Interactions of *L. monocytogenes* with Host Cellular Defenses

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Sciences
University of Toronto

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Abstract

*Listeria monocytogenes* is an intracellular bacterium that utilizes two phospholipases C (PLCs) and a pore-forming cytolysin (listeriolysin O, LLO) to escape the phagosome. However, prior to escape, the bacterium must overcome a number of phagosomal defenses, including autophagy and NOX2 NADPH oxidase production of reactive oxygen species (ROS).

Autophagy, the cellular process of self-digestion, is a key component of innate immunity. Previously, it has been shown that *L. monocytogenes* is targeted by autophagy (LC3\(^+\)) at 1 h post infection (p.i.) but the mechanism remains elusive. Here, I show that at 1 h p.i., diacylglycerol (DAG) and ROS production are required for autophagy targeting to the bacteria, which are predominantly in phagosomes. It has been shown that autophagy targeting of cytosolic *L. monocytogenes* is mediated via protein ubiquitination. However, protein ubiquitination is not associated with LC3\(^+\) bacteria at 1 h p.i.. Thus, my data suggest that distinct signals mediate autophagy targeting of *L. monocytogenes* depending on the location within host cells.
Given that ROS mediate autophagy targeting to *L. monocytogenes* and that previous studies have demonstrated that ROS production limits bacterial escape, I investigated how *L. monocytogenes* overcomes ROS production prior to phagosomal escape. I found that LLO inhibits ROS production by preventing NOX2 NADPH oxidase localization to *L. monocytogenes*-containing phagosomes. LLO-deficient bacteria can be complemented by perfringolysin O, a related cytolyisin, suggesting that other pathogens may also use pore-forming cytolsins to inhibit ROS production. While PLCs can activate ROS production, this effect is alleviated by LLO pore-formation. Therefore, the combined activities of PLCs and LLO allow *L. monocytogenes* to efficiently escape the phagosome while avoiding microbicidal ROS.

Together, this thesis provides a clearer understanding of the balance between host defense versus bacterial evasion. Greater insight into host-bacterial interaction may lead to better therapeutics that can “tip the balance” in the host’s favour.
Acknowledgments

I must first express my deepest gratitude for my supervisor, Dr. John H. Brumell, for his guidance, support and patience throughout the course of my graduate studies. I am particularly thankful for the scientific freedom I was given to pursue my own theories and hypotheses. I am also grateful to the members of my supervisory committee, Drs. Scott Gray-Owen, Nicola Jones and Philip Sherman, for their support, criticism and suggestions, pushing me beyond the boundaries of what I thought I can achieve.

This thesis would not have been possible without the input and support of all past and current Brumell Lab members. I am particularly grateful to Dr. Malina Bakowski for showing me the ropes of the lab when I first arrived, Dr. Cheryl Birmingham for her hard work on the L. monocytogenes project I was to inherit, and Dr. Ju Huang for providing both emotional and scientific advice, insight and support. I am thankful for Veronica Canadien who ensures the smooth operation of the lab. I would also like to thank Michelle Ang and Dr. Ramzi Fattouh, my lab husband, for their help with the FACS experiments and Dr. Darren Higgins from Harvard Medical School for his scientific advice and tutelage on performing in vivo L. monocytogenes infections in mice. Furthermore, I wish to express my gratitude to the staff at the SickKids imaging facility, the Mt. Sinai Hospital TEM facility as well as SIDNET at SickKids. I would also like to thank the members of the Grinstein, Jones, Klip, Robinson and Trimble laboratories for the daily laughs, reagents, and equipment usage.

Finally, I would like to thank my parents, Raymond and Elaine, for their enduring love, endless patience and unquestioning (monetary and emotional) support during my lengthy educational journey. Lastly, I must thank Dr. Mathew Estey for his insightful scientific advice (especially while on Greyhound buses), patience, emotional support, and unconditional love. You are the North star to my wandering bark; the light during the good times and the bad.
Contributions

The majority the work presented in this thesis was conducted by me. Experiments were conceived by Dr. John Brumell and myself with advice from my supervisory committee, which comprises of Drs. Scott Gray-Owen, Nicola Jones and Phil Sherman. Further advice on the direction and development of my projects was given by Drs. Sergio Grinstein, Aleixo M. Muise and Darren E. Higgins. Dr. Ramzi Fattouh helped perform the flow cytometric assays in Chapter 20 with technical assistance from Michelle Ang and David Rizutti. Mike Woodside and Paul Paroutis provided technical support for confocal microscopy. Post fixation processing of samples for transmission microscopy (TEM) in Chapter 20 was conducted by Bob Tempkin at the Mt. Sinai TEM facility.

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>APF</td>
<td>2-[(6-(4’-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid</td>
</tr>
<tr>
<td>Atg</td>
<td>Autophagy-related genes</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain-Heart Infusion</td>
</tr>
<tr>
<td>BKGD</td>
<td>Background</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophages</td>
</tr>
<tr>
<td>BMDN</td>
<td>Bone marrow derived neutrophils</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CBD-YFP</td>
<td>Cell wall binding domain – yellow fluorescent protein</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s Disease</td>
</tr>
<tr>
<td>CDC</td>
<td>Cholesterol dependent cytolysins</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CLEM</td>
<td>Correlative light microscopy-electron microscopy</td>
</tr>
<tr>
<td>CM-H₂DCFDA</td>
<td>5-(and-6)-choloromethyl-2-7-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone-mediated autophagy</td>
</tr>
<tr>
<td>CPS</td>
<td>Counts per second</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCF</td>
<td>2’-7’-dichlorofluorescein</td>
</tr>
<tr>
<td>DHC</td>
<td>Dihydrocalcein</td>
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<td>DHE</td>
<td>Dihydroethidium</td>
</tr>
<tr>
<td>DHR</td>
<td>Dihydrorhodamine 123</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyliodonium</td>
</tr>
<tr>
<td>EEA-1</td>
<td>Early endosomal autoantigen-1</td>
</tr>
<tr>
<td>Ena/VASP</td>
<td>Enabled/vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular regulated kinase 1/2</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc-gamma-receptor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GILT</td>
<td>γ-interferon inducible lysosomal thiolreductase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HPF</td>
<td>2-[(6′-hydroxy) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Inl</td>
<td>Internalin</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 3,4, 5 triphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-11-thiogalactopyranoside</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-associated membrane proteins</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated protein light chain 3</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>50% lethal dose</td>
</tr>
<tr>
<td>LLO</td>
<td>Listeriolysin O</td>
</tr>
<tr>
<td>LLR</td>
<td>Leucine-rich repeats</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NBR1</td>
<td>Neighbor of BRCA1 gene 1</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium chloride</td>
</tr>
<tr>
<td>Ndk</td>
<td>Nucleoside diphosphate kinase</td>
</tr>
<tr>
<td>NDP52</td>
<td>Nuclear dot protein 52 kDa</td>
</tr>
<tr>
<td>NETs</td>
<td>Neutrophil extracellular traps</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa-B</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post infection</td>
</tr>
<tr>
<td>Pak</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PAS</td>
<td>Phagophore assembly site</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC-PLC</td>
<td>Phosphatidylcholine specific</td>
</tr>
<tr>
<td>PAP</td>
<td>Phosphatidic acid phosphatase</td>
</tr>
<tr>
<td>PBD</td>
<td>Pak-binding domain</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>Phox</td>
<td>Phagocytic oxidase</td>
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<td>PI</td>
<td>Phosphatidylinositol</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<td>PI3P</td>
<td>Phosphatidylinositol 3-phosphate</td>
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<td>PI-PLC</td>
<td>Phosphatidylinositol specific phospholipase C</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>PR</td>
<td>Proline rich</td>
</tr>
<tr>
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</tr>
<tr>
<td>PrfA</td>
<td>Positive regulation factor A</td>
</tr>
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<td>PRR</td>
<td>Pathogen pattern recognition receptor</td>
</tr>
<tr>
<td>Px</td>
<td>PhoX domain</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SH3</td>
<td>SRC-homology 3</td>
</tr>
<tr>
<td>SLAPs</td>
<td>Spacious Listeria containing phagosomes</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SPI-2</td>
<td>Salmonella pathogenicity island 2</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded ribonucleic</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TIGAR</td>
<td>TP53-induced glycolysis and apoptosis regulator</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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<td>Ub</td>
<td>Ubiquitin</td>
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<td>UPEC</td>
<td>Uropathogenic E. coli</td>
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<td>Vcc</td>
<td>Vibrio cholera cytolyisin</td>
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<td>VPS34</td>
<td>Vacuolar protein sorting 34</td>
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<td>WASP</td>
<td>Wiskott Aldrich syndrome protein</td>
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Chapter 1
Background

1 Introduction to Phagocytosis

Host-bacterial interaction is a dynamic process where opposing measures are taken by the host and pathogen with the goal of ensuring survival. The host immune system provides defenses against invading microbial pathogen, whereas pathogens have an arsenal of virulence factors to counteract the microbicidal effects of host immunity. This interaction applies a selective pressure on both parties to evolve diverse defensive or evasion strategies. In recent years, much work has focused on the early stages of infection as it has become increasingly apparent that the course of infection can be significantly influenced by events early in an infection. As such, a number of reviews have focused solely on this critical early stage of infection (Flannagan et al., 2009; Kirkegaard et al., 2004; Kumar and Valdivia, 2009). Depending on the invading microbe, the host can mount different responses against the pathogen, be it bacterial, fungal, parasitic or viral in nature. Given the focus of this thesis, host responses to bacterial infection will be predominantly discussed.

Upon entry in the body, bacteria first encounter the early branch of the immune system: the innate immune response. The central players of this response are phagocytes, which are typically the first responders to an infection. These cells are so-termed for their ability to ingest debris, dead cells, or invading pathogens in a process called phagocytosis, “cell-eating”. These cells either reside in peripheral tissues or in the blood stream until they are activated and recruited to the site of infection.

While a class of “non-professional” phagocytes exists with the limited ability to phagocytose bacteria, a range of “professional” phagocytes, including neutrophils, monocytes, macrophages, dendritic cells and mast cells, are efficient at phagocytosis. Of these, neutrophils and macrophages are the most efficient at phagocytosis and thus are often the key cell types for studying bacterial-host interactions. Given the focus of this thesis, discussion of phagocytosis
will be restricted to that of macrophages.

1.1 Macrophages

Macrophages differentiate from hematopoetic stem cells first as the immature form, monocytes. Monocytes mainly circulate in the blood stream and exhibit limited ability to divide and phagocytose particles. Upon activation, monocytes migrate from the blood to the site of infection and depending on the type of activation signal, they can differentiate into macrophages that are either highly proliferative or efficient at phagocytosis and killing.

Phagocytosis by macrophages is initiated upon the detection of extracellular bacteria. The presence of pathogens can be identified via the activation of Toll-like receptors (TLRs), various types of opsonin receptors, or scavenger receptors. Toll-like receptors (TLRs) on phagocytes interact with bacterial cell wall components named pathogen associated molecular patterns (PAMPs). There is a diverse family of PAMPs, the best characterized of which include lipopolysaccharides (LPS), peptidoglycans (PGs), flagellin and lipoteichoic acids (LTAs). Opsonin receptors, including both Fc-gamma receptors (FcγRs) and complement receptors, can also trigger phagocytosis. Soluble factors, such as antibodies and complement, can often bind directly to extracellular bacteria. In doing so, antibodies can neutralize the invasiveness of the pathogen by physically blocking interaction between bacterial and host proteins while complement components can act in a concerted fashion to directly form holes on the bacterial surface, resulting in bacterial lysis. In addition, antibodies and complement on the surface of the bacteria can interact with FcγRs and complement receptors on macrophages to increase the efficiency of phagocytosis in a process called opsonization. Finally, scavenger receptors act in a manner similar to TLRs, binding to bacterial cell wall proteins. While the activation of these host receptors can mediate phagocytosis, it also results in a series of downstream signaling cascades that mediate the activation of a number of important cellular innate immune defenses. Many these mechanisms are involved in activating other bactericidal mechanisms, thus allowing for more efficient bacterial killing inside the phagosome.

Upon activation, macrophages mediate the rapid clearance of bacteria. This can be achieved via
either the extracellular secretion of bactericidal compounds or the intracellular degradation of ingested or phagocytosed pathogens. Extracellular killing of bacteria can occur following the secretion of reactive compounds, such as reactive nitrogen species (RNS) or reactive oxygen species (ROS), or bactericidal enzymes, such as lysozymes. Intracellular killing of bacteria involves phagocytosis of pathogens followed by digestion in the “stomach” of the cell, the lysosome. Not only is phagocytosis critical for bacterial clearance but also promotes antigen presentation, directing the establishment of the late branch of the immune system: the adaptive immune response.

### 1.2 Phagosomal Maturation

To degrade phagocytosed pathogens, phagocytes have a number of mechanisms to breakdown the internalized bacteria. One key degradative mechanism is the delivery of the bacteria to the lysosome for digestion. This is a multi-step process that begins when the bacteria is phagocytosed, creating a bacteria containing compartment called a phagosome (Figure 1). Typically, phagosomes mature through the acquisition and concerted loss of various vesicular and signaling proteins that mediate transition through different stages of maturation and ultimately fuse with the lysosome. Markers of early phagosome include early endosomal autoantigen (EEA-1) and the small GTPase, Rab5 (Bucci et al., 1992). These markers are recruited by the changes in lipid composition of the phagosomal membrane. VPS34, a class III phosphatidylinositol-3-kinase, generates phosphatidylinositol-3-phosphate (PI(3)P), which allows the binding of these early markers (Vieira et al., 2001). Next, the phagosome progresses to the late phagosome via the acquisition of V-ATPase, a proton-pump, creating an acidic luminal environment (Desjardins et al., 1994). Furthermore, lysosomal-associated membrane proteins (LAMPs) and small GTPase Rab7 are recruited (Bucci et al., 1992). Degradative enzymes, such as cathepsins and hydrolases are delivered to the lumen of the phagosome. Thus, changes to the membrane of the phagosome are associated with changes in the lumen, transitioning to a more acidic, oxidative and degradative environment as the phagosome matures. Finally, late phagosome fusion with the lysosome, which has an average pH of 4.5, results in the formation of the phagolysosome in which microbial degradation occurs (Desjardins et al., 1994) (Figure 1).
**Figure 1. Various Stages of Phagosomal Maturation.** Interaction of the bacteria containing phagosome with various subcompartments of the endocytic pathway. This mediates the transition of the phagosome from (A) early, (B) intermediate, (C) late phagosome to ultimately the phagolysosome where degradation occurs. EEA1, early endosome antigen 1; LAMP, lysosomal-associated membrane protein; PI(3)P phosphatidylinositol-3-phosphate. Adapted with permission from Macmillian Publishers Ltd: Nat. Rev. Microbiol. Flannagan et al. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. 7:5 (355-66), 2009.
2 Innate Host Defenses in the Phagosome

Prior to delivery of phagocytosed bacteria to the lysosome, there are a number of host mechanisms that are activated in the phagosome that either directly or indirectly targets the engulfed bacteria. Such strategies include the production of antimicrobial peptides, such as defensins, lactoferrins (and other metal chelators), proteases, cathepsins, RNS and ROS (Figure 2.1) (reviewed in (Flannagan et al., 2009)). Antimicrobial peptides are charged peptides that directly form pores in the bacterial membrane, resulting in lysis. Metal chelators in the phagosomal lumen can bind essential nutrients, such as iron, thus restricting bacterial growth by limiting bacterial access to free metals. Furthermore, as assortment of peptide specific proteases and cathepsins, as well as hydrolases against carbohydrates and lipids, are also present inside the phagosome, thus aiding in bacterial degradation. Finally, reactive compounds, such as RNS and ROS can interfere with bacterial survival by modifying bacterial proteins, lipids and DNA to prevent proper function or folding. It must be noted that while there are a number of antibacterial mechanisms inside the phagosome, they are activate during different stages of phagosomal maturation. For example, the optimal pH for the activity of hydrolytic enzymes is often pH < 4.0. As such, these enzymes are most active during the late phagosome stage. Conversely, NOX2 NADPH oxidase- mediated production of ROS is among the earliest and most robust defenses employed by phagocytes to combat microbial infection. The recruitment and assembly of the NOX2 NADPH oxidase complex begin as early as the formation of the nascent phagocytic cup, long before the phagosome has been fully sealed.
**Figure 2.1. Key host phagosomal defenses against invading pathogens.** Inside the phagosome, the bacteria encounters a series of bacteriocidal measures, including reactive oxygen species (ROS) generated by the NOX2 NADPH oxidase and reactive nitrogen species (RNS) generated by the inducible nitric oxide synthase (iNOS). Furthermore, antimicrobial peptides, such as defensins, and proteases all contribute to direct bacterial disruption and degradation. SOD, superoxide dismutase, MPO, myeloperoxidase. Adapted with permission from Macmillian Publishers Ltd: Nat. Rev. Microbiol. Flannagan et al. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. 7:5 (355-66), 2009.
2.1 Reactive Oxygen Species (ROS)

ROS are a group of highly reactive oxygen based free radicals that can be toxic to both the host and pathogens (Rada et al., 2008). ROS can interact with proteins, lipids and DNA, resulting in damage or structural changes to the molecule that can interfere with proper function. However, a number of important host enzymes produce ROS as a byproduct. Furthermore, ROS also have a number of important direct and indirect functions in immunity where ROS production by phagocytes can directly kill pathogens and signal the activation of further immune responses to effectively clear the infection. Thus, given the “double-edged sword” nature of ROS, there are a number of host mechanisms that regulate the level of ROS both spatially and temporally.

2.1.1 Intracellular Sources of ROS

There are a number of cellular sources that generate ROS including NOXs, the mitochondrial electron transport chain (ETC), xanthine oxidoreductase, peroxisomes, and the endoplasmic reticulum (ER) (Antonenkov et al.; Harrison, 2002; Murphy, 2009; Nauseef, 2008; Santos et al., 2009). In cells that do not express NOX2 NADPH oxidase, the main source of ROS is derived from the mitochondrial ETC. The ETC removes and transports electrons from the donor compound nicotinamide adenine dinucleotide (NADH) to generate an electrical gradient that ultimately drives the production of adenosine triphosphate (ATP) (Raha and Robinson, 2000). Two components of the ETC, NADH dehydrogenase (Complex I) and Coenzyme Q -cytochrome C reductase (Complex III), are prone to electron leakage out of the ETC. When combined with oxygen, this results in the production of superoxides (Raha and Robinson, 2000). Besides the mitochondria, there are other organelles that also harbor enzymes capable of producing ROS. Peroxisomes are organelles that contain a number of oxidases that function to catabolize long and very long fatty acid chains. Many of these enzymes, such as acyl-CoA oxidase, urate oxidase or sarcosine oxidase, produce ROS as a byproduct (Antonenkov et al.). In the ER, ER oxidoreductin (Ero1) is responsible for ROS production. Proper folding and disulfide bond formation of oxidative proteins require the maintenance of a suitable redox environment. Ero1 is a flavin-containing enzyme that produces ROS to create an oxidizing environment in the ER (Travers et al., 2000; Tu et al., 2000). In absence of Ero1, reduced and misfolded proteins
Figure 2.2. Intracellular sources of ROS. Superoxide anions (O$_2^-$) can be generated by xanthine oxidase in catalyzing the oxidation of hypoxanthine to xanthine; by NADPH oxidase (e.g. Nox2 on phagosomal membrane in phagocytic cells) in converting NADPH to NADP$^+$; and by mitochondrial electron transport chain reaction, in which electrons (e) are transferred from the mitochondria complex I (I) to CoQ via the complex II (II), then to cytochrome c via the complex III (III) and finally to O$_2$ via the complex IV (IV) and H$_2$O is produced. Electrons can leak from the electron transport chain and O$_2^-$ is generated from the complex I and III. Other sources of ROS include ER and peroxisome. Adapted with permission from Mary Ann Liebert, Inc: Antioxidants Redox Signaling, Huang J et al. Autophagy Signaling Through Reactive Oxygen Species. 14(11): 2215-31.
accumulate in the ER (Frand and Kaiser, 1998; Pollard et al., 1998). In addition to organelle-associated proteins, cytosolic enzymes can also make ROS. Xanthine oxidoreductase, a flavin-containing enzyme in the liver that catalyzes the conversion of the purine derivative, hypoxanthine, to xanthine and ROS (Harrison, 2002). Lastly, the membrane bound protein complex, NOX, also produces ROS.

2.1.2 NOX2 NADPH oxidase – Main source of ROS in Immunity

The NOX family of protein complexes, comprised of 7 members (NOX1-5 and DUOX1-2), is characterized by their ability to produce ROS by transferring an electron from NADPH to oxygen (Nauseef, 2004). However, the members of this family of complexes greatly differ in their activation signals, the kinetic of ROS production as well as the cell type in which they are expressed. Macrophages predominantly express NOX2 NADPH oxidase as other cell types express a low or negligible level of this protein complex. Compared to other NOX family members, NOX2 NADPH oxidase is the most rapid and efficient producer of ROS, making it the main source of ROS during infection.

2.1.2.1 Components of the NOX2 NADPH oxidase

NOX2 NADPH oxidase is comprised of functional transmembrane heterodimers, gp91phox and p22phox (also known collectively as the cytochrome b558), and 4 regulatory cytosolic subunits – p40phox, p47phox, p67phox and the small GTPase, Rac2 (Figure 2.3). In the inactive state, cytochrome b558 resides in either Rab5+ early endosomes (Casbon et al., 2009; Li et al., 2006) or Rab11+ recycling vesicles (Casbon et al., 2009), p40phox, p47phox and p67phox are in a dephosphorylated trimeric state in the cytosol (Lapouge et al., 2002), while cytosolic Rac2 remains inactive in the GDP bound state via interaction with RhoGDI (Figure 2.3 A) (Ando et al., 1992; Mizuno et al., 1992). Upon stimulation or the initiation of phagocytosis, rapid transit of cytochrome b558 from vesicles to the nascent phagocytic cup occurs (Figure 2.3 B) (Diebold and Bokoch, 2001). Concurrently, p47phox is phosphorylated, and undergoes a conformational change in the C-terminal “autoinhibitory domain” that now exposes two SRC-homology 3 (SH3) regions
to interact with the proline rich (PR) motif on p22phox (Dusi et al., 1993). Furthermore, Phox homology (PX) domains on p47phox bind to phosphatidic acid (PA) and phosphatidylinositol 3, 4-bisphosphate PI(3,4)P₂, transient phosphoinositides that are generated only at the plasma membrane upon phagocytosis (Bravo et al., 2001; Karathanassis et al., 2002). This further mediates p47phox localization to cytochrome b558 (Nauseef, 2004). Since p47phox, p67phox and p40phox are trimerized in the cytosol, the translocation of p47phox brings the other two regulatory subunits to the membrane as well (Figure 2.3 B) (Lapouge et al., 2002; Park et al., 1994).

However, there are specific regulatory mechanisms in place that control the activation state of both p67phox and p40phox, which are also required for the proper functioning of the NOX2 complex. Phosphorylated p67phox interacts with cytochrome b558, inducing a conformational change in the functional subunit that is necessary for ROS production (Zhao et al., 2003). The role that p40phox plays is less clear but studies suggest that phosphorylated p40phox may undergo a conformation change exposing its PB1 domain, which mediates p67phox translocation, and its PX domain, which allows the interaction with PI(3)P (Ellson et al., 2001a; Ellson et al., 2001b; Kanai et al., 2001). In the inactive state, p40phox PX and PB1 domains are bound in an intramolecular interaction, preventing interaction with p67phox or PI(3)P (Honbou et al., 2007).

As a result, NOX2 NADPH oxidase activation may also be modulated by a p40phox conformational change (Bissonnette et al., 2008). Finally, GDP-Rac2 is converted to GTP-Rac2 through the activity of a Rac guanine nucleotide exchange factor (Figure 2.3 B). Active Rac2 can now translocation to the phagosomal membrane to bind p67phox, thereby completing the assembly of a functional NOX2 NADPH oxidase complex (Figure 2.3 C).
Figure 2.3 A Schematic of NOX2 assembly and activation. A. In the resting stage, cytochrome b558 (gp91 and p22\textsubscript{phox}) resides in vesicles. Rac2, in the inactive GDP bound form, remains in the cytosol. The regulatory subunits, p47\textsubscript{phox}, p67\textsubscript{phox} and p40\textsubscript{phox}, are in a trimeric complex in the cytosol. B. Upon receiving signals for activation, cytochrome b558 is recruited to the membrane. Simultaneously, protein kinase C (PKC) phosphorylates p47\textsubscript{phox}, resulting in a conformational change thus allowing p47\textsubscript{phox} to interact with p22\textsubscript{phox} and the lipids, PA and PI(3,4)P\textsubscript{2}. RhoGDI inhibition of Rac2 is now released to allow GTP binding. ROS production commences at the nascent phagocytic cup prior to phagosome sealing. C. As the lipid composition changes during the late stages of phagosome sealing, the membrane becomes enriched with PI(3)P. p40\textsubscript{phox} undergoes a conformational change to allow interaction with PI(3)P and p67\textsubscript{phox}, thereby maintaining NOX2 NADPH oxidase function and localization to the phagosome. Adapted with permission from Mary Ann Liebert, Inc: Antioxidants Redox Signaling, Leto T et al. Targeting and Regulation of Reactive Oxygen Species Generation by NOX Family NADPH Oxidases. 2009. 11(10): 2607-19.
2.1.2.2 Activating signals for NOX2 NADPH oxidase assembly

There have been many reports about the identity of the kinases responsible for p47\textsuperscript{phox}, p67\textsuperscript{phox} and p40\textsuperscript{phox} phosphorylation, and include several PKC isoforms (PKC\textalpha{} (Bengis-Garber and Gruener, 1996; Fontayne et al., 2002), PKC\textdelta{} (Dang et al., 2001a; Fontayne et al., 2002), PKC\textbeta{} (Dekker et al., 2000; Fontayne et al., 2002), PKC\textgamma{} (Bey et al., 2004) and PKC\textzeta{} (Dang et al., 2001b; Fontayne et al., 2002)), PKA (Kramer et al., 1988), p21 activated kinase (PAK) (Martyn et al., 2005), ERK1/2 (Dang et al., 2003; Dewas et al., 2000), AKT (Chen et al., 2003), PI3K (Lehmann et al., 2009; Yamamori et al., 2004), and possibly others. The complexity of NOX2 regulation by such a large number of kinases not only suggests that perhaps a high threshold of activating signals may be required for NOX2 activity but also that there may be differing amounts of ROS produced by each individual NOX2 complex, depending on the type of local activating signal it receives. In fact, a recent study finds that NOX2 assembly and activity is highly heterogeneous - where only 50% of phagosomes formed upon Fc-\textgamma{} receptor (Fc\textgamma{}R) mediated phagocytosis have proper p40\textsuperscript{phox} localization and NOX2 function (Tian et al., 2008).

After assembly and activation, NOX2 then produces ROS in a reaction on the cytoplasmic region of the gp91\textsuperscript{phox} subunit, converting NADPH to NADP\textsuperscript{+} in a process that liberates two electrons and one H\textsuperscript{+}. Only the two electrons are transported to the lumen of the phagosome where they react with two oxygen molecules to form two superoxide ions.

While NOX2 is the main producer of ROS upon phagocytosis (Lambeth, 2004), it must be noted that other NOX family members, including NOX1, NOX3 and NOX4 are also expressed in macrophages and can be activated upon stimulation with specific signals. For example, in the field of ROS and atherosclerosis, oxidation of low-density lipoproteins (oxLDL) leads to macrophage to foam cell conversion, one of the key steps in plaque formation (Carnevale et al., 2007). It was recently found that lipopolysaccharide (LPS), a toll-like receptor (TLR)-4 agonist, can lead to increased NOX1 expression, thereby expediting macrophage conversion to foam cells (Lee et al., 2009; Park et al., 2009). Other groups have found that NOX4 also plays a critical role in the formation of oxLDL, leading to macrophage death (Lee et al.). Additionally, NOX3 expression, albeit low, has been reported in RAW macrophages (Sasaki et al., 2009). Thus, while NOX2 is the predominant form of NADPH oxidase expressed in macrophages and other phagocytes, the expression and contributions of other NOX members during an infection...
warrants further investigation. However, it must be noted that other NOX family member are unlikely to be able to compensate for NOX2 NADPH oxidase activity in the event of NOX2 dysfunction for several reasons. First, NOXes have different activation signals. NOX5, DUOX1 and DUOX2 are activated by increases in intracellular Ca\(^{2+}\) levels while NOX4 activity is not regulated by the same trimeric phox complex (Bedard and Krause, 2007). Second, other NOXes have slower kinetics of ROS production when compared to NOX2. Upon macrophage activation, NOX1 require ~90 min before ROS is produced (Lee et al., 2009) while NOX2 can produce ROS within minutes (Tian et al., 2008). Finally, other NOXes produce much lower levels of ROS when compared to NOX2. Under conditions of NOX2 transcriptional downregulation and NOX1 and NOX3 upregulation, the level of ROS production by stimulated macrophages is roughly 30x less than when NOX2 transcription is normal (Sasaki et al., 2009). Thus, while other NOXes can be upregulated to compensate for ROS production in the event of NOX2 NADPH oxidase dysfunction (Sasaki et al., 2009), they cannot, in a timely fashion, compensate for the burst of ROS required to effectively clear certain infections. As such, patients bearing mutations in NOX2 NADPH oxidase genes exhibit an inability to clear infections by organisms that have antioxidant defenses.

2.1.2.3 Loss of NOX2 NADPH oxidase function results in Chronic Granulomatous Disease (CGD)

The critical role that NOX2 NADPH oxidase-derived ROS play in host immunity was clearly demonstrated by genetic studies done in late 1980s that showed patients with mutations in NOX2 NADPH oxidase component genes develop chronic granulomatous disease (CGD) (reviewed by (Segal, 1996)). CGD is characterized by recurrent and severe infections by a particular spectrum of bacteria (such as *Staphylococcus aureus*, *Salmonella* and *Klebsiella* species) and fungi (such as *Aspergillus* and *Candida* species) as a result of the host’s inability to mount an effective innate immune response despite chronic sterile inflammation observed in the body (Winkelstein et al., 2000). On histological examination, infiltration of large amounts of immune cells at the site of infection (termed granulomas) can be characteristically observed. However, these granulomas persist as the immune cells present lack the ability to produce ROS and thus are unable to resolve the infection. Given that the NOX2 NADPH oxidase is comprised of a number of components,
the loss of any one can result in the inability to make ROS. As such, CGD refers to a group of hereditary diseases that result from a mutation in any one of the NOX2 NADPH oxidase components, characterized by a defect in ROS production. As such, CGD patients are more susceptible to catalase expressing pathogens. Since that time, much work has been done to elucidate the contribution of ROS to innate immunity and the defense against invading pathogens.

2.1.3 Types of ROS

Due to its highly reactive nature, superoxide can readily form a number of other compounds (reviewed in (Bartosz, 2009; Lam et al., 2010; Lambeth, 2004)) (Figure 2.4). Superoxide can react with nitric oxide to produce peroxynitrite, an even stronger oxidizing agent (Ramos et al., 1995). The combination of two superoxide ions, catalyzed by superoxide dismutase (SOD), results in the production of hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ acts mainly upon thiol groups in cysteine residues, leading to either oxidation (Cho et al., 2004; Rhee et al., 2000) or disulfide bond formation (Biswas et al., 2006; Georgiou, 2002). H$_2$O$_2$ can also produce hypochloric acid (HOCl) when combined with Cl$^{-}$ in a reaction catalyzed by myeloperoxidase. Both H$_2$O$_2$ and HOCl have been shown to be present in sufficient concentrations in the phagosome to kill microbes (Jiang et al., 1997; Nauseef, 2001). H$_2$O$_2$ can also interact with transition metal ions, such as ferrous and ferric ions, to produce hydroxyl radicals (OH$^-$), or with superoxides to generate singlet oxygen (O$^\cdot$). OH$^-$ in particular, while short lived, is the most highly oxidizing member of the ROS family, reacting rapidly and non-discriminately with DNA, lipids and proteins.
Figure 2.4. The generation of common ROS products in phagocytes. Reprinted with permission from Springer Science + Business Media: Seminars in Immunopathology, Lam G et al. The many roles of NOX2 NADPH oxidase-derived ROS in immunity, 32(4): 415-30
2.1.4 Functions of ROS

2.1.3.1 Microbicidal Activity of ROS

While superoxide is the main product of NOX2 NADPH oxidase, it remains controversial whether it is the main antimicrobial compound. Some researchers have noted that superoxide is relatively unreactive when compared to the rest of the ROS family and thus may not be sufficient enough of a defensive strategy on its own (Reeves et al., 2003). Conversely, others have argued that in the low pH environment of the phagosome, the majority of superoxide is protonated (HO$_2^-$), becoming a much more reactive compound (Klebanoff, 2005).

While the direct antimicrobial effect of superoxides remains contentious, the indirect antimicrobial effect of superoxides via its reactive products is certain. Due to its highly unstable nature, superoxide can readily form a number of other compounds (reviewed in (Bartosz, 2009; Lambeth, 2004)). Superoxide can react with nitric oxide to produce peroxynitrite, an even stronger reducing agent (Ramos et al., 1995). The combination of two molecules of superoxide ions, catalyzed by the activity of the enzyme, superoxide dismutase (SOD), results in the production of hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ acts mainly upon thiol groups in cysteine residues, leading to either oxidation (Cho et al., 2004; Rhee et al., 2000) or disulfide bond formation (Biswas et al., 2006; Georgiou, 2002). H$_2$O$_2$ can in turn produce hypochloric acid (HOCl) when combined with Cl$^-$ in a reaction catalyzed by myeloperoxidase. Both H$_2$O$_2$ and HOCl have been shown to be present in sufficient concentrations in the phagosome to kill microbes (Jiang et al., 1997; Nauseef, 2001). H$_2$O$_2$ can also interact with transition metal ions, such as ferrous and ferric ions, to produce hydroxyl radicals (OH'), or with superoxides to generate singlet oxygen ('O'). OH' in particular, while short lived, is the most highly oxidizing member of the ROS family, reacting rapidly and non-discriminatorily with DNA, lipids and proteins.

2.1.3.2 ROS modulation of bacterial virulence factors

In addition to its direct microbicidal effects on pathogens, ROS can also arrest pathogen survival and growth either via the inactivation of critical bacterial products or the modulation of the phagosomal or extracellular environment. For example, leukotoxin, a key virulence factor of
*Actinobacillus actinomycetemcomitans*, has been shown to induce apoptosis in human macrophages and neutrophils (Simpson et al., 1988; Tsai and Taichman, 1986). Myeloperoxidase, through the production of HOCl, has been implicated in the oxidation and subsequent inactivation of leukotoxin (Clark et al., 1986; Korostoff et al., 1998; Yamaguchi et al., 2001). Pneumolysin, a vital virulence factor secreted by *Streptococcus pneumoniae* that is required for survival in human neutrophils, has also been shown to be likewise oxidized by HOCl. Pneumolysin is a pore-forming cytolysin whose function is regulated by the ability to form disulfide bonds, without which proper oligomerization for pore formation cannot occur (Geoffroy et al., 1981; Hotze et al., 2001). Myeloperoxidase was found to be effective in inhibiting pneumolysin activity, presumably via the prevention of disulfide bond formation (Clark, 1986). Another pore forming cytolysin, listeriolysin O (LLO), has been suggested to be inhibited in much the same fashion (Myers et al., 2003; Singh et al., 2008). LLO is one of the key virulence factors produced by *Listeria monocytogenes* that mediates bacterial survival and growth in both human and murine macrophages (Portnoy et al., 1988; Schnupf and Portnoy, 2007). It has long been known that LLO requires a reducing environment for its activity (Geoffroy et al., 1987; Portnoy et al., 1992). Recent work has further identified the particular host protein - γ-interferon inducible lysosomal thiolreductase (GILT) - that is responsible for the oxidation of LLO to mediate disulfide bond formation (Singh et al., 2008). It is therefore likely that the creation of an oxidative environment by ROS can impede disulfide bond formation in LLO. However, virulence factors are not the only reported microbial target of ROS as quorum sensing by *Staphylococcus aureus* both *in vitro* and *in vivo* has also been shown to be affected (Rothfork et al., 2004). Quorum sensing is the process by which bacteria modulate their behavior once they “sense” their population has expanded above a certain threshold number. Rothfork and colleagues demonstrated that HOCl and peroxynitrite inhibited the autoinducer peptide activity, a critical player in quorum sensing, leading to an inability to upregulate virulence factor expression once the bacteria has reached sufficient numbers.
2.1.3.3 Cellular Signaling Effects

ROS, in particular H$_2$O$_2$, have a wide range of reported signaling effects that will only be briefly summarized for completeness (this topic is extensively reviewed by (Bedard and Krause, 2007; Oakley et al., 2009)).

One mechanism of ROS effect on signaling is mediated through the modification of the cysteine residue via one of two ways. First, the oxidation of the sulfur molecule to form sulfenic (Cys-SOH), sulfinic (Cys-SO2H), or sulfonic (Cys-SO3H) acid can occur (Bindoli et al., 2008). Second, the oxidation of cysteines can result in reactive thiols, leading to the formation of disulfide bonds (Biswas et al., 2006; Georgiou, 2002). The significance of the oxidation of cysteines is that ROS can influence cellular signaling via the inhibition of tyrosine phosphatases, G proteins and certain ion channels (Cho et al., 2004; Rhee et al., 2000), as well as the activation of kinases, such as mitogen-activated protein (MAP) kinases (Djordjevic et al., 2005). Another major effect of ROS on signaling is the modulation of Ca$^{2+}$ signaling. Oxidation of the ryanodine receptor, which contains ROS sensitive cysteine residues (Liu and Pessah, 1994), results in the release of intracellular Ca$^{2+}$ stores (Favero et al., 1995; Kawakami and Okabe, 1998; Suzuki et al., 1998). Similarly, ROS can also activate the inositol trisphosphate (IP$_3$) receptor family Ca$^{2+}$ release channels (Germano et al., 2004; Hu et al., 2002). An additional consequence of ROS signaling is the activation of transcription factors. Stimulation of TLR results in the increase of ROS signaling, leading to enhanced expression of NF-κB (Bubici et al., 2006). ROS signaling also results in the expression of a number of genes involved in antioxidative and tumor suppressive action, such as Nrf2 (Copple et al., 2008), forkhead box containing transcription factor O (FoxO) (Tothova et al., 2007), and p53 (Liu et al., 2008).

2.1.3.4 Other functions of ROS

In addition to the roles mentioned, ROS have a plethora of functions in immunity. ROS have been reported to participate in the formation of neutrophil extracellular traps (NETs), fibrous meshworks comprised of DNA and protein expelled into the extracellular space for the purpose of entrapping and killing pathogens (Brinkmann et al., 2004; Urban et al., 2006). ROS have also been implicated in the modulation of the immune system to help create an environment that
allows for an efficient and effective immune response. As such, there has been a wealth of research focused on the interaction between NOX2 NADPH oxidase-derived ROS and various key members of both the innate and adaptive immune system. The results of these efforts paint a complex picture of ROS function as ROS have been reported to participate in both pro-inflammatory and anti-inflammatory signaling. Finally, recent work has demonstrated a role for ROS in the induction of autophagy. The details of this ROS function are discussed later in the section 2.1.3.3.1: ROS-mediated induction of autophagy.

2.1.4 Common techniques of ROS quantification

Given the transient nature and diversity of ROS, attempts at ROS quantification or visualization can be challenging. A range of spectroscopic, chemiluminescent, electrophysiologic and other imaging-based methods have been developed to address this issue (Eruslanov and Kusmartsev, 2010; Tarpey et al., 2004; Yeung et al., 2005). Of these, only a select few are useful for quantification of intracellular ROS production. Here, I will highlight some of these commonly employed techniques (summarized in Table 2.1).

2.1.4.1 Oxidizable fluorescent probes

In general, intracellular ROS production can be assessed using cell permeant probes that fluoresce upon oxidization. This fluorescence can then be monitored via spectroscopy, microscopy or flow cytometry. Fluorescein and calcein based probes are the most commonly used indicators of ROS given their cell permeant nature.

Dihydroethidium (DHE), dihydrorhodamine 123 (DHR) and 2’-7’-dichlorodihydrofluorescein (H$_2$DCF) are among the most commonly employed fluorescein based probes for ROS visualization and quantification. DHE is a blue, cell permeant probe that is specific for the detection of superoxides (Tollefson et al., 2003). Upon oxidation by ROS, DHE is converted to ethidium bromide, a red fluorescent compound, which then intercalates into cellular DNA (Tollefson et al., 2003). However, DHE produces high background fluorescence even in resting cells, rendering this probe relatively insensitive to small changes in ROS production. Thus, while
<table>
<thead>
<tr>
<th>Name of compound</th>
<th>Type of ROS detected</th>
<th>Location of ROS detected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxidizable fluorescent dyes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydroethidium (DHE)</td>
<td>Superoxide</td>
<td>Intracellular</td>
</tr>
<tr>
<td>Dihydrorhodamine 123 (DHR)</td>
<td>Superoxide, H$_2$O$_2$, HOCl and peroxynitrite anions</td>
<td>Intracellular(Nakagawa et al., 2004)</td>
</tr>
<tr>
<td>2’-7’-dichlorodihydrofluorescein (H$_2$DCF)</td>
<td>Superoxide, H$_2$O$_2$, peroxyl radicals and peroxynitrite anions</td>
<td>Intracellular</td>
</tr>
<tr>
<td>2-[6-(4’-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF)</td>
<td>Hydroxyl radicals and peroxynitrite anions</td>
<td>Intracellular</td>
</tr>
<tr>
<td>2-[6-(4’-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF)</td>
<td>Hydroxyl radicals, peroxynitrite anions and HOCL</td>
<td>Intracellular</td>
</tr>
<tr>
<td>Dihydrocalcein (DHC)</td>
<td>H$_2$O$_2$</td>
<td>Intracellular</td>
</tr>
<tr>
<td><strong>Other oxidizable chemical compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione)</td>
<td>Superoxide, H$_2$O$_2$, HOCL, peroxyl radicals, and peroxynitrite anion</td>
<td>Intracellular and extracellular</td>
</tr>
<tr>
<td>Isoluminol (4-aminophthalhydrazide)</td>
<td>Superoxide, H$_2$O$_2$, HOCL, peroxyl radicals, and peroxynitrite anion</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Lucigenin</td>
<td>Superoxide</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Compound</td>
<td>Reactive Oxygen Species</td>
<td>Location</td>
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<td>----------------------------------------------</td>
<td>-------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Nitroblue tetrazolium chloride (NBT)</td>
<td>Superoxide</td>
<td>Intracellular</td>
</tr>
<tr>
<td>Cerium Chloride (+ TEM)</td>
<td>Superoxide, H₂O₂</td>
<td>Phagosomal</td>
</tr>
</tbody>
</table>

**Table 2.1.** Table of common compounds used for ROS detection.
DHE is highly specific for superoxides, it lacks the ability to distinguish the specific cellular location of ROS generation and is not sensitive enough to detect low levels of ROS production. In contrast, DHR can be used to detect a range of different types of ROS, including H₂O₂, HOCl and peroxynitrite anions (Crow, 1997). DHR is a non-fluorescent probe that is converted into a red fluorescent product called rhodamine 123 upon oxidation. Since both DHR and its coloured product are freely diffuseable across membranes, this technique is likewise unable to distinguish the location of ROS generation.

The limitations of DHE and DHR gave rise to a third class of fluorescent probes, H₂DCF. Similar to DHR, colourless H₂DCF is converted to the green fluorescent product, 2’-7’-dichlorofluorescein (DCF) only upon oxidation. The key advantage of DCF over rhodamine 123 is its limited permeability, thus allowing the product to be trapped inside the host cell upon generation. The limitation of this technique, however, is that H₂DCF is also poorly permeant. A number of commercially available H₂DCF probes have been generated, which bear chemical modifications that render them more specific to intracellular ROS with improved rates of cellular retention. Among the most widely used H₂DCF derivatives is H₂DCFDA, the chemically reduced and acetylated version of the probe. Chemical reduction improves the probe’s sensitivity to be oxidized, while acetylation ensures oxidation can only occur inside the host cell since the probe remains non-fluorescent until the acetate groups have been removed by intracellular esterases. Furthermore, carboxylation of H₂DCFDA (carboxy-H₂DCFDA) creates an additional negative charge, improving retention of the probe. Addition of a thiol-reactive chloromethyl group (CM- H₂DCFDA) or an amine-reactive succinimidyl ester group (H₂DCFDA, SE) allows the probe to be covalently linked to intracellular structures, giving maximal cellular retention. Depending on the modifications made, each H₂DCFDA derivative has different sensitivities to specific types of ROS. Carboxy-H₂DCFDA is sensitive to H₂O₂, peroxyl radicals and peroxynitrite anions, while CM-H₂DCFDA is sensitive to all these ROS as well as hydroxyl radicals. H₂DCFDA, SE is specific for superoxides (Myhre et al., 2003).

Recently, new generations of fluorescein-based probes have been developed for the detection of specific ROS. 2-[6-(4’-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) is specific for the detection of hydroxyl radicals and peroxynitrite anions, while 2-[6-(4’-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) is specific for both these ROS as well as hypochlorous
The fluorescence of both probes is quenched. However, upon oxidation, quenching is relieved and fluorescence can then be observed.

Of the calcein-based agents, dihydrocalcein (DHC) is one of the more popular dyes used in the field. DHC is a non-fluorescent compound that becomes green-fluorescent calcein upon oxidation. This probe is specific for H$_2$O$_2$ and has good cell permeability and retention properties. However, one major drawback of this probe is that the fluorescence can be quenched by metals (including iron, cobalt, nickel, and copper) at neutral pH (Keller et al., 2004).

2.1.4.1.1 Assessing fluorescence

Upon oxidization, all of the above-mentioned probes become fluorophores, compounds that have the potential to fluoresce (or emit light) upon excitation by the proper wavelength. To assess fluorescence, a light source is required to excite the fluorophore with light of the correct wavelength, and the resulting emission of light from the excited fluorophore is detected with a detector. Quantification of the amount of light generated by each cell gives an indication of the concentration of fluorophore present, which gives an indication of the concentration of ROS present to generate these fluorophores. However, it must be noted that one important limitation of all these probes is that they are photo-oxidizable. In other words, the very process to assess fluorescence will generate more fluorophore that can now fluoresce. Thus, repeated light-based imaging will activate the probe, giving increased fluorescence simply due to the process of imaging. This drawback may be limited by using low light conditions during fluorescence microscopy; however, given the set-up time required for microscopic imaging, the effect of photo-oxidation will be difficult to eliminate using this mode of detection. Certainly, exposure of a stained sample to light results in a noticeable increase in fluorescence within seconds.

One viable alternative to fluorescence microscopy is flow cytometric analysis of fluorescence. In flow cytometry, cells of increased fluorescence are defined or “gated” using H$_2$DCF unstained and stained controls. By comparing the histograms of fluorescence between the unstained and stained controls, the population of cells that exhibit increased fluorescence in the stained control is thus gated as ROS$^+$. This gate can then be applied to subsequent samples in the same experiment in order to give a percentage of cells that are ROS$^+$ (out of a constant number of total
25 cells examined) that have increased fluorescence as defined using the stained control. Thus, each single cell will be exposed to the excitation light once. Furthermore, the advantage of flow cytometric analysis of fluorescence allows a population based assessment of fluorescence, giving insight into the proportion of cells in a population that has increased ROS production. Additionally, if there are different populations of cells that produce high versus low levels of ROS, flow cytometric analysis will be capable of distinguishing them. While flow cytometry of H$_2$DCFDA stained cells allows for the detection of intracellular ROS, one key limitation of this technique is the inability to indicate the specific cellular location of ROS generation.

2.1.4.2 Other oxidizable chemical compounds

Given the problems of photo-oxidation, a number of compounds have been developed to assess ROS production via non-fluorescence-based methods. Among the most commonly employed reagents are luminol, lucigenin, nitroblue tetrazolium chloride, and cerium chloride.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is a light coloured compound that becomes chemiluminescent in the presence of ROS (Allen, 1986). The interaction of luminol with ROS results in the formation of luminol dianion, a reaction that is catalyzed by the enzyme, horseradish peroxidase (HRP) (Cormier and Prichard, 1968; Prichard and Cormier, 1968). Reaction of luminol dianion with O$_2$ produces unstable aminophthalate. The electrons in this unstable compound undergo decay from an excited state to the ground state, emitting a photon of light in the process. Thus, due to the 1:1 ratio of luminol and ROS to generate a luminol dianion, quantification of light generated gives an indication of the concentration of ROS in the system. Since luminol is cell permeable, both intracellular and extracellular ROS can be detected using this reagent (Briheim et al., 1984; Dahlgren et al., 1989; Lock and Dahlgren, 1988). Luminol is one of the most broad-spectrum ROS sensitive reagent, as it can detect the majority of ROS including H$_2$O$_2$, hypochlorous acid, peroxyl radicals, peroxynitrite anion, and superoxides. Derivatives of luminol, such as isoluminol, lack the ability to enter cells. As such, isoluminol is specific for the detection of extracellular ROS generation (Lundqvist and Dahlgren, 1996). While the generation of intracellular ROS can be extrapolated by comparing the results of the luminol (intra- and extracellular ROS) and isoluminol (extracellular ROS) chemiluminescence assays, direct detection of intracellular ROS production can be conducted by introducing superoxide
dismutase (SOD) to the luminol chemiluminescence assay (Chou et al., 2004; Lundqvist and Dahlgren, 1996). SOD is cell impermeable and can eliminate extracellular superoxide production. Given that both luminol and isoluminol assays are based on chemiluminescence, the quantification of ROS production is assessed on a whole population basis using a luminometer.

Specific assessment of superoxide production can be conducted in a number of ways. Lucigenin is a chemiluminescent compound commonly used to assess superoxide production. However, since lucigenin is cell impermeable, it is useful in the quantification of extracellular superoxide production (Liochev and Fridovich, 1998; Tarpey and Fridovich, 2001). To assess intracellular production of superoxide, the nitroblue tetrazolium chloride (NBT) quantification assay can be employed. NBT is a yellow, water-soluble compound comprised of two tetrazole moieties. Upon interaction with superoxides, NBT is converted to a dark blue precipitate called formazan (Beauchamp and Fridovich, 1971). NBT can freely diffuse into ROS-producing cells and insoluble formazan precipitants are formed at the site of superoxide production. Differential interference contrast imaging of fixed NBT treated cells allows for visualization of where superoxide is being produced. Alternatively, cells can be treated with DMSO and KOH to allow solublization of formazan precipitants. Subsequent spectrophotometric assessment at 570nm then allows for the quantification of formazan precipitants, which indicates the concentration of total superoxide in the system. Prior treatment of NBT treated cells with methanol will remove all extracellular formazan precipitants allowing the detection and quantification of intracellular formazan precipitants (protocol described in (Keith et al., 2009)).

2.1.4.3 Assessing phagosomal ROS by TEM

With the exception of NBT and the H$_2$DCFDA probe, the techniques discussed above cannot specifically distinguish phagosomal ROS from ROS generated elsewhere inside or outside of the cell. The H$_2$DCFDA,SE probe can be pre-conjugated to latex beads, zymosan particles or bacteria via covalent insertion into amino groups. Labeled particles or microorganisms can then be fed to macrophages. This technique has been successfully performed in the context of studying superoxide production in phagosomes of neutrophils (Ryan et al., 1990). However, the limitation of this technique is that the fluorescent probe is photo-oxidizable and therefore it is technically challenging to limit oxidization during the labeling procedure.
Perhaps the most direct method of visualization of phagosomal ROS is via cerium chloride treatment coupled with transmission electron microscopy. Much like NBT, cerium chloride also forms an insoluble precipitant, cerium perhydroxide, in presence of ROS. Cerium perhydroxide is a black compound that is deposited where it is formed, and is also easily visualized using TEM. Thus, phagosomes containing bacteria or other particles can be assessed for ROS production in the phagosomal compartment using cerium chloride. A number of studies have successfully performed this technique to visualize ROS production in phagosomes containing *Salmonella enterica* serovar Typhimurium or *Burkholderia cenocepacia* (Keith et al., 2009; Vazquez-Torres et al., 2000). While this method is not ideal for quantifying the absolute amount of intracellular ROS, it is a good method of assessing the percentage of bacteria containing phagosomes that are producing ROS in the lumen of the phagosome.

2.1.4.4 Concluding remarks on ROS quantification or visualization techniques

The development of ROS sensitive probes and chemical reagents that are sensitive to only one or two ROS members have allowed for greater insight into the physiological roles of different ROS members. It must be noted that one key limitation of all ROS sensing techniques is that they are sensitive to oxidation by air. Thus, all dyes and reagents must be kept in air tight environments, be it tightly lidded containers with moisture absorbing chips or nitrogen/argon rich environments, to reduce spontaneous oxidation. Despite efforts of controlling oxidation, ROS reagents are typically only be usable within months of opening. Thus, given the reactivity of ROS to air, diversity of ROS and the specificities of each probe, multiple assays should be done to gain a more complete and accurate picture of intracellular ROS production.
2.2 Autophagy

Autophagy is the controlled process of cellular self-digestion where cellular contents are delivered to the lysosome for degradation in order to maintain cellular homeostasis (Levine and Deretic, 2007). This process can be subdivided into three types – chaperone mediated autophagy (CMA), microautophagy and macroautophagy (reviewed in (Mizushima et al., 2008)). With respect to immunity, there has been a wealth of research demonstrating that macroautophagy, hereafter referred to as autophagy, is a key component of the innate immune defense against many pathogenic microorganisms, by removing them from the cytosol, limiting their escape from phagosomes and promoting phagosome maturation (reviewed in (Deretic and Levine, 2009; Hussey et al., 2009)). Autophagy is characterized by the presence of autophagosomes, double membranous lamellar vesicles bearing the autophagy marker, microtubule-associated protein light chain 3 (LC3) (reviewed in (Hussey et al., 2009)). LC3, or Atg8 in yeast, is a member of a family of over thirty autophagy-related (Atg) proteins that act sequentially to mediate the formation of autophagosomes. While the initial steps of autophagosome formation are currently poorly understood, autophagy proteins are thought to recruit a crescent-shaped isolation membrane (the source of which is currently unclear) that elongates around and captures the cytoplasmic cargo, such as damaged organelles, cytosol, macromolecules or pathogens.

2.3 Autophagy core machinery

Autophagy is a process that is highly conserved from yeast to drosophila to man. Autophagy is characterized by the formation of intracellular double/multi-membranous vesicles, called autophagosomes. It is typically maintained at a low basal level under non-stress condition. However, upon stress or stimulation, autophagic activity can be greatly induced (Huang and Klionsky, 2007). Over thirty key components of the autophagy machinery encoded by autophagy-related genes (ATG) function at different steps of this process (Klionsky et al., 2003), which can be grouped into several specific stages: initiation (Figure 2.5 A), elongation (Figure 2.5 B), and maturation (Figure 2.5 C), which includes autophagosome trafficking, cargo degradation and nutrient recycling.
2.3.1 Initiation

The Atg1 (homolog of mammalian ULK1) kinase complex (Atg1-Atg13-Atg17-Atg29-Atg31 in yeast, and ULK1/2-Atg13-FIP200-Atg101 in mammals) is critical for autophagy induction (Mizushima, 2010). The class III phosphatidylinositol 3-kinase (PI3K) complex comprising of VPS34 (the PI3K), Vps15, Atg14 and Beclin 1/Atg6, functions at the stage of phagophore (the autophagosome formation site or preautophagosomal structure (PAS) in yeast) initiation and assembly (Funderburk et al., 2010) (Figure 2.5 A).

2.3.2 Elongation

Phagophore elongation involves two ubiquitin-like conjugation complexes that are essential for expansion and finally autophagosome formation (Figure 2.5 B). One is the Atg12-Atg5-Atg16 complex, in which Atg12 is catalyzed by the E1-like enzyme Atg7 and E2-like enzyme Atg10, and then conjugated to Atg5 (Mizushima et al., 1998). The resultant Atg12-Atg5 conjugate further interacts with Atg16 and forms a complex that associates with autophagosomes (Mizushima et al., 2003). The other ubiquitin-like system is the Atg8 (homolog of mammalian LC3)-phosphatidylethanolamine (PE) conjugate (Ichimura et al., 2000). Under the activity of Atg7, Atg3 (another E2-like enzyme) and Atg12-Atg5 complex (acts as an E3), Atg8/LC3 is attached to the phospholipid PE and remains on the autophagosomal membrane throughout the autophagy process (Hanada et al., 2007; Ichimura et al., 2000; Kabeya et al., 2000). Before its conjugation, Atg8/LC3 needs to be processed by Atg4, a cysteine protease, to expose a glycine residue that is prerequisite for PE conjugation (Kirisako et al., 2000). Atg4 also regulates the deconjugation of Atg8/LC3-PE to recycle free Atg8/LC3 after the autophagosome is completely formed (Kirisako et al., 2000).
Figure 2.5. Steps involved in the induction of autophagy. Upon starvation or other types of stress, VPS34 and beclin 1 interaction as well as mTOR inhibition results in activation of autophagy. This sets off a chain of events that includes (A) initiation, (B) elongation, and (C) maturation of autophagy. (B) The elongation step of autophagy is regulated by two ubiquitylation systems, Atg12 and LC3II pathways. Atg 12 is first conjugated to Atg7, and then is transferred to Atg10. This allows for Atg12 conjugation to an Atg5 lysine residue. The resulting Atg12-Atg5 complex is stabilized by Atg16. This trimeric complex enhances the conversion of LC3I to LC3II. Atg4 mediates in the recycling of LC3II to LC3I. (C) The autophagosome then fuses with the lysosome, resulting in the degradation of the autophagosome and its contents. Reprint by permission from Macmillian Publishers Ltd: Nat. Rev. Immunol. Levine et al. Unveiling the roles of autophagy in innate and adaptive immunity. 7:5 (767-77), 2007.
2.3.1 Maturation: Non-selective vs selective autophagy

Autophagy can be classified into non-selective bulk autophagy and selective autophagy that targets specific cargoes. Though starvation-induced bulk autophagy has long been thought to be nonspecific, recent proteomic studies suggested a highly regulated and ordered manner of organelle degradation by autophagy under amino acid starvation condition (Kristensen et al., 2008). It was shown that at early time points after starvation, cytosolic proteins and proteasomes were degraded by autophagy, and ribosomes were degraded in lysosomes at later time points (about 12 hours post-starvation). Organelle degradation started even later (about 24 hours after starvation) (Kristensen et al., 2008).

Selective autophagy can be subdivided into many different types, depending on the nature of the cargo being degraded. Selective targeting and degradation of peroxisome (Dunn et al., 2005), mitochondria (Kim et al., 2007) and endoplasmic reticulum (ER) (Bernales et al., 2006) by autophagy are called pexophagy, mitophagy and reticulophagy, respectively. Furthermore, misfolded protein aggregates, such as Huntingtin protein aggregates, can be selectively degraded by autophagy (named aggrephagy) (Rubinsztein, 2006). Finally, autophagy is able to target invaded viruses, bacteria and parasites by a process called xenophagy (Orvedahl and Levine, 2009).

The selective cargo recognition depends on cargo receptor proteins that can be recognized by the autophagy machinery. Mitochondria-localized protein Nix (also known as Bcl2/E1B 19 kDa-interacting protein 3 (BNIP3)-like protein (BNIP3L)) was recently shown to be a receptor on mitochondria, mediating mitophagy through interaction with LC3 via the N-terminal LC3 interacting region (Novak et al., 2010). Yeast Pichia pastoris (P. pastoris), a model organism for pexophagy studies, expresses a receptor protein PpAtg30 on peroxisomal membrane and interacts with PpAtg11 for autophagy recognition (Farre et al., 2008). Moreover, several recent studies have shown that the ubiquitin (both mono- and poly-ubiquitin)-binding protein p62/SQSTM1 is a cargo receptor that links autophagy machinery to different cargo targets, including polyubiquitinated protein aggregates (Pankiv et al., 2007), monoubiquitin-tagged peroxisomes (Kim et al., 2008) and bacterial pathogens, such as Salmonella enterica serovar Typhimurium (S. Typhimurium) (Zheng et al., 2009), Shigella flexneri (S. flexneri) (Dupont et al., 2009) and Listeria monocytogenes (L. monocytogenes) (Yoshikawa et al., 2009).
2.4 Autophagy and Intracellular Pathogens

Autophagy has been extensively studied for its role in immunity. It is now believed that autophagy is an important innate immune defense mechanism, and plays multiple roles in adaptive immunity (extensively reviewed in (Deretic, 2009; Orvedahl and Levine, 2009; Virgin and Levine, 2009)). Autophagy can limit the intracellular growth of invading pathogens by degrading them through autophagosome-lysosome pathway. The mechanism of LC3 targeting in a wide range of intracellular pathogens involves, at least in part, protein ubiquitination and p62/SQSTM1 adaptor protein recruitment. P62/SQSTM1 can interact with both LC3 and ubiquitinated cargo through an LC3 interacting region and a C-terminal ubiquitin-associated (UBA) domain, respectively (Ichimura et al., 2008; Pankiv et al., 2007). Thus, p62/SQSTM1 acts in an adapter capacity to bridge the interaction between the ubiquitinated bacteria and LC3.

Recently, additional players involved in LC3 targeting of pathogens have surfaced. Kirkin and colleagues reported that another ubiquitin-binding protein NBR1 (Neighbor of BRCA1 gene 1), which shares similar domain organizations to p62/SQSTM1 and directly interacts with p62/SQSTM1, also acts as a receptor for selective autophagy targeting of ubiquitinated protein aggregates (Kirkin et al., 2009a). However NBR1 does not play a major role in autophagy of S. Typhimurium (Zheng et al., 2009), suggesting that NBR1 and p62 may have different cargo preferences. Another adaptor protein NDP52 (nuclear dot protein 52 kDa), has also been recently shown to be involved in autophagy targeting of S. Typhimurium by recognizing the ubiquitin coat on the bacteria and bringing them to the autophagosomes via binding to LC3 (Thurston et al., 2009). A focused analysis of the two adaptor proteins during S. Typhimurium infection revealed that p62/SQSTM1 and NDP52 bind to distinct microdomains on the bacteria and that the loss of either adaptors results in a decrease in autophagy targeting to the bacteria (Cemma et al., 2010). These observations suggest that while two adaptor proteins appears to be involved in autophagy targeting of S. Typhimurium, they may play different roles in the autophagy pathway that is currently unappreciated. Furthermore, examination of LC3-interacting proteins may reveal additional receptors that are responsible for selective autophagy cargo recognition.

While a wide range of bacteria, viruses and parasites have been shown to be targets of autophagy, some have evolved strategies to either evade autophagy recognition or utilize autophagy for their survival and replication (Hussey et al., 2009; Orvedahl and Levine, 2009)
Autophagy also allows the detection of certain single-stranded RNA (ssRNA) viruses through the transportation of viral replication intermediates into acidic endosomes for recognition by host pathogen pattern recognition receptor (PRR), Toll-like receptor 7 (TLR7) (Lee et al., 2007). Moreover, autophagy is involved in pro-inflammatory cytokine production via either positive or negative effects in different cell types. In the above example TLR7 activation, autophagy is required for viral infection-initiated type I interferon (IFN) production in plasmacytoid dendritic cells (Lee et al., 2007). However other studies using ATG5 knockout mouse embryonic fibroblasts (MEFs) for ssRNA virus infection found enhanced production of type I IFN in these autophagy-deficient cells, suggesting a suppression of cytokine release by autophagy (Jounai et al., 2007; Tal et al., 2009). In addition, studies of mice expressing inefficient level or a truncated Atg16L1 showed upregulated transcription of adipocytokine genes, or in the latter study elevated production of interleukin (IL)-1β and IL-18 when mice were stimulated with bacterial endotoxin, lipopolysaccharide (LPS) (Cadwell et al., 2008; Saitoh et al., 2008). Therefore autophagy has paradoxical roles in both pro- and anti-inflammatory responses.

<table>
<thead>
<tr>
<th>Autophagy targeting mechanism</th>
<th>Bacterial evasion strategy</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td><strong>Group A Streptococcus</strong></td>
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<tr>
<td>Cytosolic <em>Streptococci</em> is targeted by</td>
<td>ActA prevents ubiquitination</td>
<td>(Nakagawa et al., 2004)</td>
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<td>LC3; lack of Atg5 results in delayed</td>
<td>and LC3 targeting of</td>
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<td>bacterial clearance</td>
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<td>PLC may play an unknown</td>
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<td><em>L. monocytogenes</em></td>
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<td>PGRP-LE recognition of bacterial</td>
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<td>(Birmingham et al., 2007a; Py et al., 2007; Rich et al., 2003;</td>
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peak of LC3 targeting at 1 h p.i.; autophagy limits bacterial replication as L. monocytogenes infection of mice with ATG5\(^{-/-}\) macrophages results in higher bacterial load over control

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<th>Bacteria</th>
<th>Role in Autophagy Evasion</th>
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<tr>
<td>salmonella enterica</td>
<td>role in autophagy evasion as bacteria deficient in the PLCs exhibit increased growth in ATG5(^{-/-}) MEFs</td>
<td>Yano et al., 2008; Yoshikawa et al., 2009; Zhao et al., 2008</td>
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<td>Shigella flexneri</td>
<td>Atg5 binds to VirG</td>
<td>Bacterial IcsB masks VirG to prevent recognition and LC3 targeting</td>
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<td>Francisella tulerensis</td>
<td>Cytosolic bacteria targeted by autophagy</td>
<td>(Checroun et al., 2006)</td>
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<td>Helicobacter pylori</td>
<td>Autophagy limits the stability and toxic effect of VacA; autophagy inhibition increases bacterial replication; autophagy induction decreases replication</td>
<td>(Terebiznik et al., 2009)</td>
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<td>Vibrio cholerae</td>
<td>Autophagy targets bacterial VCC</td>
<td>(Gutierrez et al., 2004)</td>
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<td><strong>Burkholderia pseudomallei</strong></td>
<td>Cytosolic bacteria targeted by autophagy; autophagy limits bacterial replication</td>
<td>Bacterial BopA reduce LC3 targeting</td>
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| **Mycobacterium tuberculosis** | LC3 targeting to mycobacteria containing phagosomes, limiting survival | (Gutierrez et al., 2004) |

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<th><strong>Parasite</strong></th>
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<td><strong>Leishmania major</strong></td>
<td>Differentiation into infective form requires autophagy induction</td>
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| **Toxoplasma gondii** | LC3 targeting to Toxoplasma containing compartments to limit survival | (Andrade et al., 2006) |

| **Trypanosoma cruzi** | Encode orthologs of Atg8 conjugation system; autophagy induction needed for survival | (Alvarez et al., 2008) |

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<th><strong>Viruses</strong></th>
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<td><strong>Poliovirus</strong></td>
<td>Viral replication inside LC3+ double membrane</td>
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<td>Pathogen</td>
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<td>Human immunodeficiency virus (HIV)</td>
<td>Vesicles; replication dependent on autophagy (Zhou and Spector, 2007)</td>
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<td>Autophagy inhibited but autophagy components needed for replication</td>
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<tr>
<td>Hepatitis C (HCV)</td>
<td>Autophagy induction and Atg5 interaction with viral NS5B factor allows viral replication</td>
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<tr>
<td>Sindbis virus</td>
<td>Autophagy restricts viral replication</td>
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<tr>
<td>Herpesvirus (HSV-1)</td>
<td>Viral ICP34.5 factor inhibits autophagy by targeting PKR and beclin1</td>
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Table 2.3. Summary of known interactions between pathogens and autophagy
2.3.2.1 Signals for Autophagy Induction

While autophagy has been identified as a host innate immune defense, the mechanisms of autophagy signaling remain largely unknown. Autophagy regulation in immunity is summarized in Figure 2.6. Pathogen detection receptors, including TLRs and NOD-like receptors (NLRs), are crucial host immune factors that sense extra- or intracellular microbes and initiate downstream signaling pathways to activate antimicrobial immune responses (Delbridge and O'Riordan, 2007). A number of TLRs have been reported to stimulate autophagy in murine and human phagocytes (Delgado et al., 2008; Xu et al., 2007). Furthermore, agonist-stimulated receptor (such as TLRs and FcγR) signaling during phagocytosis is shown to induce autophagy targeting of the phagosome and promote phagosomal maturation (Huang et al., 2009; Sanjuan et al., 2007).

Besides TLRs, NLR members, such as Naip5, Ipaf, PGRP-LE and NOD2, can regulate autophagy as well. Naip5 is involved in promoting efficient autophagy of *Legionella pneumophila* (*L. pneumophila*) (Amer and Swanson, 2005). Ipaf has been shown to negatively regulate autophagy of *S. flexneri* in macrophages (Suzuki et al., 2007). PGRP-LE is crucial for autophagy induction and limiting bacterial growth during *L. monocytogenes* infection of *Drosophila* (Yano et al., 2008). NOD2 mediates autophagy induction and targeting of *S. flexneri* (Travassos et al., 2010). Autophagy can also be upregulated by IFN-γ and its downstream effector, Irgm1 (or human IRGM), to eliminate *M. tuberculosis* and *Toxoplasma gondii* (Orvedahl and Levine, 2009). Conversely, autophagy can also be negatively regulated by NF-κB during tumor necrosis factor (TNF)-α-stimulation (Djavaheri-Mergny et al., 2006). Viral infection can induce autophagy via the cytosolic double-stranded viral RNA-sensing kinase PKR, activating the transcription factor eIF2α (Talloczy et al., 2002). In addition, viruses can inhibit autophagy by modulating autophagy proteins. An α-herpesvirus, HSV-1, produces the neurovirulence factor, ICP34.5, to directly target Beclin 1 and block autophagy (Orvedahl et al., 2007).
Figure 2.6. Regulation of autophagy by immune signals in response to invading pathogens.

Induction of autophagy is observed upon activation by Naip5 by *L. pneumophila*, Ipaf or Atg16L1 by *S. flexneri*, PGRP-LE by *L. monocytogenes*. Some viruses can induce autophagy via eIF2α but others can inhibit autophagy via inhibition of Beclin1. Host immune modulators, such as IFN-γ, TNF-α and ROS can also induce autophagy. Reprinted by permission from Mary Ann Liebert, Inc: Antioxidants Redox Signaling, Huang J *et al*. Autophagy Signaling Through Reactive Oxygen Species. 14(11): 2215-31.
2.3.2.1.1 ROS-mediated induction of Autophagy

Mounting oxidative stress can result in damaged organelles, proteins, and DNA and the clearance of which is one of the main functions of autophagy in a number of cell types (Kaushik and Cuervo, 2006; Lemasters, 2005; Xiong et al., 2007) (reviewed in (Kiffin et al., 2006)). If proper clearance cannot take place, increasing ROS levels would then lead to autophagic cell death (Chen et al., 2008), as seen in the context of TNF-α induced autophagic cell death in Ewing sarcoma cells (Chen et al., 2008) or LPS induced autophagic macrophage cell death (Xu et al., 2006). If autophagy cannot be properly activated, cellular dysregulation in diseases of age, such as Alzheimer’s (Cataldo et al., 1996), or uncontrolled inflammation, such as Diabetes mellitus (Sooparb et al., 2004), would result. However, the mechanism by which ROS production leads to activation of autophagy is not clear.

The first direct link between ROS and autophagy was provided in context of starvation-induced autophagy and mitochondrial-derived ROS (Scherz-Shouval and Elazar, 2007; Scherz-Shouval et al., 2007a; Scherz-Shouval et al., 2007b). In response to nutrient and serum starvation, it was found that a number of cell lines – HeLa, HEK293, CHO and MEFs – greatly induced both superoxide and H$_2$O$_2$ production in a class III PI3K-dependant manner when compared to control. Furthermore, there was more ROS production in wild type MEFs over MEFs without Atg5 (Scherz-Shouval et al., 2007b). These two observations combined indicate that PI3K is somehow upstream of ROS production, leading to autophagy. ROS production was proposed to inhibit the redox sensitive cysteine protease, Atg4 - specifically the cysteine residue residing in the active site of the enzyme. Atg4 is one of the autophagy proteins involved in the regulation of LC3 phosphatidylethanolamine (PE) lipidation (LC3-II) and delipidation (LC3-I) (Nakatogawa et al., 2007), a post-translational event essential for autophagy to occur. When Atg4 encounters H$_2$O$_2$, Cys81 becomes oxidized in a reversible reaction, rendering the enzymatic activity null (Scherz-Shouval et al., 2007b). The lack of Atg4 activity is thought to allow LC3 to remain lipidated and localized to the autophagosomal membrane, leading to autophagy.

The link between mitochondrial ROS and starvation-induced autophagy was further confirmed by Chen and colleagues (Chen et al., 2009). Superoxides but not H$_2$O$_2$ were found to be the critical ROS member for the induction of autophagy in HEK293, human glioma cell line U87 and HeLa cells. This study found that pharmacological inhibition or siRNA silencing of SOD,
which converts superoxide to \( \text{H}_2\text{O}_2 \), results in increased autophagy when compared to WT controls. Conversely, the overexpression of SOD resulted in a decrease in superoxide levels and increase in \( \text{H}_2\text{O}_2 \), leading to decreased autophagosome formation. Superoxides were also found to be important for the induction of autophagy in the context of muscular atrophy. It was found that transgenic mice with a SOD deletion exhibit increased numbers of autophagosomes (Dobrowolny et al., 2008). Since the superoxides are quickly converted to \( \text{H}_2\text{O}_2 \) and other reactive products, there are limitations in ROS assays that make it hard to distinguish one particular type from another. Furthermore, ROS products are linked together in a web of reactions (Figure 2.4), thus it becomes difficult to take away any one enzyme without affecting any other products in a compensatory manner. For example, by knocking out SOD, not only is the level of \( \text{H}_2\text{O}_2 \) affected, but also the levels of HOCl, peroxynitrite and OH'. The limitation of detection techniques available, coupled with the transient nature of ROS members makes it challenging to pinpoint specific triggers of autophagy. Regardless of the type of ROS, however, it is clear that mitochondrial ROS are indeed involved in the activation of starvation-induced autophagy.

Further evidence has surfaced relating ROS to starvation-induced autophagy. One of the mechanisms of glycolysis downregulation is mediated through TIGAR, TP53-induced glycolysis and apoptosis regulator. An indirect consequence of TIGAR activity on glucose metabolism is the decrease in global intracellular ROS levels (Bensaad et al., 2006). TIGAR activity was found to block autophagosome formation in the human osteosarcoma U2OS cells, even under starvation conditions (Bensaad et al., 2009). This inhibition of autophagy was correlated with lowered ROS levels. Adding TIGAR exogenously results in an even greater decrease in ROS and autophagy. Thus, TIGAR is potentially expressed only under nutrient rich conditions to inhibit autophagy activation via the inhibition of ROS.

Unlike starvation-induced autophagy, the mechanism by which antimicrobial autophagy is activated is less clear. The first detection of invading pathogens occurs via the binding of pattern recognition receptors, such as TLRs, to its pathogen associated molecular patterns (PAMPs), resulting in the activation of autophagy in both murine RAW 264.7 macrophage cell line and primary bone marrow derived macrophages (Delgado et al., 2008; Sanjuan et al., 2009; Xu et al., 2008) (reviewed by (Delgado and Deretic, 2009)). Nucleotide-binding oligomerization domain (NOD) and NOD-like receptor (NLR) signaling has also been shown to regulate autophagy
Upon the activation of TLR, FcγR, complement receptors or others, phagocytosis of microbes is initiated and autophagy is triggered (Park, 2003; Sanjuan et al., 2007). Seminal work done by Sanjuan and colleagues showed that the activation of TLR upon phagocytosis results in the recruitment of LC3 to the phagosome (Sanjuan et al., 2007) forming a single membrane LC3+ phagosome. The induction of autophagy leads to phagosomal maturation and subsequent degradation of the phagosomal content in the autophagolysosome. TLR signaling has also been shown to be upstream of NOX2 NADPH oxidase assembly and activation (Laroux et al., 2005; Quinn and Gauss, 2004). Given that TLR activation leads to both NOX2 NADPH oxidase activation and autophagy, we hypothesized that ROS might also play a role in the activation of antimicrobial autophagy. To this end, we first examined if TLR or FcγR activated autophagy is mediated through ROS production. Using bone marrow derived neutrophils (BMDN), we challenged cells with IgG-coated latex beads or dead yeast cells, zymosan, and found that LC3 was recruited to the phagosome (Huang et al., 2009). This recruitment was further examined using either pharmacological agents to block/neutralize ROS or comparing wild type with NOX2 NADPH oxidase deficient BMDN with respect to LC3 recruitment upon stimulation. We found that in absence of NOX2 NADPH oxidase activity, LC3 recruitment to the phagosome was impaired. An analysis of the purified phagosomes revealed enhanced localization of Atg12 (an essential autophagy protein) and LC3-II on IgG-coated latex bead containing phagosome and this recruitment was inhibited by the addition of the NOX2 NADPH oxidase inhibitor, DPI. Thus, ROS was found to be critical for LC3 targeting to latex bead containing phagosomes (Figure 2.7). We further established that other cell types (epithelial cells) also employ member(s) of the NOX family for antimicrobial autophagy, suggesting that the NOX family may be a general mechanism of antimicrobial defense in a range of cell types. Using p22phox siRNA silencing in the human embryonic epithelial cells, Henle-407, we observed that the autophagy of Salmonella enterica serovar Typhimurium decreased. Recently, another independent study confirmed some of our observations in human neutrophils, suggesting that NOX2 NADPH oxidase-derived ROS induction of autophagy is not a murine only phenomenon (Mitroulis et al., 2010).
Figure 2.7. The known pathways that are dependent on ROS induction of autophagy. (A) Phagocytosis of microbes results in the recruitment of NOX2 NADPH oxidase complex to the phagosome. The presence of ROS signals the induction of autophagy through an unknown mechanism, resulting in the recruitment of autophagy protein, LC3, to the phagosome, where LC3 is then conjugated to PE. Subsequent fusion with the lysosome allows for microbial degradation in the autophagolysosome. (B) Upon nutrient starvation, ROS are generated in the mitochondria as a byproduct of electron transport chain activity. Loss of mitochondrial integrity results in the release of ROS into the cytosol, triggering autophagy. The damaged mitochondria and partial cytoplasm are then contained in an autophagosome that is likewise marked for lysosomal fusion and degradation. Reprinted by permission from Springer Science + Business Media: Seminars in Immunopathology, Lam G et al. The many roles of NOX2 NADPH oxidase-derived ROS in immunity, 32(4): 415-30
2.3.2.1.2 Diacylglycerol-mediated induction of Autophagy

Diacylglycerol (DAG) was also found to be a key signal for mediating autophagic targeting to *Salmonella enterica* serovar Typhimurium containing phagosomes at 1 h post infection (p.i.) (Shahnazari et al. 2010). Inhibition of DAG production by host DAG-producing enzymes, phospholipase D and phosphatidic acid phosphatase, results in a reduction in LC3 targeting to *S. Typhimurium*. DAG mediated autophagy targeting to the bacteria was further found to be dependent on the activity of the downstream signaling kinase, protein kinase C-delta (PKCδ) (Shahnazari et al. 2010). How PKCδ mediates autophagic targeting remains to be understood.

2.3.2.1.3 Ubiquitinated protein-mediated induction of Autophagy

Finally, ubiquitinated proteins can also be a signal for autophagy and has been extensively reviewed (Dupont et al., 2010; Kirkin et al., 2009b; Randow, 2011). Ubiquitin (Ub) is a small 76 amino acid protein that can be conjugated to proteins as single ubiquitin subunits or poly ubiquitin chains. Ubiquitinated *S. Typhimurium* is targeted by autophagy (Birmingham et al., 2006) via the recruitment of adaptor proteins, p62 (Cemma et al., 2010; Zheng et al., 2009) and NDP52 (Cemma et al., 2010; von Muhlinen et al., 2010). *L. monocytogenes* mutants that lack the ability to recruit actin, ΔActA, (see section on *L. monocytogenes* below) can also be ubiquitinated in the cytosol and subsequently targeted by autophagy (Yoshikawa et al., 2009). Finally, the remnants of the phagosome left behind after *Shigella flexneri* has escaped into the cytosol can also be ubiquitinated and targeted for autophagic clearance (Dupont et al., 2009).

It remains controversial how LC3 is targeted to pathogens. Autophagic targeting may be the result of either the fusion of LC3-positive (LC3⁺) vesicles, which elongates to form a double membrane compartment or direct LC3 conjugation to a single membrane compartment (Huang and Brumell, 2009; Sanjuan et al., 2007). Work done by Rich and colleagues indicates that cytosolic ΔActA *L. monocytogenes* mutants were found in LC3⁺, endoplasmic reticulum (ER) markers bearing, double membrane compartments in presence of the bacteriostatic agent, chloramphenicol. This observation suggests that cytosolic autophagic targeting of mutant *L.
monocytogenes is mediated by the fusion of small LC3\(^+\) vesicles from the ER (Rich et al., 2003). Similarly, a recent study using correlative light microscopy-electron microscopy (CLEM) imaging indicates that autophagy targeted Salmonella containing vacuoles are double-membrane structures (Kageyama et al., 2011). One possible explanation for these different observations may be that the mechanism of LC3 recruitment is different depending on where the pathogen is located. LC3\(^+\) vesicles may target cytosolic bacteria, while LC3 may be directly conjugated to phagosomes containing bacteria. Another possible explanation is that the mechanism of LC3 recruitment is pathogen specific (ie: Gram-positive vs. Gram-negative). Thus, many questions remain surrounding the mechanism of autophagic targeting to intracellular pathogens.

2.3.3 Common techniques used to study autophagy

Given the diversity of the field of autophagy, there is a vast range of techniques that are used to study the different types of autophagy. However, there are some key methods that are universal in the field to examine autophagic flux. Here, I will discuss these cornerstone techniques.

As mentioned, the concept of autophagy flux has evolved as the field of study matured. An observed increase in LC3-I to LC3-II conversion is no longer enough to make the argument that autophagy is increased. This is due to the fact that an accumulation of LC3-II may be due to a block in autophagosome clearance (Tanida et al., 2005), as well as an increase in the rate of autophagy. Thus, a number of other methods have been described to assess mammalian autophagy and autophagic flux (extensively reviewed in (Klionsky et al., 2008; Klionsky et al., 2007; Mizushima, 2004; Mizushima et al., 2010)).

2.3.3.1 Techniques used to quantify changes in autophagy

Currently, changes in LC3-II is most commonly assessed using western blot analysis, flow cytometry, LC3 colocalization to particle containing phagosomes, or increase in LC3\(^+\) puncta. LC3-I runs at 19KDa while LC3-II runs at 17KDa. As such, higher percentage SDS-PAGE gels will have the power to resolve these two distinct bands (Figure 2.8 A). However, it must be noted that LC3-I is often difficult to resolve for reasons that are currently unknown. Thus, LC3-II
remains the reliable indicator of autophagy. LC3-II levels have been described to increase upon autophagic induction but decrease over time (> 2h) as a result of increased turnover (Mizushima and Yoshimori, 2007). Changes in LC3 expression can also be assessed using flow cytometry. Endogenous LC3 can be stained using immunofluorescent antibodies and increased fluorescence can be assessed via flow cytometry on a population basis. Alternatively, increased cellular levels of LC3 can also be visualized by immunofluorescence imaging or transmission electron microscopy. Increases in autophagy result in increases in LC3+ puncta (Figure 2.8 B; white arrowheads). Thus, quantification of the changes in numbers of LC3+ puncta per cell can give an indication of changes in autophagy. Lastly, in the field of selective autophagy, LC3 targeting to phagosomes containing foreign particles or pathogens can likewise be used to assess changes in autophagic targeting. Quantification of the percentage of LC3+ particle containing phagosomes (Figure 2.8 C; white arrowheads) over total number of particle containing phagosomes allows an assessment of autophagic targeting to a selective target.

Autophagosomes are defined strictly as LC3+ double membrane compartments. However, LC3+ single membrane phagosomes have also been reported. Sanjuan and colleagues have previously demonstrated that TLR or FcγR-mediated phagocytosis of IgG-coated latex beads or zymosan particles results in formation of LC3+ single membrane particle containing phagosomes (Sanjuan et al., 2007). As such, these structures, deemed LC3-associated phagosomes, must be distinguished from autophagosomes. To determine if a LC3+ structure of interest is double or single membraned, CLEM is currently the gold standard for this assessment. CLEM is a process where LC3+ structures identified in immunofluorescence microscopy is matched with the corresponding electron micrograph to determine the number of membranes that are on the LC3+ structures. Other techniques, such as electron microscopy of LC3 immunogold labeling, can also be used.

2.3.3.2 Techniques used to assess flux

In tandem with studies of autophagy, techniques for assessing autophagic flux are often employed to determine if increases in autophagy is due to increased rate of autophagy or if the turnover is blocked, resulting in an accumulation of LC3-II. The rate of protein turnover can be
assessed by examining levels of p62 by western blot analysis. p62 (also known as SQSTM1/sequestome 1) is an adaptor protein that is recruited to autophagosomes through binding to LC3 and is degraded by autophagy (Bjorkoy et al., 2005). An increase in LC3-II that is accompanied with a decrease in p62 correlates well with an increase in the rate of autophagy. Conversely, an increase in both LC3-II and p62 correlates with a block in autophagic flux or clearance (Mizushima et al., 2010). However, it must be noted that p62 is not solely degraded in the autophagic pathway and thus changes in p62 levels may not solely reflect changes in autophagy (Bjorkoy et al., 2005). To address this limitation, other assays have recently been developed to assess autophagic flux.

An alternative method to measure autophagic flux is that of the RFP-GFP-LC3 tandem probe (Kimura et al., 2007). This probe was designed based on the concept of differences in lysosomal quenching of GFP versus RFP fluorescence. In the acidic environment of the lysosome, GFP fluorescence is quenched while RFP fluorescence is not. Thus, under conditions of proper autophagosome delivery to the lysosome, cells expressing the RFP-GFP-LC3 will display predominantly red puncta since GFP, but not RFP fluorescence is quenched in the lysosome. However, if a blockage in autophagosome delivery to the lysosome, cells would display predominantly yellow puncta since both GFP and RFP fluorescence will be observed in the autophagosomes prior to delivery to the lysosome. Thus, autophagic flux can be assessed by quantifying the ratio of red to yellow puncta. Cells with increased rate of autophagy will display a higher red to yellow puncta ratio while cells experiencing a block in autophagic flux will display a lower red to yellow puncta ratio. One limitation is that this technique is based on the assumption that cells retain normal rates of acidification and lysosome enzyme activity. If these parameters are altered, LC3 punctae may remain yellow for reasons other than a block in autophagic flux. As such, this assay does not give an indication of when degradation in the lysosome is occurring. One technique that can address this limitation is the GFP-LC3 cleavage assay. Upon delivery to the lysosome, the GFP-LC3 fusion protein will be partially degraded, liberating GFP (Gao et al., 2008). Thus, following free GFP accumulation via immunoblotting can give an indication of lysosome activity upon fusion with autophagosomes. While this technique has been commonly used in the study of yeast autophagy, its use in mammalian cells remains limited.
Figure 2.8. Examples of methods to assess autophagy. (A) Western blot analysis of lysates generated from RAW 264.7 macrophage infected with wild type *L. monocytogenes* for the indicated time points p.i. and probed for LC3. Bands roughly at 19 and 17 KDa, corresponding to LC3-I and LC3-II, can be resolved. (B) Henle 207 cells expressing GFP-LC3 with or without *S. typhimurium* infection. White arrowheads indicate LC3 puncta. (C) Raw 264.7 macrophages expressing GFP-LC3 infected with *L. monocytogenes*. White arrowheads indicate LC3 targeting to the intracellular bacteria.
2.4 Other Defenses

In addition to ROS and autophagy, there are a number of other key host defenses that mediate the microbicidal activity of the phagosome, including low phagosomal pH, reactive nitrogen species (RNS), antimicrobial peptides and defensins. These processes were reviewed by Flannagan and colleagues (Flannagan et al., 2009) and will only be summarized here for completeness.

Phagosomal acidification by the vacuolar ATPase is a vital step in phagosomal maturation. Not only can a low pH environment (pH 4-5) be directly bacteriocidal, but can also allow the activity of a number of pH regulated degradative enzymes. These enzymes include cathepsins, hydrolyases and other proteases. RNS are produced in macrophages by the inducible nitric oxide synthase (NOS) in response to inflammation (Fang, 2004). The main NOS product is nitric oxide. Like superoxide, nitric oxide can be converted into a number of other RNS, all of which are directly bacteriocidal. While RNS are produced in the cytosol, they can diffuse across membranes to the sites of infection (Webb et al., 2001). Together, ROS and RNS can act synergistically to damage proteins, lipids and microbial DNA (Fang, 2004). Lastly, a wide range of soluble factors such as antimicrobial peptides, bacteriocidal granules, defensins, lysozymes, lipases, proteases and peptidases target specific bacterial components and contribute to direct bacterial killing in the phagosome (Flannagan et al., 2009).

3 Bacterial Evasion Strategies in Response to Host Defenses

A number of pathogens have evolved mechanisms to evade degradation by phagocytes. Phagocytosis has been described as a two-step event requiring pathogen uptake and clearance and several pathogens have devised mechanisms to resist both these steps (Flannagan et al., 2009; Kumar and Valdivia, 2009; Steinberg and Grinstein, 2008; Woolard and Frelinger, 2008). Pathogens can hinder their uptake by phagocytes in a number of ways. Some bacteria secrete polysaccharides, which form a slippery capsule or coating around the bacteria, making phagocytosis difficult. Other pathogens, such as Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, express factors that cleave or inactivate host proteins that enhance
phagocytosis (such as antibodies or complement), thus decreasing the efficiency of uptake (Hong and Ghebrehiwet, 1992; Prasadara et al., 2002; Rooijakkers et al., 2005). Finally, *P. aeruginosa*, and uropathogenic *E. coli* (UPEC) secrete virulence factors that disrupt actin dynamics that are required for uptake (Davis et al., 2005; Garrity-Ryan et al., 2000). *Yersinia enterocolitica* also secretes virulence factors that impair uptake; however, the mechanism for this inhibition remains elusive (Grosdent et al., 2002).

Intracellular pathogens that do not resist uptake have been shown to employ other strategies to either inhibit or alter the process of phagosomal maturation to avoid lysosomal clearance. Some pathogens, such as *Mycobacterium tuberculosis*, *Coxiella burnetti*, *Legionella pneumonphila* and *Salmonella enterica* serovar Typhimurium, can withstand the low pH of the lysosomal compartment either by expressing pH insensitive proteins or enzymes that neutralize the pH in their immediate vicinity (Heinzen et al., 1996; Park et al., 1996; Sauer et al., 2005; Sturgill-Koszycki and Swanson, 2000; Vandal et al., 2008). Similarly, some pathogens encode bacterial virulence factors that can inhibit the activity or production of antibacterial peptides or small molecules (McCaffrey and Allen, 2006; Peschel et al., 2001; Schmidtchen et al., 2002; Vazquez-Torres et al., 2000). However, these survival mechanisms are inadequate if the bacteria is to colonize and flourish in the host cell. Thus, more elaborate and sophisticated mechanisms are needed in order to avoid phagosomal fusion with the lysosome.

Mechanisms of preventing phagosomal fusion with the lysosome can be classified into three categories: 1) inhibition of phagosomal maturation, 2) alteration of phagosomal maturation, and 3) escape from the phagosome. Inhibition of maturation is exemplified by *M. tuberculosis* as this bacterium prevents the recruitment of surface proteins (EEA1 and VPS34) (Fratti et al., 2001) and the generation of specific phosphoinositides (PI3P) (Fratti et al., 2001; Fratti et al., 2003), without which fusion with the lysosome cannot occur (Fratti et al., 2001; Pethe et al., 2004). Similarly, maturation of the phagosome can be altered by recruiting surface proteins that mediate phagosomal fusion with organelles other than the lysosome. For example, *L. pneumophila* expresses effectors that slow maturation and allow recruitment of host factors, Rab1 (Ingmundson et al., 2007; Murata et al., 2006) and ARF1 (Nagai et al., 2002), which mediate fusion with the endoplasmic reticulum instead of the lysosome (Robinson and Roy, 2006). Finally, some pathogens, such as *Listeria monocytogenes*, encode virulence factors that mediate escape from the phagosome (Portnoy et al., 2002).
The process of phagosomal escape is highly complex and intricate. It is one of the most efficient methods of colonization as bacterial replication occurs rapidly in the nutrient-rich cytosol. In addition to \textit{L. monocytogenes}, a number of other pathogens, including \textit{S. Typhimurium} and \textit{Shigella flexneri}, utilize this method for lysosome avoidance. Consequently, the use of \textit{L. monocytogenes} as a model pathogen to study the battle between host and bacteria will shed light on the pathogenesis of a number of other pathogens. Thus, the focus of this thesis is to gain insight into the early host defenses that are activated upon \textit{L. monocytogenes} infection and the bacterial attempts to avoid clearance.

4 \textbf{\textit{L. monocytogenes}}

\textit{Listeria monocytogenes} is a Gram-positive bacterium that is well adapted to survive both in the environment (Fenlon et al., 1995) and inside mammalian cells (Dussurget et al., 2004). This rod-shaped organism was first described in 1926 by E.G.D Murray following an epizootic outbreak in rabbits and guinea pigs in an animal care facility in England (Murray et al., 1926). The potential for this bacterium to infect humans was not realized until 1952 (Potel, 1952).

\textit{L. monocytogenes} is uniquely resistant to conventional food sterilizing techniques, such as refrigeration, low pH, high salt concentrations and other such environmental extremes (Schnupf and Portnoy, 2007). Furthermore, this organism also produces biofilms, an extracellular polymer matrix material that aids in prolonged survival (Djordjevic et al., 2002). Thus, given the ubiquitous nature of this organism, the potential for food borne outbreaks is relatively high. The first reported case of food borne \textit{L. monocytogenes} outbreak occurred in Nova Scotia in 1981, and resulted in 18 deaths (Schlech et al., 1983). Since then, there have been numerous cases of outbreaks or product recalls due to \textit{L. monocytogenes} contamination found on consumer goods including ready-to-eat meats, soft unpasteurized cheeses, and raw produce.
4.1 Genetic Diversity of \textit{L. monocytogenes}

\textit{L. monocytogenes} belongs to the genus, \textit{Listeria}, which is also comprised of \textit{L. innocua}, \textit{L. ivanovii}, \textit{L. seeligeri}, \textit{L. welshimeri} and \textit{L. grayi} (Schmid et al., 2005). Of these, only \textit{L. monocytogenes} and \textit{L. ivanovii} are pathogenic (Hitchins, 1998). However, \textit{L. ivanovii} is only pathogenic in animals. Understanding of \textit{L. monocytogenes} pathogenesis has been improved since early 2000 when the first \textit{L. monocytogenes} genome was sequenced (Glaser et al., 2001). To date, 22 different \textit{L. monocytogenes} clinical isolates have been sequenced, with each genome comprising roughly 3 Mbps, and an average of 2900 predicted protein-coding genes and an average GC content of 38% (Glaser et al., 2001; Nelson et al., 2004).

4.2 Epidemiology of Clinical Listeriosis

4.2.1 Clinical Presentation and Treatment

\textit{L. monocytogenes} infection in humans can result in listeriosis, a gastroenteritis that is self-limiting in healthy individuals but may become severe and systemic for immunocompromised individuals, the elderly and pregnant women (Rocourt and Bille, 1997). In susceptible individuals, bacterial infection can progress to septicemia, meningoencephalitis and maternofetal/neonatal listeriosis, culminating in a 20-30% mortality rate despite intervention (Swaminathan and Gerner-Smidt, 2007). Listeriosis may also present as local infections following hematogenous spread, commonly in the peritoneum, endocardium, joints or eyes (Swaminathan and Gerner-Smidt, 2007). Furthermore, these bacteria can persist asymptomatically even after the acute illness has subsided as \textit{L. monocytogenes} can be detected in the stool of 1-10% of the general population (Ramaswamy et al., 2007). The incidence rate of listeriosis ranges between 0.1 to 11.3 per million individuals in different countries (WHO, 2004). In Canada, there are on average 100 to 140 reported cases of listeriosis per year. However, it must be noted that investigation of the epidemiology of \textit{L. monocytogenes} infections can often be complicated by the variability in incubation period, which can range from 3 to 60 days.

\textit{L. monocytogenes} infection is commonly identified via blood and urine cultures. Treatment for symptomatic listeriosis in the high-risk population involves supportive therapy along with high
doses of penicillin or ampicillin in combination with an aminoglycoside. While reports of antibacterial resistance in clinical isolates remain rare (Hansen et al., 2005), more cases of resistance in animal isolates have surfaced (Srinivasan et al., 2005).

### 4.2.2 Clinical Isolates

Clinical isolates of *L. monocytogenes* can be classified into 13 serovars based on gene content and virulence. While serotypes 1/2a, 1/2b and 1/2c are the most abundant in the environment, serotypes 1/2a, 1/2b and 4b are responsible for 95% of all human infections (Swaminathan and Gerner-Smidt, 2007). Specifically, serotype 4b is responsible for the majority of *L. monocytogenes* outbreaks and is associated with more severe symptoms and greater mortality in infected individuals (Gerner-Smidt et al., 2005; Srinivasan et al., 2005). These clinical observations suggest that different serotypes may confer different levels of virulence. While there have been attempts to perform a comparative analysis between the different serotypes (Jacquet et al., 2004), a systematic attempt has yet to be made. Thus, it remains currently unclear why serotype 4b is more virulent than others.

Most notably, one serotype 1/2a strain, termed 10403S, is the most widely used strain in *L. monocytogenes* research worldwide. 10403 was originally isolated from a human skin lesion in the USA and 10403S is a streptomycin resistant mutant of the parent 10403 strain. Other common laboratory strains include LO28, a serotype 1/2c strain that is not a clinical isolate, and EGDe, a serotype 1/2a strain that was originally isolated from rabbit tissue in England.

### 4.3 *L. monocytogenes* infection and intracellular lifestyle

Upon food borne entry into the human host, *L. monocytogenes* has the ability to cross the intestinal, blood-brain and maternal-fetal barriers (Lecuit and Cossart, 2002). This organism is also a facultative intracellular pathogen that can survive and replicate inside epithelial cells or macrophages (Dussurget et al., 2004). As previously described, the cytosol of host cells is an ideal environment for pathogens since it is nutrient rich and sheltered from a number of
extracellular immune defenses, such as direct killing by complement or antimicrobial peptides and antibody neutralization and opsonization. The intracellular lifecycle of *L. monocytogenes* can be divided into four distinct stages: 1) entry into the host cell, 2) phagosomal escape, 3) cytosolic replication, and 4) secondary spread. *L. monocytogenes* expresses two proteins called Internalin A and B (InlA and B), which mediate entry into the host cell by induced endocytosis or phagocytosis.

Upon entry, *L. monocytogenes* expresses a number of virulence factors that subvert host defenses in order to avoid death in the lysosome. These include listeriolysin O (LLO) and two phospholipases (PLCs), whose activity mediates escape from the primary phagosomal vacuole. Upon escape, *L. monocytogenes* can then replicate rapidly in the nutrient rich cytosol. Another virulence factor, ActA, then mediates the nucleation of an actin tail on one end of the bacteria. Polymerization of the actin tail allows the bacteria to “rocket” into neighbouring cells entering in a double-membraned secondary vacuole. This therefore allows for secondary spread (Figure 4).

### 4.3.1 Entry into host cells

InlA and InlB mediate the uptake of *L. monocytogenes* into epithelial cells. They belong to the family of internalin proteins that are characterized by the presence of leucine-rich repeats (LLRs) (Gaillard et al., 1991). Specifically, InlA and B posses the LPXTG cell wall-sorting motif, which allows linkage of these proteins to the bacterial surface (Dussurget et al., 2004). InlA and B also have the capacity to bind to specific proteins in the host cell plasma membrane. InlA binds to host E-cadherin, an interaction that is dependent on proline 16 in E-cadherin (Mengaud et al., 1996). Mouse E-cadherin has a glutamic acid at this residue, which prevents InlA mediated entry into mouse epithelial cells (Lecuit et al., 1999). Thus, oral administration of *L. monocytogenes* is an inefficient method of establishing an in vivo infection in wild type mice. InlB binds to the hepatocyte growth factor/scatter factor, or Met, receptor (Shen et al., 2000) and the globular part of the complement component C1q receptor (C1qR) (Bierne and Cossart, 2002). A number of studies have found that InlB alone is sufficient to mediate uptake of latex beads or other noninvasive organisms (Braun et al., 1997; Braun et al., 1999; Braun et al., 1998). Interaction between InlB and the Met receptor triggers phosphorylation and activation of this tyrosine kinase receptor (Shen et al., 2000). As a result, downstream effectors are recruited (Ireton et al., 1999).
Figure 4. Intracellular lifecycle of *L. monocytogenes* with associated electron micrographs.

Intracellular survival of *L. monocytogenes* is mediated at different stages by the activity of different virulence factors. 1) Internalin A and B (InlA and InlB) mediate bacterial entry. 2) Bacterial escape from the phagosome is mediated by listeriolysin O (LLO) and PI-PLC. 3) Rapid bacterial replication occurs in the cytosol. 4) Actin rocket tail formation is mediated by ActA. 5 & 6) Secondary spread into the neighbouring cell is mediated by the actin rocket tail. 7 & 8) Escape from the double-membraned secondary phagosome is mediated by PC-PLC. Here, the cycle of *L. monocytogenes* infection repeated. Reprinted from *L. monocytogenes*, a unique model in infection biology: an overview. Pascale Cossart, Alejandro Toledo-Arana. Microbes and Infection. 10: 1041-50 (2008), with permission from Elsevier.
to mediate actin rearrangement to allow for bacterial uptake via a zipper-like mechanism (Bierne and Cossart, 2002). It must be noted that there are other factors that have been found to be involved in bacterial mediated uptake. However, since the focus of this thesis is on *L. monocytogenes* infection in macrophages and the predominant mode of bacterial uptake in this cell type is mediated via phagocytosis, further details on bacterial mediated uptake mechanisms will not be further discussed.

### 4.3.2 Escape from the phagosome

Upon entry, *L. monocytogenes* resides inside a phagosome that is destined for degradation in the lysosome. To overcome this fate, the bacteria employ three virulence factors, listeriolysin O (LLO) and two phospholipase Cs (PLCs) to escape from the phagosome. While the PLCs enhance the efficiency of escape, LLO is necessary and sufficient for phagosomal escape (Beauregard et al., 1997; Berche et al., 1987; Henry et al., 2006; Kuhn et al., 1988; Portnoy et al., 1988). Thus, LLO is the critical virulence factor for *L. monocytogenes* survival in macrophages. *L. monocytogenes* mutants that lack *hly*, the gene that encodes LLO, are avirulent in *in vivo* mouse models. While it is not clear exactly how LLO mediates escape, it has been shown that LLO inhibits fusion with the lysosome by generating small pores in the phagosome that block acidification and the accumulation of Ca\(^{2+}\) in this compartment through uncoupling of the H\(^+\) pump (Shaughnessy et al., 2006). This provides a “window of opportunity” for the PLCs to lyse the phagosomal membrane (Shaughnessy and Swanson, 2007). LLO and the PLCs have are also necessary for escape from double membrane vacuole formed after secondary cell-to-cell spread (Gedde et al., 2000).

#### 4.3.2.1 Listerialysin O (LLO)

LLO is a cholesterol dependent cytolysin (CDC) belonging to a family of pore-forming cytolysins that are produced by a number of Gram-positive bacteria (Alouf, 2000). A diverse range of pathogens secrete pore-forming cytolysins, including *Clostridium perfringens* (encodes perfringolysin O), *Streptococcus pyogenes* (encodes streptolysin O), *S. pneumoniae* (encodes
pneumolysin), *Aeromonas hydrophila* (encodes aerolysin) and *Bacillus anthracis* (encodes anthrolysin) (Tweten et al., 2001). Unlike other pore-forming cytolysins, LLO exhibits pH sensitivity, with its optimal activity at pH <6 (Schuerch et al., 2005). Under acidic conditions, LLO monomers bind to cholesterol rich regions on the membrane and undergo a conformational change, which allows for the formation of hydrogen bonds with other monomers (Gekara et al., 2005). This oligomerization results in the formation of the pre-pore complex. Via a cholesterol independent mechanism (Gekara et al., 2005; Jacobs et al., 1998), two α-helical bundles on each monomer extend to form transmembrane β-hairpins that insert into the membrane (Shatursky et al., 1999; Shepard et al., 2000). This generates a functional pore of up to 350Å in diameter that is comprised of roughly 33-50 monomers (Gilbert et al., 2000). Nascent LLO pores are thought to only allow the passage of small molecules (< 550 Da), thus rendering the phagosome permeable to Ca^{2+} and H^+ (Shaughnessy et al., 2006). Changes in calcium concentration and pH can delay phagosomal maturation, thus allowing time for the PLCs to enlarge the pores, which ultimately dissolves the phagosomal membrane (Luzio et al., 2003; Shaughnessy and Swanson, 2007).

Since pore-forming cytolysins have the potential to form pores on any cholesterol containing membrane, unregulated activity may result in host cell death. Indeed, it has been demonstrated that genetically modified *L. monocytogenes* strains that produce perfringolysin O (PFO) in place of LLO cause increased cellular toxicity compared to wild type *L. monocytogenes* (Glomski et al., 2002; Jones and Portnoy, 1994). This work suggested that *L. monocytogenes* has evolved “failsafe” mechanisms which ensure that LLO is only active at the appropriate time and cellular location (reviewed in (Schnupf and Portnoy, 2007)). Subsequent studies have shown that a number of unique factors ensure optimal LLO activity specifically in the phagosome. Acidification of the phagosome allows structural reconfigurations that render LLO stable and active. At neutral pH, LLO undergoes a rapid and irreversible alteration of its structure, rendering it unable to form pores despite being oligomerized (Schuerch et al., 2005). However, under cholesterol rich conditions, low levels of LLO activity can be observed even at neutral pH (Bavdek et al., 2007). Thus, while optimal LLO function is limited to the late acidified phagosome, LLO may still function to a lower extent at neutral pH. Furthermore, the reduction of disulfide bonds in LLO is needed for efficient formation of pores (Geoffroy et al., 1987; Portnoy et al., 1992). This is mediated by a host phagosomal protein called gamma-interferon inducible lysosomal thiolreductase (GILT) (Singh et al., 2008). LLO activity is further
modulated by other host factors in the phagosome, such as cathepsin D (Carrasco-Marín et al., 2009) and defensins (Arnett et al., 2011).

In addition to mechanisms that ensure optimal LLO activity is restricted to the phagosome, there are also mechanisms that limit LLO activity in the cytosol once the bacteria has escaped the phagosome. LLO encodes a PEST-like region (a sequence that marks proteins for proteasome degradation) which functions to limit the levels of cytoplasmic LLO to prevent cytolysis (Schnupf et al., 2006b; Schnupf et al., 2007). Cytosolic LLO levels are further controlled by a negative feedback loop between LLO concentration and LLO translation (Schnupf et al., 2006a). Finally, cytoplasmic LLO can aggregate and form protein complexes that are ubiquitinated presumably by the host and are therefore targeted for clearance by the proteasome (Viala et al., 2008). This is dependent, in part, on the N-terminal region of LLO which contains an ubiquitination site. Infection with a *L. monocytogenes* mutant that lacks this region results in higher levels of LLO and increased cytotoxicity compared to infection with wild type bacteria (Decatur and Portnoy, 2000; Glomski et al., 2003).

Different signaling events have been reported to occur in different cells upon LLO stimulation. LLO results in pro-inflammatory cytokine production in macrophages (Nishibori et al., 1996; Yoshikawa et al., 1993), apoptosis in dendritic cells (Guzman et al., 1996), and degranulation and leukotriene production in neutrophils (Sibelius et al., 1996b; Sibelius et al., 1999). In epithelial HeLa cells, LLO induces mitogen-activated protein kinase (MAPK) (Tang et al., 1996) and extracellular regulated kinase 1/2 (ERK1/2) activation (Tang et al., 1998). Studies in embryonic kidney and goblet cells all reported changes in signaling or cell function upon LLO stimulation, such as increased expression of cell adhesion molecules (Krull et al., 1997), mucin production (Coconnier et al., 1998; Coconnier et al., 2000) and NF-κB activation (Kayal et al., 2002).

In general, LLO mediated signaling can be divided into pore-dependent and pore-independent events. Pore-dependent signaling may occur as a result of a breach in membrane integrity, the formation of Ca$^{2+}$ permeable pores in the endoplasmic reticulum, and phosphoinositide (PI) metabolism (see the Phospholipase C section for details). Pore-independent signaling may result from LLO oligomerization in lipid rafts or in the cytosolic recognition of specific LLO domains. Furthermore, pore-forming cytolysins have been suggested to act as novel Toll-like receptor
(TLR) agonists. TLR activation is responsible for the induction of a proinflammatory response in immune cells. LLO, like its pore-forming cytolysin family members, pneumolysin, anthrolysin, PFO, and streptolysin O, can bind TLR4 and mediate NF-κB activation and TNF-α production (Park et al., 2004; Srivastava et al., 2005). Furthermore, TLR2−/− mice are unresponsive to LLO-deficient (ΔLLO) bacteria, displaying a deficiency in neutrophil recruitment to the site of infection (Gekara et al., 2009). Thus, it is clear that LLO is a multi-functional virulence factor that may have a number of other unappreciated functions.

4.3.2.2 Phosphatidylinositol – Phospholipase C (PI-PLC) and phosphatidylcholine – Phospholipase C (PC-PLC)

*L. monocytogenes* produces two soluble phospholipase Cs (PLCs): phosphatidylinositol (PI) specific PI-PLC and broad-spectrum phosphatidylcholine (PC)-PLC. PC-PLC is produced as a proenzyme, requiring activation by the bacterial metalloprotease after it is exported from the bacteria (Domann et al., 1991; Mengaud et al., 1991). The importance of these two PLCs to *L. monocytogenes* pathogenesis can be seen in murine infection models. While infection with single PLC deletion mutants results in a modest increase in 50% lethal dose (LD₅₀), infection with double PLC deletion mutants result in a dramatic 500-fold increase in LD₅₀ (Smith et al., 1995).

There are a number of consequences that result from PLC activity. First, PLCs can mediate membrane lysis (Goldfine et al., 1993; Smith et al., 1995), allowing for bacterial escape from the phagosome (Grundling et al., 2003; Marquis et al., 1995). Studies indicate a role for PI-PLC in mediating lysis of the single membrane primary vacuole (Camilli et al., 1993) while PC-PLC mediates the lysis of the double membrane secondary vacuole (Vazquez-Boland et al., 1992). However, both PLCs can act synergistically to allow for optimal lysis of either single or double membrane vacuole (Camilli et al., 1993; Gedde et al., 2000). Intriguingly, the requirement for PLC activity during phagosomal escape is cell-type dependent. In epithelial cells, PLCs are necessary to allow for phagosomal escape (Grundling et al., 2003), while LLO alone is necessary for escape and survival in macrophages (Gedde et al., 2000). However, it must be noted that efficient escape from the primary vacuole in macrophages requires both LLO and PI-PLC (Poussin et al., 2009; Wadsworth and Goldfine, 2002).
The second consequence of PLC activation is the activation of specific downstream signals. The generation of DAG and IP₃ by PLC mediates many changes in the cell. Most notably, DAG production results in the recruitment and activation of protein kinase Cs (PKCs). Specifically in phagocytes, DAG production requires both LLO and PI-PLC (Sibelius et al., 1999; Wadsworth and Goldfine, 2002). Generation of DAG recruits host PKCβ and PKCδ. PKCδ activation recruits host phospholipase D (PLDs) (Goldfine et al., 2000). In addition, IP₃ mediates the release of Ca²⁺ from the endoplasmic reticulum, resulting in an influx of intracellular Ca²⁺. Changes in calcium concentration results in the activation of host PLCs (Titball, 1993). These studies together suggest a model of escape in which LLO pore formation allows the passage of PI-PLC from inside the phagosome to reach the cytosolic leaflet of the phagosomal membrane (Sibelius et al., 1999). Here, PI-PLC will generate DAG and increase Ca²⁺ concentration, which then recruits host PKC and activates host PLCs. Activation of PKCs results in recruitment and activation of host PLDs, which together with bacterial PLCs can result in lysis of the phagosomal membrane, thus allowing bacterial escape. Therefore, both bacterial and host enzymes contribute to efficient bacterial escape (Poussin et al., 2009; Wadsworth and Goldfine, 2002).

### 4.3.3 Cytosolic Replication

Upon escape into the cytosol, *L. monocytogenes* undergoes a phase of rapid growth. In primary bone marrow derived macrophages, this bacterium replicates in the nutrient rich cytosol with a doubling time of roughly 40 min (de Chastellier and Berche, 1994). Quantification by transmission electron microscopy reveals that phagosomal escape begins as early as 30 min p.i.. At 1 h p.i., 20% of intracellular *L. monocytogenes* has escaped while at 4 h p.i., 50% of intracellular *L. monocytogenes* has escaped (de Chastellier and Berche, 1994).

### 4.3.4 Secondary Spread

After escape into the cytosol, another virulence factor called ActA induces polymerization of actin filaments to promote cytosolic movement and cell-to-cell spread (Domann et al., 1992; Kocks et al., 1992). ActA is anchored covalently to one pole of the bacterial surface via the C-
terminal transmembrane motif. Actin polymerization is mediated through an interaction between the central four proline-rich repeat domains in ActA and members of the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family of actin nucleators (Lasa et al., 1995). This interaction modulates bacterial speed and directionality (Auerbuch et al., 2003; Geese et al., 2002; Laurent et al., 1999). The N-terminal region of ActA mimics host Wiskott Aldrich syndrome protein (WASP) family proteins by interacting with Arp2/3, a seven-protein complex that initiates the formation of branched actin filaments (Boujemaa-Paterski et al., 2001; Cossart, 2000; Skoble et al., 2000). This interaction modulates bacterial movement via the polymerization of actin filaments (termed “actin-comet tails”). Bacteria driven by actin-comet tails can form protrusions from one cell into the next, allowing for secondary spread of *L. monocytogenes*. Upon entry into neighbouring cells, the invading bacteria insides in a double membrane vacuole which is comprised of the plasma membrane from the previous and current host cell. Here, PC-PLC is critical for mediating escape from the secondary vacuole (Grundling et al., 2003).

### 4.3.5 PrfA Regulon

The expression of the canonical *L. monocytogenes* virulence factors described above are encoded in the *prfA* regulon, regulated by a transcription factor called the positive regulation factor A, PrfA (extensively reviewed in (Freitag, 2009; Freitag et al., 2009; Gray et al., 2006)). PrfA expression is regulated by a number of environmental factors that are unique to the host environment. One such factor is temperature. PrfA transcription is directed in part by a thermosensitive promoter (Leimeister-Wachter et al., 1992) that undergoes structural rearrangements at temperatures above 30°C (Johansson et al., 2002). Independent of temperature, phagosome specific conditions have also been observed to induce expression of the *prfA* regulon. Inside the phagosome, *L. monocytogenes* is subjected to oxidative stress, reduced nutrient and metals availability and low pH. These factors have all been observed to cause increased expression of the *prfA* regulon (Becker et al., 1998; Ferreira et al., 2001; Ferreira et al., 2003; Makino et al., 2005). Consistent with the need to rapidly modulate gene expression upon changes in the environment, it must be noted that there is a pool of *prfA* transcripts inside the bacteria that allow for rapid translation of the *prfA* regulon and its virulence factors immediately upon introduction into the host (Leimeister-Wachter et al., 1992).
4.4 Phagosomal Immune Defenses and *L. monocytogenes*

A number of phagosomal defenses play an important role in limiting *L. monocytogenes* escape into the cytosol. For example, antimicrobial peptides, specifically defensins, have recently been shown to play an important role in limiting *L. monocytogenes* escape from the phagosome in macrophages (Arnett et al., 2011). Intriguingly, *L. monocytogenes* has evolved a mechanism to D-alanylate cell wall components, resulting in a more positively charged bacterial surface, thus effectively repelling cationic antimicrobial peptides (Thedieck et al., 2006). In addition to antimicrobial peptides, two other critical host defenses, ROS and autophagy, have likewise been shown to be important phagosomal defenses against *L. monocytogenes* phagosomal escape.

4.4.1 Autophagy and *L. monocytogenes*

The importance of autophagy in limiting *L. monocytogenes* replication is seen in a number of *in vivo* studies. Mice with Atg5 deficient macrophages (Atg5<sup>fl/fl</sup>-Lyz-Cre) exhibit a 50% drop in survival 21 d p.i. with *L. monocytogenes* when compared to wild type mice (Zhao et al., 2008). In particular, significantly greater bacterial load was observed in the livers at day 3 p.i. of the Atg5<sup>fl/fl</sup>-Lyz-Cre mice when compared to control. Furthermore, drosophila mutants deficient in Atg5 or the pattern-recognition receptor, peptidoglycan recognition protein (PGRP)-LE, fail to induce autophagy in response to *L. monocytogenes* infection (Yano et al., 2008). PGRP-LE recognition of the *L. monocytogenes* cell wall component, diaminopimelic acid-type peptidoglycan, results in autophagy targeting of *L. monocytogenes*, as assessed by increased LC3<sup>+</sup> double-membrane *L. monocytogenes* containing compartments (Yano et al., 2008). Drosophila lacking in PGRP-LE or expressing a mutant PGRP-LE are unable to induce autophagy, resulting in increased susceptibility to *L. monocytogenes* infection. This results in a four-fold decrease in the number of surviving drosophila mutants 8 d p.i. over the wild type (Yano et al., 2008). Thus, these *in vivo* studies provide strong evidence that autophagic induction during *L. monocytogenes* infection is a critical host defense against the bacteria.
In vitro studies reveal an extremely complex picture of how autophagy targets L. monocytogenes. Depending on the stage of infection or where the bacteria is located, autophagy targeting of L. monocytogenes may be mediated via different mechanisms. Roughly 30-40% of L. monocytogenes is targeted by autophagy with the peak of LC3 targeting occurring in the early stages of infection at 1 h p.i. (Birmingham et al., 2007a; Meyer-Morse et al., 2010; Py et al., 2007). Autophagic targeting was found to be dependent on LLO as LLO deficient L. monocytogenes does not become significantly LC3$^+$ at any point during infection (Birmingham et al., 2007a; Meyer-Morse et al., 2010; Py et al., 2007). However, during later stages of infection at 8 h p.i., when most bacteria have escaped the phagosome, only 10% of wild type and ActA deficient (ΔActA) L. monocytogenes are LC3$^+$. Interestingly, while ΔActA mutants treated with the bacteriostatic agent, chloramphenicol, become 30% LC3$^+$, wild type L. monocytogenes treated with chloramphenicol remain 10% LC3$^+$ (Birmingham et al., 2007a). This data suggests that cytosolic autophagy targeting of L. monocytogenes may be evaded by ActA (Birmingham et al., 2007a; Rich et al., 2003). It is known that the ΔActA mutant colocalizes with ubiquitinated proteins (Ub) at 8 h p.i. (Perrin et al., 2004). While it remains unclear how L. monocytogenes is ubiquitinated, a number of studies have provided evidence for LLO ubiquitination. LLO in the cytosol has been described to form Ub$^+$ and p62$^+$ aggregates (Viala et al., 2008), and is thought to be directly ubiquitinated via its N-terminal lysine residues (Schnupf et al., 2007). Recent work by Sasakawa and colleagues suggests that this protein ubiquitination event mediates recruitment of adaptor proteins such as p62/SQSTM1, which leads to autophagic targeting of the ΔActA mutant (Yoshikawa et al., 2009). Thus, these observations combined suggest that ActA may play a critical role in evading LC3 targeting by preventing ubiquitination of L. monocytogenes in the cytosol. It remains unclear if ubiquitination also plays a role in LC3 targeting during the early stage of infection.

While it is clear from in vivo observations that autophagy is capable of limiting bacterial infection, in vitro studies of L. monocytogenes replication in autophagy deficient environments have yielded less clear results. Mild increases have been reported when comparing the number of wild type L. monocytogenes recovered from ATG5 deficient over wild type MEFs at various time points p.i. (Birmingham et al., 2007a; Py et al., 2007; Yoshikawa et al., 2009). However, L. monocytogenes mutants deficient in PI-PLC exhibit markedly greater bacterial replication in ATG5 deficient when compared to wild type MEFs (Birmingham et al., 2007a). This observation
suggests that PI-PLC, or perhaps both PLCs, also play a role in the evasion of autophagy that is currently unappreciated.

Despite these studies, many questions about autophagic targeting of \textit{L. monocytogenes} still remain. Since the bacteria can rapidly escape from the phagosome, it is unclear whether \textit{L. monocytogenes} is within phagosomes or in the cytosol at the peak of autophagy targeting at 1 h p.i.. While the available data in the field suggests that ubiquitination is a process that occurs once the bacteria has entered the cytosol, there lacks direct conclusive evidence to support this claim. Given that damaged vacuoles have been shown to be a target of ubiquitination in \textit{S. flexneri} infection (Dupont et al., 2009), phagosomes containing \textit{L. monocytogenes} may be damaged by LLO pores and thus mediate ubiquitination. Furthermore, it is also unknown which host and/or bacterial factor(s) mediate autophagy targeting of \textit{L. monocytogenes} at 1 h p.i.. Thus, much less is known about the mechanism of early autophagy targeting of \textit{L. monocytogenes} (Figure 4.4 B) and how it might differ from that of later targeting of \textit{L. monocytogenes} (Figure 4.4 A).
Figure 4.4. Current model of autophagy targeting of *L. monocytogenes*. (A) In presence of chloramphenicol, *L. monocytogenes* ΔactA mutant (but not wild type) is ubiquitinated and targeted by autophagy at the later stages of infection. (B) The mechanism of autophagy targeting of *L. monocytogenes* at 1 h remains unclear. It is unknown which host or bacterial factors recruits LC3 at this early stage of infection. Prime candidates include ROS, DAG, and Ub.
4.4.2 ROS AND L. monocytogenes

Given that ROS are a potent bactericidal defense in the phagosome and they can act as a signal for autophagy targeting during S. Typhimurium infection, how does L. monocytogenes evade ROS in order to escape the phagosome? It is known that ROS are an important host defense against L. monocytogenes replication. In vivo infection of gp91phox−/− mice with wild type L. monocytogenes results in a higher bacterial load at 48 h post injection when compared to infection of wild type mice (Dinauer et al., 1997; LaCourse et al., 2002; Shiloh et al., 1999). ROS were likewise found to be important for in vitro macrophage clearance of L. monocytogenes (Ohya et al., 1998a; Ohya et al., 1998b). Furthermore, L. monocytogenes exhibits greater escape from the phagosome upon infection of gp91phox−/− bone marrow derived macrophages (BMDM) compared to wild type BMDM (Myers et al., 2003). Taken together, the observed increase in L. monocytogenes load recovered from gp91phox−/− infected mice may be attributed to increased bacterial escape from the phagosome in the absence of ROS production. Thus, it is clear that ROS is an important host defense that must be overcome in order for bacterial escape into the cytosol.

Since ROS are an effective and important host defense against infection by intracellular pathogens, it is not surprising that many intracellular pathogens have evolved mechanisms to overcome ROS production by NOX2 NADPH oxidase (Table 4.4). The Salmonella pathogenicity island 2 (SPI-2) is critical for the evasion of NOX2 NADPH oxidase targeting in Salmonella enterica serovar Typhimurium infection in murine macrophages (Vazquez-Torres et al., 2000). A similar inhibition of NOX2 NADPH oxidase targeting has likewise been reported in other infections, including Burkholderia cenocepacia (Keith et al., 2009), Chlamydia trachomatis (Boncompain et al., 2010), Coxiella burnetti (Siemsen et al., 2009), and Francisella tularensis (McCaffrey and Allen, 2006) among others. The most common effect of these bacteria on NOX2 NADPH oxidase is the mislocalization of one or multiple members of the NOX2 NADPH oxidase complex. However, it is not known how these bacteria induce mislocalization of the NOX2 NADPH oxidase.

In addition, some pathogens have evolved different means of inhibiting NOX2 NADPH oxidase activity. The inhibition of ROS by Anaplasma phagocytophilum has been attributed to the virulence factor, AnkA, which decreases transcription of gp91phox (Garcia-Garcia et al., 2009).
More recently, *F. tulerensis* production of an acid phosphatase has been shown to inhibit ROS production, presumably via dephosphorylation of the regulatory subunits of the NOX2 NADPH complex (Mohapatra et al., 2010). Likewise, the parasite *Cryptosporidium parvum* also encodes an acid phosphatase that inhibits ROS production (Aguirre-Garcia and Okhuysen, 2007). Finally, the YopD pore-forming cytolysin secreted by *Yersinia enterocolitica* inhibits ROS production; however, the mechanism by which this occurs remains unknown (Hartland et al., 1994). It should be noted that other pathogens also inhibit NOX2 NADPH oxidase activity, but the bacterial factors and mechanisms involved are not understood. Table 4.4 provides a summary of the pathogens with reported abilities and known mechanisms to inhibit ROS production.

Little work has been done to examine whether *L. monocytogenes* also combats ROS production. A previous study found that the LLO deficient *L. monocytogenes* induces higher levels of ROS than wild type at 10 min post infection in neutrophils (Sibelius et al., 1999), suggesting that LLO might modulate ROS production. While NOX2 NADPH oxidase localizes to phagosomes for efficient *L. monocytogenes* killing, LLO has been shown to alter the localization of the early phagosomal protein, Rab5α, thereby inhibiting phagosome maturation (Alvarez-Dominguez et al., 1997; Shaughnessy et al., 2006). Furthermore, work done by Makino and colleagues revealed that oxidative stress induces expression of the LLO encoding gene, *hly* (Makino et al., 2005). Specifically, exogenous addition of ROS induces *hly* expression, which is reduced upon addition of ROS neutralizing enzymes, such as superoxide dismutase or catalase (Makino et al., 2005). Together, these observations suggest that *L. monocytogenes* may overcome ROS production in an LLO-dependent manner in order to escape the phagosome.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Mechanism of NOX interaction</th>
<th>Cell Type</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td><em>Anaplasma phagocytophilum</em></td>
<td>AnkA nuclear effector protein interacts with the HL-60 transcriptional regulatory regions the gp91 gene</td>
<td></td>
<td>(Garcia-Garcia et al., 2009)</td>
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<tr>
<td><em>Bacillus anthracis</em></td>
<td>Edema toxin inhibits NOX1 production of ROS HT-29 via increase in cAMP and PKA activation</td>
<td></td>
<td>(Kim and Bokoch, 2009)</td>
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<tr>
<td><em>Burkholderia cenocepia</em></td>
<td>p22&lt;sup&gt;phox&lt;/sup&gt; and p40&lt;sup&gt;phox&lt;/sup&gt; translocation delayed</td>
<td>Raw macrophage</td>
<td>(Keith et al., 2009)</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>Mislocalization of Rac2</td>
<td>HeLa</td>
<td>(Boncompain et al.)</td>
</tr>
<tr>
<td><em>Coxiella burnetii</em></td>
<td>p47&lt;sup&gt;phox&lt;/sup&gt; and p67&lt;sup&gt;phox&lt;/sup&gt; translocation to NOX inhibited</td>
<td>Human PBMC</td>
<td>(Siemsen et al., 2009)</td>
</tr>
<tr>
<td><em>Francisella tularensis</em></td>
<td>gp91, p22&lt;sup&gt;phox&lt;/sup&gt;, p47&lt;sup&gt;phox&lt;/sup&gt; and p67&lt;sup&gt;phox&lt;/sup&gt; translocation to LVS inhibited</td>
<td>Human PBMC</td>
<td>(McCaffrey and Allen, 2006; Schulert et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>migR and fevR mutants unable to prevent ROS production</td>
<td>Human PBMC</td>
<td>(Buchan et al., 2009)</td>
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<td></td>
<td>Acid phosphatases inactivate NOX</td>
<td>Human PBMC</td>
<td>(Mohapatra et al.)</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>Early cessation of p47&lt;sup&gt;phox&lt;/sup&gt; and p67&lt;sup&gt;phox&lt;/sup&gt; translocation to NOX</td>
<td>Human PBMC</td>
<td>(Allen and McCaffrey, 2007)</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>ROS inhibition via p47&lt;sup&gt;phox&lt;/sup&gt; translocation inhibition</td>
<td>U937</td>
<td>(Harada et al., 2007)</td>
</tr>
<tr>
<td>Organism summoned</td>
<td>Mechanism of exclusion/production</td>
<td>Cell Type</td>
<td>Source(s)</td>
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<tr>
<td><em>Salmonella typhimurium</em></td>
<td>SPI-2 dependent exclusion of p22&lt;sup&gt;phox&lt;/sup&gt; and p47&lt;sup&gt;phox&lt;/sup&gt;</td>
<td>Mouse macrophage</td>
<td>(Vazquez-Torres and Fang, 2001; Vazquez-Torres et al., 2001; Vazquez-Torres et al., 2000)</td>
</tr>
<tr>
<td><em>Vibrio vulnificus</em></td>
<td>RtxA1 induce expression of Rac2 to induce ROS</td>
<td>Mouse macrophage</td>
<td>(Chung et al.)</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>YopD prevent ROS Production</td>
<td>BMDM</td>
<td>(Hartland et al., 1994)</td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>Type three secretion system dependent inhibition of ROS production</td>
<td>Human neutrophils</td>
<td>(Spinner et al., 2008)</td>
</tr>
<tr>
<td><em>Yersinia pseudotuberculosis</em></td>
<td>YopE prevent ROS Production via inactivation of Rac2</td>
<td>In vivo mouse model</td>
<td>(Songsungthong et al., 2010)</td>
</tr>
<tr>
<td><strong>Parasite</strong></td>
<td></td>
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<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>Acid phosphatase prevent ROS production</td>
<td>Human PBMC</td>
<td>(Aguirre-Garcia and Okhuysen, 2007)</td>
</tr>
<tr>
<td><em>Leishmania donovani</em></td>
<td>Lipophosphoglycan inhibition of PKC phosphorylation leads to exclusion of p47&lt;sup&gt;phox&lt;/sup&gt; and p67&lt;sup&gt;phox&lt;/sup&gt;</td>
<td>Mouse and RAW macrophage</td>
<td>(Descoteaux et al., 1992; Lodge et al., 2006)</td>
</tr>
<tr>
<td><strong>Virus</strong></td>
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<tr>
<td>Epstein Barr Virus (EBV)</td>
<td>EBNA-1 induces ROS production to increase host genetic instability</td>
<td>DG75 and BJAB (human B cell lines)</td>
<td>(Gruhne et al., 2009)</td>
</tr>
<tr>
<td>Hepatitis C Virus (HCV)</td>
<td>NS3 superoxide production in macrophages and neutrophils, via recruitment of p47&lt;sup&gt;phox&lt;/sup&gt; and p67&lt;sup&gt;phox&lt;/sup&gt;</td>
<td>Human PBMC</td>
<td>(Bureau et al., 2001; Thoren et al., 2004)</td>
</tr>
<tr>
<td>Pathogen</td>
<td>Effect</td>
<td>Cell Type</td>
<td>Reference</td>
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<td>--------------------------------------</td>
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</tr>
<tr>
<td>HIV</td>
<td>Nef directly binds to p22phox increasing ROS production</td>
<td>Human PBMC</td>
<td>(Salmen et al.; Vilhardt et al., 2002)</td>
</tr>
<tr>
<td>Japanese encephalitis virus</td>
<td>Virus induces ROS production via mechanism unknown</td>
<td>Murine neuroblastomas</td>
<td>(Raung et al., 2001)</td>
</tr>
<tr>
<td>Respiratory syncitial virus (RSV)</td>
<td>Induction of ROS production via mechanism unknown</td>
<td>Human PBMC</td>
<td>(Kaul et al., 2000)</td>
</tr>
</tbody>
</table>

**Table 4.4. List of pathogens that have been reported to actively modulate NOX activity.**

Chapter 2

Aims and Hypotheses

5 Aim

The cytosol of a host cell is a nutrient rich environment that is capable of supporting rapid microbial growth. As such, the host has a number of defensive strategies aimed at clearing invading pathogens and preventing colonization. Reactive oxygen species (ROS) production by NOX2 NADPH oxidase and autophagy are among the best-characterized host defenses against invading pathogens. Not only does ROS confer direct antimicrobial activity, but also can activate other antimicrobial defenses. Similarly, autophagy is a key player of the antimicrobial response. Autophagy is the process of controlled self-digestion upregulated in times of cellular stress. In the context of intracellular pathogens, autophagy can be activated to selectively target pathogens and mediate bacterial clearance in the lysosome.

*L. monocytogenes* is a cytosol-adapted pathogen that colonizes host cells, including macrophages, during infection (Portnoy et al., 2002). Upon invasion, a population of bacteria are able to escape from the phagosome and colonize the nutrient-rich cytosol. Phagosomal escape is mediated by the bacterial pore forming toxin Listeriolysin O (LLO) and two phospholipase C enzymes (PLCs). PLCs are thought to not only degrade the phagosome to promote escape, but can also activate signal transduction cascades within the host cell as a result of their catalytic activity (Goldfine and Wadsworth, 2002; Portnoy et al., 2002). Upon entry to the cytosol, the bacteria use a cell surface protein, ActA, to drive actin-based motility in the host cytosol, and eventual cell-cell transfer (Portnoy et al., 2002). However, prior to escape, *L. monocytogenes* will encounter a number of phagosomal host defenses that the bacteria must overcome. Among these defenses are ROS and autophagy.
5.1 Hypothesis #1:

Here, I examined the mechanisms that regulate autophagic targeting of \textit{L. monocytogenes} in macrophages at early stages of infection. I hypothesize that LC3 targets \textit{L. monocytogenes} within phagosomes at 1 h p.i. in a manner requiring both DAG production and ROS generated by the NOX2 NADPH oxidase but not protein ubiquitination.

5.1.1 Aim

Autophagy is the controlled process of self-digestion through which cellular contents are delivered to the lysosome (Levine and Deretic, 2007). There are many types of autophagy, all of which play a vital role in maintaining cellular homeostasis. Autophagy has been shown to be a key component of the innate immune defense against many pathogenic microorganisms (Levine et al.). Autophagy can promote phagosomal maturation and also limit bacterial escape from these compartments (Birmingham et al., 2006; Gutierrez et al., 2004; Sanjuan et al., 2007). Autophagy can also target cytosolic bacteria, mediating their delivery to lysosomes (Nakagawa et al., 2004; Rich et al., 2003). Despite the importance of autophagy, the mechanisms that regulate targeting of pathogens to the autophagy pathway remain unclear. Recent studies of \textit{Salmonella enterica} serovar Typhimurium (\textit{S. Typhimurium}) infection demonstrated the involvement of ubiquitinated proteins (Ub) and ubiquitin-binding adaptors (Birmingham et al., 2006; Cemma et al., 2010; Randow and Lehner, 2009; Zheng et al., 2009), reactive oxygen species (ROS) (Huang et al., 2009), and diacylglycerol (DAG) (Shahnazari et al., 2010) as key signals for the targeting of these bacteria to the autophagy pathway. Whether these signals contribute to the regulation of autophagy during infection by other bacterial pathogens is largely unclear.

Previous studies have shown that \textit{L. monocytogenes} is targeted by autophagy in host cells, as evidenced by colocalization of bacteria with the autophagy marker microtubule-associated protein light chain 3 (LC3). The peak of LC3 colocalization with bacteria occurs at 1 hour post infection (p.i.) (Birmingham et al., 2007a; Meyer-Morse et al., 2010; Py et al., 2007). However, many questions about this process still remain. Since \textit{L. monocytogenes} can rapidly escape from the phagosome, it is unclear whether bacteria are targeted by autophagy within phagosomes or in the cytosol at this early stage of infection. Furthermore, it is also unknown which host and/or
bacterial factor(s) regulate autophagy of *L. monocytogenes*. Work from the Brumell laboratory previously demonstrated that a non-motile (ΔactA) mutant of *L. monocytogenes* colocalizes with ubiquitinated proteins in the cytosol (Perrin et al., 2004). Recent work by Sasakawa and colleagues suggests that this protein ubiquitination event mediates recruitment of adaptor proteins such as p62/SQSTM1 that lead to autophagic targeting of the ΔactA mutant of *L. monocytogenes* inside the cytosol, a process initiated after 2 hours p.i. (Rich et al., 2003; Yoshikawa et al., 2009). However, it is not known if protein ubiquitination plays a role in autophagic targeting of wild type *L. monocytogenes* at early stages of infection, the time of maximal autophagy of these bacteria.

Thus, **Aim 1.1** will be to determine if LC3+ *L. monocytogenes* at 1 h p.i. is targeted within a phagosome. Given that a previously published study indicates that the majority of *L. monocytogenes* reside in phagosomes at 1 h p.i. (de Chastellier and Berche, 1994), I predict that LC3+ *L. monocytogenes* at 1 h p.i. are inside phagosomes. Next, **Aim 1.2** will be focused on understanding how LC3 is targeted to *L. monocytogenes* at 1 h p.i.. I will examine if LC3+ *L. monocytogenes* also colocalize with DAG or ubiquitin. Finally, I will modulate the levels of DAG and ROS to determine if altering the levels of DAG and ROS will influence LC3 targeting to *L. monocytogenes* at 1 h p.i..

### 5.2 Hypothesis #2:

Since *L. monocytogenes* escape from the phagosome is a slow process that begins 30 min p.i., this prompted me to examine ROS production in the *L. monocytogenes* containing phagosome prior to escape. I hypothesize that *L. monocytogenes*, via the activity of LLO, can inhibit phagosomal production of ROS by mislocalizing NOX2 NADPH oxidase components.

#### 5.2.1 Aim

The NOX2 nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (also referred to as gp91phox; phagocyte oxidase) plays a key role in immune responses via the production of reactive
oxygen species (ROS) (Lam et al., 2010). The loss of NADPH oxidase activity in mice lacking the gp91phox subunit (gp91phox−/−) results in increased replication of *L. monocytogenes* during the first 24 h of infection compared to wild type animals (Dinauer et al., 1997; Shiloh et al., 1999). *In vitro* studies suggest that the NOX2 NADPH oxidase limits escape of *L. monocytogenes* from the phagosome in macrophages (Myers et al., 2003).

To ensure survival in the phagosome, a number of pathogens have evolved mechanisms to overcome ROS production by NOX2 NADPH oxidase. Such pathogens include *Chlamydia trachomatis* (Boncompain et al., 2010), *Coxiella burnetti* (Siemsen et al., 2009), *Francisella tulerensis* (McCaffrey and Allen, 2006) among others (Lam et al., 2010). Specifically, *Salmonella enterica* serovar Typhimurium and *Burkholderia cenocepacia* inhibit ROS production by preventing NOX2 NADPH oxidase targeting to the bacteria containing phagosomes (Vazquez-Torres et al., 2000) (Keith et al., 2009). However, the mechanism by which pathogens inhibits NOX2 NADPH oxidase activity remains unknown.

Previously, it has been shown that *L. monocytogenes* infected neutrophils generate ROS 10 min post infection (p.i.) (Sibelius et al., 1999). However, dynamic studies of where ROS is produced have never been performed. Furthermore, phagosomal analysis of ROS production during *L. monocytogenes* infection has likewise never been studied. It is known that LLO can inhibit phagosome maturation by preventing fusion with the lysosome (Alvarez-Dominguez et al., 1997; Shaughnessy et al., 2006). Furthermore, work done by Makino and colleagues revealed that oxidative stress induces expression of the LLO encoding gene, *hly* (Makino et al., 2005). Specifically, exogenous addition of ROS induces *hly* expression, which is reduced upon addition of ROS neutralizing enzymes, such as superoxide dismutase or catalase (Makino et al., 2005).

Thus, **Aim 2.1** is to first compare ROS production by macrophages infected with either wild type or ΔLLO *L. monocytogenes*. Production of ROS over the course of infection will be assessed to determine the temporal changes in ROS levels. Furthermore, intracellular, extracellular, and phagosomal production of ROS will be studied separately to determine the site of ROS production. Given my hypothesis that macrophages infected with wild type *L. monocytogenes* will produce less ROS than macrophages infected with ΔLLO *L. monocytogenes*, **Aim 2.2** will be to examine how LLO can inhibit ROS production. Specifically, the effect of LLO on signaling events upstream of NOX2 NADPH oxidase activation will be examined. Furthermore,
proper assembly of the NOX2 NADPH oxidase components will also be assessed in macrophages infected with either wild type or ΔLLO *L. monocytogenes*. Lastly, **Aim 2.3** will be to assess if other LLO-related pore-forming cytolysins can also inhibit ROS production during infection.
Chapter 3
Methods

6 Bacterial strains and culture conditions

Bacterial strains used in this study were as follows: *L. monocytogenes* 10403S (Bishop and Hinrichs, 1987), ΔLLO (DP-L2161) (Jones and Portnoy, 1994), ΔLLO + LLO (DP-L4818) (Lauer et al., 2002), iLLO (DH-L1239) (Alberti-Segui et al., 2007), ΔPI-PLCΔPC-PLC (DP-L1936) (Smith et al., 1995), ΔLLOΔPI-PLCΔPC-PLC (DP-L2391) (O’Riordan et al., 2002) (gifts from Dr. D. Portnoy, UC Berkley), iPFO (DH-L1745), 10403S expressing sGFP (DH-L1039) (Shen and Higgins, 2005) and ΔLLO expressing sGFP (DH-L1137) (Agaisse et al., 2005). Both GFP expressing strains were expressed under the control of the same Hyper-SPO1 promoter fused to the 5’ UTR of the LLO gene, *hly*. LLO was purified from *Escherichia coli* encoding recombinant N-terminal-tagged His-LLO, as described (Gedde et al., 2000). Bacteria were grown in Brain-Heart Infusion (BHI) broth 14-16 h at 30°C in a standing incubator. A 1:10 dilution of the culture was grown for 2 h at 37°C in a shaking incubator prior to infection. LLO and PFO expression in iLLO and iPFO strains were induced by IPTG, as previously described (Alberti-Segui et al., 2007).

7 Macrophage generation and culture conditions

RAW 264.7 macrophages were purchased from American Type Culture Collection (Rockville, MD). All experimental protocols involving mice were approved by the Animal Care Committee of The Hospital for Sick Children. Mice were euthanized by cervical dislocation. The femur and tibia were removed, cleansed of muscle fibers and cut distally. The bone marrow was then removed via a 10 sec pulse of centrifugation at 2000 rpm. The resulting cells were centrifuged at 1500 rpm for 5 min, washed with growth medium and plated onto 10 cm tissue culture dishes. Medium was replaced with fresh RPMI growth media (see below) every 3 days. Typically, $10^8$
bone marrow-derived macrophages (BMDM) were typically recovered after 7 days. Human macrophages were prepared as previously described (McGilvray et al., 2000). RAW 264.7 cells were maintained in DMEM growth medium (HyClone) supplemented with 10% FBS (Wisent) at 37°C in 5% CO₂ without antibiotics. Murine macrophages were maintained in RPMI-1640 medium (Wisent) supplemented with 10% FBS (Wisent), 5% sodium pyruvate (Invitrogen), 5% antibiotics (Invitrogen), 5% non-essential amino acids (Invitrogen) and 0.5 µM β-mercaptoethanol (Invitrogen). BMDMs were differentiated in 30% L929 conditioned medium. L929 conditioned medium was generated by growing L929 cells (ATCC) in 150-cm² flasks at an initial density of 1x10⁸ cells per flask in growth medium as described above for use with RAW 264.7 cells. After 3 days, confluency was reached and the growth medium was substituted with DMEM alone. After 7-10 days, culture supernatant was collected and centrifuged at 1,500 rpm for 5 mins, aliquoted and stored at -20°C.

8 Plasmids and transfections

Transfections were performed 24 h prior to infection. Transfection reagents FuGene 6 and FuGene 6 HD (Roche Applied Sciences) were used according to the manufacturer’s instructions. BMDM were transfected using the Amaxa Primary Murine Macrophage Transfection kit according to the manufacturer’s instructions (Amaxa). Constructs used were GFP-LC3 (provided by Tamotsu Yoshimori, Osaka University, Japan) (Kabeya et al., 2000), RFP-LC3 (provided by Walter Beron, Universidad Nacional de Cuyo, Argentina), PKCδ-C1 (provided by Sergio Grinstein, Hospital for Sick Children, Toronto, Canada) (Tse et al., 2005) and HA-PLD1 K898R and HA-PLD2 K758R (provided by Michael Frohman, SUNY, NY, USA) (Denmat-Ouisse et al., 2001).
9 Neutrophil purification and recombinant LLO (rLLO) treatment

Neutrophils were isolated from healthy human volunteers, as described (Tole et al., 2009). Neutrophils were stimulated with 10 µg/ml PMA along with treatment of 1µg/ml, 100ng/ml or 10ng/ml of rLLO for 1 h. ROS production was assessed using the NBT quantification assay (described below).

10 In vivo infection

Three to five week old C57BL/6J and gp91phox−/− (Cybb/tm1d) mice were purchased from Jackson Laboratory. Mice were infected via intravenous injection in the lateral tail vein with wild type L. monocytogenes at 5x10⁴ CFU in 200 µl of PBS, and ΔLLO bacteria at 1x10⁹ CFU in 200 µl of PBS. Mice were sacrificed at indicated time points and the livers excised. The right lobes of the livers were fixed with neutral-buffered formalin, and embedded sections stained with hematoxylin and eosin (H&E). The left lobes were homogenized in sterile PBS for CFU quantification from serial dilutions on BHI-agar plates.

11 Macrophage replication assay and infection

After 7-10 days of differentiation, BMDM were washed twice and detached with ice cold Versene Buffer (0.8 mM EDTA, 1 mM glucose in PBS) for 20 min at 4°C and plated at 5x10⁵ cells per well in 24-well tissue culture plates, 24 h prior to infection. All strains of L. monocytogenes were infected at a multiplicity of infection (MOI) of 1. After 30 min of invasion at 37°C, cells were washed three times with phosphate buffered saline (PBS) followed by the addition of DMEM. At 1 h post-infection, medium was changed and growth medium containing...
50 µg/ml gentamicin was added. Cells were then lysed at 2, 4, 8, 12, and 24 h post-infection with 0.2% TritonX-100 in PBS. Serial dilutions of the lysates were plated on BHI-agar plates and incubated 14-16 h for subsequent quantification of colony forming units (CFUs).

12 Reagents and Antibodies

The following drugs were used as indicated: 0.3% v/v 1-butanol (Sigma), 0.3% v/v tert-butanol (Sigma), 250 µM propranolol hydrochloride (Biomol International), 10 µg/ml PMA (Sigma) and 10 µM DPI (Sigma) were used. Staining for p22\(^{phox}\), p47\(^{phox}\) and p67\(^{phox}\) was completed using mouse anti-GFP, rabbit anti-p22\(^{phox}\) (gift from Dr. Mark T. Quinn, Montana State University, Montana, USA), rabbit anti-p47\(^{phox}\) and rabbit anti-p67\(^{phox}\) (gifts from Nathalie Grandvaux, McGill University, Montreal, Canada). Rabbit polyclonal antibodies against \textit{L. monocytogenes} were a gift from Dr. Pascale Cossart (Institut Pasteur), mouse monoclonal antibodies against GFP were purchased from Invitrogen; rat monoclonal antibodies against Lamp-1 (clone ID4B) from Developmental Studies Hybridoma Bank (University of Iowa); and antibodies against mono- and poly-ubiquitinated protein were from Biomol International (FK2). All fluorescent secondary antibodies were AlexaFluor conjugates from Molecular Probes (Invitrogen).

13 Immunostaining and Immunofluorescence

Immunostaining was conducted, as previously described (Brumell et al., 2001). In brief, after infections, human macrophages were fixed using 2.5% paraformaldehyde for 10 min at 37°C. Extracellular \textit{L. monocytogenes} was detected by immunostaining prior to permeabilization. Cells were then permeabilized and blocked using 0.2% saponin with 10% normal goat serum 14-16 h at 4°C and stained for intracellular bacteria and various host proteins. All colocalization quantifications and image acquisitions of sRBC fed cells were done using a Leica DMIREE2 epifluorescence microscope equipped with a 100X oil objective, 1.4 numerical aperture. Images of WT or ΔLLO infected cells are confocal z slices taken using a Zeiss Axiovert confocal
microscope and LSM 510 software. Volocity software (Improvision) was used to analyse images. Images were imported into Adobe Photoshop and assembled in Adobe Illustrator.

14 Dynamic Luminol based assays of ROS production

$1 \times 10^5$ macrophages in PBS-g (PBS plus 7.5mM glucose) in the presence of 125µM luminol and 100U/ml horseradish peroxidase were pre-warmed for 10 min at 37°C. 15µg of superoxide dismutase (SOD) was added to quench extracellular production of ROS. Cells were then infected with WT, ΔLLO or ΔLLOΔPI-PLCΔPC-PLC bacteria at MOI 10 or treated with either 10 µg/ml PMA alone or 10 µM DPI upon ΔLLO bacterial infection. After centrifugation at 500rpm for 1 min, luminescence was recorded every 10 min for a total of 90 min at 37°C using a Envision Multimode Plate reader (Perkin Elmer). The same protocol, without the addition of SOD, was used for the isoluminol assay.

15 Nitroblue tetrazolium (NBT) quantification of superoxide production

NBT is a compound that when reduced, forms a blue insoluble precipitate called formasan. NBT quantification of global intracellular superoxide formation was conducted, as previously described (Keith et al., 2009). In brief, RAW 264.7 macrophages were cultured as described and infected with wild type, ΔLLO or iLLO or iPFO, with or without IPTG, for 30 min. For controls, 10 µg/ml PMA and 10 µM DPI were used. Cells were washed and 300 µl of freshly prepared and pre-warmed DMEM with 1.25 mg/ml NBT was added per well. Medium was replaced with growth media with 10 µg/ml gentamicin and NBT. At 4 h, cells were washed with RPMI, twice with methanol and allowed to air dry. Reduced formasan particles were solublized with 240 µl 2M KOH and 280 µl DMSO and the resulting solution analyzed spectrophotometrically at 570 nm. All readings were normalized against an uninfected control with NBT treatment. A standard curve was generated using serial dilutions of a 1 mg/ml NBT solution in 1.2:1.4 2M KOH/DMSO solution.
16 Flow Cytometric Quantification of ROS

RAW 264.7 cells were infected with *L. monocytogenes* strains or treatment conditions for 30 min as described and cells were detached from 24-well plates using 0.25% trypsin and 1 mM EDTA. Cells were then stained with CM-H$_2$DCFDA dye (Invitrogen) as per the manufacturer’s protocol. 1x10$^4$ macrophages were analyzed per condition in a FACS-Calibur flow cytometer (Becton-Dickinson). Live macrophages were identified based on a characteristic forward and side scattering property. Analysis of the flow cytometry data was performed with Flow Jo software (Tree Star Flow Jo). Sequential gating of the live population, followed by background ROS fluorescence gating on an uninfected stained control, was performed to calculate the percentage of macrophages that had an increase in ROS$^+$ fluorescence.

17 Cerium perhydroxide precipitate quantification of phagosomal ROS

RAW 264.7 cells were infected with *L. monocytogenes* strains for 30 min as described and cells washed with 0.1M Tris maleate buffer, pH 7.5 at 37°C, followed by the addition of 0.1 M Tris maleate, 7% sucrose (BioShop), 1 mM aminotriazole buffer, pH 7.5 for 10 min at 37°C. Next, cells were treated with 0.1 M Tris maleate, 7% sucrose, 1 mM aminotriazole, 1 mM CeCl$_3$, 0.71 mM NADH, 0.71 mM NADPH buffer pH 7.5 for 20 min at 37°C (all reagents were from Sigma-Aldrich unless otherwise specified). Cells were subsequently washed with 0.1 M Tris maleate, 7% sucrose and fixed in 2.5% glutaraldehyde in PBS 14-16 h, postfixed in 1% OsO$_4$, stained with 1% aqueous uranyl acetate, dehydrated in graded series of ethanols and embedded in Epoxy resin. Samples were then sectioned, stained with 2% uranyl acetate, then 0.2% lead citrate, and examined on a Tecnai 20 transmission electron microscope (FEI) operating at 200 kV.
Statistical analyses were conducted using GraphPad Prism v4.0a. In all figures, data are expressed as the mean ± standard error of the mean (s.e.m) from three separate experiments. \( P \) values were calculated using two-tailed two-sample equal variance Student’s \( t \)-test unless specified otherwise. A p-value of less than 0.05 was considered statistically significant and is denoted by *. \( p < 0.01 \) is denoted by ** and \( p < 0.005 \) is denoted by ***.
Chapter 4

Results

19 Host and bacteria factors regulate autophagic targeting of *L. monocytogenes* during early stages of infection in macrophages

**Overview:** *Listeria monocytogenes* is a bacterial pathogen that can escape the phagosome and replicate in the cytosol of host cells during infection. These bacteria can be targeted by autophagy, with up to 35% becoming LC3\(^+\) at 1 h p.i. (Birmingham et al., 2007a). Here, I show that LC3\(^+\) *L. monocytogenes* are present within phagosomes at this time, and not the cytosol. Diacylglycerol (DAG) production by both bacterial and host phospholipases is required for LC3 recruitment to bacteria. Reactive oxygen species (ROS) production by the NOX2 NADPH oxidase, a downstream consequence of DAG production, is required for LC3 recruitment to bacteria. Protein ubiquitination was recently shown to play a role in targeting cytosolic *L. monocytogenes* to autophagy (Yoshikawa et al., 2009). However, I found that protein ubiquitination is not associated with LC3\(^+\) bacteria within phagosomes at early stages of infection. My data demonstrate that distinct signals mediate targeting of *L. monocytogenes* to the autophagy pathway at different stages of infection within host cells.

19.1 Autophagy targets wild type *L. monocytogenes* within macrophage phagosomes prior to phagosomal escape.

Consistent with previous work (Birmingham et al., 2007a; Meyer-Morse et al., 2010; Py et al., 2007), I found that LC3 recruitment to *L. monocytogenes* peaks at 1 h p.i. in an LLO-dependent manner (Figure 19.1.1 A and 19.1.1 B). Therefore, I sought to characterize the mechanisms that regulate autophagic targeting at this time point. Based on published reports of *L. monocytogenes* phagosome escape kinetics (Beauregard et al., 1997; de Chastellier and Berche, 1994; Henry et al., 2006), it is unclear whether *L. monocytogenes* is in the phagosome or cytosol during the peak of autophagic targeting.
Figure 19.1.1. Autophagy targets wild type \textit{L. monocytogenes} during macrophage infection. (A) Confocal images of RAW 264.7 macrophages transfected with GFP-LC3 and infected for 1 h with wild type \textit{L. monocytogenes}. (B) Quantification of LC3 colocalization with intracellular wild type or ΔLLO bacteria over time.
To determine whether *L. monocytogenes* is present inside phagosomes or the cytosol at 1 h p.i., I first quantified the percentage of *L. monocytogenes* that were both LC3$^+$ and LAMP-1$^+$. I found that the majority (78%) of LC3$^+$ bacteria were also LAMP-1$^+$ (Figure 19.1.2 A and 19.1.2 B), suggesting that *L. monocytogenes* within phagosomes are targeted by autophagy.

To confirm this, I employed a recently described probe constructed from the cell wall binding domain (CBD) of a *Listeria* bacteriophage protein (Henry et al., 2006). A yellow fluorescent protein fusion to CBD (CBD-YFP) was developed by Swanson and colleagues to track phagosome escape by *L. monocytogenes* (Henry et al., 2006). CBD-YFP expressed within host cells binds to cytosolic bacteria, resulting in an accumulation of YFP fluorescence around the bacteria (Henry et al., 2006). Upon escape from the phagosome, *L. monocytogenes* also polymerizes actin via the ActA virulence factor. Thus, I first confirmed the ability of the CBD-YFP probe to detect cytosolic *L. monocytogenes* by quantifying the number of CBD-YFP$^+$ bacteria that were also positive for polymerized actin at 4 h p.i. when bacteria are known to be in the cytosol. As expected, the majority (60%) of CBD-YFP$^+$ *L. monocytogenes* were also actin$^+$, confirming that the CBD-YFP can detect cytosolic *L. monocytogenes* after their escape from phagosomes (Figure 19.1.2 C and 19.1.2 D). In contrast, I did not observe any accumulation of CBD-YFP to LC3$^+$ *L. monocytogenes* at 1 h p.i. (Figure 19.1.2 E and 19.1.2 F). Together, this data demonstrates that *L. monocytogenes* is targeted by autophagy within phagosomes at early stages of infection.
Figure 19.1.2. Autophagy targets wild type $L. monocytogenes$ within macrophage phagosomes during early stages of infection. (A) Confocal images of RAW 264.7 macrophages transfected with GFP-LC3 and infected for 1 h with wild type $L. monocytogenes$. Cells were then stained for Lamp-1. (B) Quantification of the percentage of LC3$^+$ bacteria that are Lamp-1$^+$ or Lamp-1$. (C) Confocal images of RAW 264.7 macrophages transfected with GFP-LC3 and infected for 1 h with wild type $L. monocytogenes$. Cells were then stained for ubiquitinated proteins (Ub$^+$). (D) Quantification of the percentage of LC3$^+$ $L. monocytogenes$ that are Ub$^+$ or Ub$. The inner panels represent a higher magnification of the boxed areas. (E) Confocal images of RAW 264.7 macrophages expressing RFP-LC3 and CBD-YFP and infected for 1 h with wild type $L. monocytogenes$. (F) Quantification of the percentage of LC3$^+$ $L. monocytogenes$ that are CBD-YFP$^+$ or CBD-YFP$^-$. 
Previous studies suggest that protein ubiquitination may mediate autophagic targeting of \textit{L. monocytogenes} in the cytosol (Yoshikawa et al., 2009). LC3 targeting of \textit{S. Typhimurium} and \textit{S. flexneri} within phagosomes has also been shown to involve protein ubiquitination (Birmingham et al., 2006; Dupont et al., 2009; Thurston et al., 2009; Zheng et al., 2009). These observations suggested the possibility that protein ubiquitination may initiate autophagic targeting of \textit{L. monocytogenes} within phagosomes. However, I observed that the majority (98%) of LC3$^+$ \textit{L. monocytogenes} did not colocalize with ubiquitinated proteins at 1 h p.i. (Figure 19.1.2 C and 20.1.3 B). These results indicated that autophagic targeting of \textit{L. monocytogenes} within phagosomes is regulated by signals distinct from those in the cytosol.

### 19.2 Host and bacterial factors promote DAG accumulation on phagosomes containing \textit{L. monocytogenes}

DAG is known to regulate autophagy of \textit{S. Typhimurium} (Shahnazari et al., 2010). However, the role of DAG in autophagy of \textit{L. monocytogenes} has not been examined. Previous studies have shown that \textit{L. monocytogenes} infection induces DAG production (Sibelius et al., 1996a; Smith et al., 1995) in a manner dependent on the activity of LLO (Sibelius et al., 1996a), bacterial PLCs (Smith et al., 1995) and possibly host phospholipases (Goldfine et al., 2000). Therefore, it was conceivable that DAG production on the phagosome may play a role in autophagic targeting of \textit{L. monocytogenes} at early stages of infection.

To visualize DAG during \textit{L. monocytogenes} infection I employed a fluorescent probe constructed from the DAG binding C1 domain of PKC$\delta$ fused to green fluorescent protein (PKC$\delta$-C1-GFP). The majority (90%) of LC3$^+$ \textit{L. monocytogenes} were observed to be DAG$^+$ (Figure 19.2.1 A). However, only 25% of DAG$^+$ \textit{L. monocytogenes} were observed to be LC3$^+$ (Figure 19.2.1 B). Furthermore, DAG colocalization with \textit{L. monocytogenes} was observed prior to the peak of autophagy at 1 h p.i. (Figure 19.2.1 C and 19.2.1 D). Together, these observations suggest that DAG may serve as an upstream signal for autophagy.
Figure 19.1.3. CBD-YFP$^+$ *L. monocytogenes* is also actin$^+$. (A) Confocal images of RAW 264.7 macrophages transfected with CBD-YFP and infected for 1 h with wild type *L. monocytogenes*. Cells were then stained with phalloidin to label F-actin. The brightness and contrast for the image (but not the inset) was enhanced to allow visualization of fluorescence inside the cell. (B) Quantification of the percentage of CBD-YFP$^+$ bacteria that are actin$^+$. Size bars = 5 µm.
Figure 19.2.1. Host and bacterial factors promote DAG accumulation on phagosomes containing *L. monocytogenes*. (A) Confocal images of RAW 264.7 macrophages co-transfected with RFP-LC3 and PKCδ-C1-GFP and infected for 1 h with wild type *L. monocytogenes*. PKCδ-C1 is a specific probe for DAG. Size bar = 5 µm. Quantification at 1 h p.i. of the percentage of LC3⁺ *L. monocytogenes* that are PKCδ-C1⁺ or PKCδ-C1⁻. (B) Cells treated as (A). Quantification at 1 h p.i. of the percentage of PKCδ-C1⁺ *L. monocytogenes* that is also LC3⁺. (C) Confocal images of RAW 264.7 macrophages transfected with PKCδ-C1-GFP and infected for 1 h with wild type or ΔLLO bacteria. Size bar = 5 µm. (D) Quantification of the percentage of intracellular wild type, ΔLLO or ΔPI-PLCΔPC-PLC *L. monocytogenes* that are DAG⁺ over time.
The kinetics of DAG colocalization with *L. monocytogenes* lacking both PLCs (ΔPI-PLCΔPC-PLC) was examined. As shown in Figure 19.2.1 D, the percentage of DAG⁺ ΔPI-PLCΔPC-PLC bacteria was approximately 50% less than wild type bacteria at 30 min and 45 min p.i.. This suggests that bacterial PLCs contribute to DAG production on phagosomes containing *L. monocytogenes*. However, the decrease in DAG localization to ΔPI-PLCΔPC-PLC bacteria was not complete, raising the likely possibility that host factors also contribute to DAG production. Indeed, host phospholipases were previously shown to contribute to DAG production during *L. monocytogenes* infection (Smith et al., 1995).

I determined the effect of inhibiting host enzymes that produce DAG. Recent studies of *S. Typhimurium* infection indicate that DAG is produced by host phospholipase D (PLD) and phosphatidic acid phosphatase (PAP) (Shahnazari et al., 2010). I, therefore, employed inhibitors of PLD (1-butanol) and PAP (propranolol hydrochloride) to determine changes in DAG production on phagosomes containing wild type or ΔPI-PLCΔPC-PLC bacteria. Inhibition of PLD or PAP resulted in a decrease in DAG colocalization to the ΔPI-PLCΔPC-PLC mutants (Figure 19.2.2). This effect was not observed with tert-butanol, an isomer of 1-butanol that has no inhibitory effect on PLD. This finding suggests that host factors PLD and PAP contribute to the accumulation of DAG at *L. monocytogenes* containing phagosomes. Surprisingly, DAG colocalization to phagosomes containing wild type *L. monocytogenes* was not altered upon addition of PLD or PAP inhibitors. This observation strongly suggests that bacterial PLCs compensate for inhibition of host DAG production. These results indicate that both host and bacterial factors contribute to DAG accumulation on phagosomes containing *L. monocytogenes*. 
Figure 19.2.2. Inhibition of host DAG producers can further decrease DAG localization to ΔPI-PLCΔPC-PLC *L. monocytogenes*. Quantification at 45 min p.i. of intracellular wild type or ΔPI-PLCΔPC-PLC *L. monocytogenes* that are DAG⁺ upon treatment with growth media (GM), DMSO, 1-butanol, propranolol, or tert-butanol.
19.3 DAG production is required for autophagic targeting of *L. monocytogenes* at early stages of infection.

To investigate whether DAG plays a role in autophagic targeting of *L. monocytogenes*, I assessed LC3 recruitment to both wild type andΔPI-PLCΔPC-PLC mutants following treatment with PLD and PAP inhibitors (Figure 19.3 A). Similar to what I observed with DAG localization in Figure 19.2.2, LC3 targeting to wild type *L. monocytogenes* did not change with PLD or PAP inhibition. In contrast, LC3 targeting of the ΔPI-PLCΔPC-PLC mutant was significantly inhibited upon treatment with either the PLD inhibitor (90% decrease) or the PAP inhibitor (73% decrease). This suggests that DAG is important for LC3 recruitment to *L. monocytogenes* containing phagosomes.

To confirm the contribution of PLD-mediated generation of DAG to autophagy, dominant negative constructs for both isoforms of PLD (PLD1 and PLD2) were employed. Similar to the pharmacological evidence, dominant negative forms of PLD reduced DAG recruitment (40-62% decrease) (Figure 19.3 B) as well as LC3 recruitment (60-61% decrease) (Figure 19.4 C) to ΔPI-PLCΔPC-PLC mutants. Taken together, both bacterial and host mediated production of DAG contribute to autophagic targeting of phagosomes containing *L. monocytogenes*.
Figure 19.3. DAG production is required for autophagic targeting of *L. monocytogenes* during early stages of infection. (A) Quantification at 1 h p.i. of intracellular wild type or ΔPI-PLCΔPC-PLC *L. monocytogenes* that are LC3\(^{+}\) following treatment with DMSO, 1-butanol, propranolol, or tert-butanol. (B) Quantification at 1 h p.i. of DAG\(^{+}\) intracellular ΔPI-PLCΔPC-PLC *L. monocytogenes* in RAW 264.7 macrophages that were co-transfected with LC3-GFP and either PLD1 DN or PLD2 DN constructs. (C) Quantification at 1 h p.i. of LC3\(^{+}\) intracellular wild type or ΔPI-PLCΔPC-PLC *L. monocytogenes* in RAW 264.7 macrophages that were co-transfected with LC3-GFP and either PLD1 dominant negative (DN) or PLD2 DN constructs.
19.4 ROS production by the NOX2 NADPH oxidase is required for autophagic targeting of *L. monocytogenes* at early stages of infection.

PLD-mediated generation of DAG is upstream of ROS production via the NOX2 NADPH oxidase (Usatyuk et al., 2009). I previously showed that NOX2-generated ROS mediates LC3 targeting to phagosomes containing *S. Typhimurium* and IgG-coated latex beads (Huang et al., 2009). Therefore, it was conceivable that DAG promotes autophagy of *L. monocytogenes* via ROS production.

To test this hypothesis, I inhibited ROS production via the addition of the NOX2 NADPH oxidase inhibitor, diphenyliodonium (DPI), which at 1 h p.i. significantly reduced LC3 colocalization with *L. monocytogenes* (Figure 19.4 A). The same reduction in autophagy was also seen when antioxidants, resveratrol and α-tocopherol, were added to decrease ROS levels (Figure 19.4 A). These findings are consistent with a role for ROS in regulating autophagy of *L. monocytogenes*.

I next evaluated autophagic targeting of *L. monocytogenes* in bone-marrow macrophages deficient in NOX2 NADPH oxidase activity (gp91\(^{phox/-}\)) (Figure 19.4 B and 19.4 C). I found that gp91\(^{phox/-}\) macrophages displayed a significant reduction in LC3 colocalization with wild type *L. monocytogenes* (Figure 19.4 B and 19.4 C). Furthermore, consistent with previous studies (Birmingham et al., 2007a; Meyer-Morse et al., 2010; Py et al., 2007), LC3 recruitment to bacteria was dependent on LLO expression. Colocalization of LC3 with ΔLLO mutant bacteria was restored upon LLO complementation (Figure 19.4 C). Taken together, ROS production by the NOX2 NADPH oxidase appears to play a key role in autophagic targeting of phagosomes containing *L. monocytogenes*. 
Figure 19.4. ROS production by the NOX2 NADPH oxidase is required for autophagic targeting of *L. monocytogenes* during early stages of infection. (A) RAW 264.7 cells were infected with wild type *L. monocytogenes* for 1 h in the presence or absence of DPI, resveratrol or α-tocopherol, as indicated. The percentage of intracellular bacteria that are LC3⁺ upon treatment with each agent is shown. (B) Confocal images of wild type or gp91phox⁻/⁻ bone marrow-derived macrophages transfected with LC3-GFP and infected for 1 h with wild type bacteria. Size bar = 5 µm. (C) Quantification at 1 h p.i. of the percentage of intracellular *L. monocytogenes* that are LC3⁺ in bone marrow-derived macrophages isolated from wild type or gp91phox⁻/⁻ mice. Wild type bacteria were compared to ΔLLO bacteria (negative control), as well as a ΔLLO mutant complemented with LLO.
19.5 LC3 targeting is dependent on LLO pore-formation

Given that LLO is necessary and sufficient to induce LC3 targeting, I next examined whether LLO-mediated signaling, pore-formation or both is responsible for facilitating LC3 recruitment. To address this question, PFA or heat-killed wild type *L. monocytogenes* was fed to macrophages. Regardless of opsonization with IgG, non-viable *L. monocytogenes* did not recruit LC3 at 1 h post addition (Figure 19.5 A). Given that LC3 is not recruited to ΔLLO *L. monocytogenes* (Figure 19.1 and 19.4), these results suggest that active LLO production by viable *L. monocytogenes* is required for LC3 targeting.

To directly test the contribution of LLO pore-formation to LC3 targeting, a series of *L. monocytogenes* LLO point mutants exhibiting varying degrees of hemolytic activity were next employed. The C484A mutant has 75% of wild type hemolytic activity, the C484S mutant has 20%, the W491A mutant has 5% while the W492A mutant has 0.1% (Michel et al., 1990). These mutants are identical other than their point mutation and thus they likely share LLO signaling properties, but not the pore-forming property. At 1 h p.i., roughly 30% of wild type *L. monocytogenes* were LC3⁺ while the mutant bearing a transposon insertion in *hly* (Tn916) did not colocalize with LC3 (Figure 19.5 B). However, roughly 20% of C484A mutants were LC3⁺, 17% of C484S mutants were LC3⁺, 5% of W491A mutants were LC3⁺, while 3% of W492A mutants were LC3⁺. These observations indicate that LC3 targeting is dependent on LLO hemolytic or pore-forming activities.
Figure 19.5. LC3 targeting is dependent on LLO pore-forming ability secreted from live *L. monocytogenes*. (A) PFA fixed or heat killed wild type *L. monocytogenes* (PFA-WT or HK-WT) with or without opsonization with IgG (IgG PFA-WT or IgG HK-WT) were added to LC3-GFP expressing RAW 264.7 macrophages. Percentage LC3 colocalization to intracellular bacteria at 1 h post addition was quantified. (B) RAW 264.7 macrophages were infected with a panel of LLO point mutants: C484A, C484S, W491A, and W492A. The wild type is the background strain used to generate the point mutants and the Tn916 mutant is an LLO disrupted mutant. The brackets following each mutant denote the percentage of wild type *L. monocytogenes* hemolytic activity exhibited by the mutant. LC3 targeting of intracellular mutants was quantified at 1 h p.i..
19.6 ROS production by the NOX2 NADPH oxidase is required for SLAP formation at later stages of infection.

Here I focused on autophagy during the early stages of infection prior to phagosomal escape into the cytosol. The outcome of this autophagic targeting event depends on many factors. Previously, our laboratory showed that one outcome of this early autophagic targeting event is the formation of spacious *Listeria* containing phagosomes (SLAPs), compartments that are non-degradative, LAMP-1$^+$ and LC3$^+$ (Birmingham et al., 2007b). Formation of SLAPs was found to require autophagy in the host cell, and expression of LLO by bacteria. It was proposed that SLAPs represent a “stalemate” between the host and bacteria, allowing slow bacterial replication in SLAPs that allows chronic *L. monocytogenes* infection in a host (Birmingham et al., 2007b).

Indeed, compartments resembling SLAPs have been observed in a mouse model of *L. monocytogenes* chronic infection (Bhardwaj et al., 1998). Consistent with the notion that early autophagic targeting leads to SLAP formation, pharmacological inhibition of ROS production by the NOX2 NADPH oxidase reduced the formation of SLAPs (Figure 19.6). SLAP formation was decreased in gp91$^+_{phox-/-}$ bone marrow-derived macrophages compared to wild type macrophages. These findings demonstrate that ROS-mediated autophagy at 1 h p.i. is required for the later formation of SLAPs. Thus, signals that mediate early autophagic targeting of *L. monocytogenes* may also are likely to be important in determining events that lead to establishment of chronic infection.
Figure 19.6 ROS production by the NOX2 NADPH oxidase is required for the generation of SLAPs. (A) Confocal images of RAW 264.7 macrophages infected for 4 h with wild type *L. monocytogenes*, with or without DPI. Cells were stained with LAMP-1 antibodies. Size bar = 5 µm. White arrowheads indicate Spacious Listeria phagosomes (SLAPs) while the white arrow indicates LAMP-1 colocalization with bacteria that are not in SLAPs (B) Quantification at 4 h p.i. of the percentage of infected macrophages that form SLAPs in the presence or absence of DPI, resveratrol or a-tocopherol, as indicated. (C) Confocal images of wild type or gp91phox−/− bone marrow-derived macrophages infected for 8 h with wild type *L. monocytogenes*. Cells were stained with LAMP-1 antibodies. Size bar = 5 µm. White arrowheads indicate SLAPs while the white arrows indicate LAMP-1 colocalization and not SLAPs. (D) Quantification at 8 h p.i. of the percentage of infected wild type or gp91phox−/− bone marrow-derived macrophages that form SLAPs from C.
19.7 Summary

In my graduate studies, I examined the signals that mediate autophagic targeting of *L. monocytogenes* during the early stages of infection in host cells. I showed that *L. monocytogenes* is largely confined within LAMP-1⁺ phagosomes at the peak of autophagy (1 h p.i.). A recent study by Sasakawa and colleagues indicates that cytosolic *L. monocytogenes* deficient in ActA are coated with ubiquitinated proteins and subsequently targeted by autophagy 4 h p.i. (Yoshikawa et al., 2009). Within the phagosome, however, my studies indicate that autophagic targeting is not dependent on protein ubiquitination but is instead dependent on DAG and ROS mediated signaling. Thus, *L. monocytogenes* can be targeted by autophagy by distinct mechanisms at different stages of infection within host cells. These findings highlight the complex nature of autophagy regulation and its versatility in targeting pathogens within different compartments in eukaryotic cells.
20 Listeriolysin O suppresses PLC-mediated activation of the microbicidal NADPH oxidase to promote *Listeria monocytogenes* infection

**Overview:** *Listeria monocytogenes* is an intracellular bacterial pathogen that utilizes two phospholipases C (PI-PLC and PC-PLC) and a pore-forming cytolysin (listeriolysin O, LLO) to escape the phagosome and replicate within the host cytosol. Here, I show that PLCs are capable of activating the phagocyte NOX2 NADPH oxidase during *L. monocytogenes* infection, a response that would adversely affect pathogen survival. However, secretion of LLO inhibits the NADPH oxidase by preventing its localization to phagosomes. LLO-deficient bacteria can be complemented by perfringolysin O, a related cytolysin, suggesting that other pathogens may also use pore-forming cytolysins to inhibit the NOX2 NADPH oxidase. My studies demonstrate that while the PLCs induce antimicrobial NOX2 NADPH oxidase activity, this effect was alleviated by the pore-forming activity of LLO. Therefore, the combined activities of PLCs and LLO on membrane lysis and their opposing effects on the NOX2 NADPH oxidase allow *L. monocytogenes* to efficiently escape the phagosome while avoiding the microbicidal respiratory burst.

### 20.1 LLO inhibits ROS production by the NOX2 NADPH oxidase

I performed dynamic measurements of intracellular and extracellular ROS production in bone marrow-derived macrophages (BMDM) using luminol or isoluminol, respectively (Figure 20.1.1 A-D). During infection by wild type *L. monocytogenes*, both intracellular and extracellular ROS production peaked at 10 min p.i. and decreased at later time points (Figure 20.1.1 A and 20.1.1 C). Treatment of cells with phorbol 12-myristate 13-acetate (PMA), a known activator of the NOX2 NADPH oxidase, served as a positive control (Figure 20.1.1 B and 20.1.1 D). These findings demonstrate that *L. monocytogenes* can induce rapid activation of ROS in leukocytes, consistent with previous observations (Sibelius et al., 1999).
Next, I examined ROS production in cells infected with LLO-deficient bacteria (ΔLLO, lacking the hly gene). I observed higher intracellular ROS production in ΔLLO infected cells compared to wild type infected cells at 40-70 min post p.i. (Figure 20.1.1 A and 20.1.1 C; Table 20.1 A). Addition of the flavoprotein inhibitor diphenyliodonium (DPI, a known NADPH oxidase inhibitor) prevented ROS production in ΔLLO infected cells (Figure 20.1.1 B and 20.1.1 D; Table 20.1 A and 20.1 B). The kinetics of extracellular ROS production were similar upon infection with wild type or ΔLLO L. monocytogenes (Figure 20.1.1 C, Table 20.1 B). These observations suggest that LLO inhibits intracellular ROS production.

Since I observed a significant difference in ROS production between wild type and ΔLLO infected cells during 40-70 min p.i. (Figure 20.1.1 A and 20.1.1 C; Table 20.1 A and 20.1.1 B), I further examined intracellular ROS production in this window of infection using the 5-(and-6)-chloromethyl-2-7-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) dye. RAW 264.7 macrophages were infected with either wild type or ΔLLO bacteria and CM-H2DCFDA dye was added 30 min p.i. and examined at 1 h p.i. As expected, treatment of cells with PMA resulted in robust ROS production (Figure 20.1.2 A and 20.1.2 B). Consistent with the luminol assay, I observed little ROS production in wild type infected cells but high levels of ROS production in ΔLLO infected cells, comparable to those attained by stimulation with PMA (Figure 20.1.2 A and 20.1.2 B).

I also assessed NOX2 NADPH oxidase activity by specifically measuring its product, superoxide, using the nitroblue tetrazolium (NBT) reduction assay (described in (Keith et al., 2009)). ΔLLO bacteria induced a marked production of superoxide, while infection with wild type bacteria did not (Figure 20.1.3). These results indicate that LLO inhibits ROS production during infection. To further define the role of LLO, I employed an LLO-deficient strain that inducibly expresses LLO (iLLO) in response to isopropyl β-D-1-thiogalactopyranoside (IPTG). In the absence of IPTG, iLLO bacteria triggered ROS production. However, in the presence of IPTG, iLLO bacteria did not trigger ROS production upon infection (Figure 20.1.3).
Figure 20.1.1. ΔLLO mutants induce greater ROS production compared to WT or ΔLLOΔPI-PLCΔPC-PLC *L. monocytogenes* during infection in macrophages. (A-B)

Intracellular ROS production was assessed via the luminol assay. BMDMs were (A) infected at an MOI of 10 with wild type (WT) or ΔLLO bacteria or (B) treated with PMA or DPI upon ΔLLO bacteria infection. Background ROS production from uninfected cells (BKGD) was also assessed. Time 0 represents the first reading shortly after the addition of bacteria or drugs. The figure shows data from one representative experiment done in duplicate where each data point represents the average and range. The experiment was performed a total of three independent times (see Table 20.1 A). 

(C-D) Extracellular ROS production was assessed via the isoluminol assay. BMDMs were (C) infected at an MOI of 10 with wild type (WT) or ΔLLO bacteria or (D) treated with PMA or DPI in presence of ΔLLO bacteria infection. ROS production from uninfected cells (BKGD) was also assessed. Time 0 represents the first reading shortly after the addition of bacteria or drugs. The figure shows data from one representative experiment done in duplicate where each data point represents the average and range. The experiment was performed a total of three independent times (see Table 20.1 B).
Table 20.1. Mean and SEM of luminol and isoluminol readings from primary BMDM infected with WT or ΔLLO bacteria. (A) Mean and SEM of three independent luminol assays done on BMDM that were infected at MOI 10 with WT or ΔLLO bacteria, or treated with PMA or DPI upon ΔLLO infection. (B) Mean and SEM of three independent isoluminol assays done on BMDMs that were infected with WT or ΔLLO bacteria, or treated with PMA or DPI upon ΔLLO infection.

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<th>Time (mins)</th>
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<th>ΔLLO (CPS) Mean ± SEM</th>
<th>ΔLLOΔPI-PLCΔPC-PLC (CPS) Mean ± SEM</th>
<th>PMA (CPS) Mean ± SEM</th>
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# WT is not significantly different from the ΔLLOΔPI-PLCΔPC-PLC mutants at any time point.

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<th>Time (mins)</th>
<th>WT (CPS) Mean ± SEM</th>
<th>ΔLLO (CPS) Mean ± SEM</th>
<th>ΔLLOΔPI-PLCΔPC-PLC (CPS) Mean ± SEM</th>
<th>PMA (CPS) Mean ± SEM</th>
<th>ΔLLO + DPI (CPS) Mean ± SEM</th>
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<td>200 ± 28.28</td>
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# WT is not significantly different from the ΔLLOΔPI-PLCΔPC-PLC mutants at any time point.
Figure 20.1.2. LLO inhibits PLC-mediated ROS production by the NOX2 NADPH oxidase. (A) RAW 264.7 macrophages were treated with PMA or infected with the indicated bacterial strain (MOI 10) with or without DPI. ROS production was assessed via flow cytometry with CM-H2DCFDA. The CM-H2DCFDA dye was added 30 min p.i. and cells were analyzed at 60 min p.i.. Flow cytometry analysis was conducted on live populations, gated against a stained unstimulated control (red). Percentages indicated on the plots refer to the portion of cells that are ROS⁺. Representative plots are shown. (B) Quantification of the percentage of ROS⁺ cells that were infected with the indicated bacterial strains. Graph represents mean ± SEM from five independent experiments.
Figure 20.1.3. LLO inhibits ROS production by NOX2 NADPH oxidase. RAW 264.7 macrophages were either treated with PMA or infected at a MOI of 10:1 with the indicated bacterial strain. Superoxide production was assessed using NBT, a compound that upon reduction, forms a black formasan precipitate. Formasan formation was assessed spectrophotometrically in cell lysates (see Methods). NBT was added 30 min p.i.
20.2 LLO can inhibits ROS production in other cell types

Addition of purified LLO to human neutrophils was sufficient to inhibit PMA-induced ROS production (Figure 20.2 A) at concentrations that did not cause cell toxicity (Figure 20.2 B). Therefore, LLO is both necessary and sufficient for inhibition of intracellular ROS production by the NADPH oxidase.

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**Figure 20.2. LLO is capable of inhibiting ROS production by PMA activated human neutrophils.** (A) NBT quantification assay of PMA stimulated human neutrophils treated with varying doses of recombinant LLO (rLLO) for 60 min. (B) Percentage neutrophil viability was assessed using the trypan blue exclusion assay at 60 min post treatment with the various doses of rLLO.
20.3 LLO inhibits phagosomal production of ROS

To visualize the localization of ROS production during *L. monocytogenes* infection, I employed transmission electron microscopy (TEM) with cerium chloride (Vazquez-Torres et al., 2000). IgG-coated 3.8 µm latex beads were fed to RAW 264.7 macrophages as positive controls of phagosomal ROS production. A dark cerium precipitate was observed in phagosomes containing latex beads, indicative of ROS production in this compartment (Figure 20.3 A, 20.3 B and 20.3 G), which was inhibited by DPI treatment (Figure 20.3 C, 20.3 D and 20.3 G). ROS production was not observed in phagosomes containing wild type *L. monocytogenes* (Figure 20.3 F and 20.3 G). However, marked production of ROS was observed in phagosomes containing ΔLLO bacteria (Figure 20.3 E and 20.3 G), but not in phagosomes containing ΔLLO ΔPI-PLC ΔPC-PLC bacteria (Figure 20.3 G). These results indicate that LLO inhibits PLC-mediated NOX2 NADPH oxidase-dependent ROS production in the phagosome.

20.4 *L. monocytogenes* PLCs contribute to the induction of ROS production by the NOX2 NADPH oxidase

The observation that ΔLLO bacteria induced high levels of ROS production, comparable to those observed in cells treated with PMA, suggested that bacterial products have the capacity to promote NOX2 NADPH oxidase activity in the absence of LLO. It was previously shown that PLCs from *Clostridium perfringens*, *Pseudomonas aeruginosa* and *Bacillus cereus* are linked to activation of the NOX2 NADPH oxidase (Styrt et al., 1989; Titball, 1993). These enzymes generate diacylglycerol, a cofactor for activation of protein kinase C (PKC), which contributes to NOX2 NADPH oxidase activation via the phosphorylation of p47phox (Fontayne et al., 2002). Similarly, *L. monocytogenes* PLCs generate diacylglycerol and activate PKC during infection of macrophages (Camilli et al., 1993; Goldfine et al., 1993; Wadsworth and Goldfine, 2002). Therefore I hypothesized that *L. monocytogenes* PLCs could activate NOX2 NADPH oxidase in the absence of LLO.
Figure 20.3. LLO inhibits ROS production in phagosomes. (A, B) Phagosomal production of ROS was assessed via transmission electron microscopy (TEM). RAW 264.7 macrophages were treated with IgG-coated latex beads in the presence of cerium chloride. Reduction of cerium chloride results in the formation of cerium perhydroxide precipitate. The presence of these electron-dense products in TEM was indicative of NOX2 NADPH oxidase production of ROS. (B) is a higher magnification view of the boxed region in (A). (C, D) Experiment conducted as in (A) and (B) but in the presence of DPI. (D) is a higher magnification view of the boxed region in (C). RAW 264.7 macrophages were infected with either WT or ΔLLO bacteria at MOI 10. (E) Representative TEM of a ΔLLO-containing phagosome at 60 min p.i.. (F) Representative TEM of a wild type-containing phagosome at 60 min p.i.. (G) The percentage of cerium perhydroxide⁺ phagosomes at 60 min p.i. were quantified. One hundred phagosomes were assessed per sample from four independent experiments. Bars represent mean ± SEM. All scale bars are 1 µm.
To test this hypothesis, I examined the effect of *L. monocytogenes* PLCs on ROS production. In contrast to ΔLLO bacteria, a strain lacking LLO and both PLCs (ΔLLO ΔPI-PLC ΔPC-PLC) produced little ROS (Figure 20.1.1 A, Table 20.1 A and 20.1.2 B). Infection of RAW 264.7 macrophages with a strain that lacks both PLCs (ΔPI-PLC ΔPC-PLC, lacking *plcA* and *plcB* genes) resulted in ROS production comparable to that of uninfected controls and cells infected with wild type bacteria (Figure 20.1.2 B). Thus, these observations suggest that PLCs promote low levels of ROS production during infection by wild type bacteria.

Next, I treated cells with purified PI-PLC and observed a significant induction of ROS production in macrophages that was inhibited by the broad spectrum PKC inhibitors Rottlerin and GÖ6983 (Figure 20.4 A). ROS production by PI-PLC was also inhibited by concurrent treatment with purified LLO. These results suggest that PI-PLC is sufficient to drive NOX2 NADPH oxidase activation by stimulating PKC and that LLO is sufficient to inhibit this deleterious consequence of PLC activity. This is consistent with a previous finding that *L. innocua* expressing PI-PLC induces greater ROS production than wild type *L. innocua* in neutrophils (Sibelius et al., 1999). However, *L. monocytogenes* mutants that only express PI-PLC (and not PC-PLC or LLO) or PC-PLC (and not PI-PLC or LLO) do not induce significant ROS production (Figure 20.4 B). One possible explanation for these observations is that physiologically both PLCs are needed to mediate ROS production. However, higher doses of PI-PLC may be sufficient to drive NOX2 NADPH oxidase activation by stimulating PKC.

Collectively, my results indicate that bacterial PLCs induce ROS production and that LLO inhibits this deleterious consequence of PLC activity.
Figure 20.4. Both PLCs are required to induce ROS production by macrophages during infection. (A) RAW 264.7 macrophages were treated with the indicated strains with or without 15µM Rottlerin or 10nM Go6983, PKC inhibitors, for 60 min and CM-H2DCFDA dye was used to measure ROS production. Quantification of the percentage of ROS+ cells was done using flow cytometry. Bars represent mean ± SEM from three independent experiments. (B) RAW 264.7 macrophages were infected of MOI 10:1 with WT, ΔLLO, ΔLLOΔPC-PLC, ΔLLOΔPI-PLC bacteria for 30 min. Quantification of the percentage of ROS+ cells that were infected with the indicated bacterial strains. Graph represents mean ± SEM from five independent experiments.
20.5 LLO deficient bacteria survive in NOX2 NADPH oxidase deficient macrophages

The relevance of LLO-mediated inhibition of the NOX2 NADPH oxidase on intracellular *L. monocytogenes* survival/replication was determined by gentamicin protection assays using bone marrow-derived macrophages (BMDM) isolated from C57BL/6 and gp91\(^{phox/-}\) mice. The number of intracellular ΔLLO bacteria decreased over time in C57BL/6 BMDM (Figure 20.5 A), consistent with previous observations (Alberti-Segui et al., 2007). In contrast, ΔLLO bacteria displayed extended survival in gp91\(^{phox/-}\) BMDM. Wild type *L. monocytogenes* replicated at a similar rate in both C57BL/6 and gp91\(^{phox/-}\) BMDM (Figure 20.5 B). ΔLLO bacteria that were complemented by expression of LLO (ΔLLO + LLO) displayed a similar replication profile as that of wild type bacteria (Figure 20.5 C).

20.6 LLO deficient bacteria survive in NOX2 NADPH oxidase deficient mice

The *in vivo* relevance of LLO-mediated inhibition of the NOX2 NADPH oxidase was examined in C57BL/6 and gp91\(^{phox/-}\) mice via infection by wild type *L. monocytogenes* or ΔLLO bacteria. Wild type *L. monocytogenes* replicated significantly over the course of 24 h in both C57BL/6 and gp91\(^{phox/-}\) mice (Figure 20.6 A). The bacterial load at 24 h was higher in gp91\(^{phox/-}\) compared to C57BL/6 mice (Figure 20.6 B), as previously reported (Dinauer et al., 1997). Histological examination of the liver revealed a greater number of granulomas in the gp91\(^{phox/-}\) mice (Figure 20.6 C). However, clearance of ΔLLO bacteria was significantly impaired in the gp91\(^{phox/-}\) mice as compared to C57BL/6 mice (Figure 20.6 D and 20.6 E). Consistent with this result, granulomas were observed at 24 h post-infection in the livers of gp91\(^{phox/-}\) mice infected with ΔLLO bacteria, but not C57BL/6 mice (Figure 20.6 F). These results indicate that LLO-mediated inhibition of NOX2 NADPH oxidase activity is required for *L. monocytogenes* survival both *in vitro* and *in vivo.*
Figure 20.5. ΔLLO bacteria survive in NOX2 NADPH oxidase-deficient macrophages in vitro. (A, B, C) BMDM from C57BL/6 or gp91phox−/− mice were infected with WT, ΔLLO or ΔLLO + LLO bacteria. The number of intracellular bacteria (colony forming units) was quantified by gentamicin protection assay at the indicated times. Data represent the mean ± SEM for three independent experiments.
Figure 20.6. \(\Delta LLO\) bacteria survive in NOX2 NADPH oxidase-deficient macrophages \textit{in vitro} and \textit{in vivo}. (A) C57BL/6 and gp91\(^{phox-/-}\) mice were intravenously infected with wild type \textit{L. monocytogenes}. Mice were sacrificed at the indicated times and livers removed for quantification of bacterial load. Graphs represent bacterial CFUs from three independent experiments for a total of six mice per condition per time point. Statistical analyses were performed using a non-parametric Mann-Whitney test to assess significance. (B) Comparison of bacterial CFUs (mean \pm SEM) recovered at 24 h post-infection from C57BL/6 and gp91\(^{phox-/-}\) mice from three independent experiments were compared using the non-parametric Mann-Whitney test. (C) Liver sections from both C57BL/6 and gp91\(^{phox-/-}\) mice 24 h post-infection were embedded and mounted. Tissues were stained with H&E and representative images were acquired. Scale bar represents 100 \(\mu m\). (D) C57BL/6 and gp91\(^{phox-/-}\) mice were intravenously infected with \(10^9\) \(\Delta LLO\) bacteria in a volume of 200\(\mu l\). Mice were sacrificed at the indicated times and livers removed for quantification of bacterial load. Data represent results from three independent experiments with a total of six mice per condition per time point. Statistical analyses were performed using a non-parametric Mann-Whitney test to assess significance. NS = not significant. (E) Bacterial CFU in the liver (mean \pm SEM) recovered at 48 h post-infection from C57BL/6 and gp91\(^{phox-/-}\) mice from three independent experiments were compared using the non-parametric Mann-Whitney test. (F) Liver sections from C57BL/6 and gp91\(^{phox-/-}\) mice 24 h post-infection were embedded and mounted. Tissues were stained with hematoxylin and eosin (H&E) and representative images are shown. Scale bar, 100 \(\mu m\).
20.7 LLO inhibits NOX2 NADPH oxidase assembly at the phagosome

The NOX2 NADPH oxidase is composed of a transmembrane heterodimer of gp91phox and p22phox, and 4 regulatory cytosolic subunits: p40phox, p47phox, p67phox and the small GTPase, Rac2. I hypothesized that LLO inhibits ROS production by preventing proper localization of NOX2 NADPH oxidase components to the phagosome. To test this hypothesis, human peripheral blood mononuclear cells (PBMCs) were infected with GFP-expressing wild type or ΔLLO bacteria and immunostained for p22phox (Figure 20.7.1), p47phox or p67phox (Figure 20.7.2). IgG-opsonized sheep red blood cells were employed as a positive control for localization of the NOX2 NADPH oxidase to phagosomes (Figure 20.7.1A, 20.7.2A and 20.7.2 C). I observed minimal colocalization between intracellular wild type bacteria and the NOX2 NADPH oxidase components tested (Figure 20.7.1 B, 20.7.1 C, 20.7.2 B and 20.7.2 D). In contrast, I observed more than a three-fold increase in co-localization between intracellular ΔLLO bacteria and all of the NOX2 NADPH oxidase components tested (Figure 20.7.1 B, 20.7.1 C, 20.7.2 B and 20.7.2 D). These observations demonstrate that LLO prevents proper NOX2 NADPH oxidase assembly at the phagosome.

20.8 Perfringolysin O (PFO) expression can complement NOX2 NADPH oxidase inhibition by LLO deficient bacteria

I next determined whether other bacterial pore-forming cytolysins could also inhibit the NOX2 NADPH oxidase, similar to LLO. I used ΔLLO bacteria expressing perfringolysin O (PFO) from C. perfringens under a tightly controlled IPTG-inducible promoter (iPFO). In the absence of IPTG, iPFO infected cells generated significant amounts of superoxide, similar to ΔLLO (Figure 20.8 A). However, upon IPTG induction, superoxide production was inhibited.

The same effect was observed in bacteria-containing phagosomes via TEM analysis of cerium perhydroxide precipitates (Figure 20.8 B). Since PFO can complement LLO for inhibition of NOX2 NADPH oxidase, these results suggest that the inhibition of ROS production may be a common strategy used by other pore-forming cytolysin secreting bacteria.
Figure 20.7.1. LLO inhibits NOX2 NADPH oxidase assembly at the phagosome. (A) Human macrophages were treated with IgG-opsonized sheep red blood cells (sRBC) for 60 min and fixed. Cells were then stained with anti-p22\textsubscript{phox} antibodies. Differential interference contrast (DIC) microscopy and epifluorescence images were acquired. Arrows indicate colocalization of p22\textsubscript{phox} with phagosomes. (B) Human macrophages were infected at MOI 10 with either wild type or ΔLLO bacteria for 60 min and fixed. Cells were then stained with anti-p22\textsubscript{phox} antibodies in red and for bacterial-expressed GFP in green (to ‘boost’ this signal). Representative confocal z slices are shown. Insets are higher magnifications of the boxed areas. (C) Quantification of p22\textsubscript{phox}, p47\textsubscript{phox} or p67\textsubscript{phox} colocalization with sRBC or bacteria containing phagosomes. One hundred phagosomes were assessed from three independent experiments. Bars represent mean ± SEM.
Figure 20.7.2. Wild type *L. monocytogenes* inhibit ROS production through LLO to mislocalize p47\textsubscript{phox} and p67\textsubscript{phox} to the phagosome. (A, C) Human macrophages were treated with IgG-opsonized sheep red blood cells (sRBC) for 1 h and then fixed. Cells were then stained with either anti-p47\textsubscript{phox} (A) or anti-p67\textsubscript{phox} antibodies (C). Differential interference contrast (DIC) microscopy and epifluorescence images were acquired. (B, D) Human macrophages were infected with either wild type or ΔLLO bacteria for 1 h and fixed. Cells were then stained with either anti-p47\textsubscript{phox} (B) or anti-p67\textsubscript{phox} antibodies (D), in red. Bacteria expressing GFP were visualized with anti-GFP antibodies. Representative confocal z slices are shown. Insets in (B) and (D) are higher magnifications of the boxed areas. All scale bars are 10 µm.
Figure 20.8. LLO inhibition of ROS production is complemented by perfringolysin O (PFO). (A) Superoxide production by RAW 264.7 macrophages was measured by NBT reduction assay. Cells were either treated with PMA or infected at MOI 10 with wild type or ΔLLO bacteria for 30 min, followed by NBT treatment. Cells were also infected with iPFO, a complemented strain of ΔLLO that expresses PFO under an IPTG inducible promoter. Graph represents mean ± SEM from three independent experiments. (B) Cells were either treated with 10 µg/ml PMA or infected at MOI 10 with wild type or ΔLLO bacteria for 30 min, followed by cerium chloride treatment. TEM analysis was employed to quantify the percentage of phagosomes that were cerium perhydroxide precipitate positive. One hundred phagosomes were assessed from four independent experiments. Bars represent mean ± SEM.
20.9 LLO inhibition of NOX2 NADPH oxidase assembly is not mediated through altering key signaling events that mediate NOX2 NADPH oxidase assembly

It remains to be determined how LLO can selectively block delivery of NADPH oxidase components to the phagosome. To begin to explore this question, I examined signaling events linked to activation of the NADPH oxidase in *L. monocytogenes* infected cells. I observed phosphorylation of the p40<sub>phox</sub> subunit of the NADPH oxidase occurs in macrophages infected with both wild type and ΔLLO bacteria (Figure 20.9 A). Given that p38, AKT and ERK1/2 activation results in ROS production (Chen et al., 2003; Dang et al., 2003; Dewas et al., 2000), I next examined the activation of these signaling kinases during wild type and ΔLLO bacterial infection. I observed no major differences in activating phosphorylation between wild type or ΔLLO infected macrophages (Figure 20.9 B-D). Finally, I examined the phosphorylation state of a number of protein kinase C (PKC) isoforms since PKC<sub>α</sub>, PKC<sub>β</sub>, and PKC<sub>δ</sub> have been shown to activate NOX2 NADPH oxidase via phosphorylation of the regulatory subunits (Bey et al., 2004; Dekker et al., 2000; Fontayne et al., 2002). However, no significant differences in the phosphorylation states of any PKC isoform examined were observed between wild type or ΔLLO *L. monocytogenes* infected cells (Figure 20.9 E).
Figure 20.9. LLO mediated inhibition of ROS production is not dependent on altering the phosphorylation states of key signaling molecules responsible of NOX2 NADPH oxidase activation. BMDMs were infected with WT or ΔLLO bacteria for 15, 30, 45, 60 or 120 min. Cells were lysed and western blot analysis was performed to examine changes in (A) phospho-p40phox, (B) phospho-p38, (C) phospho-AKT, (D) phospho-ERK1/2 and (E) phospho-PKCα/β or phospho-PKCδ.
20.10 Summary

Here I demonstrate, for the first time, that *L. monocytogenes* modulates NOX2 NADPH oxidase activity during infection. Upon invasion, *L. monocytogenes* produces PLCs and LLO, which mediate bacterial phagosome escape. Production of diacylglycerol by PLCs has been implicated in phagosome escape by *L. monocytogenes* (Grundling et al., 2003). However, diacylglycerol can also activate the NOX2 NADPH oxidase through the activation of PKC (Dang et al., 2001b). Therefore, the induction of antibacterial ROS by PLCs is a potentially deleterious consequence of the mechanism that these enzymes utilize to promote phagosome escape in host cells. Countering this deleterious effect, LLO plays a key role in inhibiting the NOX2 NADPH oxidase by blocking localization to phagosomes. Therefore, one consequence of LLO pore formation is inhibition of PLC-driven ROS production, in addition to its role in phagosome escape and other virulence functions (Goebel and Kuhn, 2000).
Chapter 5
Discussion

The host and *L. monocytogenes* interactions are a complex series of events that can be either directed by the host to clear the infection or by the bacteria to evade toxic host defenses. Here, I provide insight to further current understanding of this dynamic interplay during *L. monocytogenes* intracellular infection. Autophagy is a key host defense against a multitude of pathogens. Past work has shown that autophagy can target *L. monocytogenes* at both early and late stages of infection. While a number of studies have shed light into the mechanism of autophagy targeting during the late stages of infection, the mechanism of how early targeting occurs has remained elusive. In this thesis, I provide evidence indicating that LC3 targeting of *L. monocytogenes* during early and late stages of infection is mediated via two different mechanisms, depending on where the bacterial is located inside the cell. LC3 targeting of cytosolic *L. monocytogenes* is mediated via ubiquitination of the bacteria and p62 binding (Figure 21.1 A) while early LC3 targeting to *L. monocytogenes* containing phagosomes is mediated via ROS and DAG (Figure 21.1 B).

Given that ROS can induce autophagy and is in itself bactericidal, I next examined the possibility of bacterial evasion of ROS. Here, I show that *L. monocytogenes*, via the activity of LLO, inhibits local ROS production by NOX2 NADPH oxidase in bacteria containing phagosomes. In addition, I found that other LLO related pore forming cytolysins are also capable of inhibiting ROS production, suggesting that this mechanism of host defense subversion may be a common bacterial strategy for intracellular survival.

Together, this thesis provides a clearer model of the “push and pull” of host defense versus bacterial evasion (Figure 21.2). Host defensive strategies, which include autophagy and ROS, target intracellular pathogens, resulting in their clearance in the phagolysosome. However, *L. monocytogenes* encodes a number of virulence factors, including LLO, which is capable of shutting down host defenses, such as ROS production, resulting in bacterial survival (Figure 21.2). While this thesis provides new insights into *L. monocytogenes* pathogenesis, some intriguing observations have generated a number of new hypotheses that are worthy of further discussion and future exploration.
Figure 21.1. Model of autophagy targeting to *L. monocytogenes* at early and late stages of infection, which are mediated by different mechanisms. (A) LC3 targeting to cytosolic *L. monocytogenes* is mediated via ubiquitination of the bacteria and p62 binding to recruit autophagy. (B) LC3 targeting to *L. monocytogenes* containing phagosomes at 1 h p.i. is dependent upon NOX2 NADPH oxidase production of ROS as well as both bacterial and host PLCs production of DAG.
Figure 21.2. The “Push and Pull” dynamics of host and bacteria interaction. (A) Host defenses, such as autophagy and ROS, target invading pathogens, resulting in bacterial clearance in the phagolysosome. (B) Bacteria, such as *L. monocytogenes*, encode virulence factors that are capable of shutting down host defenses (such as LLO inhibition of ROS production), resulting in intracellular bacterial survival.
21 Deciphering the complexity of *L. monocytogenes* and autophagy interaction

My work described in this thesis sheds light into the signals that mediate autophagic targeting of *L. monocytogenes* at 1 h p.i. (Figure 21.1). While future studies will be needed to fully understand the complexity of autophagic induction upon *L. monocytogenes* infection, my studies provide novel insight into the unique phagosomal targeting of bacteria that is likely relevant to a number of other intracellular organisms.

21.1 Different downstream signaling consequences of DAG production by bacteria or host

From my studies, I found evidence to suggest that DAG is produced by both the bacteria and host. This observation suggests that DAG production has beneficial consequences for both bacteria and host. DAG production by bacterial PI-PLC and PC-PLC has been extensively documented (Camilli et al., 1991; Goldfine et al., 1993; Poussin et al., 2009; Sibelius et al., 1999; Smith et al., 1995). PLCs production of DAG at the phagosome mediates PKC recruitment and activation, which in turn is capable of recruiting host phospholipases, including host PLCs and PLDs. Since both host phospholipases are implicated in allowing more efficient bacterial escape from the phagosome, the production of DAG by the bacteria allows ultimately for phagosome lysis and escape. Here, I provide evidence that in addition to its role in mediating escape, DAG production may be also responsible for the recruitment of LC3.

Since LLO is required for DAG generation (Sibelius et al., 1996), it has been hypothesized that LLO allows bacterial PLCs to travel from the lumen of the phagosome to the outer leaflet of the phagosomal membrane. Here, bacterial PLCs drive the production of DAG, which in turn recruits host phospholipases to generate more DAG. This model remains controversial since it is unclear whether bacterial PLCs, which are 30-35kDa, can freely passage through an LLO pore (~350Å) (Gilbert et al., 2000).
Another potential model can be proposed based on the findings of Goldfine and colleagues. In this model, LLO, even in absence of bacterial PLCs, recruits host PKCs, which in turns recruits host PLCs and PLDs (Goldfine et al., 2000). As host PLCs convert phosphatidylinositides into inositide triphosphate and DAG, the phagosomal membrane becomes more porous, thus allowing the diffusion of bacterial PLCs from the lumen to the outer leaflet of the phagosomal membrane. However, my results argue against this model since wild type *L. monocytogenes* displayed unaltered DAG production at the phagosome despite treatment with various host PLC inhibitors. While previous studies suggest that host PLCs are upstream of PLD recruitment, it is possible that bacterial PLCs can also recruit PLD. Given that PI-PLC specifically recruits and activates PKCβ (Poussin et al., 2009), which is specific for the recruitment and activation of host PLD (Dieter and Fitzke, 1995), it can be argued that PI-PLC activity can recruit and activate host PLD.

Here, I propose a new model based on my findings. During wild type *L. monocytogenes* infection, LLO recruits and activates PKC, which recruits host PLCs. The concerted activity of both bacterial and host PLCs break down the phagosomal membrane to allow bacterial PLCs to travel from the lumen of the phagosome to the outer leaflet. Here, PI-PLC production of DAG recruits host PKCβ, which then recruits host PLD. Together, bacterial and host phospholipases continue to lyse the phagosomal membrane, generating large amounts of DAG, which recruits LC3.

### 21.2 Induction of Autophagy – Advantage to the cell or bacteria?

In light of my proposed model, it is possible that the recruitment of host PLCs and DAG production is part of an autophagic response that is activated in presence of membrane damage. Thus, host PLCs are recruited to sites of membrane damage in an effort to remodel and repair the membrane via a concerted process of breakdown (by the PLCs) and rebuild (by DAG mediated targeting of autophagy). The fact that host PLCs assist in bacterial escape from the phagosome may be an unintended outcome of membrane degradation during the remodeling process. Previously, membrane damage induced by *Vibrio cholera* cytolysin, Vcc, been reported to
induce autophagy in an attempt to protect cells from Vcc intoxication (Gutierrez et al., 2007). More recently, alpha-toxin (secreted by *S. aureus*), streptolysin O (secreted by *S. pyrogenes*), and hemolysin (secreted by *E. coli*) were all shown to induce autophagy via the AMP-activated protein kinase (AMPK) pathway (Kloft et al., 2010). AMPK is a pathway that monitors cellular ATP levels. Pore-formation can result in leakage of ATP into the extracellular milieu, thus creating “energy stress”. This then activates the AMPK pathway, resulting in the inhibition of the mTOR complex. The work presented here suggests that the degree to which LLO can form pores influences autophagic targeting at 1 h p.i. (Figure 19.5 B). Thus, these observations combine to argue for the possibility of autophagy targeting to *L. monocytogenes* containing phagosomes in response to LLO pore-formation, which then damages the phagosomal membrane. In the absence of membrane damage, ΔLLO mutants therefore do not trigger an autophagy response (Figure 19.1 and Figure 19.4 A).

As mentioned above, other pore-forming cytolysins have the ability to induce autophagy via activation of AMPK, which activates protein kinase A (PKA), resulting in subsequent phosphorylation of its substrate, cyclic-AMP response element binding (CREB). Therefore, I examined changes in phospho-CREB levels via western blot analysis of cells infected with wild type or ΔLLO *L. monocytogenes*. Preliminary studies indicate that wild type infected cells showed greater levels of phospho-CREB over ΔLLO mutant infected cells at 1 h p.i.. Thus, this result suggests that LLO may also activate AMPK. This could explain, at least in part, the induction of autophagy at 1 h p.i.. However, just how LLO pore-formation targets LC3 to the phagosomal membrane is less clear.

### 21.3 How is LC3 targeted to *L. monocytogenes* containing phagosomes?

The question of how LLO recruits LC3 is a complex one and requires examination of the origin of the LC3. Knowing how LC3 is recruited would then give insight into how LLO may be triggering this process. There are two models of LC3 recruitment. The classical model of autophagy targeting involves the fusion of LC3\(^+\) vesicles with the damaged organelle to create a double-membraned compartment. It is theorized that *de novo* conjugation of LC3-I to PE to form
LC3-II, mediated by the Atg12-Atg5-Atg16L1 complex, is possible at the phagosomal membrane (Figure 21.3). This would give rise to a LC3\(^+\) single-membraned compartment. However, the issue of how LC3 is recruited to phagosomes remains controversial in the field. Here, I propose that LC3 targeting to \textit{L. monocytogenes} is mediated by a \textit{de novo} mechanism. Examining wild type \textit{L. monocytogenes} containing phagosomes at 1 h, I observed mainly single-membraned phagosomes and thus it appears unlikely that LC3\(^+\) phagosomes are double-membraned. However, to confirm this observation, CLEM should also be performed to examine if LC3\(^+\) \textit{L. monocytogenes} is likewise contained in a single-membraned phagosome.

\section*{21.4 \textit{L. monocytogenes} possesses the ability to limit autophagic targeting}

It stands to reason that if bacterial replication is enhanced when autophagy is inhibited, then autophagic induction is a host-mediated event since the host benefits from the induction of autophagy. Conversely, if bacterial replication is decreased when autophagy is inhibited, then autophagic induction is likely a bacterial-mediated event since the bacteria benefits from the induction of autophagy. This distinction is less clear in the case of autophagic targeting of \textit{L. monocytogenes}. Previous reports of \textit{L. monocytogenes} \textit{in vitro} survival in mouse embryonic fibroblasts (MEFs) show no significant difference in \textit{L. monocytogenes} replication between infection in wild type or Atg 5\(^{-/-}\) MEFs (Birmingham et al., 2007a; Py et al., 2007). These findings suggest that autophagy does not play a role in either limiting or enhancing bacterial infections or that \textit{L. monocytogenes} has a mechanism of overcoming autophagic clearance. Certainly, in absence of LLO, bacterial replication is limited in both wild type and Atg5\(^{-/-}\) MEFs. However, it is hard to ascribe autophagy evasion activity to LLO since LLO is required for bacterial escape and thus, the drop in bacterial replication may not necessarily be due to inhibition of autophagy but an inability to escape into the cytosol. As such, the search for this putative \textit{L. monocytogenes} encoded factor which limits autophagic targeting is hampered by the limitation that a number of factors can influence bacterial replication in addition to autophagic targeting. Thus, to fully appreciate the role of LLO on autophagy evasion, LC3 targeting of wild type and \textit{ΔLLO} \textit{L. monocytogenes} must be examined in a cell type that does not require LLO for
Figure 21.3. Proposed model of autophagic targeting of latex beads or bacteria. FcγR/TLR signaling mediates NOX2 NADPH oxidase assembly and activation, resulting in phagosomal production of ROS. ROS acts as a signal for the recruitment of autophagic machinery, mediating the de novo conversion of LC3-I to LC3-II directly at the phagosome. Maturation of the LC3+ phagosome results in the fusion with lysosome. Reprinted from NADPH oxidase contribute to autophagy regulation. Huang et al. Autophagy 5:6 887-9, 2009, with authors’ permission.
escape (ie: in Hela or Henle-407 cells). In addition to LLO, \textit{L. monocytogenes} PLCs have also been implicated in autophagy evasion. \(\Delta PI\)-PLC mutants exhibit increased replication in \textit{Atg5}\(^{-/}\) MEFs when compared to control MEFs suggesting that PI-PLC may play either a direct or indirect role in the evasion of autophagy targeting (Birmingham et al., 2007a). A kinetic study of \(\Delta PI\)-PLC mutants indicated that 30% of these bacteria remain LC3\(^+\) up to 8 h p.i. while only 10% of wild type \textit{L. monocytogenes} remains LC3\(^+\). This finding suggests that PI-PLC plays a role in the evasion of autophagy targeting. However, since LC3 targeting to either wild type or \(\Delta PI\)-PLC mutants never increases beyond 30% of the population, it is possible that additional \textit{L. monocytogenes} factors may be involved to limit autophagy targeting. Thus, to overcome activation of autophagy, I hypothesize that \textit{L. monocytogenes} may encode an as yet unknown factor, in addition to PI-PLC, which can inhibit autophagy to promote bacterial survival.

21.4.1 Future Directions

Previously, my work and work by others in the Brumell laboratory demonstrated that roughly 20-30% of wild type (strain named 10403S) and \(\Delta actA\) \textit{L. monocytogenes} are LC3\(^+\) at 2 h p.i. (Birmingham et al., 2007a). Intriguingly, work done by Yoshikawa and colleagues using \textit{L. monocytogenes} strains from a different background (EGDe) indicated that 10% of wild type \textit{L. monocytogenes} are LC3\(^+\) and 60% of \(\Delta actA\) bacteria are LC3\(^+\) at 2 h p.i.. Collectively, these observations suggest the possibility that in absence of ActA, EGDe \textit{L. monocytogenes} is targeted by LC3 while 10403S \textit{L. monocytogenes} expresses an unknown factor to evade LC3 targeting. Thus, a comparison of genes expressed in 10403S but not in EGDe may reveal the putative autophagy-inhibiting factor. In collaboration with John Parkinson of the University of Toronto, we have compared the genomes of both wild type strains of \textit{L. monocytogenes} and compiled a list of 62 genes that are unique to 10403S. Of these genes, 12 are expressed only in virulent strains of \textit{Listeria}. Thus, future efforts to search for the putative autophagy-inhibiting factor should begin with the generation of 10403S \textit{L. monocytogenes} knock outs of each of these 62 genes and LC3 targeting should then be examined. It is predicted that a greater percentage of mutants deficient in the autophagy-inhibiting factor would be targeted by LC3 compared to the wild type. Conversely, EGDe strains expressing each of these 12 genes should also be constructed and LC3 targeting then examined. It is my prediction that a lower percentage of the
mutant EGDe strain expressing the autophagy-inhibiting factor would be targeted by LC3 compared to the wild type.

21.5 Multiple signals acting in the same pathway could mediate autophagy targeting

Given that DAG, ROS and LLO pore-formation all exert significant individual impact on autophagic targeting of *L. monocytogenes* at 1 h, these three signals of autophagy may act in the same pathway. DAG is required for the activation of protein kinase Cs (Mellor and Parker, 1998), which have been reported to activate NOX2 NADPH oxidase activity (Raad et al., 2009). Thus, DAG may act upstream of ROS.

21.5.1 Future Directions

To test the hypothesis is that DAG is upstream of ROS production, I quantified DAG localization to *L. monocytogenes* containing phagosomes in the presence or absence of ROS inhibition by DPI. In cells infected with ΔPI-PLCΔPC-PLC *L. monocytogenes*, DAG localization to the phagosome was unaltered in presence of DPI inhibition, suggesting that host production of DAG is upstream of ROS production. Surprisingly, localization of DAG to wild type *L. monocytogenes*-containing phagosomes decreased upon treatment of DPI, suggesting that bacterial production of DAG is downstream of ROS production. Given the confusing nature of these preliminary data, more work is needed to fully understand the difference between host and derived and bacterial derived DAG.

Furthermore, I hypothesize that bacterial-derived DAG is located on the inner leaflet of the phagosomal membrane immediately post infection, whereas host-derived DAG is located on the outer leaflet of the phagosomal membrane. This localization would, presumably, change as the membrane becomes permeabliized. DAG specific antibodies derived from PKCδ and PKCε DAG binding regions have been employed in the context of EM to study DAG production (Babazono et al., 1998). Thus, I propose that DAG production on both leaflets of the phagosomal
membrane should be examined using immuno-gold electron microscopy. This technique can be employed to study DAG production over time. Cells infected with wild type or ΔPI-PLCΔPC-PLC L. monocytogenes can be fixed at 15, 30, 45 and 60 mins p.i. and DAG production on the inner versus outer leaflet of the phagosomal membrane assessed at each time point. Presumably, in cells infected with wild type bacteria, DAG production would occur on both leaflets of the membrane. In cells infected with ΔPI-PLCΔPC-PLC L. monocytogenes, DAG production would occur first in the outer leaflet of the phagosomal membrane, followed by production on the inner leaflet of the membrane.

22 Walking a tight rope: The importance of L. monocytogenes virulence factor antagonism in presence of NOX2 NADPH oxidase activity

The work presented in this thesis describes the previously unknown function of LLO to inhibit local ROS production by NOX2 NADPH oxidase inside L. monocytogenes containing phagosomes. Furthermore, I provide evidence to suggest that the inhibition of ROS production may be a general function of other LLO related pore-forming cytolysins. As such, this thesis provides new insights that further advance current understanding L. monocytogenes pathogenesis.

22.1 Multiple peaks of ROS production may be due to different activation signals

Previous studies have reported that at 10 min p.i., both wild type and ΔLLO bacteria induce ROS production. Here, I confirm these findings of an early wave of ROS production and further show that there is a second wave of ROS production from 40 min – 70 min p.i.. The dynamic studies with luminol indicate that these two waves are induced by different mechanisms, where the first wave is PLCs-independent induction of ROS while the second wave is PLCs-dependent
induction of ROS. Furthermore, the second wave of ROS production can be inhibited by LLO, whereas the first wave at 10 min cannot. These observations suggest that a third bacterial factor can trigger the early wave of ROS production. Given that TLRs have been shown to be upstream of NOX2 NADPH oxidase activation (Bae et al., 2009; Elsen et al., 2004; Lee et al., 2008; Suliman et al., 2005; Vulcano et al., 2004), a potential source of this initial wave of ROS production may be TLR activation in response to an unidentified L. monocytogenes PAMP. Thus, I hypothesize that TLR activation mediates both intra- and extracellular ROS production at 10 min p.i. where LLO cannot effectively inhibit ROS production. In contrast, PLC-mediated ROS production at 40 – 70 min p.i. occurs locally inside the phagosome, where LLO effectively mislocalizes the NOX2 NADPH oxidase to inhibit ROS production.

An alternate explanation to consider is that two different populations of phagosomes are responsible for generating the first and the second wave of ROS production. It has been observed that there is a degree of heterogeneity in phagosome formation within a given cell (Henry et al., 2004). Henry and colleagues showed there appears to be two distinct populations of phagosomes that are formed upon addition of IgG-coated latex beads or sheep red blood cells to RAW 264.7 macrophages. One population loses PI3P localization at the phagosome after 10 min while the other population continues PI3P acquisition for up to 20 min. Consistent with this, other laboratories have observed NOX2 NADPH oxidase assembly on only 50% of phagosomes (personal communication with Dr. Mary Dinauer). Thus, given that NOX2 NADPH oxidase localization to the phagosome is dependent on its binding to PI3P (Lambeth, 2004), it is possible that the wave of ROS production at 10 min p.i. is derived from all phagosomes prior to the loss of PI3P, while ROS production at 40 – 70 min p.i. occurs in the population of phagosomes that retain PI3P over time.

### 22.1.1 Future Directions

To examine the possibility that TLR recognition of bacterial PAMP results in ROS production at 10 min p.i., cells incapable of mounting TLR-mediated signaling should be examined. Myeloid differentiation primary response gene 88 (MyD88) is a critical adapter protein in the signaling pathway of all TLRs (with the exception of TLR3) (Arancibia et al., 2007). Thus, mice deficient in MyD88 would exhibit an inability to mount the majority of TLR mediated responses. BMDM
from wild type and MyD88 knockout mice should be compared for their ability to produce ROS upon *L. monocytogenes* infection. A luminol assay can be used to compare dynamic ROS production. If ROS production is MyD88 dependent, then the burst of ROS at 10 min p.i. would be absent in the MyD88 knockout. If ROS production is dependent on MyD88, BMDM from specific TLR knockout mice can be examined to determine which TLR is responsible for ROS production.

Phagosomal heterogeneity may also be responsible for ROS production by different phagosomes at different times. Thus, this possibility should also be tested. Given that only half of phagosomes remain PI3P+ after 10 min, immunofluorescence microscopy analysis can be employed to quantify the percentage of PI3P+ and PI3P− *L. monocytogenes* containing phagosomes that are also p22phox, p40phox or p67phox. This experiment will give an indication of whether the NOX2 NADPH oxidase complex is present exclusively on PI3P+ phagosomes. However, the presence of the NOX2 NADPH oxidase complex on PI3P+ phagosomes will not directly confirm the presence of ROS in those specific phagosomes. One technically challenging but highly informative experiment that can directly show ROS production at PI3P+ phagosomes is correlative light microscopy – electron microscopy (CLEM). To do this, human peripheral blood mononuclear cells (PBMCs) can be cultured and transfected with PI3P specific probes (either PX-GFP or 2-FYVE-GFP constructs). 24 h later, cells can be infected with either wild type or ΔLLO *L. monocytogenes* and cerium chloride treatment can be employed as described in section 17. After fixation, cells could imaged via using confocal microscopy to identify PI3P+ *L. monocytogenes* containing phagosomes followed by TEM imaging to quantify the percentage of PI3P+ *L. monocytogenes* containing phagosomes that have cerium perhydroxide precipitates. It is predicted that PI3P+ *L. monocytogenes* containing phagosomes are cerium perhydroxide+ while PI3P− *L. monocytogenes* containing phagosomes are cerium perhydroxide−.

22.2 Relationship between ROS and LLO provides insight into *L. monocytogenes* pathogenesis in different cell types

Interestingly, it must be noted that while LLO is a critical virulence factor to ensure *L. monocytogenes* survival in murine macrophages, it is the PLCs that are required for bacterial
survival in other cell types, such as in human laryngeal cancer cells (Hep-2), human cervical epithelial cells (HeLa), human embryonic intestinal epithelial cells (Henle-407) (Grundling et al., 2003) and human embryonic kidney cells (Burrack et al., 2009). It is currently unclear whether the difference in virulence factor importance is due to a difference of species (mouse vs. humans) or of cell type (macrophages vs non-phagocytes). Furthermore, it is also unclear why this distinction of virulence factor dependence exists. In light of my work, one possible explanation emerges. Given that macrophages produce a substantially higher level of ROS than epithelial cells, it is possible that a virulence factor that can decrease the level of oxidative stress (ie: LLO) may be critical for *L. monocytogenes* survival, while virulence factors that inadvertently induce more oxidative stress (ie: PLCs) may be dispensable in this cell type. Conversely, in an environment with little ROS production (ie: epithelial cells), counteracting oxidative stress is not a priority, so LLO is dispensable. Thus, the evolution of LLO and PLCs may be important for mediating bacterial survival in different cell types.

### 22.2.1 Future Directions

To test whether LLO dependency differs between cell type or host species, a replication assay of wild type and ΔLLO *L. monocytogenes* in human PBMCs can be performed. Presumably, if LLO is required for bacterial survival in macrophages regardless of species, then ΔLLO *L. monocytogenes* in human PBMCs should be effectively cleared in the same fashion as it is in murine BMDMs (Figure 20.5).

An alternate experiment would be to perform replication assays in both murine epithelial cells and BMDMs. If LLO is critical for bacterial survival in macrophages but not epithelial cells, then ΔLLO replication should exhibit wild type *L. monocytogenes*-like growth kinetics in murine epithelial cells. If LLO is not needed for survival in murine epithelial cells, then this would support the hypothesis that *L. monocytogenes* survival in high ROS producing cell types require LLO to inhibit ROS while survival in low ROS producing cell types do not require LLO.

Recently, Clark and colleagues created a K562 human erythroleukemia cell line that stably expresses functional NOX2 NADPH oxidase (Clark and Valente, 2004). Thus, to directly test this hypothesis, K562 cells with or without NOX2 NADPH oxidase can be infected with either
wild type or ΔLLO *L. monocytogenes* in replication assays. If the hypothesis is valid, then K562 cells expressing NOX2 NADPH oxidase should rapidly clear ΔLLO *L. monocytogenes* in a fashion that is similar to macrophages while K562 cells without NOX2 NADPH oxidase would permit similar replication kinetics by both wild type and ΔLLO *L. monocytogenes*.

### 22.3 Understanding the mechanism of LLO mislocalization of NOX2 NADPH oxidase

The question of how LLO mislocalizes NOX2 NADPH oxidase remains unanswered. While LLO may not be influencing the phosphorylation states of PKCs, p38, AKT, p40 or ERK1/2, I cannot rule out the possibility that LLO is altering the activation of other signaling molecules upstream of NOX2 NADPH oxidase activation. However, it can be concluded that pore formation is critical in mediating NOX2 NADPH oxidase mislocalization. It remains to be determined whether pore formation has a direct or indirect effect on NOX2 NADPH oxidase mislocalization. Certainly, previous studies have indicated that LLO can modify the phagosomal compartment, resulting in a delay in phagosomal maturation (Alvarez-Dominguez et al., 2008; Alvarez-Dominguez et al., 1997; Henry et al., 2006; Prada-Delgado et al., 2005). In particular, studies conducted by Prada-Delgado and colleagues found that LLO excludes Rab5a recruitment to the *L. monocytogenes* containing phagosome. Rab5a was found to participate in the recruitment of Rac2 to the phagosome (Prada-Delgado et al., 2005). Since activated Rac2 is one of the key NOX2 NADPH oxidase components that mediate proper localization to the phagosome, it is possible that LLO-dependent exclusion of Rab5a prevents Rac2 recruitment and therefore prevents NOX2 NADPH oxidase localization (Prada-Delgado et al., 2001). Certainly, in the context of *Yersinia pseudotuberculosis*, an outer membrane protein, YopE inactivates Rac2, thus preventing ROS production during *Y. pseudotuberculosis* infection in macrophages (Songsunthong et al., 2010). To address this hypothesis, I compared levels of activated Rac2 between wild type and ΔLLO infected RAW 264.7 macrophages using a pulldown assay. PAK-PBD, which binds specifically to activated Rac, was used the bait. However, I did not detect any differences between wild type and ΔLLO infected cells. This may be due to the fact that
pulldowns reflect the level of activated Rac2 in the entire cell and the phenotype may be localized and limited to the phagosome.

If LLO is mediating NOX2 NADPH oxidase mislocalization via the exclusion of Rab5a, it remains unclear how a pore-forming toxin can be excluding a phagosomal protein. Under normal conditions, Rab5 is transiently recruited to the phagosome via binding to phosphatidylinositol (3) phosphate (PI3P), where it subsequently recruits a number of effectors required for the maturation of the early phagosome (Duclos et al., 2000; Fratti et al., 2001). Since PI3P is also required for the proper localization of NOX2 NADPH oxidase components (Lambeth, 2004), I examined if there are differences in PI3P colocalization to wild type or ΔLLO L. monocytogenes containing phagosomes. At 1 h p.i., I observed similar recruitment of PI3P to wild type and ΔLLO L. monocytogenes, suggesting that LLO-dependent NOX2 NADPH oxidase mislocalization is not mediated via alterations in PI3P enrichment at the phagosome.

There are few clues that exist in the field to direct the search for the mechanism behind Rab5 exclusion by a pore-forming toxin. Legionella pneumophila is another intracellular microorganism that induces a similar exclusion of Rab5c (Clemens et al., 2000). However, the mechanism is likewise unknown. Mycobacterium tuberculosis also has the ability to alter phagosomal maturation by modifying the bacteria containing phagosome. Unlike L. monocytogenes, M. tuberculosis compartments are Rab5+ but Rab7-. Despite the presence of Rab5, both Rab5 and Rab7 are inactive upon M. tuberculosis infection, in a manner that is dependent on the bacterial GTPase activating protein, nucleoside diphosphate kinase (Ndk) (Sun et al., 2010). Ndk mediates the conversion of the active GTP-bound Rab protein to the inactive GDP-bound form. Interestingly, M. tuberculosis mislocalizes both the NOX2 NADPH oxidase and the inducible nitric oxide synthase (iNOS) (Katti et al., 2008). Thus, exclusion of NOX2 NADPH oxidase by L. monocytogenes may also be mediated by the inactivation of Rab5a or other Rab proteins.

It must be noted that there are a number of different functions already attributed to LLO and there likely exists many more. Thus, it is possible that LLO inhibits NOX2 NADPH oxidase via multiple mechanisms. The difficulty in studying these other roles of LLO lies in the fact that LLO is required for phagosomal escape and is critical for survival in macrophages. Thus, the other roles of LLO can be hidden since bacteria lacking LLO are rapidly cleared. Furthermore,
cytosolic roles or consequences of LLO activity are difficult to assess since efficient escape does not occur in absence of LLO. To get around this limitation, a number of studies have utilized exogenous addition of LLO or LLO point mutants to understand the biochemical changes that occur upon exposure to LLO.

Another potential method to study LLO function is to study non-phagocytic cells infected with wild type and ΔLLO *L. monocytogenes*. Since LLO is not required for efficient phagosomal escape in these cells, the limitation of LLO dependent escape can be eliminated. Therefore, future studies should be directed towards a better understanding of LLO function. Given that current understanding of the many potential roles of LLO is quite limited, I expect that the mechanism by which LLO inhibits NOX2 NADPH oxidase may be much more complex than what we can currently appreciate.

### 22.3.1 Future Directions

Further examination of whether differential Rac2 recruitment could explain differences in ROS production between wild type and ΔLLO infected cells should be pursued. My attempts to examine the levels of activated Rac2 at the whole cell level were not fruitful. Thus, Rac2 localization and activation at the level of the phagosome should be tested. Previously, fluorescent fusion constructs for murine Rac1 (YFP-Rac1), Rac2 (YFP-Rac2) and activated Rac (CFP-PBD) have been successfully employed in RAW 264.7 macrophages to study red blood cell phagocytosis (Hoppe and Swanson, 2004). Therefore, one can express these constructs in RAW 264.7 macrophages and infect for 1 h with either wild type or ΔLLO bacteria. If the inhibition of NOX2 NADPH oxidase assembly by wild type bacteria is mediated via Rac2 mislocalization, the percentage of Rac2+ or PBD+ wild type *L. monocytogenes*-containing phagosomes should be significantly lower than that of ΔLLO *L. monocytogenes*-containing phagosomes.

If Rac2 is found to be mislocalized, then it is possible that Rab5a mislocalization in wild type *L. monocytogenes* containing phagosomes might be cause of Rac2 mislocalization and ROS inhibition. To examine a possible link between Rab5a and Rac2, immunofluorescence co-staining with Rab5a and Rac2 should be done to determine if Rab5a+ ΔLLO *L. monocytogenes* containing phagosomes are also Rac2+. If Rab5a+ compartments are also Rac2+, then the link
between Rab5a and ROS production should next be examined using CLEM. If Rab5a allows the recruitment of Rac2, which mediates the assembly of the rest of the NOX2 NADPH oxidase components, then Rab5a\(^+\) *L. monocytogenes*-containing phagosomes should be cerium perhydroxide\(^+\). Wild type and Rab5a deficient BMDM can be infected with either wild type or ΔLLO bacteria followed by the examination of ROS production using each of the ROS quantification methods that are described in this thesis. If Rab5a is required for NOX2 NADPH oxidase assembly at the phagosome, then infection with ΔLLO *L. monocytogenes* would not induce ROS in the Rab5a deficient BMDM.

In addition to Rab5a and Rac2, other mechanisms of LLO mediated mislocalization of the NOX2 NADPH oxidase should also be examined. My preliminary work, as well as a recent publication, demonstrate that LLO mediates protein kinase A (PKA) activation as evidenced by increases in the phosphorylation level of the PKA substrate, cyclic-AMP response element biding (CREB) (Gonzalez et al., 2011). Given that PKA also phosphorylates the inhibitory site of p47, resulting in the inhibition of ROS production, it is possible that LLO mediates NOX2 NAPDH oxidase mislocalization via the activation of PKA. I propose that further work should be done to fully understand how LLO activates PKA.

Previously, pore-forming cytolysins have been shown to activate PKA by increasing cellular cAMP levels (Gonzalez et al., 2011). Thus, the effect of LLO on cAMP levels should be examined. To do so, western blotting techniques can be applied to compare cAMP levels between macrophages infected with wild type or ΔLLO *L. monocytogenes*. Next, the question of how LLO can influence cAMP levels should be examined. cAMP is produced by adenyl cyclase and degraded by phosphodiesterase (PDE). *In vitro* alterations of cAMP levels can be achieved either by inhibition its production (inhibiting adenyl cyclase) or decreasing its degradation (inhibiting PDE). The modulation of cAMP levels or PKA activity should thus be examined in context of macrophages infected with wild type or ΔLLO *L. monocytogenes*. If LLO inhibition of NOX2 NADPH oxidase is mediated by PKA activation, treatment with pharmacological inhibitors of PKA, such as H-89 dihydrochloride or KT5720, or compounds that inhibit cAMP production, such as 2\(^{\prime}\), 5\(^{\prime}\)-dideoxyadenosine, should increase ROS production during wild type *L. monocytogenes* infection. Conversely, drugs that either enhance PKA activity directly, such as forskolin or 6-bnz-cAMP (cAMP mimetic), or inhibit cAMP degradation, such as 3-isobutyl-1-methylxanthine (IBMX), should inhibit ROS production during ΔLLO *L. monocytogenes*
infection. My preliminary studies involving the NBT quantification assay demonstrate that treatment with either 6-bnz-cAMP, forskolin or IBMX can significantly reduce ROS production in macrophages infected with ΔLLO L. monocytogenes. Thus, these observations suggest that LLO modulation of PKA activity may be a key mechanism of NOX2 NADPH oxidase inhibition.

Lastly, the functional effect of PKA modulation on bacterial replication and survival should be examined. *In vitro* replication assays of wild type and ΔLLO infected macrophages infected with wild type and ΔLLO L. monocytogenes should be conducted in the presence of both PKA activating and inhibiting drugs. It is predicted that PKA activation should inhibit ROS production in macrophages infected with ΔLLO bacteria, thus enhancing bacterial survival and replication over time. Conversely, it is predicted that PKA inhibition should allow ROS production in macrophages infected with wild type bacteria, thus decreasing bacterial survival and replication over time. The rate of survival of both *L. monocytogenes* strains in wild type or PKA deficient animals should be examined. I hypothesize that survival of wild type *L. monocytogenes* should decrease in absence of PKA.

### 22.4 Multiple mechanisms may be involved in NOX2 NADPH oxidase inhibition

Given that all of the NOX2 NADPH oxidase components examined were mislocalized from the wild type *L. monocytogenes*-containing phagosome, the assumption was made that a single mechanism universal to all components might be responsible for the inhibition of ROS production. However, it is possible that *L. monocytogenes* may have multiple mechanisms to mediate NOX2 NADPH oxidase mislocalization. In the case of *F. tulerensis*, a number of different mechanisms of NOX2 NADPH oxidase inhibition have been reported. ROS production by neutrophils in response to *F. tulerensis* infection is inhibited by decreasing phosphorylation of p47^phox^ and mislocalizing gp91^phox^ from phagosomes containing *F. tulerensis*. Furthermore, this bacterium also encodes a phosphatase, AcpA, which contributes to ROS inhibition. Finally, cells infected with the *F. tulerensis* mutants deficient in the virulence operon regulator, MigR, display
a partial increase in ROS production. Together, these observations suggest that \textit{F. tulerensis} employs multiple mechanisms to downregulate NOX2 NADPH oxidase activity. Given the importance of ROS as an antibacterial defense for all intracellular pathogens, it is likely that \textit{L. monocytogenes} also has multiple mechanisms of ROS inhibition. Since different PKCs are responsible for phosphorylation of different NOX2 NADPH oxidase subunits, it is possible that specific bacterial virulence factors may target a specific PKC. Furthermore, \textit{L. monocytogenes} encodes a manganese superoxide dismutase (MnSOD) as well as a catalase. Mutants deficient in MnSOD display attenuated survival \textit{in vitro} in macrophages and \textit{in vivo} in a manner that is similar to ΔLLO mutants (Archambaud et al., 2006). Furthermore, ΔMnSOD \textit{L. monocytogenes} also displays a deficiency in phagosomal escape. Thus, \textit{L. monocytogenes} possesses at least three different mechanisms to defend against ROS, acting at different steps in the pathway to produce ROS (Figure 22.5). It remains to be seen if these ROS inhibition mechanisms are synergistic or cooperative in nature.

22.5 Opposing consequences of LLO and PLCs activity allows for ROS production in the phagosomal compartment

Why would \textit{L. monocytogenes} have evolved two virulence factors that have antagonistic effects on NOX2 NADPH oxidase activity? There are two potential explanations for this conundrum. One possible explanation would be that PLCs activation of NOX2 NADPH oxidase is an unintended consequence of PLCs activity. Given that PLCs play an important role in \textit{L. monocytogenes} escape from the phagosome, the deleterious effect of ROS induction can thus be counterbalanced by LLO to decrease local oxidative stress. The opposing consequences of LLO and PLCs activity can be termed “virulence factor antagonism”. Thus, while these two virulence factors are seemingly antagonistic to each other, they function together to allow for bacterial survival. An alternate explanation could be because limited host production of ROS is somehow beneficial for \textit{L. monocytogenes} survival. Thus, bacterial PLCs may drive ROS production while LLO ensures that ROS in the phagosome, or in the immediate vicinity of the bacteria, is kept to a minimum. It is known that ROS participate in a number of signaling events and perhaps the
induction of these events may be advantageous for bacterial survival. However, since *L. monocytogenes* displays an increased replication rate in mice deficient in gp91<sup>phox</sup> compared to wild type mice, it does not appear that ROS improve bacterial survival.
Figure 22.5. Methods of ROS inhibition employed by *L. monocytogenes*. *L. monocytogenes* possesses three different mechanisms against the deleterious effects of ROS. LLO acts to inhibit local production of ROS by mislocalizing the NOX2 NADPH oxidase. Residual ROS is eliminated by bacterial superoxide dismutase (SOD) and catalase.
22.6 NOX2 inhibition by pore forming cytolysins and virulence factor antagonism may be common strategies for intracellular survival

The observation that PFO, a pore-forming cytolysin secreted by *C. perfringens*, can also mediate a similar inhibition of NOX2 NADPH oxidase as LLO suggests that inhibition of ROS production may be a conserved consequence of pore-forming cytolysin activity. A number of pathogens that encode pore-forming cytolysins can also inhibit ROS production, including *C. perfringens, Y. pestis, B. anthracis, H. pylori* among others. Thus, further examination of the mechanism of LLO inhibition of NOX2 NADPH oxidase activity may reveal a common function for all pore-forming cytolysins.

Furthermore, the work presented in this thesis reveals how *L. monocytogenes* initiates a dynamic, and in this case antagonistic, set of signals in host cells via its virulence factors to allow for optimal bacterial growth and survival during infection. Like *L. monocytogenes, C. perfringens* is capable of escaping from the phagosome (O'Brien and Melville, 2000). This process is dependent on the activity of the bacterial PLC in addition to PFO (O'Brien and Melville, 2004). Another intracellular organism that also produces both a bacterial PLC and a pore-forming cytolysin is *Bacillus anthracis*. As mentioned, this bacteria can inhibit NOX1 generated ROS in colonic epithelia in a manner that is dependent on the pore-forming cytolysin, edema toxin (Kim and Bokoch, 2009). While edema toxin belongs to a different class of pore-forming cytolysins from either LLO or PFO, the potential for virulence factor antagonism between *B. anthracis* PLC and pore-forming cytolysin remains a viable possibility. Finally, both *H. pylori* and *F. tularensis* have the ability to inhibit ROS production (Table 2.3), and encode bacterial PLCs. Given that a number of pathogens secrete PLCs and are also able to inhibit ROS production, this model of virulence factor antagonism may be applicable to several pathogenic species.

While perhaps counterintuitive at first glance, virulence factor antagonism is potentially an extremely powerful mechanism that would allow two opposing responses to be regulated both spatially and temporally. The induction of ROS production by PLCs is detrimental to the bacteria. However, the presence of LLO allows for local inhibition of ROS while global ROS
production may continue. In this light, LLO can modulate the effect of PLC spatially such that *L. monocytogenes* inside the phagosome can be protected from the production of ROS.

### 22.7 Understanding LLO pore-formation and ROS in the targeting of autophagy

Given that LLO can inhibit ROS production and that ROS mediates autophagy targeting, cells infected with ΔLLO should produce significant level of ROS, thus recruiting LC3. However, my work (Figure 19.1.1), and that of others (Birmingham et al., 2007a; Meyer-Morse et al., 2010; Py et al., 2007) clearly indicates that LLO is required for autophagy targeting. How then can LLO inhibit ROS yet the lack of ROS prevents autophagy targeting? There are a number of possible explanations for this apparent paradox. First, it is possible that ROS need to be transported from the phagosomal lumen to the outer membrane of the phagosome (perhaps via the LLO pores) in order to be detected by the autophagic system. Thus, while ROS is needed for autophagic targeting, production of ROS inside the lumen of the ΔLLO containing phagosome will not be detected by the autophagic machinery in the cytosol. Thus, ΔLLO is not targeted by autophagy. In cells infected with wild type *L. monocytogenes*, low levels of ROS production was observed (Figure 20.1.2 B). Thus, it may be possible that this low level of ROS can traverse the phagosomal membrane via LLO pores and be detected by the autophagic machinery.

A second possibility is the idea of bacterial virulence heterogeneity. Indeed, it has been theorized that not every single wild type *L. monocytogenes* express the same level of LLO (Birmingham et al., 2008). Previously, work from the Brumell laboratory suggests that there are three possible fates that can occur upon *L. monocytogenes* infection. One population of internalized *L. monocytogenes* can escape from the phagosome, replicate inside the cytosol and achieve secondary spread into neighbouring cells via the formation of the actin rocket tail. A second population of *L. monocytogenes* fails to escape from the phagosome and is degraded in the lysosome in the conventional phagolysosomal pathway. A third population of bacteria is targeted by autophagy inside a non-degradative compartment. These compartments, termed spacious *Listeria* associated phagosomes (SLAPs), cannot fuse with the lysosome but the bacteria can also
not escape. SLAP are non-degradative, LAMP-1+ and LC3+ (Birmingham et al., 2007b). Formation of SLAPs was found to require autophagy in the host cell, and expression of LLO by the bacteria. Modulation of LLO expression via an inducible promoter which expresses a moderate level of LLO (compared to wild type expression level of LLO) can increase SLAP formation (Birmingham et al., 2007b). Birmingham and colleagues proposed that SLAPs allow slow bacterial replication that may allow chronic L. monocytogenes infection in a host (Birmingham et al., 2007b). Indeed, compartments resembling SLAPs have been observed in a mouse model of L. monocytogenes chronic infection (Bhardwaj et al., 1998). Thus, these observations suggest that there may be variable LLO expression even in the wild type L. monocytogenes population. Bacteria that express high level of LLO can efficiently escape from the phagosome while those that express low levels of LLO are degraded in the lysosome. However, the population of bacteria expressing a moderate level of LLO may be targeted by autophagy and form SLAPs.

Incorporating my observations into this model, it is possible that the population of bacteria expressing high levels of LLO is effectively inhibiting ROS production (and thus preventing autophagic targeting), thus allowing for escape into the cytosol. The population of bacteria expressing low levels of LLO cannot effectively inhibit ROS production and thus is cleared. The population of bacteria expressing moderate levels of LLO does not inhibit ROS production efficiently and thus recruits autophagy (Figure 22.7). This moderate level of ROS production is likely insufficient to clear the bacteria but is sufficient to recruit autophagy. Consistent with this model, my preliminary work indicates that SLAPs are also dependent on ROS production (Figure 19.6).

### 22.7.1 Future Directions

One direct way of testing this model is via the use of the iLLO mutant. Using the iLLO mutant, one can control the expression level of LLO by altering the concentration of IPTG. Immunofluorescence microscopy and cerium chloride TEM methods or CLEM can then be employed to examine ROS production and autophagic targeting. I predict that in the absence of IPTG, the iLLO mutant would be predominately cleared in the phagolysosomal pathway with
most phagosomes exhibiting cerium perhydroxide precipitates. Moderate expression of iLLO would show greater autophagic targeting at 1 h with moderate cerium perhydroxide precipitation inside the phagosome. High or over expression of LLO would result in enhanced autophagic targeting at 1 h with no cerium perhydroxide precipitation inside the phagosome. The same study should be conducted with the iPFO mutant. This experiment would then confirm the importance of pore formation on modulating the intracellular fate of *L. monocytogenes*. 
Figure 22.7. Proposed model of *L. monocytogenes* interaction with early host defenses against phagocytosed pathogens. Upon phagocytosis, *L. monocytogenes* encounters a number of host defenses and depending on the level of LLO, one of three potential fates can occur. One population of bacteria with low LLO expression cannot inhibit NOX2 NADPH oxidase production of ROS, and is thus readily degraded in the phagolysosome. Another population of bacteria with medium LLO expression mediates a moderate inhibition of ROS production. It is hypothesized that this moderate level of ROS is insufficient to kill the bacteria but does prevent phagosomal escape and mediate LC3 targeting to the *L. monocytogenes* containing phagosome. Over time, this “stalemate” compartment becomes a SLAP where neither the host can clear the bacteria or nor the bacteria escape. Finally, the population of bacteria with high LLO expression efficiently inhibits ROS production, allowing the bacteria to escape into the cytosol. Modified from *Listeria* evades killing by autophagy. Birmingham *et al.* Autophagy 4:3 368-71, 2008, with authors’ permission.
23 Concluding Remarks

In this thesis, I have provided insight into the signals that mediate autophagic targeting of *L. monocytogenes* at 1 h p.i.. In addition, my work provides evidence for a novel function of LLO in the inhibition of ROS production by the NOX2 NADPH oxidase. These observations led to the proposal of a new model that furthers the current understanding of *L. monocytogenes* intracellular survival and targeting by host defenses. Despite these advances, more questions remain to be answered before we will fully understand *L. monocytogenes* pathogenesis. Future research should continue to focus on understanding the intracellular lifestyle of *L. monocytogenes*, as this bacterium is a useful model pathogen to study the host and bacterial processes that are activated upon infection. Given the rise of antibiotic resistance, new approaches are necessary in combating infections. Insights into how host defenses are activated in response to intracellular infections may allow the creation of novel therapeutics that enhance host defenses to target pathogens. Such therapies could be less susceptible to bacterial resistance, since they would act on host proteins and not bacterial factors. Thus, administration of drugs that enhance host immunity, such as autophagy inducers, and those that weaken bacterial attack, such as pore-forming cytolysin inhibitors, may together provide a solution to a wide range of infections.
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