the endocytic pathway and that inhibits the fusion with the lysosome. After being internalized into host cells, *S*. Typhimurium mostly resides and replicates inside a modified phagosomal compartment known as *Salmonella*-containing vacuole (SCV) (Brumell et al. 1999). Proteins associated with the early (Rab GTPase Rab5) and late (Rab GTPase Rab7) endosomes localize to the SCV (Asrat et al. 2014). The bacterium then secretes a virulence factor called SopB, that alters the charge of the membrane surface of the SCV, affecting the recruitment of Rab35, a GTPase involved in endocytic recycling. In line, fusion of the SCV with the lysosome is inhibited (Bakowski et al. 2008; Bakowski et al. 2010).

In contrast to phagocytosis, autophagy (discussed in detail in the next section) targets intravacuolar and intracytoplasmic bacteria. It is one of the earliest defense responses encountered by intracellular bacterial pathogens and many bacteria have evolved mechanisms to evade killing by this pathway (Baxt et al. 2013).

1.3 Autophagy

1.3.1 Background

The word autophagy is derived from the Greek words, *auto* "self" and *phagein* "to eat" and describes an evolutionarily conserved and adaptive catabolic and energy-generating process by which cells deliver proteins and organelles from the cytosol to lysosomes for degradation (Yang & Klionsky 2010). This process was first observed in the mouse kidney more than 50 years ago, where mitochondria appeared within membrane-bound compartments called "dense bodies" at that time (Clark 1957; Novikoff 1959). But it lasted until 1963, at a symposium on lysosomes, where de Duve created the term "autophagy" to describe the presence of vesicles that contain parts of the cytoplasm and organelles (De Duve & Wattiaux 1966).

The early studies of autophagy were based on morphological and pharmacological studies, but in the late 1990s the molecular era of autophagy started, which revolutionized the ability to detect and genetically manipulate this process. Although autophagy was initially identified in mammals, a significant breakthrough in the understanding of how autophagy is controlled came from analysis in yeast models. This allowed the characterization of the Atg ("autophagy-related") proteins and the connection of the autophagic process with diseases (Klionsky 2007; Yang & Klionsky 2010).

Autophagy is a homeostatic process that operates at a basal level. By autophagy, cells remove potentially harmful protein aggregates and control their cytoplasmic biomass and the abundance of organelles (Deretic 2008a). It is essential for energy metabolism during starvation (Boya et al. 2005; Lum et al. 2005). Autophagy also plays a role in type II programmed cell death, or autophagic cell death, where autophagy is needed for the execution of death in apoptosis-defective cells (Galluzzi et al. 2008; Scarlatti et al. 2009). It has furthermore function in cell death during embryonic development for the clearance of apoptotic cells (Qu et al. 2007). Autophagy also plays a role in

longevity, which is promoted by caloric restriction, an important inducer of autophagy (Bergamini et al. 2007). Moreover, autophagy participates in both innate and adaptive immunity, protecting cells against intracellular pathogens (Section 1.3.2.3) and contributing to antigen presentation in the context of the MHC class II complex (Levine & Deretic 2007; Schmid et al. 2007).

On the other side, autophagy also has a role in many human pathophysiologies. For instance, it has been shown that autophagy irregularities may result in malignancies. The monoallelic expression of the Atg gene *beclin1* is implicated in breast, prostate and ovarian cancers (Liang et al. 1999; Qu et al. 2003; Won et al. 2010). However, in advanced cancers, autophagy may have the opposite effect on the tumor development, promoting the progression of tumors because it can provide nutrients during starvation (Mathew et al. 2007; White & DiPaola 2009). Autophagy is also important in neurodegenerative processes as it affects the degradation of certain aggregate proteins that are toxic and can disrupt neuronal function (Ravikumar et al. 2002; Webb et al. 2003; Yu et al. 2005). The performance of genome-wide association (GWA) scans has implicated single nucleotide polymorphisms (SNP) in the autophagy genes *atg16L1* and *IRGM* in autoimmune and inflammatory disorders such as Crohn's disease and inflammatory bowel disease (Massey & Parkes). The pathogenesis of many of these multifactorial syndromes is not completely understood but it has been suggested for Crohn's disease that a defective autophagy process alters the immune responses in the gut. As consequence, the clearance of pathogenic adherent-invasive *E. coli* is altered, which may lead, at least in part, to pathogenesis of Crohn's disease (Lapaquette et al. 2010; Lapaquette et al. 2012).

1.3.2 Types of autophagy

Several autophagy pathways have been described so far, based on their mechanism of activation, site of cargo sequestration, and the type of cargo: 1) microautophagy, 2) macroautophagy (or simply autophagy), and 3) chaperone-mediated autophagy (**Fig. 1.6**). All of them deliver their substrates to the lysosome (Legakis & Klionsky 2006). Some of the sequestration events occur at the lysosomal membrane, these are denoted by the prefix "micro" (Ahlberg et al. 1982; Mizushima 2007). In other cases,

the substrate is enclosed inside a specialized organelle, the autophagosome, and this occurs spatially away from the lysosomal membrane. These pathways begin with the prefix “macro” (Legakis & Klionsky 2006). Microautophagy is a nonspecific degradation pathway, while macroautophagy, can work as a non-selective bulk process induced in response to starvation or, alternatively, be a selective and regulated process that requires cargo recognition (Stolz et al. 2014). Chaperone-mediated autophagy is a receptor-driven degradative pathway in which proteins possessing a specific sequence signal are transported from the cytoplasm, through the lysosomal membrane, to the lysosomal lumen (Cuervo & Dice 1996).

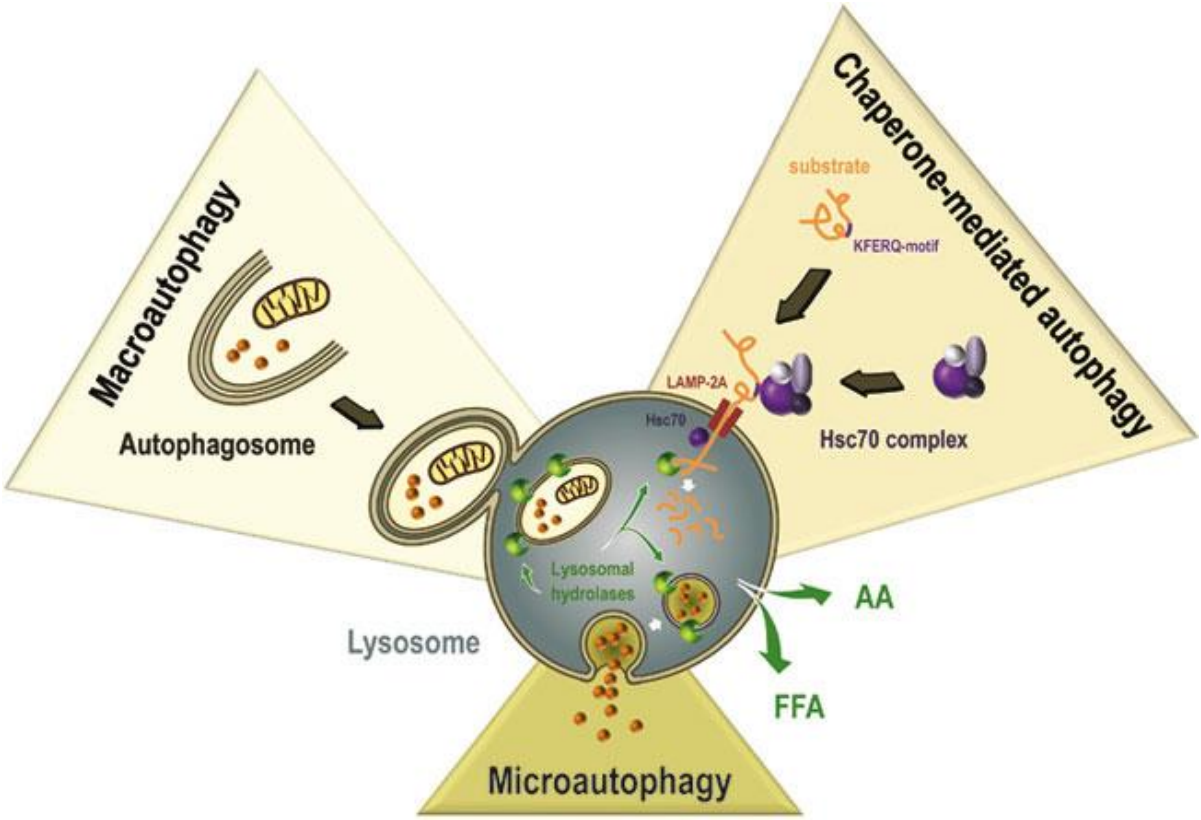


Fig. 1.6: Schematic presentation of the different types of autophagy.
During microautophagy the lysosomal membrane invaginates to engulf portions of the cytoplasm which are consequently degraded once entirely enclosed. During macroautophagy specialized vacuoles, called autophagosomes, are formed for cargo transportation. The autophagosomes deliver proteins, lipids and organelles to the lysosome. Chaperone-mediated autophagy sequesters proteins harbouring a KFERQ-like motif that, mediated by the Hsc70 complex, are directly targeted to the lysosomes for degradation. AA, amino acids; FFA free fatty acids (from Wirawan, 2012).

1.3.2.1 Canonical autophagy

The best characterized autophagy pathway is macroautophagy (hereafter called autophagy). Canonical autophagy, which is induced under starvation conditions, involves the stages of (1) initiation, (2) nucleation, (3) elongation and closure, (4) recycling and (5) degradation (**Fig. 1.7**).

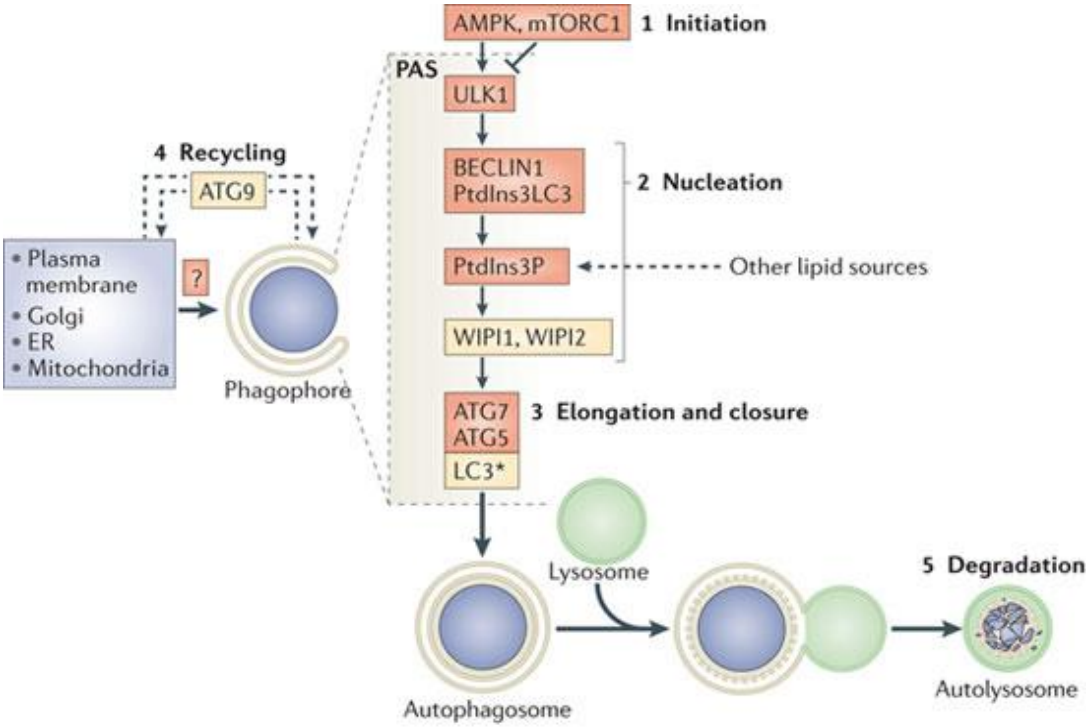


Fig. 1.7: Canonical and non-canonical macroautophagy in mammals.
Canonical autophagy involves the steps of: (**1**) initiation, (**2**) formation, (**3**) elongation and closure, (**4**) recycling and (**5**) degradation. Non-canonical routes to autophagosome formation, which bypass some of the canonical steps, have emerged and the proteins that may be bypassed are highlighted in red boxes. AMPK, AMP-activated protein kinase; BECLIN1, BCL-2 interacting myosin/moesin-like coiled-coil protein 1; ER, endoplasmic reticulum; LC3, light chain 3; mTORC1, mammalian target of rapamycin complex 1; PtdIns3P, phosphatidylinositol 3-phosphate; ULK1, UNC51-like kinase 1; WIPI1, WD repeat domain phosphoinositide-interacting 1 (from Codogno, 2012).

In mammalian cells, autophagosomes are generated from a double membrane called phagophore, which is assembled at the phagophore assembly site (PAS) (Codogno et al. 2012). A consensus is emerging that the phagophore membrane originates from endoplasmic reticulum membranes (Hayashi-Nishino et al. 2009; Ylä-Anttila et al. 2009), although several other cellular compartments, such as the Golgi (Yamamoto et al. 1990; Bodemann et al. 2011), the plasma membrane (Ravikumar et al. 2010) and

mitochondria (Hailey et al. 2010) may contribute to the expansion of the nascent autophagosome. Once canonical autophagy is induced, a series of 18 Atg proteins and other important elements are hierarchically recruited to the PAS (Mizushima et al. 2011). Following the elongation of the phagophore, the cytoplasmic cargo is wrapped, leading to the formation of a double-membrane autophagosome that matures by fusion with the lysosomes, to form an autolysosome in which the degradation process takes place (Mizushima 2007).

The initiation phase of autophagy requires the ULK complex, which contains ULK1 and ULK2 (UNC51-like Ser/Thr kinases), Atg13, FIP200 (FAK family kinase interacting protein of 200 kDa) and Atg101 (Hosokawa, Hara, et al. 2009; Hosokawa, Sasaki, et al. 2009). The activity of the ULK complex is negatively regulated by mTORC1 (mammalian target of rapamycin complex 1) (Hosokawa, Hara, et al. 2009; Jung et al. 2009), and positively by AMPK (AMP-activated protein kinase) (J. W. Lee et al. 2010; Egan et al. 2011; Kim et al. 2011; Shang et al. 2011), among other regulatory signaling pathways. In the absence of starvation, the ULK complex is bound to mTORC1 and is thus inactived by phosphorylation of ULK1 and Atg13 (Ganley et al. 2009). Upon amino acid starvation, mTORC1 is inactivated and dissociates from the ULK complex, which leads to dephosphorylation and activation of the ULK1 complex. The activated ULK1 complex translocates from the cytosol to PAS structures in the ER (Itakura & Mizushima 2010).

During nucleation (**Fig. 1.8**), the ULK complex interacts with the Beclin1 complex, which is composed of the class III PI3 kinase Vps34 (vacuolar protein sorting 34), Beclin1, p150 and Atg14L (also known as Atg14 and Barkor) (Itakura et al. 2008; Sun et al. 2008; Zhong et al. 2009). ULK1 phosphorylates Vps34, and this enhances the activity of the Beclin1 complex (Russell et al. 2013). The activated ULK and Beclin 1 complexes then phosphorylate unknown proteins containing Ser and Thr residues, respectively, and produce an autophagosome-specific pool of phosphatidylinositol-3-phosphate (PtdIns3P). PtdIns3P is essential for canonical autophagosome formation. It allows the recruitment of additional Atg proteins and autophagy-specific PtdIns3P effectors, such as DFCP1 (double FYVE-containing protein 1) (Axe et al. 2008) and WIPI (WD-repeat domain phosphoinositide-interacting) proteins (Jeffries et al. 2004; Proikas-Cezanne et

al. 2004). The portions of the ER that are PtdIns3P-enriched and positive for DFCP1 are called omegasomes. They provide a platform for expansion of the isolation membrane (Axe et al. 2008). The WIPI proteins 1 and 2 are present on the isolation membrane developing from the omegasome and play a critical role in maturation of the omegasomes into autophagosomes (Polson et al. 2010).

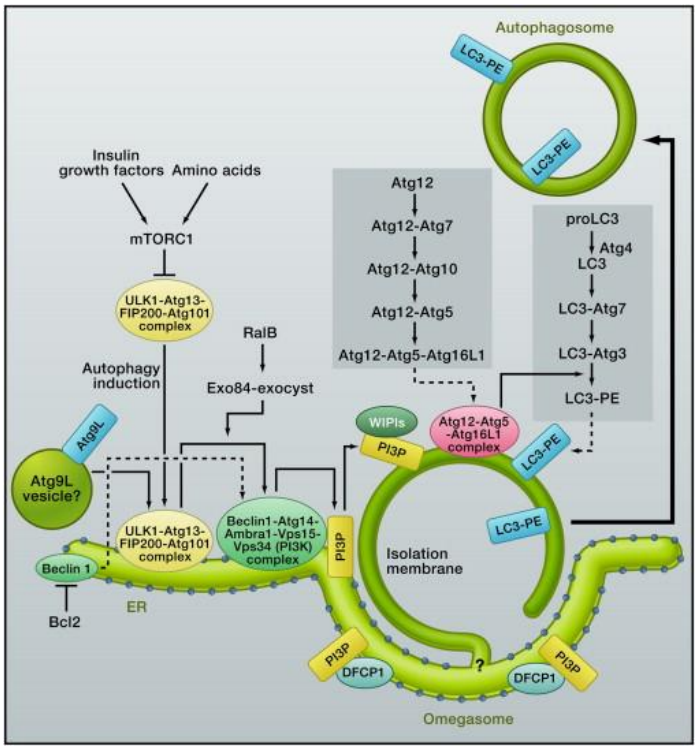


Fig. 1.8: Autophagosome formation and Atg proteins in mammalian cells.
Upon autophagy induction, the ULK complex is activated and translocates to the ER, where it regulates Beclin1 complex formation. The generation of PtdIns3P recruits DFCP1 and promotes the formation of the omegasome. WIPIs are also crucial for the maturation of the omegasome. The Atg12-Atg5-Atg16L1 complex and the LC3-PE conjugate play important roles in the elongation and closure of the isolation membrane. DFCP1, FYVE-containing protein 1; WIPI, WD repeat domain phophoinositide-interacting 1 (from Mizushima, 2011).

The next step in autophagy formation is the elongation and closure (**Fig. 1.8**) of the autophagosome. At this stage, WIPIs may interact with further PtdIns3P effectors to promote the recruitment of two autophagosomal, ubiquitin-like conjugation systems (Mauthe et al. 2011). One of those complexes is the Atg16L1 complex that comprises Atg16L1, Atg5 and Atg12. The Atg16L1 complex predominantly localizes to the outer surface of the isolation membrane and dissociates from the membrane immediately after the completion of the autophagosome (Mizushima et al. 2001). Atg12 is covalently attached to Atg5. This conjugation is catalyzed by Atg7 and Atg10 (Mizushima et al. 1998; Tanida et al. 1999; Mizushima et al. 2002). Atg5 is further bound noncovalently to Atg16L1 to form an Atg12-Atg5-Atg16L multimeric structure through homo-oligomerization of Atg16L (Mizushima et al. 2003). The Atg12-Atg5-Atg16L complex mediates the lipidation of the ubiquitin like protein LC3 (microtubule-

associated protein 1 light chain 3) and its family members GATE16 and GABARAP. These proteins are synthesized as precursors that are processed at the C termini by the cysteine protease Atg4 (Kabeya et al. 2004). The resulting C-terminal glycine-exposed form of LC3 (called LC3-I) is present throughout the cytoplasm. It is activated by Atg7 and transferred to Atg3 to be finally covalently linked to an amino group of phosphatidylethanolamine (PE) to form LC3-II (Ichimura et al. 2000). The lipidation of LC3 is important for normal development of the isolation membrane, likely for its closing step. In this regard, the LC3 and the GABARAP/GATE-16 subfamilies might play different roles in autophagosome formation, being involved in the expansion of the isolation membrane or in its further maturation, respectively (Weidberg et al. 2010).

Another important stage of autophagosome formation is the recycling of membranes through Atg9L1 trafficking. Atg9L1 is the only transmembrane protein among the core Atg proteins. It localizes to omegasomes as well as to the trans-Golgi network endosomes (Young et al. 2006). The Atg9L1 vesicles shuttle back and forth to the omegasome, although the Atg9L1 vesicles do not seem to integrate with the growing isolation membrane (Orsi et al. 2012). The exact function of Atg9L1 is unclear, but it may act to supply the PAS and the elongating isolation membrane with critical factors or lipid components (Mizushima et al. 2011).

The last step in the autophagy pathway is the maturation of the autophagosome and the degradation of its cargo inside the autolysosome. The maturation of autophagosomes includes several fusion events with vesicles originating from early and late endosomes, as well as from lysosomes. The fusion with endosomes forms the so called amphisomes, allowing convergence of the endocytic and autophagic pathways; subsequent fusion of autophagosomes or amphisomes with lysosomes generates autolysosomes (Tooze et al. 1990; Berg et al. 1998). Specific membrane fusion is normally accomplished by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes. The fusion events between autophagosomes and lysosomes requires the autophagosomal Qa-SNARE syntaxin 17 that interacts with SNAP29 (Qbc-SNARE) upon starvation, and the lysosomal VAMP8 (R-SNARE) (Itakura et al. 2012). Rab7 also plays a role in the final maturation of late

autophagic vacuoles, participating in the fusion with late endocytic/lysosomal compartments (Gutierrez et al. 2004; Jäger et al. 2004). Finally, it was proposed that autophagosome-lysosome fusion depends on the activity of H^+ATPases (Kawai et al. 2007). After digestion, the degraded cargo is released in the host cell cytoplasm and reused in anabolic processes. The LC3-II molecules placed on the cytoplasmic face of autolysosomes can be delipidated by Atg4 and recycled while the LC3-II found on the internal surface of autophagosomes is degraded within autolysosomes (Noda et al. 2009).

1.3.2.2 Non-canonical autophagy

In non-canonical macroautophagy (**Fig. 1.7**), the biogenesis of conventional autophagosomes proceeds either in the absence of key Atg proteins that originally defined the canonical process, or originates from membrane sources that differ from the traditional phagophore assembly site (Codogno et al. 2012).

Non-canonical Beclin1-independent autophagy has been reported after treatment with pro-apoptotic compounds (Zhu et al. 2007; Scarlatti et al. 2008; Tian et al. 2010; Grishchuk et al. 2011; Mauthe et al. 2011), during differentiation (Arsov et al. 2011), and following bacterial toxin uptake (Mestre et al. 2010). Another path of non-canonical autophagy bypasses the ULK initiation step in response to glucose deprivation (Cheong et al. 2011). Furthermore, there are forms of non-canonical autophagy that bypass the AMPK-mTORC1-ULK1 initiation complex, as autophagy does not always require AMPK activity and is not always inhibited by mTORC1 (Sarkar et al. 2005; Yamamoto et al. 2006; Grotemeier et al. 2010). It is also possible to observe formation of autophagosomes in Atg5-deficient cells after a prolonged treatment with etoposide. Unlike in conventional autophagy, autophagosomes in this case seem to be generated in a Rab9-dependent manner by fusion of the isolation membranes with vesicles derived from the trans-Golgi and from late endosomes (Nishida et al. 2009).

Finally, it is worth to mention that many pathogens have access into cells by phagocytosis and that autophagy and phagocytosis are connected processes (Shui et al. 2008). In this context, LC3-associated phagocytosis (LAP) is an interesting phenomenon in which LC3 is recruited to single-membrane phagosomes. LAP

promotes a more rapid maturation of the phagosome (Sanjuan et al. 2007). It proceeds independently of the normal ULK1 initiation complex and of PtdIns3P, but it requires the presence of Beclin-1, Atg5-Atg12-Atg16, Atg7 and LC3 (Sanjuan et al. 2007; Martinez et al. 2011). Even though many aspects of LAP still need to be determined, it is known that it is triggered by the engagement of Toll-like receptors. How LC3 is incorporated into the phagosomal membrane is still a matter of debate and several models have been proposed depending on the nature of the particle within the phagosome. However, in order to distinguish between LAP and canonical autophagy, electron microscopy analyses are mandatory to exclude or confirm the formation of double membrane autophagosomes (Lai & Devenish 2012; Mehta et al. 2014).

1.3.2.3 Selective autophagy

Starvation-induced autophagy is a non-selective degradation pathway that breaks down cellular components to provide energy and amino acids for anabolic processes (Mizushima 2007). Alternatively, selective autophagy is a highly selective and tightly regulated process that requires cargo recognition by the autophagy machinery. It can target aggregated proteins (aggrephagy), endosomes (heterophagy), secretory granules (crinophagy), lipids (lipophagy), mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), endoplasmic reticulum (reticulophagy) and pathogens (xenophagy) (Yamamoto & Simonsen 2011; Mousavi et al. 2001; Weckman et al. 2014; Weidberg et al. 2009; Mijaljica et al. 2007; Oku & Sakai 2010; Kraft et al. 2008; Bernales et al.; Knodler & Celli 2011).

Xenophagy, or antibacterial autophagy, involves the selective recognition of intracellular pathogens and their delivery to the autophagic machinery for degradation (**Fig. 1.9** (**a**)) (Knodler & Celli 2011). In this way, autophagy has emerged as an innate immune mechanism against intracellular pathogens, and can target intracellular bacteria either in the cytosol or in vacuoles (Levine & Deretic 2007; Schmid & Münz 2007; Levine & Kroemer 2008; Deretic 2011; Mostowy 2013). The selective targeting of intracellular bacteria is achieved through autophagy receptors which recognize special

signals, also called “eat-me” signals, on the surface of the cytoplasmic microorganism or on the bacteria-containing-vacuole, connecting them to the autophagy pathway.

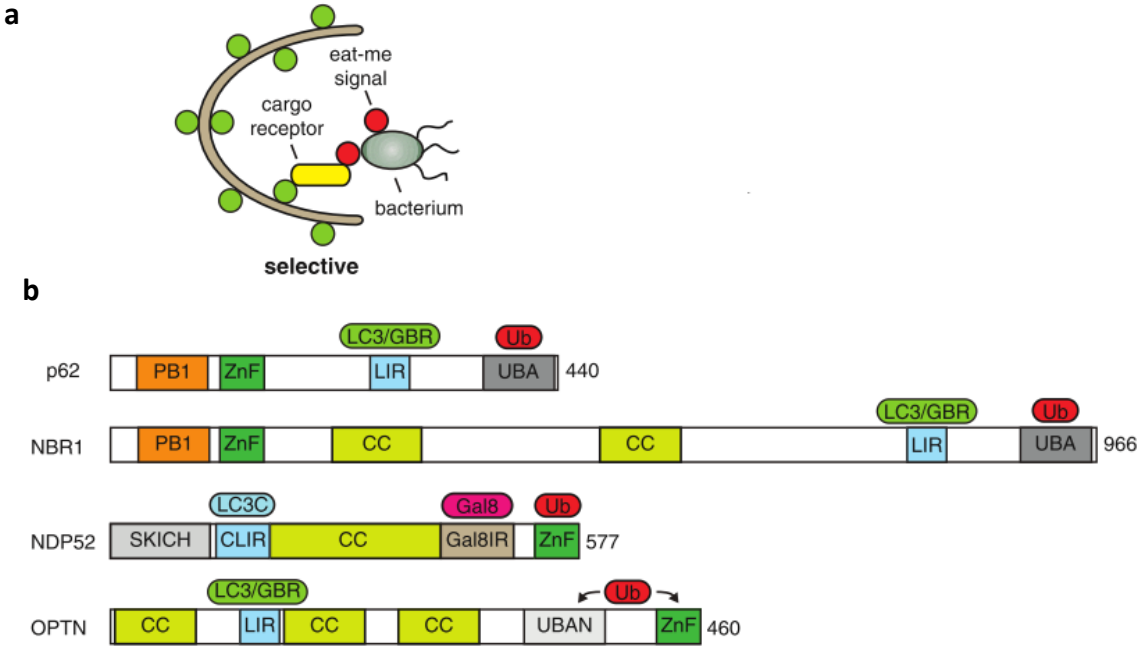


Fig. 1.9: Xenophagy and selective recruitment of the phagophore to its cargo.
(**a**) In xenophagy, the autophagic receptors recognize polyubiquitylated bacteria. Red circles represent “eat-me” signals, like ubiquitin; green circles, LC3 and GABARAP proteins; yellow ellipse, autophagy/cargo receptor (**b**) Domain structures of autophagy receptors. Highlighted are binding sites for “eat-me” signals and LC3 family members. All autophagy receptors bind ubiquitin-labelled cargo; only NDP52 detects the “eat-me” signal Galectin-8 (Gal8). p62, NBR1 and Optineurin (OPTN) bind non-selectively LC3 and GABARAP proteins via their LC3-interacting regions (LIRs), while NDP52 preferentially interacts with LC3C via a LC3C-specific binding site (CLIR). CC, coiled-coil; CLIR, LC3C-specific LIR; Gal8, Galectin-8; Gal8IR, Galectin-8 interacting region; LIR, LC3-interacting region; OPTN, Optineurin; PB1, Phox and Bem1P; SKICH, skeletal muscle and kidney enriched inositol phosphatase carboxyl homology; Ub, ubiquitin; UBA, ubiquitin-associated domain; UBAN, ubiquitin binding in ABIN and NEMO domain; ZnF, ubiquitin zinc-finger domain (modified from Boyle & Randow, 2013).

The “eat-me” signals mark autophagic cargos. One of these signals is ubiquitin (Ub), a small protein characterized as a label that also may target proteins for degradation by the proteasome (Shahnazari & Brumell 2011). The conjugation of ubiquitin to its cargo (in the case of xenophagy, a bacterial substrate) requires the activity of three enzymes: Ub-activating E1, Ub-conjugating E2 and an Ub-ligase E3. Ubiquitin can be present as a monomer or as a polymer chain, but poly-ubiquitin is the form associated with proteasomal degradation and selective autophagy. Galectin-8 is another “eat-me” signal, which specifically binds the host cell sugar β-galactoside, which normally

localizes to the plasma membrane surface. Moreover, it may be exposed from damaged bacteria-containing vacuoles to the cytosol when the pathogen induces membrane damage. Ubiquitin and Galectin-8 are recognized by specific domains of the autophagy receptors (**Fig. 1.9** (**b**)). The autophagy receptors may contain an ubiquitin associated domain (UBA) or an ubiquitin binding zinc-finger domain (ZnF) which bind ubiquitin, or a Galectin-8 interacting region (Gal8IR) that binds to Galectin-8. Additionally, the autophagy receptors have an LC3-interacting region (LIR) which allows the interaction of the autophagy receptors with proteins of the LC3/GABARAP family in order to recruit autophagosomal membranes to the element targeted for elimination (Johansen & Lamark 2011; Boyle & Randow 2013). Up to now, four autophagy receptors have been identified: NBR1 (neighbour of Brca1 gene); NDP52 (nuclear dot protein 52), unique in the way that it has a Gal8IR additionally to a ubiquitin-binding ZnF domain; p62 (Nucleoporin p62, also known as SQSTM1, sequestosome 1), and Optineurin (Pankiv et al. 2007; Kirkin et al. 2009; Wild et al. 2011).

Many bacteria are targeted for degradation by xenophagy (**Fig. 1.10** (**a**)), in example *S*. Typhimurium (Birmingham et al. 2006), *Mycobacterium tuberculosis* (Gutierrez et al. 2004), *Mycobacterium marinum* (Collins et al. 2009), and Group A *Streptococcus* (GAS) (Nakagawa et al. 2004). Among them, *S*. Typhimurium is the most well studied model. As it was mentioned in the Section 1.2, *S*. Typhimurium mainly resides and replicates inside a *Salmonella*-containing vacuole (SCV). However, a subset of bacteria has pore-forming activity that damages the SCV. SCV membrane damage exposes specific molecules to the cytosol, either on the bacterial surface (Perrin et al. 2004), or on the inner face of the SCV membrane, that in turn recruit the autophagy receptors NDP52 (Thurston et al. 2012), p62 and Optineurin (Thurston et al. 2009; Zheng et al. 2009; Wild et al. 2011) which then deliver the pathogen to the autophagy pathway for degradation. Interestingly, membrane damage by *Salmonella* can also generate a stress response that is characterized by local and transient amino acid starvation. This inhibits the mTORC1 activity, thereby favouring the metabolic switch needed for the induction of autophagy (Tattoli, et al. 2012).

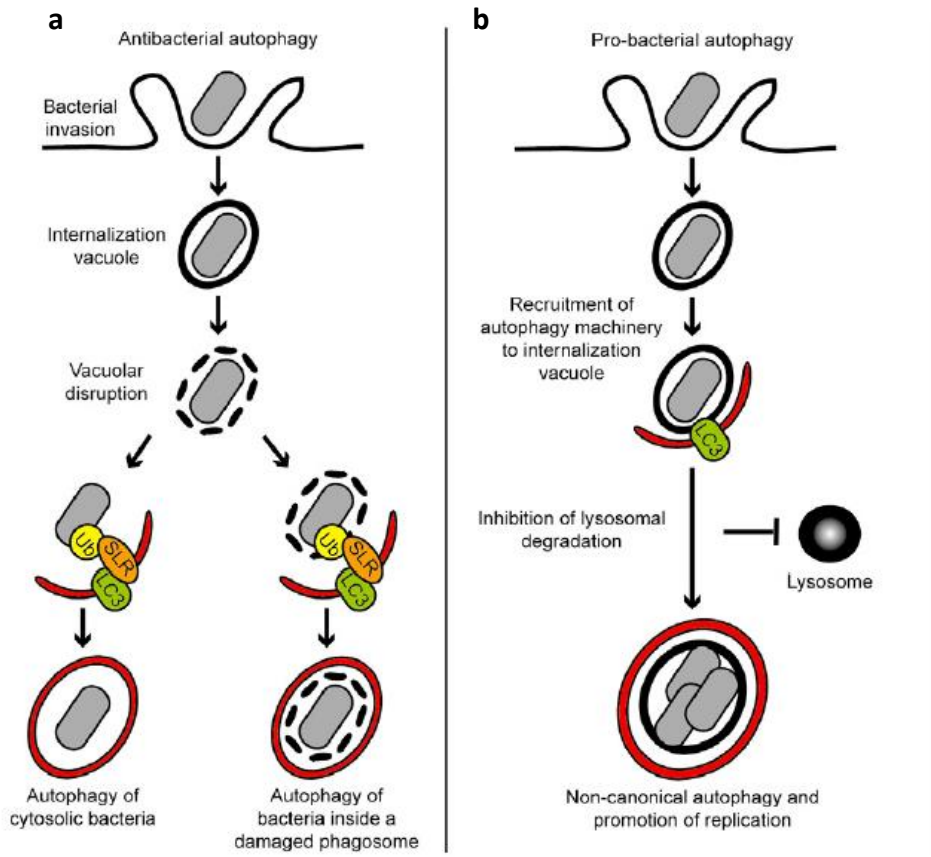


Fig. 1.10: Autophagy pathways triggered by bacterial invasion.
(**a**) Antibacterial autophagy. After entry into cells, bacteria are localized inside an endosome/phagosome. Upon vacuolar disruption, autophagy receptors may recognize ubiquitin or other signals on bacteria located in the cytosol (left) or inside a damaged vacuole (right). Then, they are enclosed within an autophagosome and delivered to the lysosome for degradation. (**b**) Pro-bacterial autophagy. Some internalized bacteria may recruit a subset of the autophagy machinery and create a replicative niche inside an autophagosome-like structure. These bacteria subvert the autophagy machinery to avoid degradation in a lysosomal compartment which supports bacterial replication. Ub, ubiquitin; SLR, autophagy adaptor (e.g. p62, NDP52); LC3, (proteins of the LC3/GABARAP family) (modified from Mostowy, 2013).

Although xenophagy is a host immune response to restrict bacterial replication some bacteria may avoid or subvert this pathway to support their intracellular replication (**Fig. 1.10** (**b**)) (Mostowy 2013). *Listeria monocytogenes* (Birmingham et al. 2007) and *Shigella flexneri* (Ogawa et al. 2005), two pathogens that use actin-based motility to move within the host cell cytoplasm, are pathogens that could be recognized by autophagy after rupture of their phagosomes and invasion of the host cell cytosol. However, they have evolved mechanisms to directly interfere with the activity of autophagy components, avoiding in this way ubiquitylation and activation of the autophagic defense system (Huang & Brumell 2014). *Listeria* possesses ActA, an

essential protein that promotes the actin-based motility by recruitment of host proteins Arp2/3 complex and Ena/VASP. The decoration with those host proteins inhibits the mobilization of ubiquitin and p62 to the surface of the bacteria, thereby preventing autophagy recognition (Yoshikawa et al. 2009). Like *Listeria*, *Shigella* expresses the surface protein IcsA to polymerize host actin at the bacterial surface (Asrat et al. 2014). IcsA can be recognized by Atg5, which targets the bacterium to the autophagy pathway. In order to avoid this, *Shigella* secretes IcsB by its TTSS that competitively binds to IcsA and prevents its recognition by Atg5 (Ogawa et al. 2005). Another interesting example of evasion of autophagic degradation is given by *Legionella pneumophila. Legionella* is a pathogen that avoids autophagy through inducing a global block of this pathway. *Legionella* creates an ER-derived vacuolar niche, called *Legionella*-containing vacuole (LCV), which is protected from autophagy by the action of the effector protein RavZ secreted by its type four secretion system. RavZ acts as a cysteine protease that irreversibly deconjugates PE from LC3 (Choy et al. 2012). Interestingly, *Legionella* lacking RavZ can still survive and replicate within the LCVs, indicating that even more mechanisms of autophagy evasion function during infection (Baxt et al. 2013).

While *Listeria*, *Shigella* and *Legionella* are examples of bacteria that evade or inhibit autophagy, it is also known that other bacteria may hijack autophagy components to promote their replication (**Fig. 1.10** (**b**)). *Yersinia pseudotuberculosis* may take advantage of autophagy for replication within a non-acidic *Yersinia*-containing-vacuole (YCV) in macrophages, and inhibition of *Yersinia*-induced autophagy leads to trafficking of *Yersinia* inside lysosomes (Moreau et al. 2010). Recently, the interaction between *Y. pseudotuberculosis* and autophagy in HeLa cells was investigated. It was observed that bacteria can take different autophagosomal pathways, triggering either single- or double- membranous compartments positive for LC3. However, it seems that *Y. pseudotuberculosis* replicates inside single-membrane YCVs that display not only autophagosomal markers but also VAMP3 (vesicle associated membrane protein 3). VAMP3 is a R-SNARE that regulates amphisome formation by controlling the fusion between late endosomes and autophagosomes, and it is responsible for the localization of *Y. pseudotuberculosis* within single-membrane YCVs in HeLa cells (Ligeon et al. 2014). In contrast to *Y. pseudotuberculosis*, *Coxiella burnetti* needs the

establishment of an acidic autophagolysosome-like vacuole, called *Coxiella*-containing vacuole (CCV) to replicate within host cells. By a mechanism not very well understood, it accumulates autophagic markers such as LC3 and manipulates the Rab GTPases Rab5 and Rab7 to traffic within the endocytic pathway in order to reach a low pH and become metabolically active (Hardiman et al. 2012). The *Coxiella* type four secretion system is necessary for LC3 targeting to the CCV, but specific bacterial effectors that play a role are unknown (Asrat et al. 2014). Additionally, autophagy can also be necessary for the intercellular spread of the bacteria from infected cells to neighbouring cells as shown for *Brucella abortus* (Starr et al. 2012). *Brucella* is an intracellular bacterium that, after being internalized by the host cell, resides inside a *Brucella*-containing vacuole (BCV). Then it manipulates intracellular membrane trafficking processes: it undergoes a limited fusion with lysosomes and traffics towards the ER. *Brucella* replicates inside the ER-derived BCVs and subsequently converts its vacuole into a compartment with autophagic features called aBCV. The aBCV has a double-membrane structure and its formation depends on Beclin1 and ULK1, but it is independent of Atg5, Atg7 or LC3. The generation of the aBCV is important for bacterial release, by a mechanism not known yet but that could involve exocytic processes or cell death and concomitant bacterial egress (Starr et al. 2012).

1.4 Project objective

The invasive phenotype of enteropathogenic *Y. enterocolitica* relies on the presence of the bacterial surface protein invasin. Intestinal epithelial cells are supposed to be the primary target cells of the cell-binding activity of invasin to trigger uptake and transmigration of the bacteria through the intestinal epithelium by β1-integrin activation. Autophagy has recently emerged as an important host innate immune defense mechanism against invading microbes that eliminates intracellular bacteria. As such, autophagy of epithelial cells may protect against invasion and dissemination of intestinal bacterial pathogens. On the other hand, autophagy might also provide nutrients, membranes and a safe intracellular niche that could support bacterial replication.

In this study, we wondered whether autophagy-related events are triggered in epithelial cells infected with *Y. enterocolitica*, and which consequences autophagy may have for the *Yersinia* infection process.

2 RESULTS

2.1 Autophagic events are triggered after uptake of *Yersinia enterocolitica* by epithelial cells

The epithelial cells of the small intestine are the first cells that interact with *Y. enterocolitica* after being ingested by the host. This interaction is mediated by the protein invasin on the surface of the bacteria, which engages β1-integrin receptors on the epithelial cells, triggering the uptake of the bacteria into the cells and the eventual translocation to the basal side on the epithelial monolayer. It is also known that the invasin-β1-integrin receptor interaction induces autophagy in macrophages infected with a strain of *Y. enterocolitica* that is devoid of the virulence plasmid pYV (strain WAC, the virulence plasmid-cured derivative of the wild-type *Y. enterocolitica* strain WA-314, serotype O:8). In contrast, infection of macrophages with wild-type *Y. enterocolitica* suppresses autophagy by the action of the TTSS encoded by pYV (Deuretzbacher et al. 2009).

We first attempted to address if *Y. enterocolitica* WAC also induces autophagic responses in epithelial cells after being internalized. For this purpose, we used HeLa and mIC_{cl2} cells (mouse intestinal cells) as models of epithelial cell infection, and monitored the appearance of autophagosomes in non-infected and infected cells by three popular techniques: biochemical detection of the membrane-associated form of the autophagosomal marker LC3, light microscopy for detection of the subcellular localization of GFP-LC3, and electron microscopy to study the ultrastructure of the bacteria-containing vacuoles (Mizushima et al. 2010).

2.1.1 Conversion of LC3-I to LC3-II

The conversion of LC3-I to LC3-II is a hallmark of autophagy and indicates autophagosome formation. As lipidated LC3-II has a faster electrophoretic mobility

than unprocessed LC3-I, the conversion can be detected by SDS-PAGE and immunoblotting with antibodies against LC3 (Mizushima & Yoshimori 2007). These experiments showed that the avirulent WAC strain (cultivated at 27 °C) triggered the generation of LC3-II in HeLa cells in a time-dependent manner (**Fig. 2.1**). Treatment with chloroquine served as positive control mediating LC3-II accumulation through the blockage of autophagosome maturation into autolysosomes.

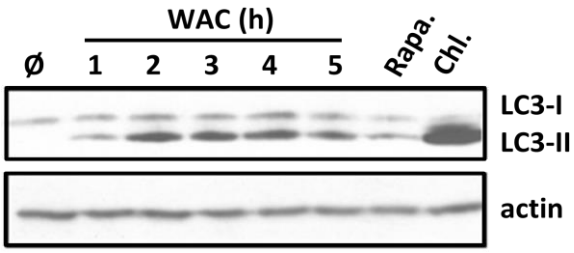


Fig. 2.1: LC3-II conversion in HeLa cells infected with *Y. enterocolitica*.
The *Y. enterocolitica* virulence plasmid-cured strain WAC triggers LC3-II conversion in a time dependent manner in HeLa cells. Chloroquine (Chl., 75 µM) and Rapamycin (Rapa, 20 mM) served as a positive controls for LC3-II accumulation. Equal loading of the gel was controlled by immunoblotting against actin.

In contrast to WAC-infected cells, almost no conversion of LC3-I to LC3-II was observed in cells infected with the wild-type strain WA-314 (cultivated at 37 °C) (**Fig. 2.2**). It is worth to remark that WA-314 harbours the virulence plasmid pYV that, at 37 °C, expresses Yops, which provides phagocytosis resistance to the bacteria. This result indicates that type three protein secretion may prevent internalization and thus induction of autophagy otherwise conferred by avirulent WAC, as it was observed previously for macrophages (Deuretzbacher et al. 2009).

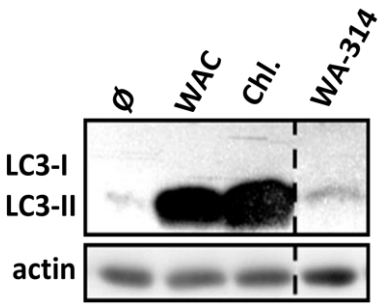


Fig. 2.2: Wild-type *Y. enterocolitica* prevents the induction of autophagy in infected HeLa cells.
The *Yersinia* wild type strain WA-314 prevents the conversion of LC3-I to LC3-II in HeLa cells. Chloroquine (Chl., 75 µM) served as a positive control. Cell lysates were prepared 4 h 30 min after onset of infection. Equal loading of the gel was controlled by immunoblotting against actin.

The infection with WAC cultivated at 27 °C may match the physiological initial stage of infection in the gut, as invasin is maximally expressed at that temperature and the

TTSS is inactive. For this reason, the infection with WAC cultivated at 27 °C was extended to transimmortalized mouse intestinal cells (mIC_{cl2} cells) (Bens et al. 1996) derived from the bases of small intestinal villi of a mouse fetus and provided by Dr. Alain Vandewalle (INSERM U773, Centre de Recherche Biomédicale Bichat Beaujon, Université Paris 7). A model with epithelial cells of intestinal origin is interesting because it is reported that autophagy in intestinal epithelial cells protects against dissemination of intestinal bacteria (Benjamin et al. 2013). We observed that avirulent WAC triggered the transition of LC3-I to LC3-II also in mIC_{cl2} cells (**Fig. 2.3**) which indicates that the mechanism of autophagy induction by *Yersinia* is operative in epithelial cells of intestinal origin too. Treatment with chloroquine, rapamycin and starvation served as positive controls mediating LC3-II accumulation.

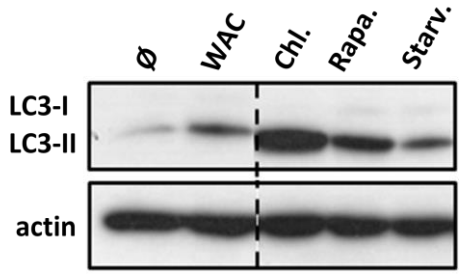


Fig. 2.3: LC3-II conversion in mIC_{cl2} cells infected with *Y. enterocolitica*.
WAC triggers LC3-II conversion in mIC_{cl2} cells. Chloroquine (Chl., 75 µM), rapamycin (Rapa., 20 mM) and starvation (Starv.), served as positive controls. Cell lysates were prepared 4 h 30 min after onset of infection. Equal loading of the gel was controlled by immunoblotting against actin.

2.1.2 Analysis of colocalization of GFP-LC3 with *Yersinia*-containing vacuoles in epithelial cells

Another important method to monitor the induction of autophagy is to study the sub-cellular localization of LC3. The conversion of LC3-I to LC3-II correlates with the recruitment of LC3 to autophagosomal membranes. GFP-tagged LC3 is therefore commonly used to label and detect autophagosomes. HeLa and mIC_{cl2} cells were transfected with a plasmid encoding GFP-LC3 and the mobilization of GFP-LC3 into autophagosomal structures and vacuoles upon *Yersinia* infection was analyzed by fluorescence microscopy. After 2 h 30 min of infection with WAC (labelled by RFP expression) some *Yersinia* bacteria started being decorated with GFP-LC3 in HeLa cells (**Fig. 2.4**). This indicates that GFP-LC3 is directed to *Yersinia*-containing vacuoles (YCVs) after autophagy induction. The population of intracellular *Yersinia* that colocalized

with GFP-LC3 increased up to 30 % at 4 h 30 min post infection, in concordance with the strong accumulation of endogenous LC3-II observed at that time point by immunoblotting (Section 2.1.1).

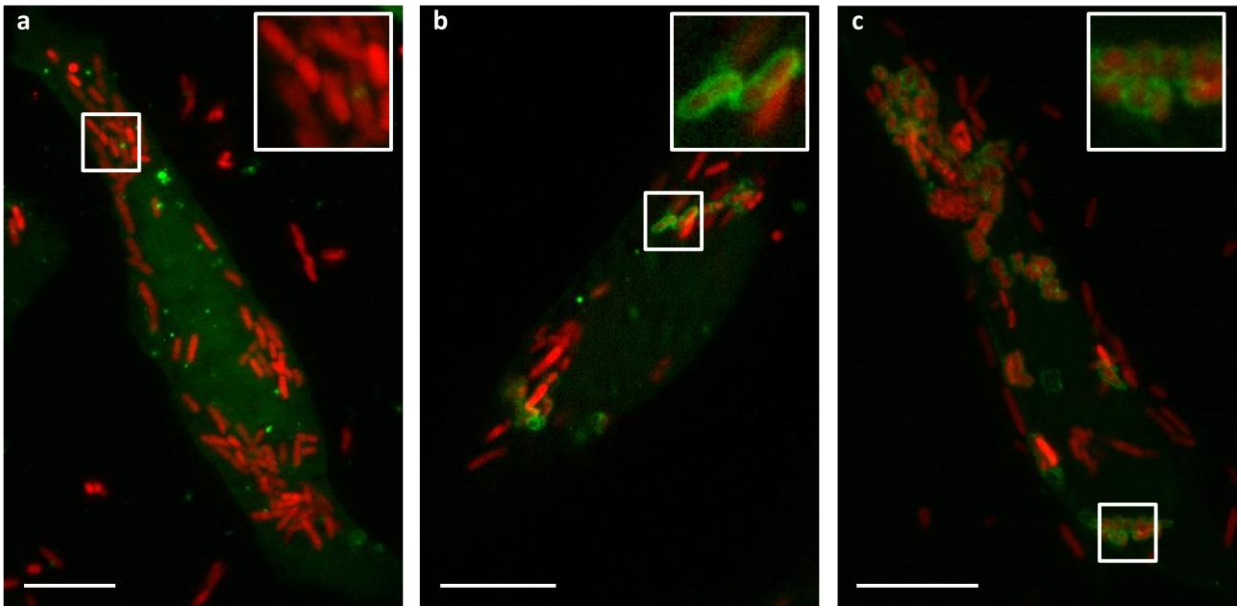


Fig. 2.4: GFP-LC3 is recruited to *Y. enterocolitica* following internalization by HeLa cells.
HeLa cells were transfected to express GFP-LC3 and infected with WAC labelled by RFP expression. The cellular localization of GFP-LC3 was microscopically monitored at different time points after infection. (**a**) 90 min post infection, (**b**) 2 h 30 min post infection, (**c**) 4 h 30 min post infection. Bars, 10 µm.

Y. enterocolitica triggered recruitment of GFP-LC3 also in infected mIC_{cl2} cells, in correlation with the conversion of LC3-I to LC3-II analyzed by western blotting (**Fig. 2.5**). The colocalization with GFP-LC3 was related to almost 30 % of the total intracellular bacteria at 4 h 30 min post infection, comparable with the results obtained in HeLa cells. These observations made by microscopical analysis of the subcellular localization of GFP-LC3 support the idea that invasion of epithelial cells by *Y. enterocolitica* triggers autophagic events towards the bacteria.

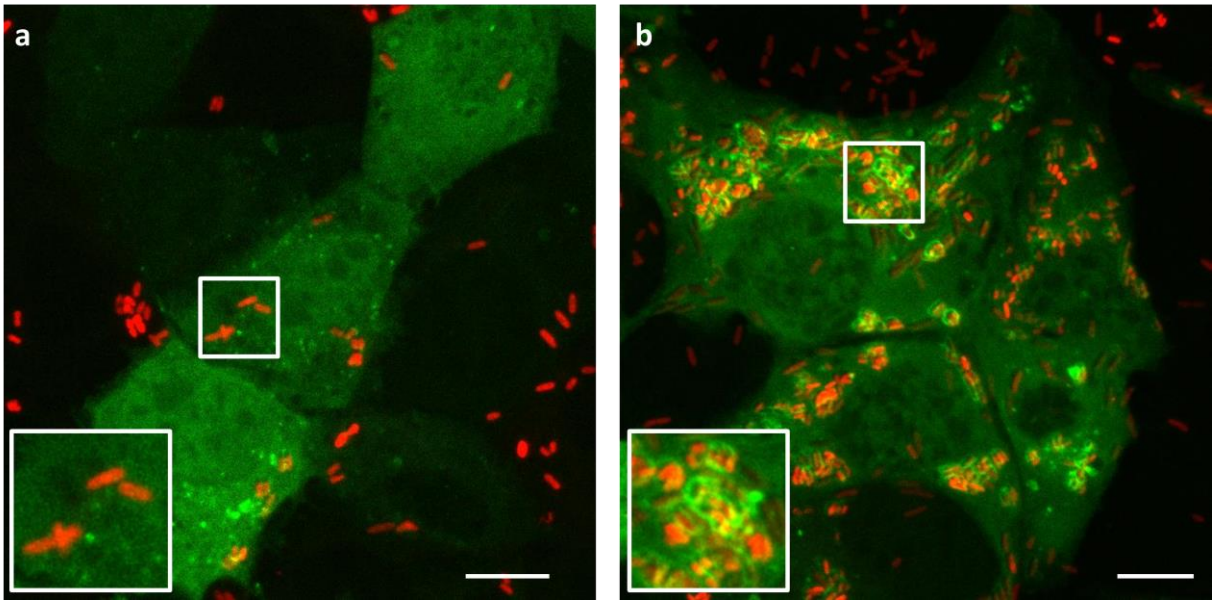


Fig. 2.5: GFP-LC3 is recruited to *Y. enterocolitica* following internalization by mIC$_{cl2}$ cells.
mIC$_{cl2}$ cells were transfected to express GFP-LC3 and infected with WAC labelled by RFP expression. The cellular localization of GFP-LC3 was microscopically monitored at different time points after infection. (**a**) 90 min post infection, (**b**) 4 h 30 min post infection. Bars, 10 µm.

2.1.3 Ultrastructural analysis of the morphology of *Yersinia*-containing vacuoles in epithelial cells

The mobilization of LC3 to vacuoles may be a general process during uptake of particles by phagocytosis. This phenomenon is called LC3-associated phagocytosis (LAP) and is not necessarily related to autophagy (Sanjuan et al. 2007). For this reason, it was important to determine whether LC3-II induction and GFP-LC3 recruitment by *Yersinia* may result from canonical autophagosome formation or may simply follow phagocytosis in a LAP process. This was investigated by transmission electron microscopy (TEM) in collaboration with Dr. Reimer from the Heinrich-Pette-Institute for Experimental Virology in Hamburg. TEM is one of the most sensitive methods to detect autophagic compartments, which are characterized by double or sometimes multiple limiting membranes (Eskelinen et al. 2011).

In these experiments, HeLa cells were infected with WAC for 4 h 30 min before fixation for TEM. The ultrastructure of the YCVs was analyzed in order to discriminate bacteria within single- or double-membrane vacuoles (**Fig. 2.6**). The results of the TEM analysis

showed the presence of bacteria within multiple or double-membrane vacuoles inside the cells, indicative of canonical autophagy. The existence of such multiple membrane-bound vesicles is consistent with wrapping of single-membrane endosomes/phagosomes enclosing bacteria by autophagic sequestration membranes in terms of xenophagy (Klionsky et al. 2012). As the presence of double and multilamellar structures by TEM suggested that WAC was delivered to the autophagy pathway, the next step was to confirm the identity of such vacuoles by performing correlative light and electron microscopy (CLEM).

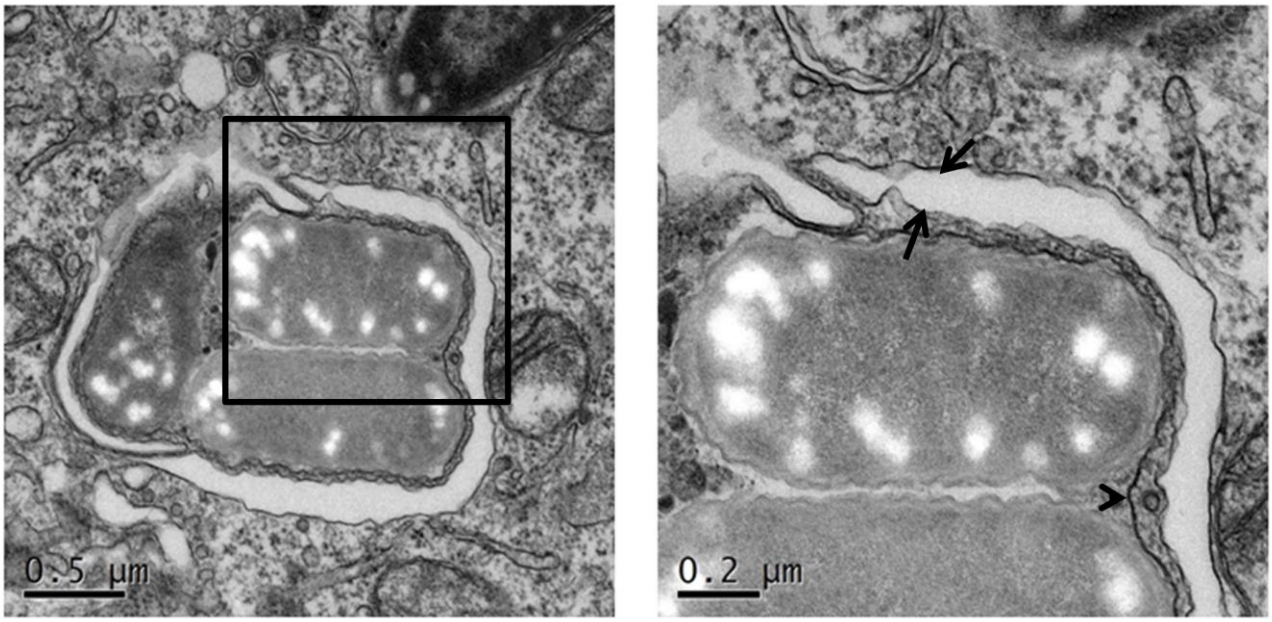


Fig. 2.6: Ultrastructure of the *Yersinia*-containing vacuole in HeLa cells.
HeLa cells were infected for 4 h 30 min with WAC and then processed for TEM. Lower and higher magnification image of a YCV. Arrows indicate double membrane-bound compartments surrounding bacteria. The arrowhead indicates a third layer of membrane which may correspond to the endosomal membrane that initially wrapped the bacteria after internalization.

By CLEM, the membranous structures labelled by GFP-LC3 are first characterized at the fluorescence light level with a confocal microscope, followed by a TEM analysis (Ligeon et al. 2015). Localization of GFP-LC3 at the fluorescent light level allows the broad definition where the protein is found and where it colocalizes with bacteria. More detailed information is gained by higher-resolution analysis of the structures seen in the fluorescent light microscope by electron microscopy.

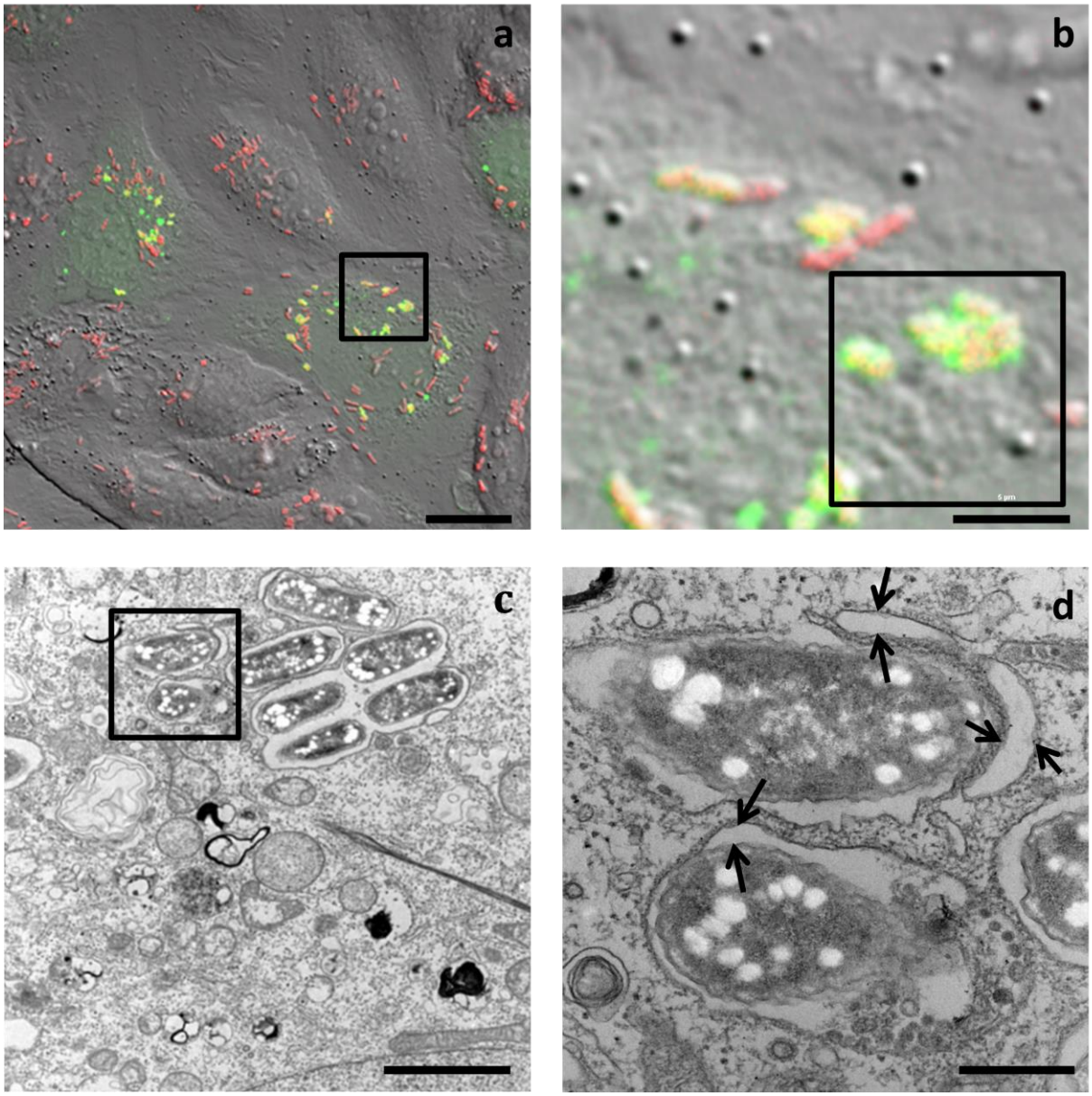


Fig. 2.7: Ultrastructure of LC3-positive vacuoles containing *Yersinia*.
HeLa cells were transfected to express GFP-LC3, infected for 4 h 30 min with WAC (labelled by RFP expression) and then processed for CLEM. (**a**) HeLa cells observed by confocal laser microscopy; bar, 25 µm. (**b**) Magnification of the insert from (**a**); bar, 5 µm. (**c**) Section of a single HeLa cell observed by TEM; bar, 2 µm, magnification of the insert from (**b**). (**d**) Magnification of the insert from (**c**); bar, 400 nm. Arrows denote outer and inner membranous autophagic compartments containing bacteria which correspond to the LC3-positive bacteria detected by fluorescence microscopy. Bar, 200 nm.

To this end, HeLa cells were transfected with a GFP-LC3 construct and infected with WAC for 4 h 30 min before fixation and CLEM analysis. We observed that GFP-LC3-positive bacteria detected by fluorescence microscopy (**Fig. 2.7** (**a**) and (**b**)) corresponded to bacteria that started to be surrounded by multiple or double membranes in electron microscopy (**Fig. 2.7** (**c**) and (**d**)). This supported the idea that

multiple and double-membranous YCVs represent canonical autophagosomes. However, some GFP-LC3-positive vacuoles did not display clear autophagosomal structures at that time point, which suggests that also LAP may initially occur. We further analyzed the ultrastructure of the YCV in mIC_{cl2} cells after 4 h 30 min post infection (**Fig. 2.8**) and also observed bacteria within multiple or double-membraned vacuoles. These results support the idea that *Y. enterocolitica* activates canonical autophagy in both epithelial cells lines.

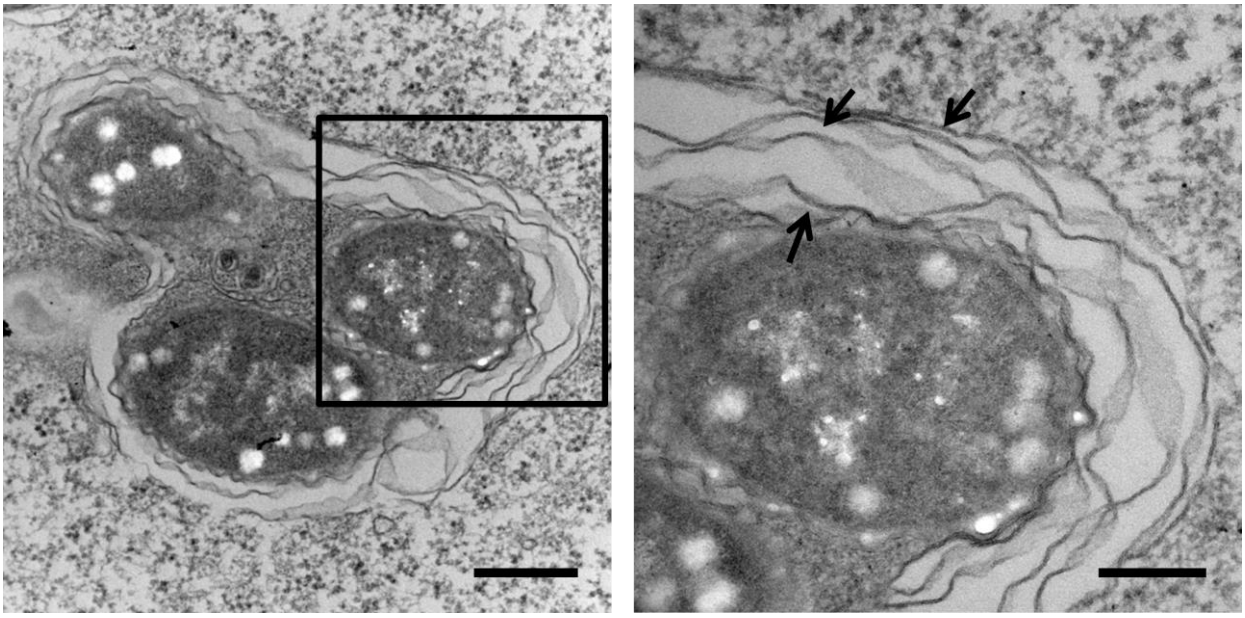


Fig. 2.8: Ultrastructure of the *Yersinia*-containing vacuole in mIC_{cl2} cells.
mIC_{cl2} cells were infected for 4 h 30 min with WAC and then processed for EM. Lower (bar, 400 nm) and higher (bar, 200 nm) magnification image of a YCV. Arrows indicate multiple membrane-bound compartments surrounding a bacterium.

As the results obtained with HeLa and mIC_{cl2} cells are comparable, and due to the fact that mIC_{cl2} require longer replication time, highly complex fresh media and expensive transfection reagents, we continued working on HeLa as an adequate epithelial cell model to gain more insights into the targeting of autophagy by *Y. enterocolitica*.

2.2 Epithelial autophagy is induced by different serotypes of *Y. enterocolitica*

We demonstrated that the internalization of *Y. enterocolitica* triggered autophagic events in epithelial cells. We next attempted to analyze if this response was a general feature observed for different serotypes of *Y. enterocolitica* or, on the contrary a specific event induced by *Y. enterocolitica* serotype O:8 (biogroup 1B). With this purpose we investigated two other strains of *Y. enterocolitica* belonging to the serogroups O:5 (strain NF-O, from nosocomial origin) and O:36 (strain IP2222, from environmental origin). Both strains are part of the 1A biogroup and are generally considered apathogenic because they are devoid of some classical virulence-associated determinants of pathogenic biogroups such as the virulence plasmid pYV. However, they still possess the gene encoding the invasin protein, which hypothetically allows them to invade cells by β1-integrin engagement and could explain, in part, their role as an opportunistic pathogen (Batzilla et al. 2011).

The autophagic responses to NF-O and IP2222 in HeLa cells were studied by the analysis of the conversion of endogenous LC3-I to LC3-II and the sub-cellular localization of GFP-LC3. It was observed that NF-O did not trigger a significant conversion of LC3 in comparison with non-infected cells (**Fig. 2.9** (**a**)). In concordance, the microscopical analysis of HeLa cells expressing GFP-LC3 and infected with NF-O revealed no GFP-LC3-positive YCVs. This could be related to the finding that barely any intracellular bacteria were detected within the infected cells (**Fig. 2.9** (**b**)). Comparable results were obtained for the IP2222 strain (data not shown). The fact that, in spite of carrying an *inv* gene (Batzilla et al. 2011), almost no bacteria were found inside the cells was surprising as it was supposed that the presence of invasin confers an invasive phenotype to *Yersinia*. To provide the strains with comparable invasive properties, we transformed the NF-O and IP2222 strains with the plasmid pInv1914. The plasmid pInv1914 encodes for the invasin protein of an invasive *Y. enterocolitica* O:9 strain. The transformation of NF-O and IP2222 strains with pInv1914, to create NF-O-*inv*$^+$ and IP2222-*inv*$^+$, is expected to provide those strains with a highly invasive phenotype. In order to test our hypothesis, HeLa cells expressing GFP-LC3 were infected with NF-O-*inv*$^+$ (**Fig. 2.9** (**c**)) and analyzed by fluorescence microscopy. It was observed that, in

contrast to wild-type NF-O, NF-O-*inv*$^{+}$ strongly invaded HeLa cells. Furthermore, intracellular bacteria were capable of recruiting GFP-LC3 to the YCVs. Additionally, westernblotting analyses for LC3 conversion showed that NF-O-*inv*$^{+}$, in contrast to NF-O, induced a clear conversion of endogenous LC3-I to LC3-II in infected HeLa cells (**Fig. 2.9** (**a**)). Similar results were observed for IP2222-*inv*$^{+}$ in HeLa cells (data not shown).

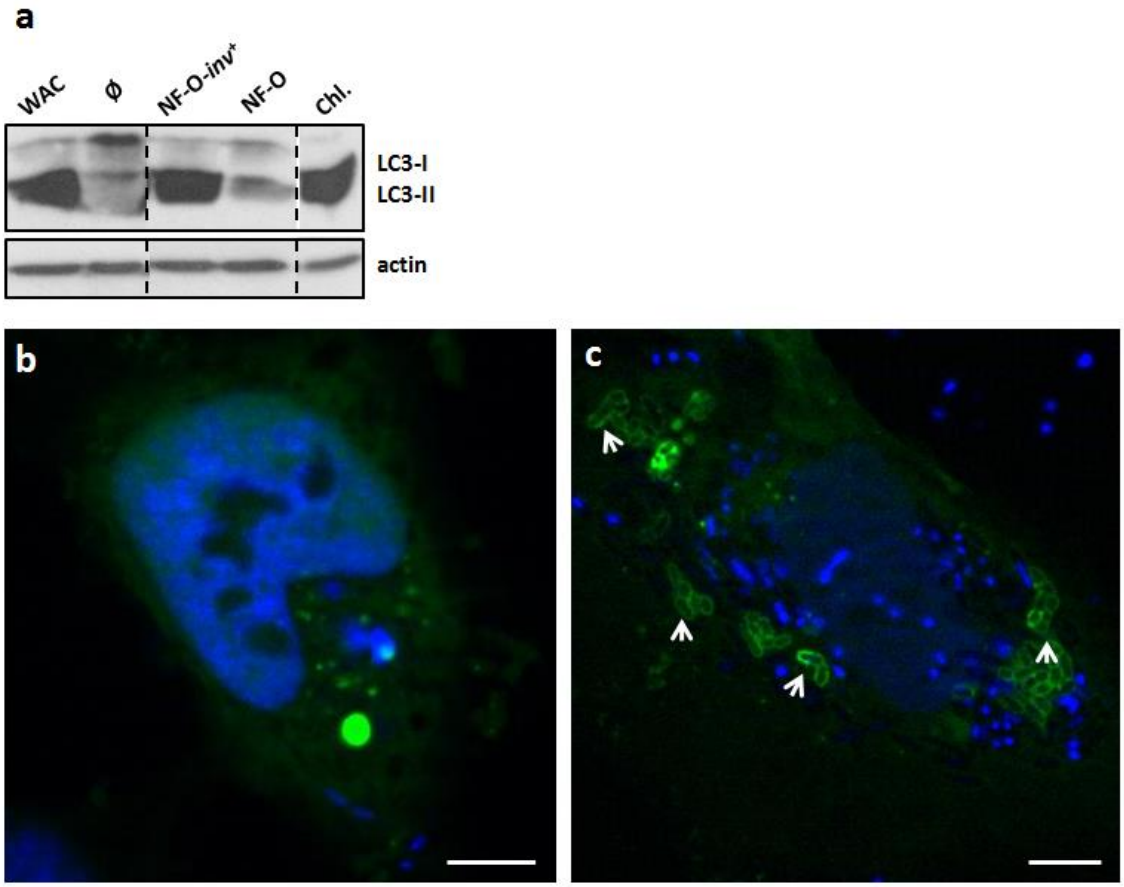


Fig. 2.9: Epithelial autophagy is triggered by serogroup O:5 of *Y. enterocolitica* after internalization into HeLa cells.
(**a**) HeLa cells were infected with WAC, *Y. enterocolitica* serogroup O:5 strain NF-O, or NF-O expressing the *inv* gene of an invasive strain of *Y. enterocolitica* (NF-O-*inv*$^{+}$). The activation of autophagy was analyzed by monitoring the conversion of endogenous LC3 by immunoblotting. Chloroquine (Chl., 75 µM) served as positive control. Cell lysates were prepared 4 h 30 min after onset of infection. Equal loading of the gel was controlled by immunoblotting against actin. (**b**) and (**c**), HeLa cells were transfected to express GFP-LC3, infected with NF-O (**b**) or NF-O-*inv*$^{+}$(**c**), and stained with DAPI to visualize bacteria. Arrows denote bacteria that are surrounded by GFP-LC3. Bar, 5 µm.

These results suggest that, although the *inv* gene is present in the bacteria, the invasin protein of *Y. enterocolitica* of the biogroup 1A was not efficient to facilitate the uptake by the cells. Furthermore, it became clear that invasion into cells was a necessary step to induce autophagic events and that *Yersinia* invasin from invasive strains essentially contributes to both processes. Finally, the fact that *Y. enterocolitica* belonging to the

O:5 and O:36 serotypes induce autophagic responses after internalization into epithelial cells shows that this response is not restricted to *Y. enterocolitica* serotype O:8 (WAC strain used in this study), but it might be a more general characteristic of this species.

2.3 Y*ersinia*-induced autophagy does not involve mTOR inhibition

Autophagy is a very tightly regulated process. One of its key regulators is the mTORC1 complex. At normal nutrient supply, the activated mTORC1 complex, which is formed by the kinase mTOR, phosphorylates and thus inactivates the initiation complex of autophagy (ULK1 complex). In contrast, mTORC1 is inactivated under starvation, allowing activation of the autophagy pathway. This is possible because mTORC1 is an important sensor of the metabolic status of the cells. It integrates multiple pathways triggered by glucose, growth factors, oxygen tension and ATP levels. When mTOR is activated it not only represses autophagy but also controls key cellular functions, such as mRNA translation, cell growth and ribosomal biogenesis, in part through the phosphorylation of p70S6K and 4EBP1 (Tattoli et al. 2012; Abdel-Nour et al. 2014). Due to its role in the activation of autophagy, the impact of bacterial infection on mTOR-dependent signaling has already been studied for *Shigella*, *Salmonella* (Tattoli et al. 2012), *Listeria* (Tattoli et al. 2013) and *Legionella* (Ivanov & Roy 2013). It has been shown that pathogens capable of causing membrane damage to the bacteria-containing vacuole, either through the action of the TTSS or the release of pore-forming toxins, induce a transient and local amino acid starvation state (Tattoli et al. 2012; Tattoli et al. 2013). Such a condition causes rapid inhibition of mTOR signaling, which is associated with the induction of autophagy (Tattoli et al. 2012; Tattoli et al. 2013), thus showing that xenophagy can occur independently from the global nutrient status of the infected cells. A comparable mechanism might be involved in autophagy activation by *Yersinia*. For that reason, we aimed to characterize the interplay between mTOR signaling and autophagy induction in *Y. enterocolitica*-infected HeLa cells. The activation of mTOR signaling is often evaluated by measuring changes in the phosphorylation of the Thr389 residue in the hydrophobic motif of the ribosomal S6

kinase 1 (p70S6K), a major target of the kinase activity of mTOR. Then, to gain insights into the involvement of mTOR signaling in *Yersinia*-induced autophagy, the phosphorylation of p70S6K was assessed by immunoblotting with antibodies targeting p70S6K (Thr389) (**Fig. 2.10**). Strikingly, and in contrast to *Shigella*, *Salmonella* and *Listeria*, we observed that the generation of LC3-II by WAC infection was not accompanied by a change in the phosphorylation pattern of p70S6K, showing that *Yersinia*-induced xenophagy did not involve mTOR inactivation. This supports the idea that a different pathway might be operating to activate autophagy during *Yersinia* infection.

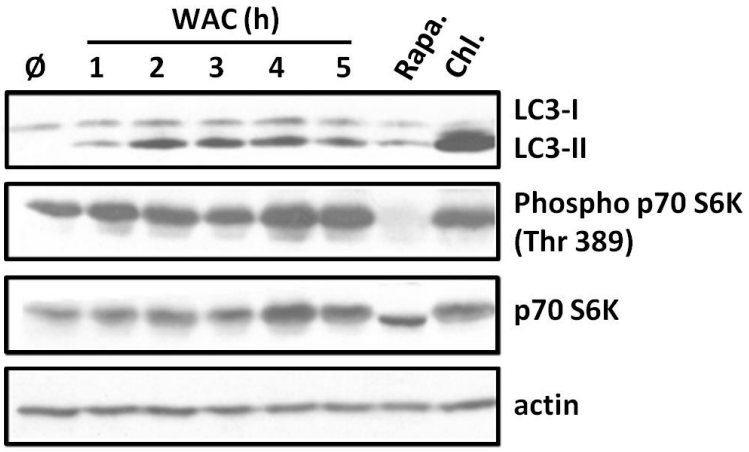


Fig. 2.10: *Y. enterocolitica* does not inactivate mTOR in HeLa cells.
WAC induces the conversion of endogenous LC3-I to LC3-II in HeLa cells without inhibition of mTOR signaling. Cell lysates were prepared at the indicated times after onset of infection. Equal loading of the gel was controlled by immunoblotting against actin. Rapamycin (Rapa, 20 mM) was used as positive control for mTOR inhibition. Chloroquine (Chl, 75 µM) served as a positive control for LC3-II accumulation.

2.4 *Y. enterocolitica* inhibits autophagosome acidification

Another important issue to address was the exploration of the consequences of *Yersinia*-induced autophagy for the bacteria. Bacterial autophagy has been highlighted as a fundamental host cell response to bacterial invasion. In agreement with this, it is expected that the formed bacteria-containing autophagosomes mature by fusion with lysosomes to generate autolysosomes (Mostowy & Cossart 2012). The autolysosomes provide an acidic environment containing active proteolytic enzymes where the

bacteria are degraded. However, as it was addressed in section 1.3.2.3, some pathogens may avoid xenophagy-mediated degradation, whereas others may exploit the autophagy machinery for intracellular survival. To learn more about the maturation of the *Yersinia*-containing autophagosomes and the consequences of xenophagy to *Y. enterocolitica* within epithelial cells, we investigated the presence of some markers of the vesicular trafficking pathway at the YCV. During the normal trafficking pathway, early endosomes and phagosomes begin to fuse with late endosomes and lysosomes. Late endosomes and lysosomes are characterized by the presence of lysosome-associated membrane proteins (LAMPs) and lysosomal proteases (cathepsins B, D and L). Only lysosomes and phagolysosomes, but not late endosomes, contain important amounts of mature cathepsin proteases. Additionally, the pH of lysosomes and phagolysosomes is significantly lower than in late endosomes. The decrease in the pH is due to the action of the vacuolar proton ATPase (v-ATPase).

To understand the fate of the YCVs, we first analyzed the acidification of the vacuoles, as a fundamental step for autophagosome maturation. Accordingly, we used LysoTracker Red DND-99, a fluorescent acidotropic probe that emits red fluorescence at a pH below 5.5. This cell membrane permeable dye is useful for labelling and tracking acidic organelles in cells. We examined the colocalization of LysoTracker with either the classical marker of autophagosomes, LC3 (**Fig. 2.11** (**a**) and (**c1-2**)), or with LAMP-1 (lysosomal-associated membrane protein-1) (**Fig. 2.11** (**c3-4**) in time course experiments. For this purpose, cells were transfected with GFP-LC3 or with YFP-LAMP-1, infected with WAC, incubated with LysoTracker Red for 30 min before sample fixation and stained with DAPI (4',6'-diamidino-2-phenylindole, a cell membrane permeable fluorescent molecule that binds DNA). The results showed that a population of YCVs was delivered to acidic compartments as it is expected for the normal degradation pathway of internalized extracellular pathogens: between 0.1-8 % of intracellular WAC was LysoTracker-positive at 1.5 h, and 22-24 % at 6 h post infection (**Fig. 2.11** (**c2**) and (**c4**)). However, the LysoTracker-positive population of YCVs did not colocalize with GFP-LC3 (**Fig. 2.11** (**a**)) at any of the time points of infection (**Fig. 2.11** (**c2**)), indicating that GFP-positive bacteria might avoid acidification. In contrast, a subpopulation of YFP-LAMP-1-positive YCVs colocalized with LysoTracker already at early time points of infection (**Fig. 2.11** (**c4**)), suggesting that some YCVs may

undergo acidic degradation. Importantly, the marker of late endosomes/lysosomes YFP-LAMP-1 was recruited earlier than GFP-LC3 to the YCVs. At 1.5 h post infection, 48 % of the intracellular bacteria were already positive for YFP-LAMP-1 while no bacteria were positive for GFP-LC3. Furthermore, at 6 h post infection, more than 90 % of the intracellular bacteria were positive for YFP-LAMP-1 (**Fig. 2.11** (**c3**)) but only 43 % were positive for GFP-LC3 (**Fig. 2.11** (**c1**)), suggesting that GFP-LC3-positive YCVs might colocalize with YFP-LAMP-1. This observation was confirmed using HeLa cells that were transfected with GFP-LC3, infected with WAC, and stained with DAPI and antibodies targeting LAMP-1 (**Fig. 2.11** (**b**)). These experiments showed that GFP-LC3-positive YCVs always colocalized with LAMP-1 (**Fig. 2.11** (**c5**)) though an important number of bacteria remained positive only for LAMP-1, but was GFP-LC3-negative (**Fig. 2.11** (**b**)). These results support the idea that, preceding the induction of autophagy, most of the YCVs underwent maturation steps by fusion with late endosomes early after internalization, thereby acquiring LAMP-1. Then, the LAMP-1-positive bacteria could be divided into two populations: one LysoTracker-positive population that was delivered to acidic compartments (such as lysosomes), and another that followed a fate different from degradation, being recognized by the autophagic machinery and enclosed in a neutral autophagosome.

In addition, we analyzed the marker of proteolytic activity DQ-BSA (**Fig. 2.12** (**a**)) and presence of the lysosomal protease cathepsin D (**Fig. 2.12** (**b**)) in the GFP-LC3-positive YCVs. DQ-BSA is an albumin protein marked with a fluorogenic group that becomes fluorescent upon proteolysis. We observed no association of cathepsin D or DQ-BSA with the GFP-LC3-positive YCVs which supports the results obtained with LysoTracker.

Taken together, these results show that the bacteria-comprising LC3-positive compartments did not acidify, whereas bacteria within LC3-negative compartments were frequently subjected to acidification, which suggests that the LC3-positive YCVs might have evolved a way to block the autophagy flux and thus the maturation of the autophagosomal vacuole into a degradative autolysosome.

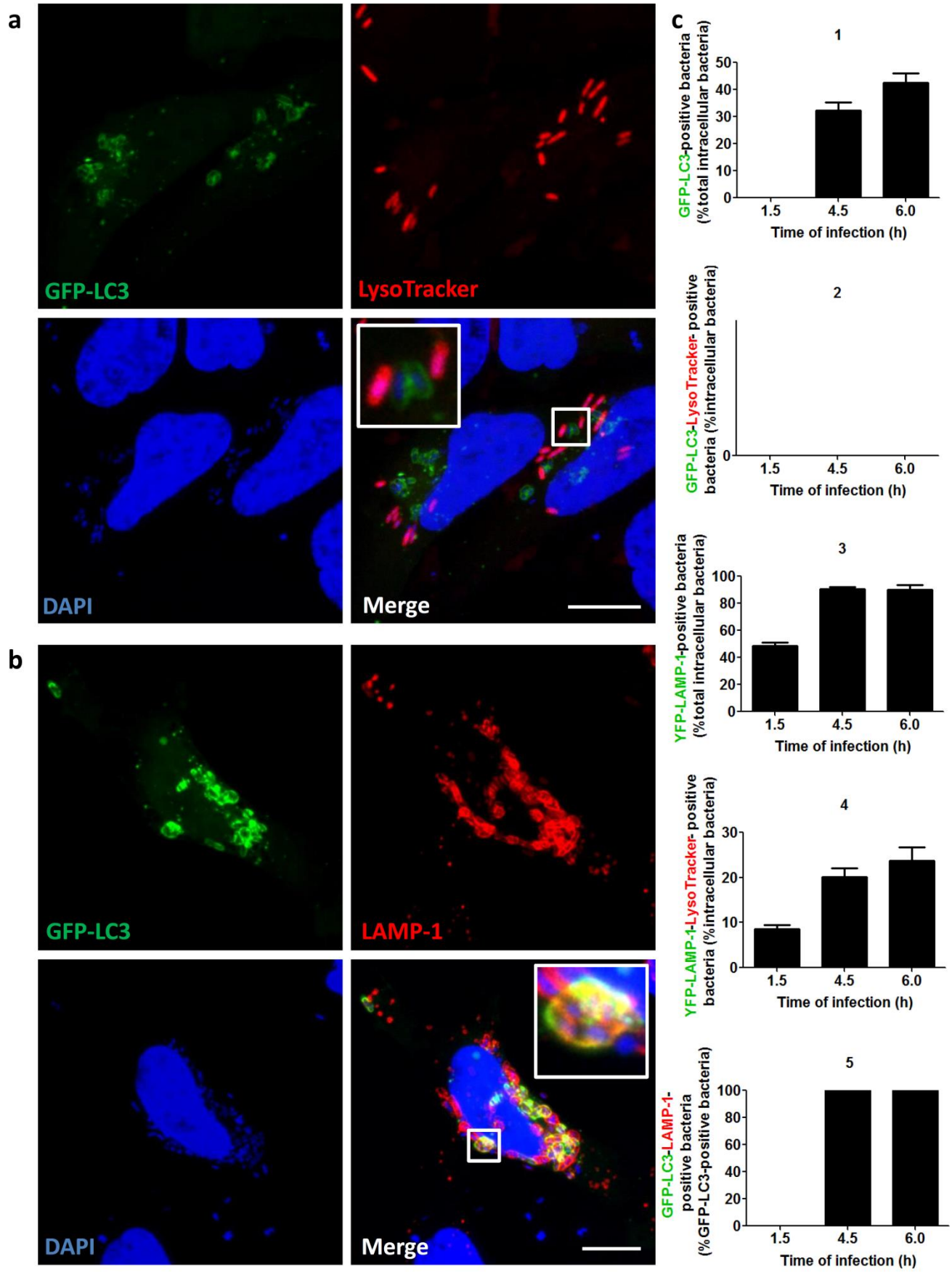


Fig. 2.11: GFP-LC3 positive YCVs do not acidify but contain LAMP-1.
(continues on next page)

(**a**) HeLa cells expressing GFP-LC3 at 6 h post infection with WAC and stained with LysoTracker and DAPI. Bar, 10 µm. (**b**) HeLa cells expressing GFP-LC3 at 6 h post infection with WAC, stained with anti-LAMP-1 and DAPI. Bar, 10 µm. (**c1-2**) HeLa cells transfected with GFP-LC3, infected with WAC and stained with LysoTracker. (**c-3-4**) HeLa cells tranfected with YFP-LAMP-1, infected with WAC and stained with Lysotracker. (**c5**) HeLa cells transfected with GFP-LC3, infected with WAC and stained with anti-LAMP-1 and DAPI. 30 cells from randomly selected fields were counted for each time point. Results are expressed as means ± SEM. n = 2.

To confirm that *Yersinia* was imposing a blockage on the autophagic flux, a RFP-GFP tandem fluorescent-tagged LC3 expression plasmid (called tfLC3, from "tandem fluorescent LC3") was used (**Fig. 2.13** (**a**)) (Kimura et al. 2007). The tandem fluorescent protein allows the dissection of the maturation of the autophagosome to the autolysosome during the autophagic flux. It combines an acid-sensitive GFP with an acid-insensitive RFP, allowing the visualization of the maturation of an autophagosome (neutral pH) to an autolysosome (acidic pH) due to the loss of the GFP signal upon acidification following lysosomal fusion. The autophagosomes are therefore structures positive for both GFP and RFP. Once the lysosome has fused, the acidic pH quenches the GFP. Thus, autolysosomes appear red due to the RFP signal that still persists under low pH. HeLa cells were transfected with tfLC3, infected with WAC and the emitted fluorescence was analyzed (**Fig. 2.13** (**b**)). Torin 1, a potent inhibitor of mTOR, and thus of the mTORC1 complex that regulates autophagy initiation, was used as a control for the normal autophagic flux (**Fig. 2.13** (**c**)). When autophagy is induced by Torin 1 treatment and the autophagic flow works normally, it is possible to observe populations of autophagosomes (visualized in yellow in **Fig. 2.13** (**c**)) as well as of autolysosomes (visualized in red in **Fig. 2.13** (**c**)). On the contrary, if the flux is blocked at the step of fusion of autophagosomes with lysosomes, no autolysosomes would be observed. Our results showed that almost 100 % of the LC3-positive YCVs displayed both GFP and RFP signals (**Fig. 2.13** (**b**)), suggesting that autophagosomes could not fuse with lysosomes and hence, the autophagic flux was blocked at the step of autolysosome formation. In contrast, red puncta representing conventional autolysosomes without bacteria could be detected (**Fig. 2.13** (**b**)). Importantly, the detection of both yellow YCVs and red puncta within the cytoplasm of the same infected cell indicated that the blockage of the autophagic flux that inhibited the acidification of LC3-positive YCVs was a local effect that did not affect conventional constitutive autophagy.

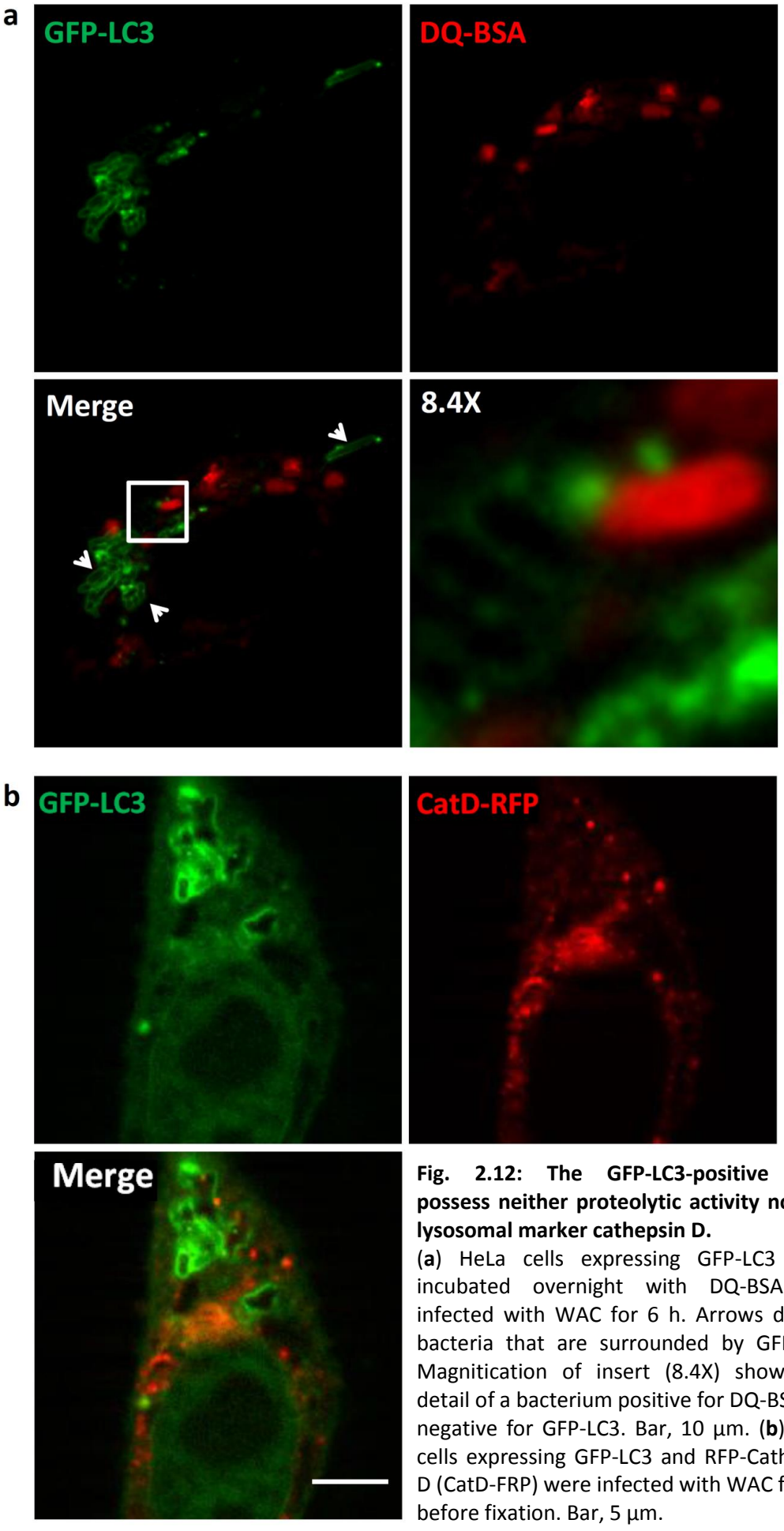


Fig. 2.12: The GFP-LC3-positive YCVs possess neither proteolytic activity nor the lysosomal marker cathepsin D.
(**a**) HeLa cells expressing GFP-LC3 were incubated overnight with DQ-BSA and infected with WAC for 6 h. Arrows denote bacteria that are surrounded by GFP-LC3. Magnitication of insert (8.4X) shows the detail of a bacterium positive for DQ-BSA but negative for GFP-LC3. Bar, 10 µm. (**b**) HeLa cells expressing GFP-LC3 and RFP-Cathepsin D (CatD-FRP) were infected with WAC for 6 h before fixation. Bar, 5 µm.

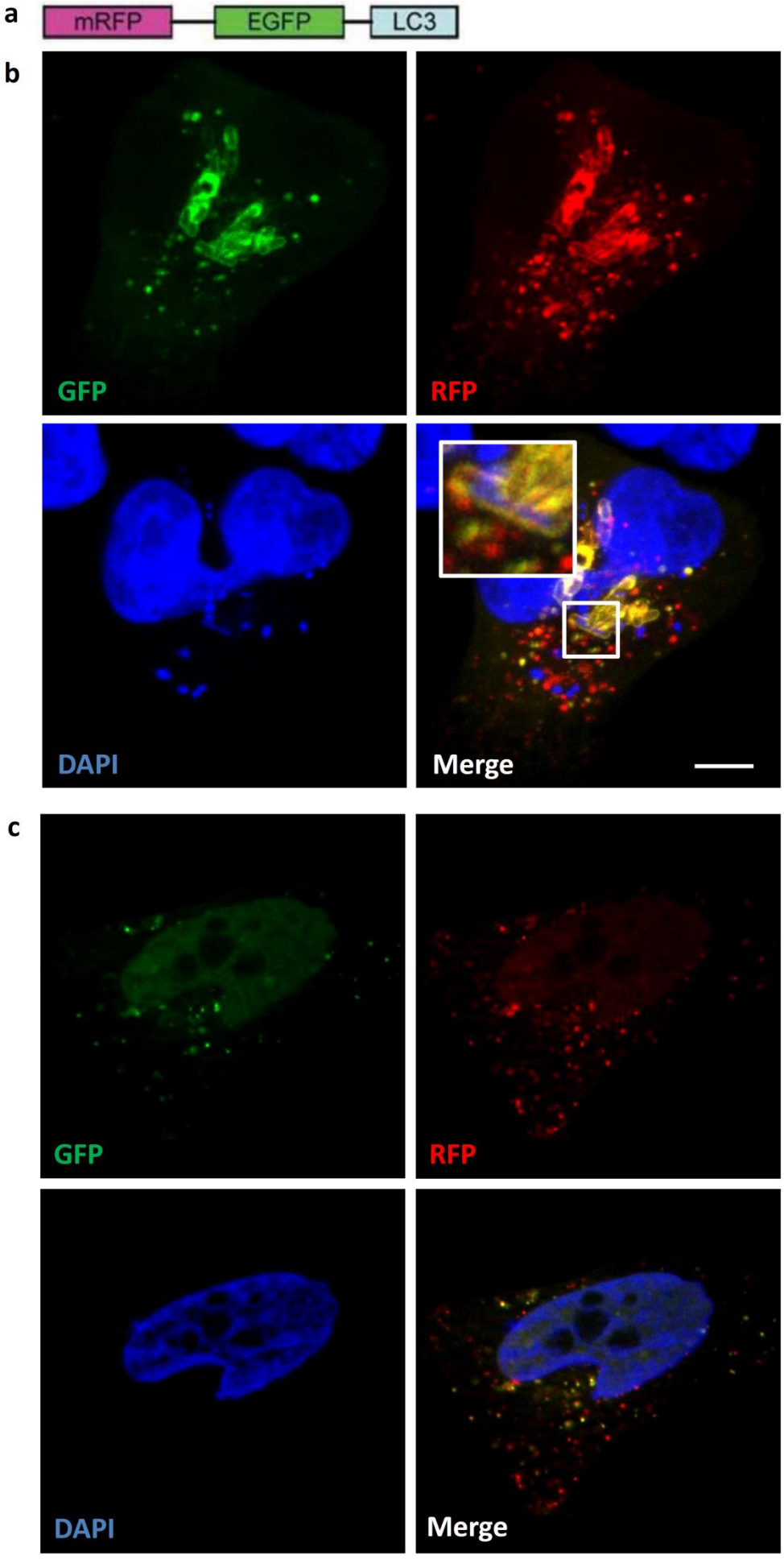

a
mRFP
EGFP
LC3
b
GFP
RFP
DAPI
Merge
c
GFP
RFP
DAPI
Merge

Fig. 2.13: The LC3-positive YCVs are neutral compartments due to blockage of the autophagic flux.
(previous page) (**a**) Diagram of the tfLC3 structure. (**b**) HeLa cells expressing tfLC3 at 6 h of infection with WAC and stained with DAPI. Bar, 5 µm (previous page). (**c**) HeLa cells expressing tfLC3 and treated with the mTOR inhibitor Torin 1, 250 nM, for 6 h prior fixation. Bar, 10 µm.

Pathogenic bacteria that survive within vacuole structures utilize different strategies to avoid being killed in a compartment that could potentially mature into an acidic phagolysosome/autolysosome (Duclos & Desjardins 2000). One possibility for the inhibition of the acidification of the LC3-positive YCVs could be that the accumulation of v-ATPase in the YCVs is prevented. Alternatively, the v-ATPase could associate with the YCV but it is inactivated by a factor released from the bacteria. In order to discriminate between these two possibilities, the presence of the v-ATPase was microscopically analyzed in the YCV using anti-v-ATPase antibodies on cells infected by WAC (**Fig. 2.14**). These experiments showed that LC3 did not colocalize with the v-ATPase, which suggests that the failure to acidify the YCVs could be explained by a block in membrane trafficking responsible for the recruitment of the v-ATPase to the LC3-positive YCV.

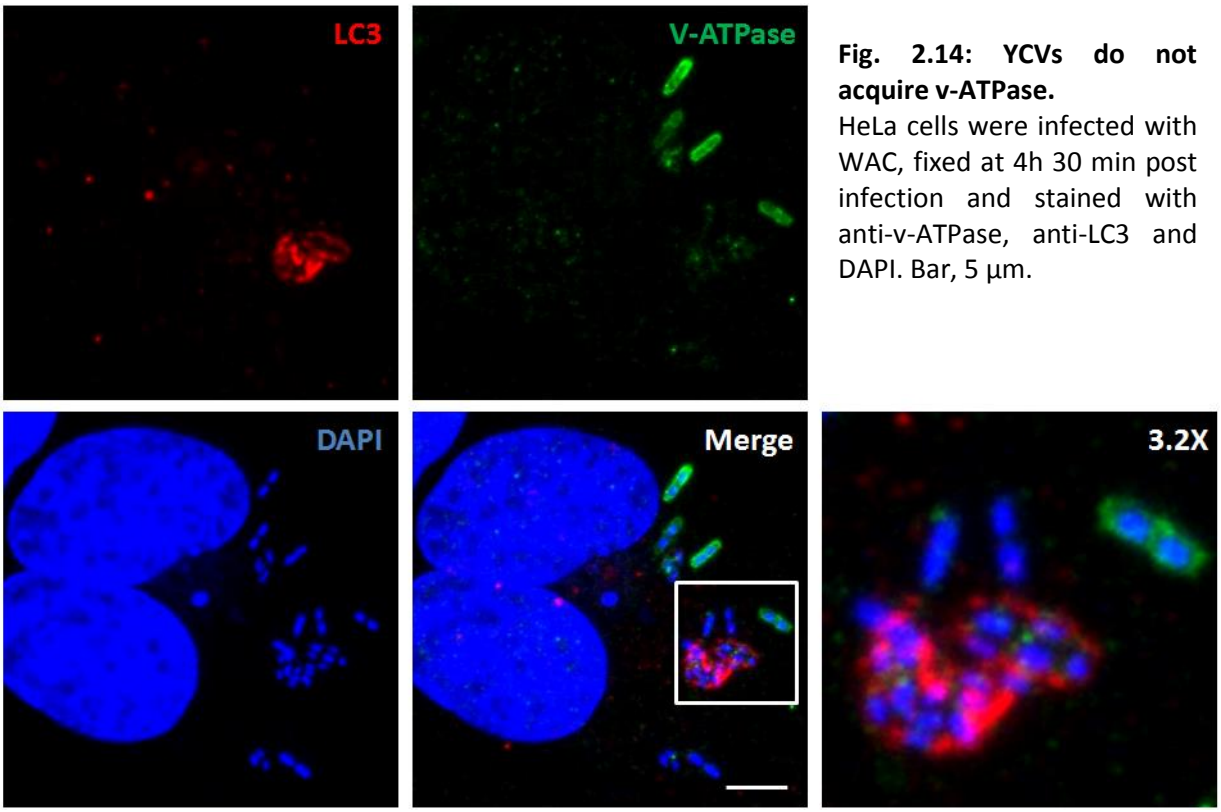


Fig. 2.14: YCVs do not acquire v-ATPase.
HeLa cells were infected with WAC, fixed at 4h 30 min post infection and stained with anti-v-ATPase, anti-LC3 and DAPI. Bar, 5 µm.

All together, these results defined a population of YCVs as autophagosome-like structures which firstly acquired some contents from late endosomes (as they possess LAMP-1 but not cathepsin D) and subsequently were targeted by the autophagic machinery. The LC3-positive YCVs were characterized by their ability to avoid degradation within acidic compartments by imposing a blockage on the autophagic flux. Finally, the avoidance of the acidification of the LC3-positive YCVs could be explained, at least in part, by the prevention of the recruitment of the v-ATPase to the LC3-positive YCVs.

2.5 Blockage of the autophagic flux is an active process specific for *Y. enterocolitica*

We next wanted to investigate if the inhibition of the acidification of the LC3-positive YCVs was an active process that required viable yersiniae. In this case, HeLa cells expressing GFP-LC3 were infected with killed, formaldehyde-fixed WAC (kWAC) and stained with LysoTracker. It was observed that, although the bacteria were efficiently internalized by the cells, kWAC was not able to induce GFP-LC3 recruitment to its surface in contrast to WAC (**Fig. 2.15** (**a**)). kWAC was not either capable to induce conversion of LC3-I to LC3-II by immunoblotting (**Fig. 2.15** (**b**)). Thus, the triggering of autophagic responses not only required the presence of the invasin protein on the surface of the bacteria (section 2.2) and bacterial internalization, but also vital yersiniae. Notably, kWAC localized mostly within LysoTracker-positive compartments even at early time points of infection (1 h 30 min post infection). In contrast, no viable WAC bacteria were inside acidic compartments at 1 h 30 min post infection and no LC3-positive YCV colocalized with LysoTracker at 4 h 30 min or 6 h post infection, confirming that the vital WAC population within LC3-positive YCVs was protected from acidification (**Fig. 2.15** (**a**)). These results demonstrated that the induction of autophagy and the inhibition of acidification of the LC3-positive YCVs are active processes that require viable bacteria.

We also analyzed if the blockage of the autophagic flux and the prevention of the acidification of the bacteria-containing vacuole were specific for *Y. enterocolitica* or if

another microorganism capable of invading cells could show a similar phenotype. To answer this question, HeLa cells expressing GFP-LC3 were infected with a strain of *E. coli* that was genetically modified to express the *inv* gene (*E. coli-inv*$^+$) of an invasive strain of *Y. enterocolitica*. The samples were then stained with LysoTracker for 30 min before fixation, and the colocalization of LysoTracker and GFP-LC3 with the bacteria was examined (**Fig. 2.15** (**a**)). *E. coli-inv*$^+$ could easily invade the cells, but GFP-LC3 was mainly found in autophagosomal punctate structures and not surrounding the bacteria, probably representing conventional autophagosomes. Analysis of the conversion of endogenous LC3-I to LC3-II by immunoblotting in contrast showed an accumulation of LC3-II after *E. coli-inv*$^+$ infection comparable to WAC-infection (**Fig. 2.15** (**c**)). We concluded that *E. coli-inv*$^+$ induced autophagy in a different manner than WAC, as it did not recruit LC3 to the bacterial surface but it apparently enhanced conventional autophagy. *E. coli-inv*$^+$, like WAC, was not found within acidic compartments positive for LysoTracker at 1 h 30 min. However, at later time points most of the bacteria were subjected to acidification. This indicates that *E. coli-inv*$^+$ was not protected against acidification and likely degraded by the phagosomal-lysosomal pathway.

This set of results showed that the presence of invasin on the surface of the bacteria was necessary to invade the cells, but not sufficient to trigger autophagic events, as an active interaction of vital bacteria with host cells was required. Additionally, this mechanism might in some way be specific to inhibit acidification of the YCV, as *E. coli-inv*$^+$, but not *Y. entercolitica*, was always found within acidic compartments after 4h 30 min post infection.

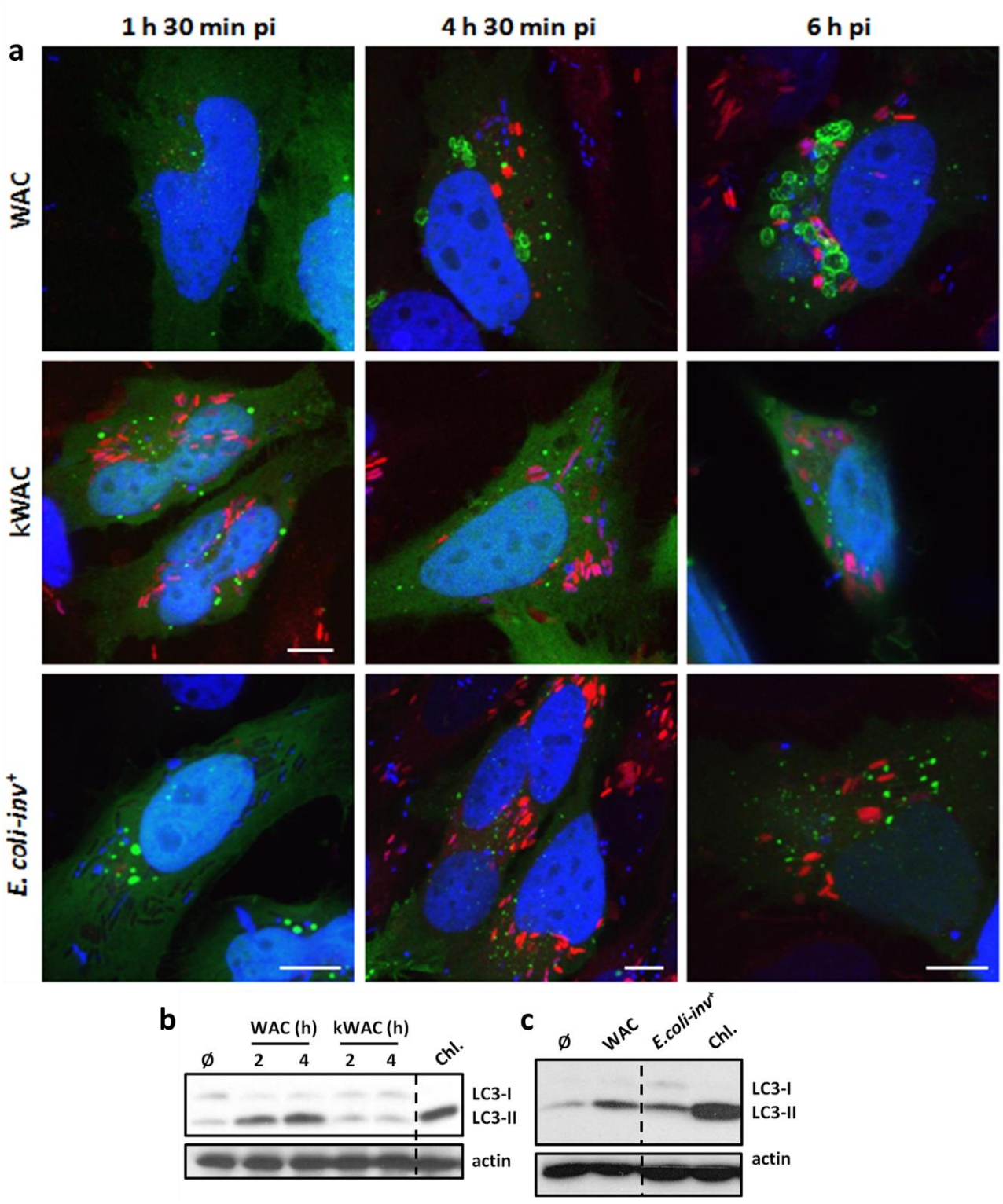


Fig. 2.15: The prevention of acidification of the LC3-positive bacteria is an active process specific for *Y. enterocolitica*.

(**a**) HeLa cells expressing GFP-LC3 (green), infected with WAC, killed formaldehyde-fixed WAC (kWAC), or *E. coli* expressing the *inv* gene of an invasive strain of *Y. enterocolitica* (*E. coli-inv*$^+$) were stained with LysoTracker (red) and DAPI (blue) 30 min before fixation. Bar, 10 µm. pi, time post infection. (**b**) and (**c**) HeLa cells were infected with WAC, kWAC and *E. coli-inv*$^+$. The activation of autophagy was analyzed by monitoring the conversion of endogenous LC3 by immunoblotting. Chloroquine (Chl., 75 µM) served as positive control. Cell lysates were prepared 4 h 30 min after onset of infection. Equal loading of the gel was controlled by immunoblotting against actin.

2.6 *Y. enterocolitica* replicates in epithelial cells

Due to the finding that WAC localizes into an autophagic vacuole where it blocks its maturation and acidification, we hypothesized that autophagy might support the survival of WAC in HeLa cells. This may be in contrast to *E. coli-inv*$^{+}$ which was delivered to lysosomes and not protected from acidification. To test our hypothesis, and to learn more about the fate and the viability of the internalized bacteria in epithelial cells, we performed gentamicin protection assays for WAC or *E. coli-inv*$^{+}$. HeLa cells were infected with WAC or *E. coli-inv*$^{+}$ for 30 min to allow binding and invasion to occur. Then, the cells were washed to remove extracellular non-adherent bacteria and incubated with fresh medium with gentamicin. Gentamicin cannot enter the mammalian cells, so it only kills extracellular bacteria, but not bacteria that have already entered the cell. Thus, internalized bacteria are protected from the antibiotic and therefore survive and can be quantified after lysis of the cells. The infected cells were incubated with the gentamicin-containing medium for different periods of time. Then the cells were lysed and serial dilutions of the lysates were plated on agar dishes to determine numbers of colony forming units that reflect intracellular bacterial counts (**Fig. 2.16**). It was observed that *Yersinia* could survive and replicate inside HeLa cells, as the total number of intracellular bacteria increased 4.5-fold over a period of 8 h 30 min post infection. In contrast the amount of intracellular *E. coli-inv*$^{+}$ decreased 5-fold during the same time, indicating that the bacteria were killed by the cells, which may fit to the observation that the bacteria localized to an acidic compartment (section 2.5). In addition, the replication of WAC within epithelial cells was also examined by live cell imaging of HeLa cells transfected with GFP-LC3. In this movie, a subset of intracellular bacteria seemed to be able to replicate inside GFP-LC3-positive YCVs (**Fig. 2.17** and **Video 2.1**). These results support our hypothesis and suggest that WAC may specifically survive and replicate within epithelial cells, while *E. coli-inv*$^{+}$ is killed. Thus, *Y. enterocolitica* could potentially benefit from host cell autophagy as a niche for intracellular replication.

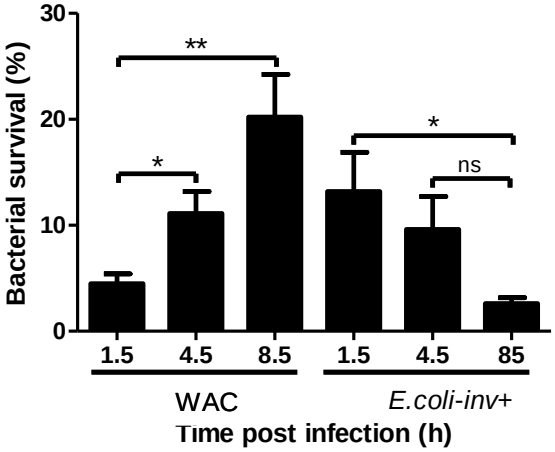


Fig. 2.16: *Y. enterocolitica* replicates inside epithelial cells.
Gentamicin protection assay for determination of survival and replication of WAC and *E. coli-inv*$^{+}$ in HeLa cells. HeLa cells were infected with WAC or *E. coli-inv*$^{+}$ for 30 min. Then, the cells were washed and incubated for different time periods with medium with gentamicin to kill extracellular bacteria. At the indicated time point the cells were lysed, and serial dilutions were plated to enumerate CFU. Results are expressed as means ± SEM. n = 5.

To determine if the replication of WAC observed in HeLa cells was a phenomenon dependent on autophagy, we performed gentamicin killing assays using wild-type MEF cells or MEFs deficient for the *atg5* gene, an essential protein for autophagy that is required for the formation of the phagophore. MEFs were infected with WAC and colony forming unit assays (CFU) were performed at different time points after infection to assess the influence of autophagy on the viability of the internalized bacteria (**Fig. 2.18**). We observed that the number of internalized bacteria was almost comparable between both cell lines but the amount of intracellular bacteria increased stronger in the wild-type MEFs compared to the Atg5$^{-/-}$ MEF cells over a period of 8 h 30 min. Therefore, although the autophagy pathway in MEFs did not appear to be required for survival of *Y. enterocolitica*, the bacteria could multiply within autophagosomes in wild-type MEF cells more efficiently than in Atg5$^{-/-}$ MEF cells, which supports our idea that *Yersinia*-induced autophagy facilitates intracellular bacterial replication.

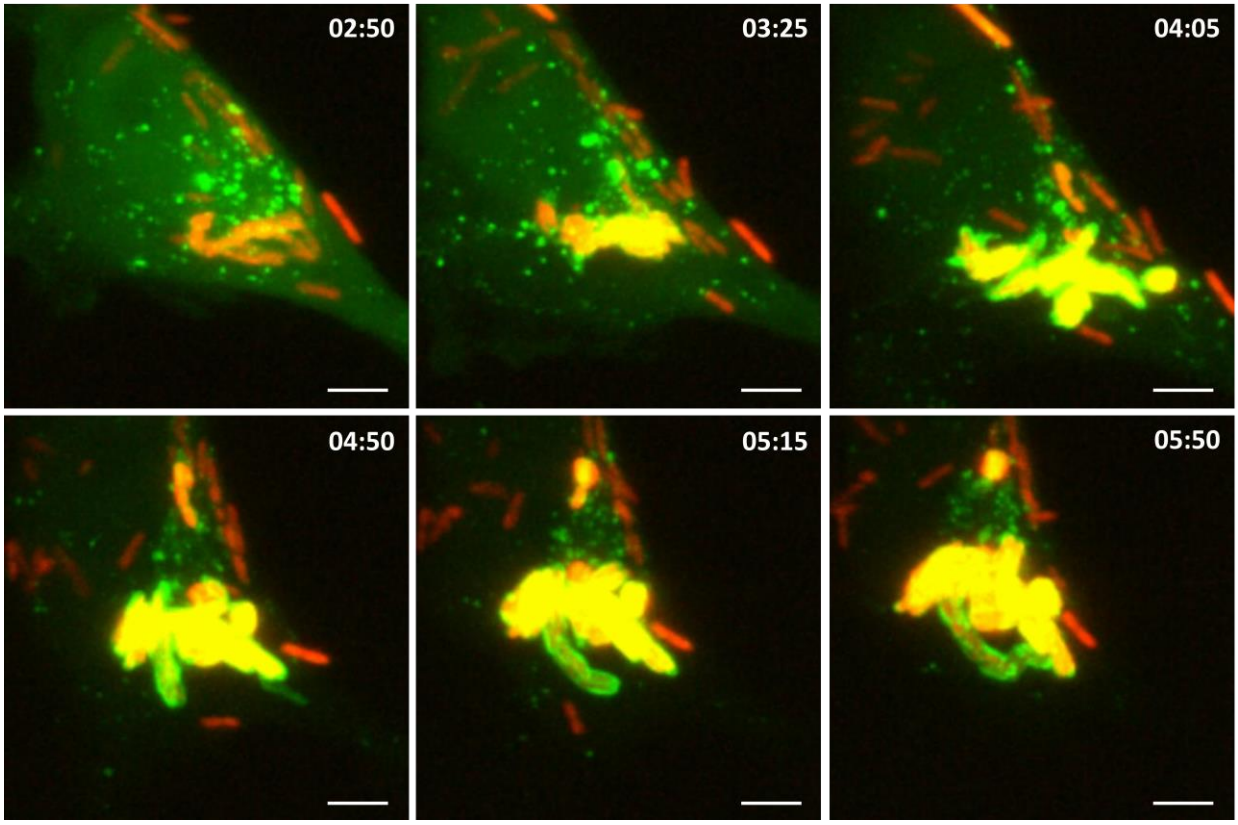


Fig. 2.17: *Y. enterocolitica* replicates within GFP-LC3-positive YCVs.
HeLa cells were transfected to express GFP-LC3 and infected with WAC labelled by RFP expression. After 2 h of infection, cells were imaged every 5 min intervals with a spinning-disc confocal microscope. Series of live-cell imaging data and the elapsed time are shown (hours:minutes). Bar, 5 µm.

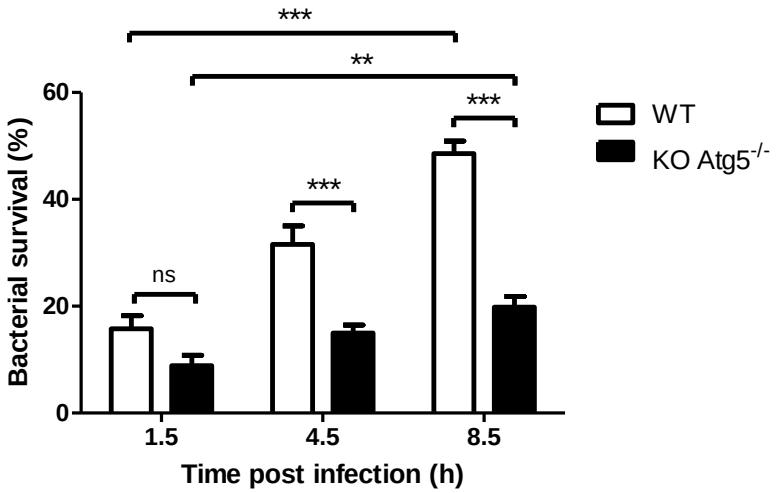


Fig. 2.18: Autophagy supports replication of *Y. enterocolitica*.
Gentamicin protection assay for determination of the survival and replication of WAC in wild-type or Atg5$^{-/-}$ MEF cells. MEFs were infected with WAC for 30 min. Then, the cells were washed and incubated for different periods of time with medium with gentamicin to kill extracellular bacteria. At the indicated time points the cells were lysed, and serial dilutions were plated to enumerate CFU. Results are expressed as means ± SEM. n = 5.

2.7 Autophagy supports release of *Y. enterocolitica* from infected cells

Some bacteria subvert the autophagy pathway to promote cell-to-cell transmission, as it is the case for *Brucella* and *M. marinum* (Starr et al. 2012; Gerstenmaier et al. 2015). We then wanted to determine whether autophagy could play a similar role in *Y. enterocolitica* infected cells. In concordance, the release of WAC from wild type and $Atg5^{-/-}$ infected MEF cells into the culture medium was investigated over 8 h 30 min of infection. MEFs were infected with WAC for 30 min. Then, the cells were washed to remove non-adherent bacteria and incubated for one hour with medium with gentamicin to kill adherent bacteria on the surface of the cells. Subsequently, the cells were washed again and incubated with medium without antibiotics. In time periods of one hour, the medium of the cells was taken and serial dilutions thereof were plated on agar dishes to enumerate CFU (**Fig. 2.19** (**a**)). We observed that during infection of $Atg5^{-/-}$ MEFs the amount of bacteria released from the infected cells to the culture medium increased only marginally between 3 h and 8 h post infection. In contrast, the number of bacteria released from wild-type MEF cells increased 4-fold within that time frame. Interestingly, the release of WAC from wild-type MEFs started to be evident at 5 h post infection, a time when autophagy was already activated and *Yersinia* located within LC3-positive YCVs. These results suggest that *Yersinia* may exploit the autophagic pathway to egress from the epithelial cells to the extracellular environment. This could be a mechanism to cross the epithelial monolayer of cells in the small intestine and to gain access to the lymphoid tissue of the Payer´s patches. To discriminate if the release of bacteria was a result of an unconventional secretion process mediated by autophagy or a consequence of cell death, we performed the lactate dehydrogenase (LDH) release assay. This is an assay for cytotoxicity that measures the release of the intracellular protein LDH by cells undergoing cell death as they lysed and release their cellular contents to the extracellular environment. The results showed that WAC infected wild-type MEFs released 6-fold more LDH to the extracellular environment than $Atg5^{-/-}$ MEF cells after 24 h of infection (**Fig. 2.19** (**b**)). The higher level of cytotoxicity observed in wild-type cells correlated with the stronger release of the bacteria from those cells. This suggests that the bacteria might be

released from dying wild-type MEFs. One possible explanation for that could be that WAC promotes cell death by the stress imposed on the cells due to stronger bacterial replication in wild-type MEFs. A different explanation would be that a virulence factor of *Yersinia* actively induces cell death with the aim to promote egress of the bacteria from the cells.

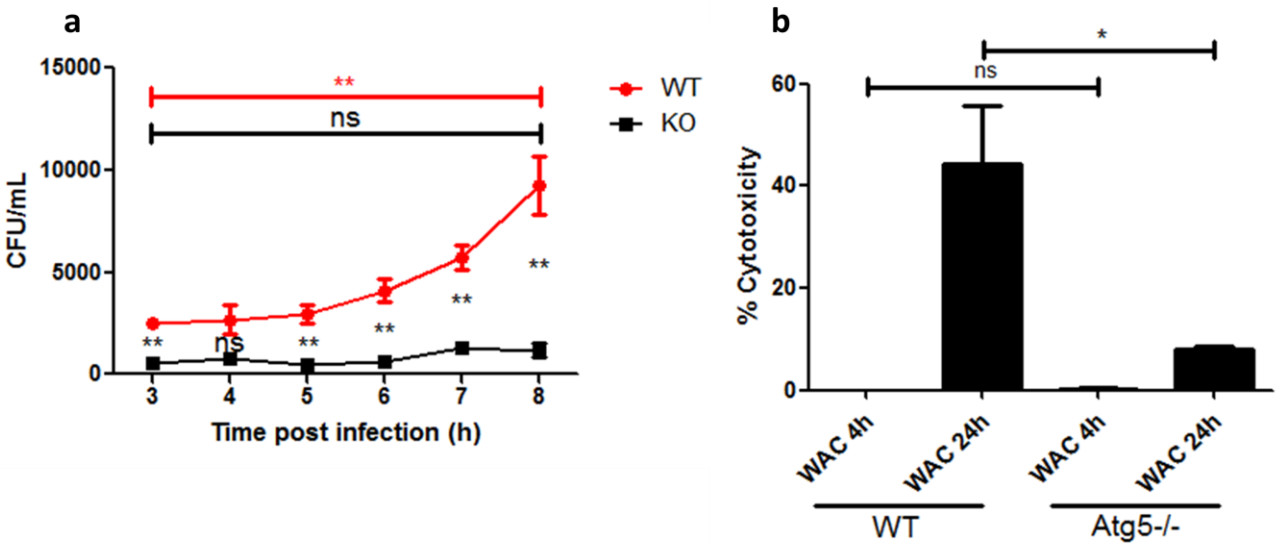


Fig. 2.19: Autophagy contributes to the release of *Yersinia* from infected cells.
(**a**) Yersiniae released from infected wild-type and Atg5$^{-/-}$ cells into the cell culture medium were counted at several time points after infection. (**b**) Cells infected with *Y. enterocolitica* were analyzed for cytotoxicity by measuring the release of lactate dehydrogenase (LDH) from infected cells. The % cytotoxicity was determined by comparison of LDH released from infected samples with LDH released from completely lysed cells (100 %) and from untreated samples (0 %). Results are expressed as means ± SEM. n = 5

2.8 Recognition of *Y. enterocolitica* by autophagy receptors

In general, autophagy is a non-selective cellular process in response to starvation. But autophagy may also operate selectively, which is essential for the cell-autonomous defense against bacteria invading the cytosol. For that reason, it is important to understand the mechanisms responsible for the specific targeting of bacteria and other cellular constituents by autophagy. It was shown that accumulation of ubiquitin on the surface of bacteria serves as a signal that recruits the autophagy adaptors p62 and NDP52 (Boyle & Randow 2013). We were therefore interested whether those autophagy receptors and ubiquitin were involved in the recognition of *Y. enterocolitica* and the induction of the autophagy pathway. To answer this question we performed

colocalization experiments of LC3 with ubiquitin, p62 and NDP52. HeLa cells were co-transfected to express mCherry-LC3 and GFP-Ubiquitin, mCherry-LC3 and GFP-NDP52, or transfected with GFP-LC3 and stained with anti-p62 antibodies after infection with WAC and fixation. We found that after 4 h 30 min of infection a clear colocalization of ubiquitin, p62 and NDP52 with LC3-positive bacteria was observed, while LC3-negative bacteria were rarely positive for ubiquitin or any of the autophagy adaptors tested (**Fig. 2.20**). This data pointed out that ubiquitin, as well as the adaptors p62 and NDP52 may play a role in the recognition of the population of *Yersinia* which is targeted to the autophagy pathway.

2.9 *In vivo* effects of autophagy on *Y. enterocolitica* infection

It was previously reported from murine models that intestinal epithelial autophagy protects against invasion of *S*. Typhimurium (Benjamin et al. 2013) and may relieve from cellular stress associated with host cell death and inflammation caused by *Shigella flexneri* (Chang et al. 2013). Based on our *in vitro* results, we also wondered about the relevance of *Yersinia*-mediated autophagy in an *in vivo* infection model. We aimed to perform *in vivo* assays in collaboration with the lab of Dr. Hooper at the University of Texas Southwestern Medical Center in Dallas (United States) in order to determine whether autophagy in intestinal epithelial cells supports wild-type *Y. enterocolitica* (WA-314) infection and dissemination to extraintestinal organs (liver and spleen) after an oral challenge with the bacteria. We used C57Bl/6 mice with an intestinal epithelial cell-specific deletion of the gene *atg5* (*Atg5*$^{\Delta IEC}$). The reason to use the WA-314 strain instead of the plasmid-cured version WAC relied on the fact that the virulence factors encoded by the plasmid pYV of the wild-type strain (including the adhesin YadA and the TTSS) are needed to assure successful persistence of the bacteria within PPs and dissemination to liver and spleen in the murine host. In contrast, WAC shows impaired ability to persist within the PPs and to disseminate to extraintestinal organs (Di Genaro et al. 2003).

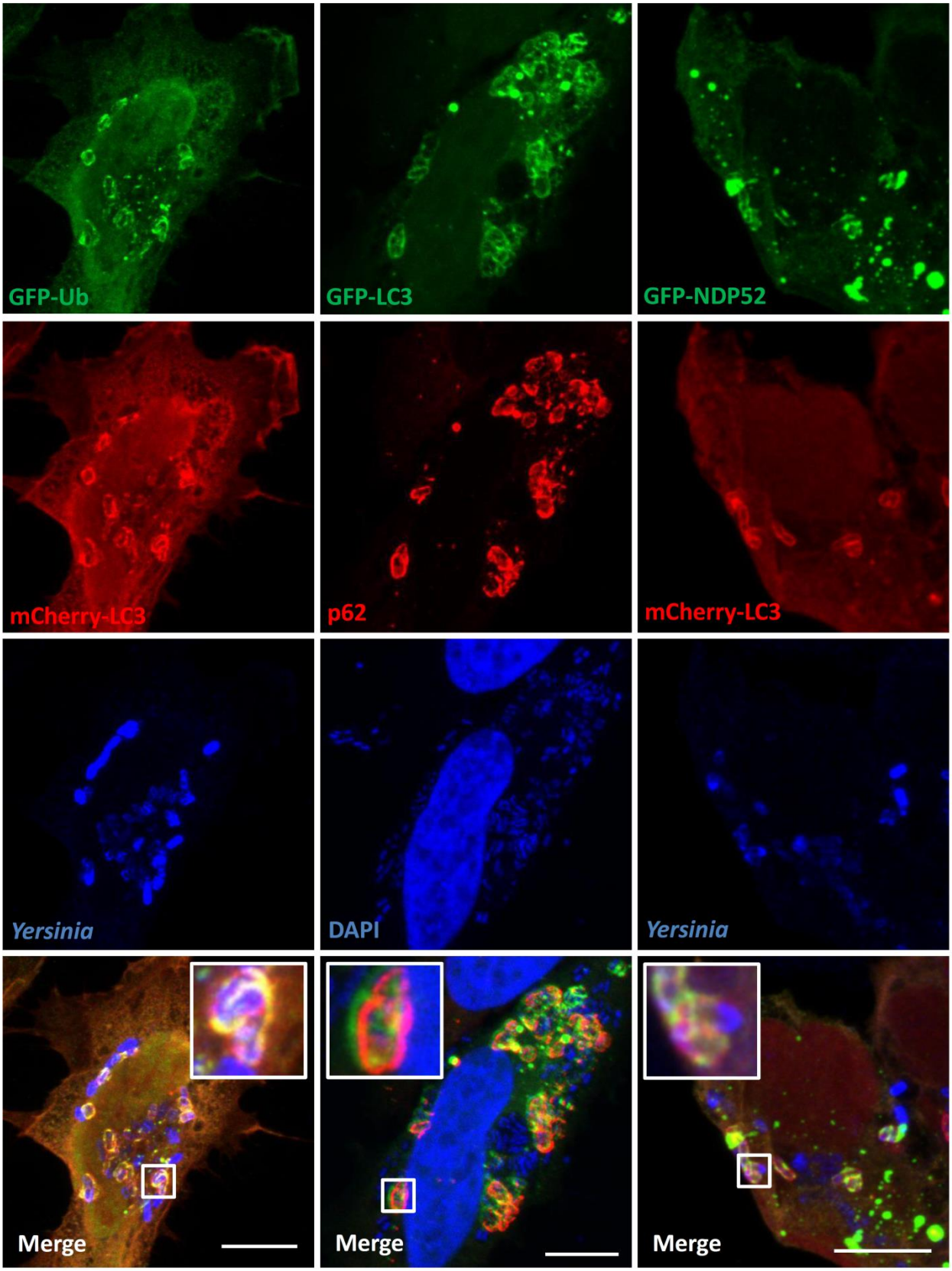


Fig. 2.20: Ubiquitin, p62 and NDP52 colocalize with LC3-positive *Yersinia*.
HeLa cells expressing GFP-Ubiquitin (Ub) and mCherry-LC3, GFP-LC3 or GFP-NDP52 and mCherry-LC3 were infected with WAC for 4 h 30 min, then stained with anti-*Yersinia* (**left and right panels**), or DAPI and anti-p62 antibodies (**middle panel**), and processed for fluorescence microscopy. Bar, 10 µm.

At first, we verified the intestinal colonization with WA-314 to determine the optimal time point of infection for *Y. enterocolitica* in the *in vivo* model. For this purpose, wild type C57Bl/6 mice were orally infected with $1x10^{10}$ wild-type *Y. enterocolitica* and CFUs were counted from contents of the small intestine and from homogenates of liver and spleen at 0.5, 1, 2, 3 and 5 days post infection (**Fig. 2.21**). As we were interested in an event that depends on bacterial entry into epithelial cells likely occurring at a very early step of infection, and in order to avoid analysis of effects that may be influenced by other immune evasion mechanisms that could operate later and compensate for an initial failure of autophagy, we selected the earliest time point post infection at which we observed stable infection of animals as well as an adequate dissemination to liver and spleen. For these reasons, 12 h post infection was the time point chosen for the subsequent set of experiments in which we sought to determine

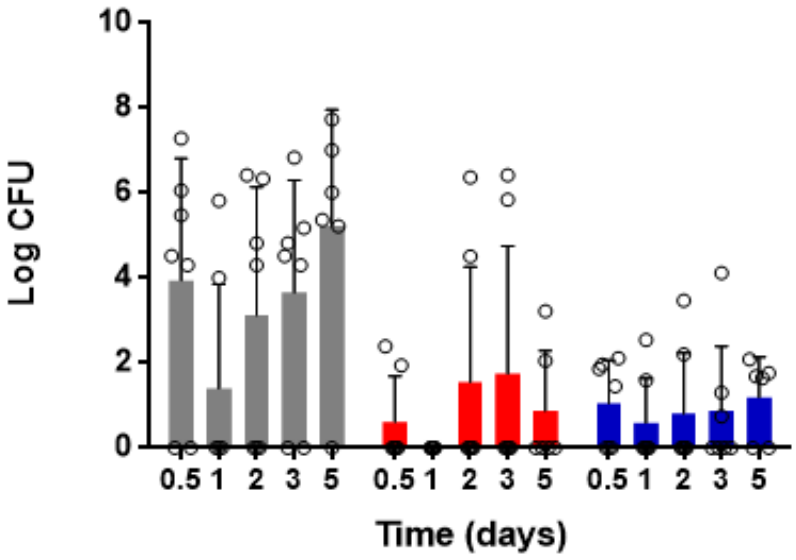


Fig. 2.21: Colonization and dissemination of *Y. enterocolitica* after oral infection of C57Bl/mice.
Bacterial burdens (CFU) in spleen (red), liver (blue), and contents of the small intestine (gray) after oral infection with $1x10^{10}$ *Y. enterocolitica*. Each point represents an individual mouse. Data are from two independent experiments and represented as mean ± SEM. n = 2.

the role of autophagy in intestinal epithelial cells on invasion and dissemination of *Y. enterocolitica*. With this purpose we used $Atg5^{\Delta IEC}$ mice which were created by crossing mice with a loxP-flanked *atg5* allele ($Atg5^{fl/fl}$) with villin-*Cre* transgenic mice (Benjamin et al. 2013). The $Atg5^{\Delta IEC}$ and $Atg5^{fl/fl}$ (wild-type control) littermates were

orally infected with 1x10^{10} wild-type *Y. enterocolitica* and bacterial burdens in the liver, spleen and the contents of the small intestines were counted (**Fig. 2.22**). We observed that there were no statistical significant differences in the colonization of the small intestines and the bacterial burdens in liver and spleen in *Atg5*$^{\Delta IEC}$ mice compared to wild-type *Atg5*$^{fl/fl}$ mice at the time point investigated. Thus, autophagy seems not to influence the dissemination of the bacteria at that stage of infection. Additional experiments with differing time points and bacterial loads may be required to specify this point in more detail.

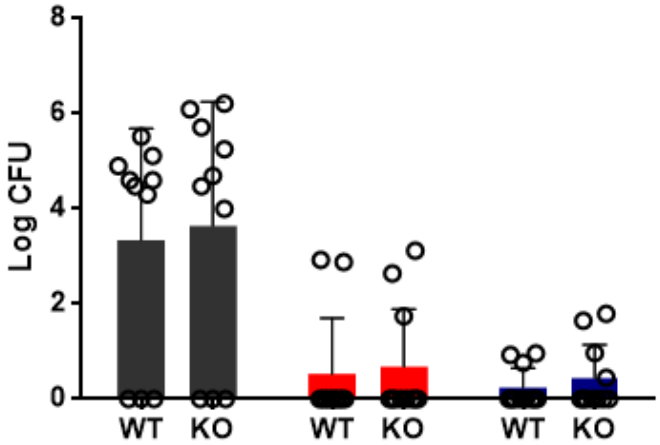


Fig. 2.22: Colonization and dissemination of *Y. enterocolitica* after oral infection of *Atg5*$^{\Delta IEC}$ and *Atg5*$^{fl/fl.}$ mice.
Bacterial burdens (CFU) in the spleen (red), liver (blue) and the small intestines (gray) of *Atg5*$^{\Delta IEC}$ (knockout) and *Atg5*$^{fl/fl}$ (wild-type) littermates at 12 h after oral infection with 1x10^{10} CFU of *Y. enterocolitica*. Each point represents an individual mouse; data are from two independent experiments and represented as mean ± SEM. n = 2.

3 DISCUSSION

Autophagy has been elucidated as an important innate immune response that fights invading microbes (Deretic & Levine 2009). It is one of the earliest defense mechanisms encountered by pathogens within minutes or hours after entry into the host cell cytosol (Baxt et al. 2013). During this process, the intracellular microbes are enveloped into an autophagosome that delivers them to lysosomal degradation (Deretic 2011). However, many pathogenic bacteria avoid autophagy-mediated degradation, whereas others may exploit the autophagy machinery for intracellular survival within their host (Mostowy 2013). *Yersinia enterocolitica*, the causative agent of yersiniosis, is an enteropathogen that can manipulate several immune responses of the host to promote its survival (Galindo et al. 2011). The virulence factors of pathogenic strains allow the bacteria to invade epithelial cells which permits the subsequent translocation of the bacteria through the intestinal epithelial monolayer at very early stages of infection. Furthermore, *Yersinia* virulence factors subvert phagocytosis and block the activation of proinflammatory pathways to avoid killing of bacteria at later phases of the infectious process (Cornelis 1994). In this context, the bacterial surface protein invasin seems to play a critical role as invasion factor as it is required for attachment to and internalization by epithelial cells of the intestine through the engagement of β1-integrin receptors on the cell surface (Pepe & Miller 1993). However, the events that happen inside the epithelial cells infected by *Yersinia*, and their significance for the infectious process, are not well understood. It has been reported that autophagy within the intestinal epithelium plays an important defensive role against invasive bacteria that reach enterocytes. Thus, autophagy prevents the dissemination of enteropathogenic bacteria to extraintestinal organs (Benjamin et al. 2013; Conway et al. 2013). Additionally, it has been previously observed that *Yersinia*-mediated immunomodulation also targets autophagy in macrophages (Pujol et al. 2009; Moreau et al. 2010; Deuretzbacher et al. 2009).

In this study, we have analyzed the role of autophagy in *Y. enterocolitica* infection of epithelial cells. This aimed to shed light on how this host cell defense mechanism affects the pathogenicity of *Yersinia* and the course of *Yersinia* infection.

We observed that following β1-integrin mediated uptake of virulence-plasmid cured *Y. enterocolitica* into intestinal epithelial cells, the bacterium could enter the autophagy pathway. During a normal autophagic flux, LC3 is processed from cytoplasmic LC3-I to autophagosome-related LC3-II, and subsequently degraded in autolysosomes, which prevents LC3-II accumulation. We observed by immunoblotting that the LC3-II levels were increased in *Y. enterocolitica*-infected epithelial cells as compared to uninfected controls. Furthermore, by fluorescence microscopical analysis we corroborated that GFP-LC3 was decorating a population of about 30 % of the intracellular pool of *Y. enterocolitica* (LC3-positive YCVs). Finally, by correlative light electron microscopy we examined the ultrastructure of the YCVs and observed that the majority of the GFP-LC3-positive YCVs corresponded to internalized yersiniae captured in typical, multiple or double-membranous autophagic vacuoles, which indicates xenophagy occurring on *Y. enterocolitica* (Levine 2005; Knodler & Celli 2011). Similar findings were reported for several other pathogens, and also for *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* in macrophages (Pujol et al. 2009; Deuretzbacher et al. 2009; Moreau et al. 2010). Moreover, engulfment of *Y. pseudotuberculosis* by epithelial cells was recently reported to display features of autophagy, however, in contrast to our results, the bacteria were located mainly within single vacuoles (resembling LAP) instead of classical multiple or double-membrane-bound autophagosomes (Ligeon et al. 2014). Thus, there seem to be differences in the induction of autophagic events between *Y. pseudotuberculosis* and *Y. enterocolitica*. We also showed that the LC3-positive YCVs share an unusual trafficking pattern as they first acquired certain markers of late endosomes (like LAMP-1, but not cathepsin D or v-ATPase) and later markers of autophagosomes (LC3) (**Fig. 3.1**). Besides that, the LC3-positive-YCVs do not undergo acidification which demonstrates a blockage in the normal flux of autophagy. It is thought that both the maturation of endosomes/phagosomes and autophagosomes, require a final fusion step with lysosomes (Huynh & Grinstein 2007; Kawai et al. 2007). We speculate that the YCVs fuse with several vesicular compartments, including late

endosomes, before interacting with the autophagy pathway. It is actually possible that *Y. enterocolitica* blocks fusion of lysosomes with the YCV to stall the maturation of the LC3-positive YCV and to engage the autophagy pathway as a survival mechanism (**Fig. 3.1**). In fact, *Y. enterocolitica* replicated in autophagy-competent cells better than in cells defective for autophagy, as specified later. Interestingly, we observed that intracellular yersiniae that were free of autophagic membranes (corresponding to LC3-negative YCVs) fused with lysosomes, indicating that vesicle maturation and acidification were specifically inhibited for the *Yersinia*-containing autophagosomes. Furthermore, infection with killed formaldehyde-fixed *Y. enterocolitica* WAC did not inhibit the acidification of the YCVs and epithelial cells infected with killed bacteria exhibited yersiniae mostly within acidic compartments. These observations suggest that an active interaction between the host cell and *Yersinia* is required to trigger autophagy and to inhibit acidification. Additionally, after infection of epithelial cells with *E. coli* expressing the *inv* gene of a pathogenic invasive strain of *Y. enterocolitica,* the bacteria mostly localize inside acidic compartments. This suggests that the inhibition of the acidification is specific for *Yersinia* and requires a factor present in *Yersinia,* but not in *E. coli*, independently from invasin.

Pathogenic bacteria that survive within phagosome-like structures exploit several strategies to avoid being killed in a vacuole that could become acidic by fusion with lysosomes (Méresse et al. 1999). For example, *M. tuberculosis* survives within macrophages by preventing the accumulation of v-ATPase at the phagosomal membrane which would lead to acidification. This hampers the maturation of the phagosome at an early stage (Sturgill-Koszycki et al. 1994; Rohde et al. 2007). Our results suggest that *Y. enterocolitica*, similar to *M. tuberculosis*, has evolved a tactic to avoid the association of the v-ATPase with the YCV. However, the exact mechanism and the bacterial factor/s responsible for the prevention of the acidification of the LC3-positive-YCVs need to be deciphered. Additionally, it has been reported that during nutrient-independent, ubiquitin-selective autophagy, actin promotes the fusion of autophagosomes with lysosomes (J.-Y. Lee et al. 2010). The molecular mechanism involves the histone deacetylase-6 (HDAC6) that is not required for autophagy activation but that rather controls autophagy by recruiting the actin-remodelling machinery. This in turn assembles an F-actin network that stimulates the fusion of

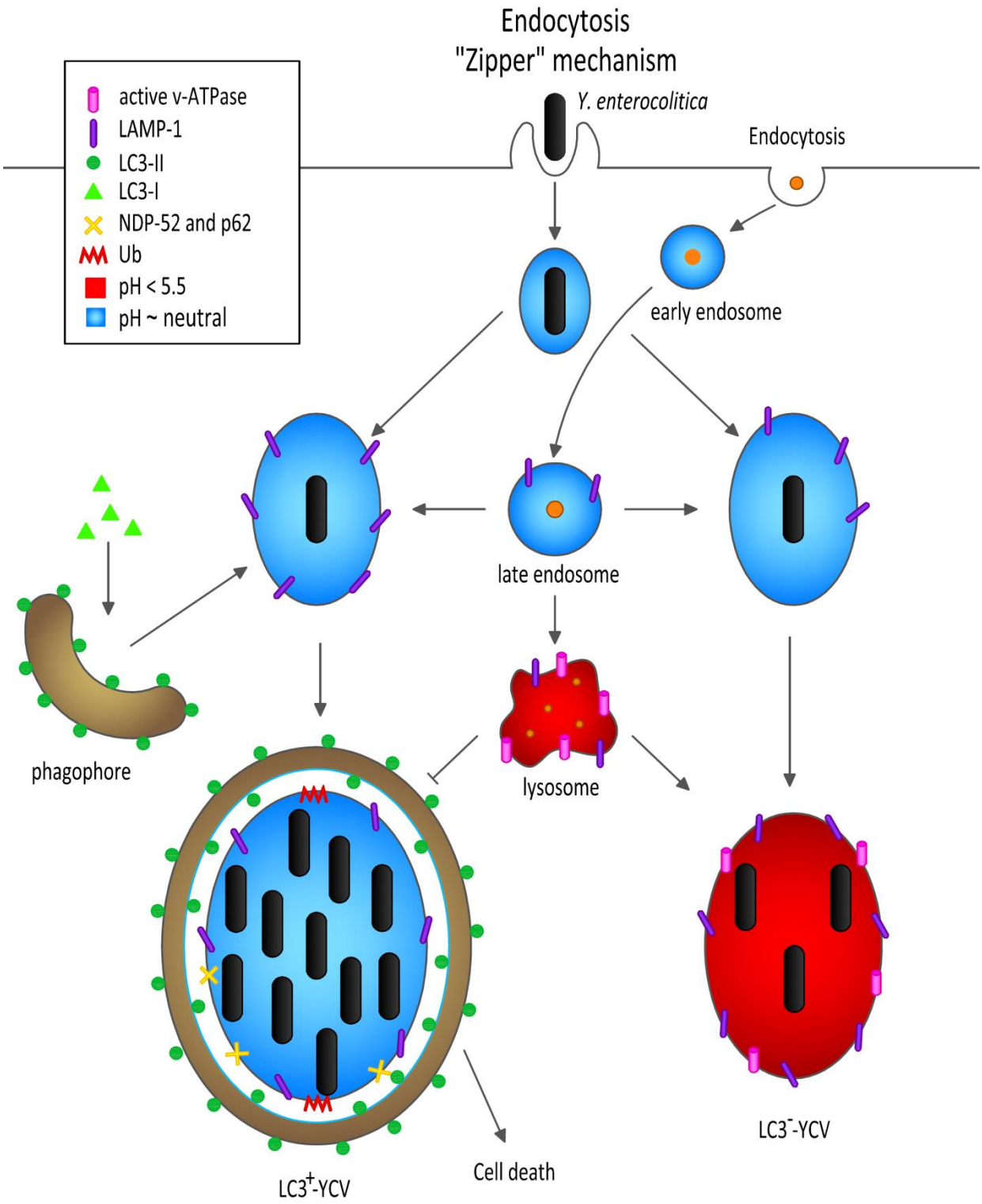


Fig. 3.1: Proposed model on the trafficking of the *Yersinia*-containing vacuole and its intersection with endocytic and autophagy pathways in epithelial cells.
Following endocytosis by a "zipper" mechanism, *Y. enterocolitica* resides in a YCV that can fuse with late endosomes to acquire LAMP-1. Prevention of the recruitment of the v-ATPase blocks acidification of the $LC3^+$-YCV. Furthermore, the fusion with lysosomes is blocked. After conversion from LC3-I, LC3-II localizes to the membranes of the phagophore and autophagosome. LC3-II, normally degraded and recycled in acidic autolysosomes, accumulates in YCVs due to the neutral pH and the lack of active proteases. The resulting bacterial replication in the autophagosome may, over time, result in cell death and release of bacteria. In contrast, a second population of $LC3^-$-YCV fuses with lysosomes and the bacteria are killed.

autophagosomes with the lysosomes and the subsequent degradation of the substrates. So it is feasible that a bacterial factor could interact with HDAC6 to block autophagosme maturation. *Y. enterocolitica* biogroup 1B posses several chromosomal virulence factors that should be further studied in the context of host-bacterium interaction and autophagy. For example, it is known that *Y. enterocolitica* biogroup 1B has a chromosome-encoded type three secretion system, called Ysa, for the delivery of protein effectors into host cells (Haller et al. 2000; Matsumoto & Young 2009). Studies in mice demonstrated that the Ysa TTSS plays a role in the colonization of the gastrointestinal tissues by *Yersinia* in the earliest stages of infection (Venecia & Young 2005). Furthermore, pathogenic and non-pathogenic *Yersinia* species possess one or two type two secretion systems, called Yts1 and Yts2 (von Tils et al. 2012). In *Y. enterocolitica*, Yts1 is speculated to be related with the interaction of free-living bacteria with their environment (Shutinoski et al. 2010). Additionally, it was described that Yts1 is involved in the dissemination and colonization of liver and spleen in orally infected mice, although the secreted substrates responsible for these effects are not determined (Iwobi et al. 2003). On the other side, it has recently shown that Yts2 is important for intracellular survival of *Y. enterocolitica* within macrophages (Bent et al. 2015). Therefore, it is possible that effector/s proteins delivered by the Ysa system or the type two secretion systems may interfere with the degradation of yersiniae within epithelial cells to favour the subsequent steps of the infection process. Finally, studies using in vivo expression technology (IVET) and signature-tagged mutagenesis (STM) have identified bacterial genes required for growth within the host (Darwin 2005) that could be also be important for *Yersinia*-induced autophagy and/or the blockage of the autophagic flux imposed by yersiniae. These studies have shown that chromosomal factors like the protease HreP, and the phospholipases PldA and YplA, among others, are specifically induced after infection of mice (Darwin 2005).

The generation of autophagic YCVs with neutral pH might interfere with the *Yersinia*-induced immune response: Blocking autophagosome maturation may allow the bacteria to establish a replicative niche which is continuously supplied with nutrients via fusion with autophagosomal vesicles as a consequence of autophagy induction. We verified that hypothesis using $Atg5^{-/-}$ MEF cells. When autophagy was impaired by the use of Atg5 knockout cells, the bacteria multiplicated intracellularly with a

considerably lower rate than in wild-type cells, suggesting that autophagy supports bacterial replication, although it is not mandatorily required for the intracellular survival of the bacteria. This result differs from previous observations of our group that described the degradation of *Y. enterocolitica* in the autophagosomes of macrophages (Deuretzbacher et al. 2009). A possible explanation for this discrepancy could be that the autophagosome pathways taken by the YCVs vary depending on the cell type infected. Alternatively, the different methods used to impair autophagy in those cell lines could account for that difference. To inhibit autophagy in macrophages the inhibitor 3-methyladenine (3-MA) was applied to the cells before infection (Deuretzbacher et al. 2009). 3-MA blocks the class III PI3 kinase Vps34 which is part of the complex that mediates phagophore nucleation. However, this inhibitor, as most chemical inhibitors of autophagy, is not entirely specific and can to some extent also inhibit the class I PI3 kinase, leading to autophagy induction in some systems, as well as affect cell survival through AKT1 and other kinases (Klionsky et al. 2012). Accordingly, it is generally preferable to analyze specific loss of function effects in autophagy with *atg* mutant cells, as with the $Atg5^{-/-}$ MEFs that we used in the present study. Lafont and colleagues studying *Y. pseudotuberculosis* in macrophages and HeLa cells (Moreau et al. 2010; Ligeon et al. 2014) also found that this bacterium can replicate in autophagic YCVs. However, and as mentioned before, LC3-positive *Y. pseudotuberculosis*-containing vacuoles show mainly single membrane structures in HeLa cells. Hence, interaction of *Y. enterocolitica* with the autophagy pathway may differ from the strategy employed by *Y. pseudotuberculosis*. We do neither know the reason underlying that discrepancy nor the consequences thereof but, even when *Y. enterocolitica* and *Y. pseudotuberculosis* are pathogens that cause very similar syndromes, the differences in the autophagic processes that they trigger could be related to different survival strategies developed by these pathogens due to distant evolution and pathogenicity of both bacteria (Wren 2003).

Pathogenic bacteria can take advantage of autophagy for more than to promote bacterial replication. A role of autophagy in bacterial egress and cell-to-cell transmission has been previously reported. For instance, *Brucella* is a bacterium that has evolved a strategy to manipulate intracellular membrane trafficking processes, being able to convert its replicating vacuole into one with autophagic features (called

aBCV, for “autophagic *Brucella*-containing vacuole”), independently of Atg5 and Atg7 (Starr et al. 2012). Interestingly, this conversion is necessary for effective bacterial transmission to neighbouring cells and it does not involve host cell death, even though no molecular mechanism is yet proposed. In addition, autophagy is necessary for nonlytic cell-to-cell transmission of *Mycobacterium marinum* in *Dictyostelium* cells (Gerstenmaier et al. 2015). *M. marinum* egress depends on a mechanism of ejection through an F-actin based structure called the ejectosome. Engagement of the autophagic machinery at the distal pole of ejecting bacteria helps to seal the membrane damages generated by the ejection, preventing the lysis of the host cell. The group of Quinn has reported that *M. tuberculosis* is internalized in A549 cells (a human type II pneumocyte cell line) into an autophagic vacuole that has markers of late endosomes but that does not fuse with lysosomes. The bacteria replicate within this compartment, which seems to be associated with lytic cell death that may promote bacterial dissemination (Fine et al. 2012). In the case of *Y. enterocolitica* we have shown that the interplay between the bacterium and the autophagic machinery promotes the release of a higher number of bacteria from infected wild-type MEFs to the extracellular milieu in comparison to $Atg5^{-/-}$MEF cells. Furthermore, we have observed that the spreading of the bacteria, in contrast to *Brucella* and *Mycobacterium*, may be related to lytic host cell death, as indicated by LDH assays. Therefore, it is plausible that the localization of *Y. enterocolitica* within autophagosome-like structures could have important consequences for the cell fate and outcome of infection. Indeed autophagy has been shown to be involved in a number of different regulatory pathways, in particular in the regulation of cell death (Liu & Levine 2014). Hijacking of the autophagy pathway by recruiting the autophagy machinery and sequestering membranes for the enlargement of the YCVs during bacterial replication could be detrimental for the cells due to the inability to use autophagy for its normal function such as the clearance of damaged organelles and cell survival. Thus, we hypothesize that the modulation of autophagy by *Y. enterocolitica* infection could compromise the cellular viability, allowing the bacteria to escape from the cell to either infect other cells or to survive extracellularly at the basal side of the intestinal epithelium. The subjacent molecular mechanism as well as the cellular and bacterial factors involved in this process still need to be deciphered and more work is

needed to examine the connection between autophagy and cell death in the context of intracellular *Y. enterocolitica* infection of epithelial cells.

The data obtained with *Y. enterocolitica* infecting macrophages have demonstrated that autophagy stimulation is mediated by the *Yersinia* adhesin invasin and the plasmid encoded YadA, depending on the engagement of β1-integrins (Deuretzbacher et al. 2009). Our results indicated also a critical role of the invasin protein in *Y. enterocolitica*-mediated autophagy induction in epithelial cells as it confers an invasive phenotype to the bacteria. We found out that apathogenic strains of the serogroups O:5 and O:36 (both belonging to biogroup 1A), although they are supposed to possess *inv*-like genes, could not efficiently invade cells and therefore they were not able to induce autophagy unless they were transformed to express the *inv* gene of invasive *Y. entercolitica*. It has been reported that the *inv* gene of serogroup O:8 is related to those of the biogroup 1A serogroups O:5 and O:36, but the identity level is only about 50%. A 99-bp region of the *inv* gene is absent in serogroup O:8 (biogroup 1B) but present in O:5 and O:36 serotypes (Batzilla et al. 2011). The impact of that region remains unclear, but it could explain the differences in the efficiency of invasion between biogroups 1A and 1B and thus the induction of autophagy responses triggered by O:8, but not by the other two serotypes. However, the presence of an invasive invasin on the surface of the bacteria is not the only factor required to induce autophagy in epithelial cells as we did not observe autophagy following infection with invasin-expressing *E. coli* or invasin-expressing killed *Yersinia*. Thus, other factors expressed by the intracellular bacteria might be playing a role in *Yersinia*-induced autophagy in an active host cell-pathogen interaction requiring vital yersiniae. The mechanisms by which internalization of *Yersinia* is coupled to autophagy activation are not yet understood. The stimulation of β1-integrins by *Yersinia* activates several signaling intermediates that could also function in autophagy. Invasin-mediated cell invasion involves the recruitment of focal adhesion complex components that link integrin signaling to actin cytoskeleton reorganization (Alrutz & Isberg 1998; Dersch & Isberg 1999; Isberg & Barnes 2001). Some focal adhesion-related proteins have been shown to be important in autophagosome biogenesis. For instance, the focal adhesion FAK family kinase interacting protein of 200 kDa (FIP200) regulates cell size and

migration but is also required for autophagosome generation through its interaction with ULK1 to form the autophagy initiation complex (Hara et al. 2008). Another focal adhesion protein important for autophagy is paxillin, a signal transduction adaptor protein that is phosphorylated by FAK and Src upon β1-integrin engagement. Paxillin interacts with Atg1 (the homolog of ULK1 in mammals) in *Drosophila* and also seems to be important for autophagosome formation during nutrient deprivation (Chen et al. 2008). Additionally, it has been reported that the actin cytoskeleton participates in the very early stages of autophagosome formation, promoting the generation of PtdIns3P under starvation induced autophagy. Moreover, the RhoGTPases RhoA and Rac1 play important regulatory roles in this process (Aguilera et al. 2012) as well as in signaling of the β1-integrin receptors. Invasin-mediated actin polymerization furthermore involves the Arp2/3 complex, an actin nucleator (Alrutz et al. 2001; McGee et al. 2001; Wiedemann et al. 2001) which is necessary for the trafficking of Atg9 and autophagosome formation (Monastyrska et al. 2008; Zavodszky et al. 2014). These findings suggest several critical signaling branches at which *Yersinia*-mediated cell invasion could overlap with autophagy induction. However, since our results mentioned above indicate that invasin is not sufficient to induce autophagy, this suggests that complementary mechanisms may contribute to the activation of autophagy by pathogenic *Yersinia* in epithelial cells. Some studies have highlighted a role of host cell membrane damage in the triggering of autophagy. Damage of cellular membranes generated by some pathogens induces amino acid starvation that leads to mTOR inactivation and the activation of autophagy (Tattoli et al. 2012). The breaks in the membranes can be generated in several ways: *Shigella* causes damage to the plasma membrane while entering into the host cells, *Salmonella* presumably damages the SCV by action of its TTSS, and *Listeria* seems to damage the *Listeria*-containing vacuole by action of the pore-forming toxin Listeriolysin O (LLO) (Tattoli, Matthew T Sorbara, et al. 2012; Tattoli et al. 2013). In addition, the cellular disturbances arising from the invasion process of *Salmonella*, and its escape from the SCV to the cytosol, exposes host molecules of damaged membranes such as β-galactoside. These recruit Galectin-8 and the autophagic machinery to the bacteria (Thurston et al. 2012). Therefore, damage of host membranes would connect the recognition of pathogens to the induction of autophagy. Our results on the analysis of the mTOR activity during *Y.*

entercolitica infection showed no inhibition of mTOR, suggesting that a different pathway might be operating to activate autophagy. Furthermore, fluorescence microscopy did not show a clear colocalization between mCherry-Galectin 8 and intracellular *Y. enterocolitica*, which may imply that host β-galactoside is not exposed to the cytosol indicating that there is no damage in the YCV (data not shown). Both results support the idea that a phenomenon different than host cell membrane damage is the cause of *Y. enterocolitica*-induced autophagy. Alternatively, there are several mTOR-independent pathways that may regulate the initiation of autophagy, including the inositol signaling pathway, the Ca2+/calpain pathway, the cAMP/EPAC/RAP2B pathway, etc (Sarkar 2013). For instance, the intracellular pathogen *Anaplasma phagocytophilum* secretes a type four secretion effector called Ats-1 (*Anaplasma* translocated substrate 1) which binds Beclin1 and hijacks the Beclin1 nucleation complex for autophagy initiation independently of mTOR signaling (Niu et al. 2012). Then, *Anaplasma* forms a membrane vacuole resembling an autophagosome that is needed for the replication of the bacteria. As mentioned before, autophagy is also regulated by the second messenger cAMP (cyclic adenosine monophosphate) and increased levels of intracellular cAMP have inhibitory effects on autophagy (Noda & Ohsumi 1998). *S. aureus* triggers autophagy by a pathway that presumably does not involve mTOR activity, as it is independent of the Beclin1 complex. In this case, the activation of autophagy relies on the secretion of α-hemolysin which seems to be responsible for the decrease of the levels of cellular cAMP after infection (Mestre et al. 2010; Mestre & Colombo 2012).

The recognition of pathogens by the autophagy pathway is an essential step for selective xenophagy. The results of this study show that the autophagic machinery selectively targets a population of *Y. enterocolitica* infecting epithelial cells. We observed that only LC3-positive YCVs recruit ubiquitin, p62 and NDP52. Thus these molecules might mediate the surveillance of *Y. enterocolitica* and the delivery of the bacteria to the autophagy pathway. *Salmonella* also requires ubiquitin, p62 and NDP52 for its targeting to autophagy. However, the mechanism involved in the induction of autophagy by *Y. enterocolitica* may be different from the one that targets *Salmonella* because the later is recognized by ubiquitin, p62 and NDP52 when the bacteria escape

from the SCV to the cytosol, while no apparent damage is made by *Y. enterocolitica* to the YCVs. If these adaptors are strictly required for the recruitment of the autophagy machinery to the YCV and their specific roles in bacterial survival remain elusive. How *Yersinia* is targeted with ubiquitin, the identity of the ubiquitylated host or bacterial proteins, and the responsible ubiquitin ligases are also currently unknown and should be a subject for futures studies. In the case of *Salmonella*, a host ubiquitin E3 ligase called LRSAM1 (leucin-rich sterile alpha motif 1) is responsible for autophagy-associated ubiquitylation. Finally, alternative molecules could target yersiniae to the autophagy pathway, independently from ubiquitylated signals, as it is the case for the lipid second messenger diacylglycerol (DAG) for *Salmonella*. DAG is observed on SCVs after *Salmonella* infection and is generated via the actions of phospholipase D and phosphatidic acid phosphatase (PAP) (Shahnazari et al. 2010). The mechanism by which DAG promotes autophagy remains to be determined (Shahnazari & Brumell 2011).

It has been reported that epithelial autophagy is a critical mechanism of cell-intrinsic innate immunity that eliminates invading bacteria before they can access deeper tissues. Two groups have explored the role of autophagy *in vivo* with *Salmonella*-infected mice, using knockout animals in which a gene essential for autophagy (*atg5* or *atg16L1*) was absent in the intestines (Benjamin et al. 2013; Conway et al. 2013). Both groups reported that *Salmonella* infection in control mice resulted in colocalization of autophagy-associated proteins with the bacteria, analyzed by fluorescence microscopy. In contrast, intestinal epithelial cells without *atg5* or *atg16L1* did not demonstrate such colocalization. Furthermore, infected knockout mice also exhibited increased dissemination to extraintestinal organs, from which they concluded that knockout mice are defective in their ability to process *Salmonella* within autophagosomes, supporting the hypothesis that autophagy serves as a host defense mechanism against microorganisms *in vivo*. From our *in vitro* results we hypothesized that *Y. enterocolitica*, in contrast to *Salmonella*, may benefit from autophagy to replicate in the intestinal epithelium and, eventually, to cross this barrier to reach the submucosa. For this reason, we expected to get higher burdens of bacteria in extraintestinal organs of wild-type mice in comparison with mice with a conditional deletion of the *atg5* gene in the intestinal epithelium. Contradictorily, we observed no

significant differences in the dissemination of bacteria to liver and spleen between the two groups of mice which may suggest that autophagy does not have a strong influence on the dissemination of *Yersinia* at that stage of infection in mice. Moreover, we were neither able to detect activation of autophagy by immunostaining with antibodies targeting LC3 nor RFP-labelled *Yersinia* within the infected tissues at time points that varied from 6 h to 2 days post infection (data not shown). Thus, additional experiments that include earlier time points and different bacterial loads may be needed to specify more clearly if autophagy plays a role in *Y. enterocolitica* infection *in vivo*.

In the present study, we analyzed whether *Yersinia enterocolitica* targets autophagy in epithelial cells. We investigated the trafficking of the autophagosomal *Yersinia*-containing vacuole within epithelial cells and the function of epithelial autophagy in the course of *Yersinia* infection. We showed that yersiniae manipulate this host defense pathway to promote the intracellular replication of the bacteria. Although important conclusions about the interplay of autophagy with internalized *Y. enterocolitica* were drawn, many aspects remain to be elucidated. One main point to be clarified is how *Y. enterocolitica* controls the induction of autophagy in epithelial cells. The factor or factors responsible for the blockage of the autophagy flux imposed by yersiniae also remain to be identified. Finally, further characterization of the interaction between the bacteria and autophagy will be required to elucidate the physiological role of autophagy in *Y. enterocolitica* infection in the mouse model. Overall, this study provides important insights into the autophagic response against *Y. enterocolitica* in epithelial cells which may add a new piece to the puzzle how *Yersinia* manipulates the host innate immune response.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 General equipment

Table 4.1: Technical and mechanical devices

Equipment	Source
Balance	R 160P and Pt 1200, Sartorius (Göttingen, Germany) 440-47N, Hartenstein (Würzburg, Germany)
Biological safety cabinet	HERA safe, Thermo Fischer Scientific (Waltham, USA)
Centrifuges	3K30, Sigma (Harz, Germany) 5810R, Eppendorf (Hamburg, Germany)
Electrophoresis-SDS-PAGE	Mini-Protean-II Cell, Bio-Rad Laboratories (Munich, Germany) Hoefer SE 400, Amersham Biosciences (Freiburg, Germany)
Film processor	Curix 60, AGFA (Cologne, Germany)
Freezing container	Mr. Frosty, Hartenstein (Würzburg, Germany)
Incubator	BBD 6220, B5090E, Heraeus (Hanau, Germany) Certomat BS-1, Sartorius (Göttingen, Germany)
Microplate reader	Infinite M200, TECAN (Männedorf, Switzerland)
NanoDrop 1000	peqLab Biotechnology (Erlangen, Germany)
Neubauer chamber	Hartenstein (Würzburg, Germany)
pH Meter	320 pH Meter, Mettler Toledo (Hamburg, Germany)
Photometer	Ultraspec 3000, Amersham Pharmacia Biotech (Freiburg, Germany)
Pipettes	2, 10, 20, 100, 200 and 1000 µL, Gilson (Middleton, USA) accu-jet pro, BRAND (Wertheim, Germany)
Powersupply	Power Pac 1000, Bio-Rad Laboratories (Munich, Germany) BluePower 500, Serva (Heidelberg, Germany)
Scanner	CanoScan 4400F, Canon (Amsterdam, The Netherlands)

Thermo block	TDB-120, Hartenstein (Würzburg, Germany)
Tweezers	Hartenstein (Würzburg, Germany)
Vortex	Labinco L46, Hartenstein (Würzburg, Germany)
Water bath	GFL Typ 1013, GFL (Würzburg, Germany)
Western-blot-chamber	Hartenstein (Würzburg, Germany)
X-ray cassette	Dr. Goos Suprema, Hartenstein (Würzburg, Germany)

Table 4.2: Microscopic devices

Live Cell Spinning Disk	Type, Provider
Provider	Improvision (Coventry, United Kingdom)
Microscope	Axiovert 200M, Zeiss (Jena, Germany)
Objective	Plan-Apochromat 63X / 1,4 Ph3 oil immersion
Confocal unit	Spinning Disk CSU22 (Yokogawa, Japan)
Camera	EM-CCD C9100-02, (Hamamatsu, Japan)
Laser	Colbot Calypso CW 491nm, Cobolt Jive 561nm (Stockholm, Sweden)
Laser combiner	LMM5, Spectral Applied Research (Richmond Hill, Canada)
Emission filters	ET 525/50 (green), ET 620/60 (red), Chroma Technology (Rockingham, USA)
UV lamp	X-cite series 120W with Hg-lamp, EXFO (Mississauga, Canada)
Halogen Lamp	Standard housing 100W, Zeiss (Jena, Germany)
Incubation chamber	Temperature/humidity/CO2 control, Solent Scientific (Regensworth, United Kingdom)
Stage	Motorized BioPrecision inverted XY stage and PiezoZ stage, Ludl Electronic Products (Hawthorne, USA)
Software	Volocity, Perkin Elmer (Waltham, USA)

Confocal Laser Scanning Microscope	Type, Provider
Provider	Leica (Wetzlar, Germany)
Stand	Leica DM IRE2, Leica (Wetzlar, Germany)
Objective	Plan-Apochromat 63X / 1,4 Ph3 oil immersion
Confocal unit	Leica TCS SP2 AOBS confocal point scanner
Lasers	Ar/Kr (488nm, 514nm), HeNe (543nm, 594nm, 633nm)
Emission filters	Filtersystems: I 3, blue ecx. (BP 450-490, LP515); N 2.1, green exc. (BP 515-560, LP590); A, UV exc. (BP 340-380, LP425)
UV lamp	Standard housing, 50W HBO mercury
Halogen lamp	Standard housing, 100W, 12V
Stage	POC perfusion chamber, CO2: PeCon CTI-Controller 3700 digital, Temp.: PeCon tempcontrol 37-2 digital; Z-drive: Piezo focus drive
Software	Leica LCS, Leica (Wetzlar, Germany)

4.1.2 Labware and disposables

Table 4.3: Labware and disposables

Type	Source
Cell culture dishes (Ø 10cm)	Sarstedt (Nümbrecht, Germany)
Cell culture plates 24-wells	Sarstedt (Nümbrecht, Germany)
Conical centrifuge tubes	Greiner (Frickenhausen, Germany)
Eppendorf tubes	Eppendorf (Hamburg, Germany
Glass bottom culture dishes	WillCo Wells (Amsterdam, The Netherlands)
Glass bottom culture dishes with grid	ibidi (Munich, Germany)
Glass cover slips, round (Ø12 mm)	Hartenstein (Würzburg, Germany)
Microslides	Marienfeld (Lauda-Königshofen, Germany)
Mounting medium with DAPI	SouthernBiotech (Birmingham, USA)
Parafilm M	Bemis (Neenah, USA)
Pipette tips	Sarstedt (Nümbrecht, Germany)
PVDF membrane	Roth (Karlsruhe, Germany)
Syringes	Braun (Melsungen, Germany)
Syringe filters	Thermo Scientific (Waltham, USA)
X-ray film	Super RX, FUJIFILM (Tokyo, Japan)

4.1.3 Chemical agents and buffers

Chemicals were obtained from AppliChem (Darmstadt), Biomol (Hamburg), Biozym (Hessisch Oldendorf), Fermentas (St. Leon-Roth), Fluka (Neu-Ulm), GE Healthcare (Freiburg), Invitrogen (Karsruhe), JIR Dianova (Hamburg), Merck (Darmstadt), Perkin Elmer (Waltham, USA), Roche (Mannheim), Roth (Karlsruhe) and Sigma (München).
Culture media for bacteria and PBS were autoclaved 20 min at 120 °C and 1.2 bar. Solutions of antibiotics were filtrated using 0.22 µm filters.

4.1.4 Antibiotics

Table 4.4: Antibiotics

Antibiotic	Dissolved in	Working concentration	Source
Ampicillin	H_2O	100 µg/mL	Sigma (#A9518)
Chloramphenicol	70 % EtOH	20 µg/mL	ROTH (#3886.1)
Gentamicin	H_2O	100 µg/mL	SERVA (#22185.02)
Kanamycin	H_2O	50 µg/mL	ROTH (#T832.2)
Nalidixic acid	1M NaOH	60 µg/mL	Sigma (#N8878)

4.1.5 Inhibitors, stimulators, cell stains

Table 4.5: Inhibitors and stimulators

Inhibitors and stimulators			
Name	Dissolved in	Working concentration	Source
Chloroquine	H_2O	75 µM	Sigma (#C6628)
Rapamycin	DMSO	20 µM	LC Laboratories (#R-5000)
Torin 1	DMSO	250 nM	TOCRIS (#4247)

Table 4.6: Dyes

Dye	Working concentration	Source
LysoTracker Red DND-99	75 nM	Life Technologies (#L-7528)
DQ-Red BSA	5 nM	Life Technologies (#D12051)
Trypan Blue	0.35 %	Sigma (#93595)

4.1.6 Plasmids

Table 4.7: Prokaryotic and eukaryotic expression plasmids

Prokaryotic expression		
Name	Expressed protein	Reference
pLAC-RFP	Red fluorescent protein	Oellerich et al., 2007
Eukaryotic expression		
Name	Expressed protein	Reference
CathepsinD-RFP	Cathepsin D (human) with RFP	Yuseff et al., 2011
EGFP-LC3	LC3 (mouse) with EGFP	Martens et al., 2005
h-EGFP-LC3	LC3 (human) with EGFP	Rabinovitch et al., 2005 (Addgene #11546)
mCherry-LC3	LC3 (mouse) with mCherry	Provided by Lena Novikova
ptf-LC3	LC3 (rat) with EGFP and mRFP	Kimura et al., 2007 (Addgene #21074)
GFP-NDP52	NDP52 (human) with GFP	Morriswood et al., 2007
GFP-Ub	Ubiquitin C (human)with EGFP	Dantuma et al., 2006 (Addgene #11928)
YFP-LAMP-1	LAMP-1 (human) with YFP	Henry et al., 2006

4.1.7 Culture media

Table 4.8: Culture media for mammalian cells

Cell culture media and additives for mammalian cells		
Name	Details	Source
Media		
DMEM	Dulbecco's Modified Eagle Medium + GlutaMAX	Gibco (#31966-021)
DMEM	Dulbecco's Modified Eagle Medium	Gibco (#11880-028)
Ham's F12	Nutrient mixture F-12	Gibco (#21765-029)
OptiMEM	Reduced serum medium	Gibco (#11140-050)
Media additives		
Dexamethasone	Prepared in 95 % EtOH	D8893 (#S8893)

EGF	Epidermal Growth Factor from murine submaxillary gland. Prepared in H_2O	Sigma (#E4127)
FBS	Fetal Bovine Serum, heat inactivated	Gibco (#10500)
Glucose	D-(+)-Glucose solution, 100 g/L in H_2O, sterile	Sigma (#G8644)
L-Glutamine	200mM	Gibco (#25030-024)
HEPES	HEPES Buffer, 1 M, pH 7.0-7.6	Gibco (#15630-056)
Insulin	Insulin from bovine pancreas, ≥25 units/mg. Prepared in H_2O, 1 % acetic acid	Sigma (#I1882)
MEM NEAA	MEM non-essential amino acids (100X)	Gibco (#11140)
Selenium	Prepared in H_2O	Sigma (#9133)
Transferrin	apo-Transferrin bovine, prepared in H_2O	Sigma (#T1428)
Triiodothyronine	3,3',5-Triiodo-L-thyronine sodium salt. Prepared in 95 % EtOH	Sigma (#T5516)
Other solutions		
DPBS	Dulbecco's Phosphate Buffered Saline (without Ca^{2+}, Mg^{2+})	Gibco (#14190-094)
Trypsin	025 % trypsin, EDTA 1X	Gibco (#25200-056)

Table 4.9: Culture media for bacteria

Culture media for bacteria		
Name	Details	Source
2xYT	Broth for cultivating recombinant strains of *E. coli*	Roth (#X966.1)
CIN Agar	CIN Agar Base, Modified	Difco (#218172)
LB Broth	Luria-Bertani Broth	Roth (#X968.2)
LB Agar	Luria-Bertani Agar	Roth (#X969.2)
SOC	SOC Outgrowth Medium	New England BioLabs (#B9020S)

4.1.8 Kits

Table 4.10: Kits

Name	Source
Cytotoxicity Detection KitPLUS	Roche (#04744926001)
FemtoLUCENT PLUS-HRP	G-Biosciences (#786-10)
Lipofectamine LTX Reagent with PLUS Reagent	Life Technologies (#15338100)
Nuclobond PC 100	Macherey Nagel (#740573)

4.1.9 Antibodies

Table 4.11: Primary and secondary antibodies

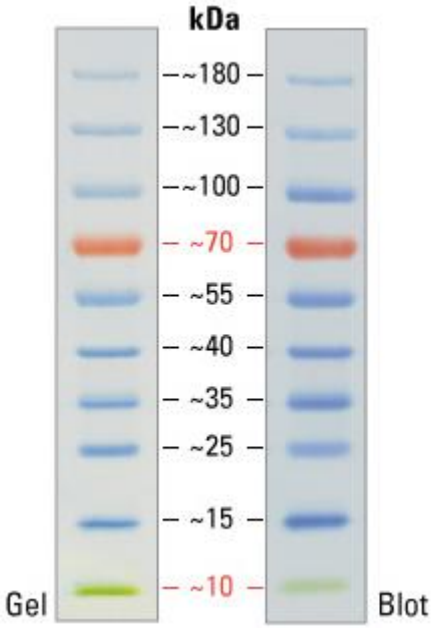
Specificity				
Primary antibodies	Host species	Dilution	Application	Source
Actin	Mouse	1:2000	WB	Millipore (#MAB1501)
LC3B	Rabbit	1:2000	WB	Cell Signaling (#2775)
LC3	Rabbit	1:200	IF	Novus Biologicals (#NB100-2220)
LAMP-1	Mouse	1:200	IF	DSHB (#H4B4)
P62/SQSTM1	Rabbit	1:1000	IF	Sigma-Aldrich (#P0067)
Phospho-p70 S6 Kinase (Thr389)	Rabbit	1:1000	WB	Cell Signaling (#9234)
p70 S6 Kinase	Rabbit	1:1000	WB	Cell Signaling (#9202)
v-ATPase C1	Rabbit	1:100	IF	Santa Cruz (#sc-20944)
Secondary antibodies				
Alexa Fluor 568 anti-rabbit IgG	Goat	1:1000	IF	Invitrogen (#A11011)
Peroxidase-conjugated anti-rabbit IgG	Goat	1:20000	WB	Dianova (#111-035-003)
Peroxidase-conjugated anti-	Goat	1:20000	WB	Dianova (#111-035-003)

mouse IgG				

4.1.10 Protein ladder

PageRuler Plus Prestained Protein Ladder

Thermo Scientific (#26619)



4.1.11 Bacterial strains

Table 4.12: Bacterial strains

Bacterial strains	Description	Reference
Y. enterocolitica		
WA	Wild-type WA-314, biogroup 1B, serogroup O:8, Nal^R	Heesemann & Laufs, 1983
WA-C	Virulence plasmid-cured derivate of WA, Nal^R	Heesemann & Laufs, 1983
RFP-WAC	WA-C transformed with pLAC-RFP, Nal^R, $Chlor^R$	Deuretzbacher et al., 2009
NF-O	Clinical nosocomial outbreak isolate, biogroup 1A, serogroup O:5	Ratnam et al., 1982
IP2222	Non-clinical environmental isolate, biogroup 1A, serogroup O:36	Grant et al., 1999
NF-O-*inv*$^+$	NF-O harbouring pInv1914, Amp^R	this study
IP2222-*inv*$^+$	IP2222 harbouring pInv1914, Amp^R	this study

E. coli		
E. coli-inv^{+}	HB101 harbouring pInv1914, Amp^{R}	Schulte et al., 1998
NEB 10-beta	DH10B^{TM} derivative	New England BioLabs (#C3019)

4.1.12 Cell lines

Table 4.13: Cell lines

Name	Description	Culture conditions	Reference
HeLa	Epithelial cell line from human adenocarcinoma	DMEM, 10 % FBS, 1 % MEM Non-Essential Amino Acids	Scherer et al., 1953
mIC_{cl2}	Epithelial cell line from murine intestine	50 % Ham's F12, 50% DMEM, 2 % FBS, 2 mM Glutamine, 20 mM HEPES, 5 µg/mL Insulin, 10 ng/mL EGF, $1x10^{-9}$M Triiodothryronine, 5 µg/mL Transferrin, 60 nM Selenium, $5x10^{-8}$ M Dexamethasone, 2.24 % D-Glucose (10 % solution)	Bens et al., 1996
MEF WT	Wild-type murine embryonic fibroblasts	DMEM, 10 % FBS, 1 % MEM Non-Essential Amino Acids	Mizushima et al., 2001
MEF $Atg5^{-/-}$	Murine embryonic fibroblasts knock-out for a*tg5*	DMEM, 10 % FBS, 1 % MEM Non-Essential Amino Acids	Mizushima et al., 2001

4.1.13 Software

Images were analyzed with Volocity Version 6.0 software (Perkin Elmer).

Statistical analyses were performed in GraphPad Prism 5 using unpaired Student's t-test.

4.2 Methods

All experiments performed in this study were done in safety level 2 (S2) laboratories.

4.2.1 Molecular and cell biological methods

4.2.1.1 Cultivation of bacteria

4.2.1.1.1 E. *coli*

NEB-10 beta or *E. coli-inv*$^{+}$ were cultivated overnight at 37 °C under aerobic conditions either in liquid (120 rpm) or on solid LB-medium. Antibiotics were added according to the particular plasmids that should be expressed (Table 4.4).

4.2.1.1.2 Y. *enterocolitica*

Y. enterocolitica strains were grown overnight at 27 °C under aerobic conditions either in liquid (120 rpm) or on solid LB-medium with selective antibiotics (Table 4.12).

4.2.1.2 Preparation of electrocompetent *Y. enterocolitica*

The preparation of electrocompetent bacteria was modified from Conchas & Carniel (Conchas & Carniel 1990). A 10 mL starter culture was inoculated with a colony of *Y. entercolitica* (strains NF-O and IP2222) and grown overnight at 27 °C in LB broth (120 rpm) under aerobic conditions. 2 mL of the overnight culture was used to further inoculate 100 mL LB broth which was incubated for 2 h 30 min. The bacteria were pelleted (4000 g, 15 min, 4 °C) and the pellet was resuspendend in 20 mL of ice-cold sterile water and incubated on ice for 1 h 30 min. The bacteria were pelleted again and washed twice with ice-cold 10 % glycerol in sterile water (20 mL volume). The final pellet was carefully resuspended in 600 µL of ice-cold 10 % glycerol in sterile water. Aliquots of 60 µL were frozen in dry ice and stored at -80 °C.

4.2.1.3 Transformation of electrocompetent *Y. enterocolitica*

An aliquot of competent bacteria was thawed on ice and 1 µL of plasmid DNA (~0.5 gµ/µL) was added. The cell suspension was loaded into a chilled 1 mm cuvette. The pulse was triggered immediately according to the following parameters:

Voltage 2.5 kV

Resistance 200 Ω

Capacitor 25 µF

As soon as possible the cells were resuspended in 1 mL of SOC medium and transferred into an eppendorf tube. After incubating 2 h at 37 °C and 300 rpm, transformed bacteria were plated on LB agar dishes containing 600 µg/mL of ampicillin to select the positive clones.

4.2.1.4 Isolation of plasmid DNA

Plasmid DNA was isolated from 50 mL bacterial cultures expressing the plasmids, using the Nucleobond PC 100 Kit according to the manufacturer's instructions.

4.2.1.5 Preparation of chemically competent bacteria *E. coli*

A 20 mL starter culture was inoculated with one colony of *E. coli* and grown overnight at 37 °C. 2 mL of the overnight culture was used to further inoculate 200 mL of LB medium with 0.8 mL $MgSO_4$ 1 M and 2 mL of KCl 1 M, which was incubated until the culture reached an OD_{600} of 0.3- 0.5. The culture was cooled down on ice for 15 min, the bacteria were pelleted (3000 rpm, 5 min, 4 °C) and the pellet was resuspended in 15 mL of Tfb1 and incubated on ice for 1 h 30 min. After pelleting the bacteria again (3000 rpm, 5 min, 4 °C), the supernatant was removed, 8 mL Tfb2 were added and bacteria were carefully resuspended by turning the tubes on ice. Aliquots of 50-100 µl were snap frozen in liquid nitrogen and stored at -80 °C.

Table 4.14: Tfb1 and Tfb2 buffers

1X Tfb1 buffer, pH 5.8	
22.5 mL	Glycerol
1.5 mL	$CaCl_2$, 1 M
0.441 g	KOAc
1,814 g	$RbCl_2$
1,485 g	$MnCl_2$
Up to 150 mL with ddH_2O	

1X Tfb2 buffer	
3.75 mL	Glycerol
1875 mL	$CaCl_2$, 1 M
250 µL	MOPS, 1M
0.03 g	$RbCl_2$
Up to 25 mL with ddH_2O	

4.2.1.6 Transformation of chemically competent *E. coli*

One aliquot of competent bacteria was thawed on ice for 10 min and 1-100 ng of plasmid DNA were added. After incubating 30 min on ice, a heat shock was performed at 42 °C for 30 sec to introduce the DNA into the bacterial cells. After an incubation period of 5 min on ice, 1 mL of SOC medium was added. Transformed bacteria were incubated at 37 °C for 60 min with shaking at 300 rpm and then plated on LB agar dishes containing the antibiotics corresponding to the transfected plasmid to select for positive clones.

4.2.1.7 Cell culture

All eukaryotic cells were cultured in incubators at 37 °C, 5 % CO_2 and 90 % humidity.
HeLa and MEF cells were passaged every two days. mIC_{cl2} cells were passaged only once per week. For passaging, cells were washed with DPBS and trypsinized for 5 min at 37 °C. Then, cells were divided in a proportion 1:10 in 10 cm dishes with fresh culture media to keep a working stock of cells.

35 mm and 24-well formats were used to perform experiments with bacteria. Cell suspensions were prepared as described above and the number of cells was counted using trypan blue and a Neubauer chamber. 2-5x10^4 cells (for 24-well format) or 2x10^5 cells (for 35 mm format, glass bottom) were seeded onto the wells and incubated 24 h prior to transfection or infection.

4.2.1.8 Freezing and thawing of cells

4.2.1.8.1 Freezing of cells

A confluent monolayer of cells on a 10 cm culture dish was trypsinized and the cells were harvested by centrifugation (5 min at 200 g). The cell pellet was resuspended in 1.6 mL of freezing medium (FBS containing 10% DMSO) and transferred into a sterile cryo-vial into a freezing container (Mr. Frosty) in order to be frozen at -80 °C.

4.2.1.8.2 Thawing of cells

The frozen vial was removed from the -80 °C freezer and transferred to a 37 °C water bath for thawing (1-2 min). 10 mL of fresh medium was added to the thawed cells and they were directly plated onto a culture dish.

4.2.1.9 Cell transfection

4.2.1.9.1 HeLa and MEF cells

One day before transfection, $5x10^4$ cells in 500 µL of growth medium were seeded on cover slips in 24-well-plates and incubated for 24 h. Then, the medium was removed and replaced with new medium and the transfection complexes were prepared for a 24-well format, as follows: 300 ng of plasmid DNA were diluted in 30 µL of OptiMEM and mixed thoroughly; 2.3 µL of PEI solution was added to the diluted DNA and the final mix was incubated for 15 min at room temperature to allow the formation of the transfection complexes. 30 µL of the DNA-PEI complexes were added dropwise to the cells in the well. The plate was incubated 24 h at 37 °C in the CO_2 incubator prior to infection.

Preparation of PEI for transfection: 1 mg of Polyethylenimine (PEI) was dissolved per 1 mL of high purity water previously adjusted to pH 2 with HCl. The solution was stirred for 2-3 h until PEI was completely dissolved. NaOH was added to adjust the pH to 7.0. The solution was filtered through a 0.22 µm filter and aliquots were frozen at -80 °C.

4.2.1.9.2 mIC_{cl2}

mIC_{cl2} were transfected using Lipofectamine LTX and PLUS Reagents (Invitrogen), according to the manufacturer's instructions, using 750 ng DNA, 1.125 µL of Lipofectamine LTX and 0.375 µL of PLUS Reagent for a 24-well format.
A list of the plasmids used for transfection is found in Table 4.7.

4.2.1.10 Infection of cells

One day before infection, cells were seeded into wells or transfected with DNA, and bacteria were cultivated in liquid LB medium as described in 4.2.1.1.2. At the day of infection, the medium of the cells was replaced by 200 µL of fresh medium (in the case of a 24-well format) or 1 mL (35 mm format, glass bottom), and the bacteria were harvested by centrifugation (3000 g, 5 min, 4 °C). The pellet was resuspended in sterile PBS. The Optical Density (OD) of a 1:10 dilution of the bacterial suspension was measured at 600 nm (OD_{600}) with a spectophotometer and adjusted to 0.33. 5 µL of the bacterial suspension (or 1 µL only in the case of microscopical studies) were added to each well of a 24-well format (2 µL for all experiments in 35 mm format, glass bottom) to add the bacteria at a multiplicity of infection (MOI) of 500 per cell (or 100 in the case of microscopical studies). The plate or dish was then gently rocked back and forth to spread bacteria equally over the cells. After 1 h of infection, the wells were washed 3 times and incubated with 100 µg/mL of gentamicin in fresh medium to prevent bacterial overgrowth of the cells. The experiment was then stopped at the desired time point after infection.

4.2.1.11 Chemical treatments

To block the autophagic flux or to induce autophagy, chloroquine (75 µM) or rapamycin (20 µM) were added to the cells for at least 4 h. The cells were seeded one day before the chemical treatment.

4.2.1.12 Assay for cell death analysis

Lactate dehydrogenase (LDH) is an intracellular protein which is released from cells undergoing cell death and lysis. LDH activity released from the cytosol of damaged cells can be measured in order to quantify cytotoxicity by a non-radioactive colorimetric assay (Weiss & Zychlinsky 2002).

After 4 and 24 h of infection, 100 µL samples of supernatant were removed from the wells and added to a new 96-well plate. The activity of LDH in the samples (triplicate) was measured using the Cytotoxicity Detection KitPLUS (Roche) according to the manufacturers' instructions. The data were analyzed using Graph Pad Prism 5.0 (Graph Pad Software).

4.2.1.13 Bacterial killing assay

The survival of *Y. enterocolitica* and *E. coli-inv*$^{+}$ within epithelial and MEF cells was measured using the gentamicin protection assay. $5x10^4$ cells were seeded per well (24-well format) and infected 24 h later with 5 µL of bacteria in 200 µL of fresh medium for 30 min to allow bacterial invasion. Then, a set of wells was washed 3 times with medium to remove any non-adherent bacteria and the cells were subsequently lysed in 1 mL of 1 % Triton X, 0.1 % SDS in PBS, and serial dilutions of the suspension were plated onto agar dishes with antibiotics to determine the number of cell associated bacteria (t_{30min}, in CFU). The remaining wells were washed as described above to remove the non-adherent bacteria, and fresh culture medium containing 100 µg/mL of gentamicin was added to each well. Gentamicin cannot enter mammalian cells, so it killed only extracellular bacteria and not bacteria that have already been taken up by the cells. The cells were incubated with the gentamicin-containing medium for 4 ($t_{4h30min}$) and 8 h ($t_{8h30min}$). Then, the medium was removed, and the cells were washed and lysed as described above. Serial dilutions were plated to determine the number of intracellular bacteria (gentamicin-resistant CFU). Dishes were incubated for 48 h and the numbers of colonies were counted. Each set of samples contained 3 wells. The data was analyzed using Graph Pad Prism 5.0 (Graph Pad Software).

4.2.1.14 Bacterial release assay

To determine the number of Y. *enterocolitica* bacteria that were released from wild-type and Atg5$^{-/-}$ MEF cells, 5x10^4 cells were seeded per well (24-well format) and infected 24 hours later with 5 µL of bacteria in 200 µL of fresh medium at a MOI of 500 for 30 min to allow bacterial invasion. Then, the wells were washed 3 times with medium to remove any non-adherent bacteria and 500 µL of fresh culture medium containing 100 µg/mL of gentamicin was added to each well. Cells were incubated for another 1 h (37 °C, 5 % CO_2) and after that were washed 3 times with medium to remove the antibiotics. Fresh culture medium without antibiotics was added to the wells. One hour later, the medium of the wells was transferred to eppendorf tubes and replaced with fresh medium in the wells. Serial dilutions in the medium of the eppendorf tubes were plated onto agar dishes with antibiotics to determine the numbers of bacteria that were released to the medium. This procedure was repeated every hour up to 8 h 30 min post infection. Dishes were incubated for 48 h and the numbers of colonies were counted. The data were analyzed using Graph Pad Prism 5.0 (Graph Pad Software)

4.2.1.15 Fluorescence microscopy methods

4.2.1.15.1 Immunostaining

To visualize proteins in fixed cells using fluorescence microscopy, cells were subjected to immunostaining. At the indicated time points after infection, the culture medium was removed, the cells were washed three times with DPBS (with Ca^{2+}, and Mg^{2+}) and the cover slips were fixed with formaldehyde 3.7 % for 15 min at room temperature. Afterwards, cells were washed three times with PBS and permeabilized with ice-cold methanol for 10 min at -20 °C. Then, cells were washed three times and re-hydrated with PBS for 10 min. Subsequently, 2 % BSA / PBS was added for 30 min to block free epitopes. Primary antibodies were diluted in 2 % BSA / PBS. Cover slips were incubated with 30 µL of primary antibody solution inside a humid chamber for one hour at room temperature. After incubation, the cover slips were washed three times with PBS / 0.05 % Tween-20 (PBST) for 5 min each time. Dilutions of secondary antibodies were

prepared in 2 % BSA / PBS. Cover slips were then incubated for 1 h at room temperature in a humid chamber in the dark. After that, they were washed three times with PBST for 5 min each time. After the last wash, excess of liquid was removed, 5 µL of DAPI Fluoromount-G mounting medium was added to the slide and cover slips were placed with cells facing down. Slides were dried at room temperature for 24 h before imaging.

4.2.1.15.2 Staining of acidic compartments

For analysis of lysosomal markers, the medium of infected (for LysoTracker) or not yet infected cells (for DQ-BSA) was replaced by fresh medium with either LysoTracker Red or DQ-BSA. After the indicated incubation time (Table 4.15), cells were washed three times with culture medium and fixed with formaldehyde 3.7 % for 15 min at room temperature. Samples were immediately analyzed by confocal microscopy.

Table 4.15: Dyes for staining of acidic compartments

Dye	Working concentration	Time	Temperature
LysoTracker Red DND-99	75 nM	30 min	37 °C
DQ-Red BSA	5 nM	Overnight	37 °C

4.2.1.15.3 Life-cell imaging

For time lapse imaging of GFP-LC3, $2x10^5$ of HeLa cells were seeded in 1 mL of medium on a 35 mm glass bottom dish. At the following day, cells were transfected for the expression of GFP-LC3. One day later, the medium of the dish was replaced by 0.5 mL of fresh medium and the cells were infected with 2 µL of WAC labelled for the expression of red fluorescent protein (RFP) at a MOI of 50. One hour post infection, cells were washed three times and incubated with 100 µg/mL gentamicin for another hour. Cells were directly imaged using a spinning disk system. Images of the red and green fluorescence channels were taken every 5 min with an inverted Zeiss Axiovert 200M microscope with a temperature- and CO_2- controllable environmental chamber,

an oil immersion 63X objective, and a CCD camera. Acquisition and processing of images was performed with Volocity Software (Improvision).

4.2.2 Biochemical methods

4.2.2.1 Preparation of cell lysates

For the protein biochemical analysis of treated and infected cells, cell lysates were prepared using RIPA lysis buffer (Table 4.16).

The culture medium was removed from the wells and the plate was store on ice. Immediately, 40 µL of ice-cold RIPA lysis buffer was added to each well (24-well format) and the plate was incubated on ice for 10 min with rocking. Then, the cells were scraped vigorously using plastic pipette tips (100/200 µL) and the lysates were transferred to a fresh eppendorf tube and store on ice in order to continue with the next step (4.2.2.2).

Table 4.16: RIPA lysis buffer

1X RIPA lysis buffer	
2.5 mL	Tris-HCl pH 8, 1 M
1.5 mL	NaCl, 5 M
5 mL	NP-40, 10 %
0.5 mL	SDS, 10 %
2.5 mL	DOC, 10 %
0.1 mL	EDTA, 0.5 M
0.05 mL	EGTA, 1 M
1 tablet	cOmplete (protease inhibitor cocktail)
Up to 50mL with ddH_2O	

4.2.2.2 SDS-Page

Cell lysates for SDS-PAGE were mixed with 4X SDS loading buffer (Table 4.17), 10 min at 95 °C and centrifuged 30 min at 14000 rpm, 4 °C (at this point, samples might have been frozen at -80 °C). The samples were loaded on polyacrylamide mini gels. Gels were run at 150 V during stacking and separation. The PageRuler Plus Prestained

Protein Ladder (Thermo Scientific) was used to monitor the progress of electrophoresis.

Table 4.17: SDS sample buffer

4X SDS sample buffer (10mL)	
1.2 g	SDS
4.7 mL	Glycerol
2 mL	TrisHCl pH 7, 1M
0.4 mg	Bromophenol blue
0.5 mL	2-β mercaptoethanol
Up to 10 mL with ddH_2O	

Table 4.18: Buffers for preparing SDS-gels

Name	Description	Source
Resolving gel buffer	1.5 M Tris-HCl buffer, pH 8.8	Biorad (#161-0798)
Stacking gel buffer	0.5 M Tris-HCl buffer, pH 6.8	Biorad (#161-0799)

Table 4.19: Running buffer

1X Running buffer pH 8.3	
3 g	Tris base
14.4 g	Glycine
1 g	SDS
Up to 1 L with ddH_2O	

4.2.2.3 Western blot and immunodetection

Proteins separated by SDS-PAGE (section 4.2.2.2) were transferred onto a PVDF (0.2 mm) membrane by electrophoretic transfer using a semi-dry Western Blot system. From bottom (anode) to top (cathode), we stacked two pieces of filter papers soaked in Transfer Buffer A, followed by a piece of filter paper soaked in Transfer Buffer B, a piece of methanol-activated PVDF membrane, the SDS gel placed directly on top of it,

and finally three pieces of filter paper soaked in Transfer Buffer C. Transfer of proteins was performed at 5.7 mA/cm^2 for 15 min.

Table 4.20: Buffers for semi-dry western blot

1X Buffer A pH 10.4	
30 mL	Tris 1M, pH 10.4
10 mL	Methanol
Up to 100 mL with ddH_2O	

1X Buffer B pH 10.4	
2.5 mL	Tris 1M, pH 10.4
10 mL	Methanol
Up to 100 mL with ddH_2O	

1X Buffer C pH 9.4	
2.5 mL	Tris 1M, pH 10.4
0.26 g	6-aminohexanoic acid
10 mL	Methanol
Up to 100 mL with ddH_2O	

Subsequently, the membrane was blocked with PBST or TBST 5 % fat-free milk powder or BSA (depending on the primary antibody) for 1 h at room temperature. The membrane was then incubated with the primary antibody in blocking solution overnight at 4 °C. After washing three times with PBST or TBST for 15 min each, the membrane was incubated with the secondary HRP (horseradish peroxidase)-coupled antibody in PBST for 1 h followed by three washing steps with PBST. Detection solution was added to the membrane according to manufacturer's instructions (SuperSignal West Femto, Thermo Scientific). Emitted light was detected by exposing the membrane to an X-ray film which was developed in a X-ray film-processor (Agfa Curix 60).

Table 4.21: Buffers for dilution of antibodies

1X PBS-0.05 % Tween	
0.2 g	KCl
0.2 g	KH_2PO4
8 g	NaCl
1.1 g	Na_2HPO4
0.5 mL	Tween20
Up to 1 L with ddH_2O	

1X TBS-0.05 % Tween, pH 7.6	
8 g	NaCl
2.4g	Trizma HCl
0.5mL	Tween20
Up to 1L with ddH_2O	

4.2.3 *In vivo* studies

4.2.3.1 Mice

C57BL/6 wild-type mice, $Atg5^{fl/fl}$ (Hara et al. 2006) and $Atg5^{\Delta IEC}$ mice (Benjamin et al. 2013) were kindly provided by the Dr. Hooper Laboratory, University of Dallas Southwestern Medical Center (UTSW), USA. 8-12 week old mice were used for all experiments. Experiments were performed using protocols approved by the Institutional Animal Care and Use Committees of the UT Southwestern Medical Center.

4.2.3.2 Mouse infection

Mice were infected intragastrically by gavage with $1x10^{10}$ WAC-RFP in a 200 µL volume. Mice were not subjected to fasting before infection. The dose actually administrated was determined by plating serial dilutions on LB agar dishes for 48 h and counting CFU.

4.2.3.3 Colonization and dissemination assays

Mice were sacrificed by inhalation of isoflurane (Isothesia) and material from the lumen of the small intestine, liver and spleen were removed, weighed and homogenized in 1 mL (material from small intestine) or 5 mL (liver and spleen) of PBS, respectively. To determine the numbers of CFU per organ, serial dilutions of homogenates were plated on *Yersinia* CIN agar.

5 FIGURES

6 TABLES

7 REFERENCES

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ACKNOWLEDGEMENTS

I want to thank my supervisor Dr. Klaus Ruckdeschel for the opportunity to work in his laboratory, for his patience and support, and for introducing me to the interesting world of host-pathogen interaction.

I would also like to thank Prof. Dr. Aepfelbacher for accepting me as a member of the Institute for Microbiology and for giving me the opportunity, together with Prof. Dr. Braulke, to be part of the graduate school GRK 1459.

Thanks also to Dr. Pommerening-Röser for being the second reviewer of the thesis and the constant feedback and support along these years.

I also appreciate the support that I have received from the actual and former members of our lab: Julia, Rike, Manja, Claudia, Melanie, Rosa, Lena and Nicole. And additionally thanks to all the members from other groups at the Institute for Microbiology for the good times inside and outside work. Specially thanks to Claudia Trasak for taking lot of care about my questions and for help, and also to the UKE Microscopy Imaging Facility for their pieces of advice. At this point, I want to explicitely thank Clemens, Xenia and Liane for all the tricks they gave to me, the support and the great companionship during our graduate school days.

Finally, I want to thank my family and friends. Especialmente a mi mamá y mi abuela que me apoyaron todos estos años de forma incondicional. Las quiero mucho y les debo todo lo que tengo y lo que soy. Gracias también a mis amigos de Uruguay y del mundo: Ale, Mari, Clau, Rossotti de mi corazón, Pipi, Rodrigo, Sonia, Majo, Xarly, Loreto, Moni (mención especial, sin vos la autofagia no hubiese sido lo mismo!), Sureka (you saved my life darling!), Pao, Flor, Xime, Jime, Lauri, Vivi, Rati, Gonza, Diego, Marina y Caro. Y muy especialmente a German y Paola (en orden alfabetico para que no se peleen!) por todas las terapias vocacionales y no vocacioneles y a Shovy y Erikin por ser mi familia hamburguesa inolvidable :)

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

María José Valencia López

Hamburg, den 27.04.2015