

DYNAMIC REGULATION OF VACUOLE HOMEOSTASIS DURING INFECTION
BY INTRACELLULAR BACTERIAL PATHOGENS

by

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Dedication

I dedicate this thesis to Thomas Delberti, Thomas McNally, Michael McDnough, and Tracy Bigelow. These are four of my past high school science teachers whom first introduced me to the world of science. I want to thank them for their excitement, support, and inspiration that resulted in my success and love of science.

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Abstract

Pathogen-containing vacuoles (PCV) are a rich interface for interaction of intracellular pathogens and their mammalian host cells. This thesis investigates mechanisms by which host and pathogen regulate the vacuolar environment to manipulate the outcome of infection. A novel role for the mammalian IKK-family kinase, TANK-binding-kinase-1 (TBK1), in maintaining integrity of PCV during an intracellular bacterial infection was identified. In the absence of TBK1, invading pathogens were released from the phagosome and entered into the host cytosol. Transcriptional analysis revealed elevated levels of Aquaporin-1 (AQP1) in mouse embryo fibroblasts (MEFs) deficient in TBK1. AQP1, a water channel that regulates swelling of secretory vesicles, was associated with PCV. Overexpression of AQP1 led to PCV destabilization and bacterial release into the cytosol in a manner dependent on bacterially-induced membrane damage and ion flux. Inhibition of AQP1 physiological function in multiple cell types also led to increased instability of PCV. These results highlight aquaporins as key mediators of vacuole integrity and homeostasis to control bacterial infection. From these data, it led to the hypothesis that while homeostatic regulation of vacuolar membrane channels may be utilized by host cells to control bacterial infection, they might also be exploited by cytosolic bacterial pathogens such as *Listeria monocytogenes* (*L. monocytogenes*), to escape the vacuole. Listeriolysin O (LLO) is a critical *L. monocytogenes* virulence factor that forms pores in the phagosomal membrane, altering the vacuolar environment. LLO is required for *L. monocytogenes*

cytosolic entry, but host mechanisms that contribute to vacuole rupture are poorly defined. Upon inhibition of chloride flux, *L. monocytogenes* was unable to escape the phagosome. Functional inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) also suppressed *L. monocytogenes* escape into the cytosol. From these results suggest the potential that in response to *L. monocytogenes* infection, CFTR transports chloride into the vacuole, enhancing LLO stability and activity, resulting in catastrophic rupture of the vacuolar membrane. *In vitro* data support this hypothesis, as LLO hemolytic activity increased upon exposure to high levels of sodium chloride. These results demonstrate an unanticipated role for CFTR and chloride transport in *L. monocytogenes* pathogenesis. Overall, these findings emphasize the importance of host homeostatic mechanisms in host-pathogen interactions.

Chapter 1

Introduction

1. Introduction to the biology of the thesis

This thesis examines interactions that occur between intracellular pathogens and host cells, and identifies regulatory factors that govern host compartmentalization during intracellular infection. The introduction reviews the mechanisms by which the intracellular pathogens *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), group A *Streptococcus* (GAS; *Streptococcus pyogenes*), and *Listeria monocytogenes* (*L. monocytogenes*) invade, traffic, and establish a replicative niche within a host cell to cause infection. Also addressed in the introduction is the host cell response to intracellular bacterial infection, highlighting the regulation of cellular homeostasis. The general concepts and mechanisms of homeostasis and its fundamental role in cellular physiology will be developed and defined further in the introduction.

2. Interactions of intracellular pathogens with the host cell

Intracellular pathogens are a diverse grouping of microorganisms, including bacteria, viruses, and parasites, that utilize mammalian cells as their replicative niche (Figure 1.1). Each type of pathogen has evolved mechanisms to invade the host cell, acquire and utilize host nutrients, replicate, and cause infection. In this thesis, the focus is solely on intracellular bacteria and their interactions with host cells.

2.1 Endocytic compartment

The endocytic compartment is a complex network of membrane vesicles that are continuously trafficked between the plasma membrane and the internal environment of the mammalian cell. Endocytosis plays a critical role in regulating neuronal, metabolic, and proliferative signals, nutrient uptake, interactions with the external environment, and mounting defenses against invading microorganisms (Mellman, 1996, Haas, 1998). The pathways of endocytosis are depicted in Figure 1.2; in short, plasma membrane derived vesicles can either take the form of phagosomes that engage in large particle (phagocytosis), fluid/small particle (pinocytosis), and receptor bound-ligand uptake, or become molecular sorting endocytic vesicles with targeted destinations (Mellman, 1996). In both cases, the newly formed vesicles fuse with early endosomes (EEs), and as a result of an ATP-driven proton pump, the vacuolar ATPase, EEs have a slightly acidic pH of ~6.0-6.8 (Yamashiro *et al.*, 1984, Mellman *et al.*, 1986). The vesicles then either recycle back to the plasma membrane in recycling vesicles (RV), or fuse with late endosomes (LEs) and lysosomes (Mellman, 1996). The LEs and lysosomes are the primary sites of

exogenous and endogenous macromolecule breakdown because of their acidic pH of ~5 and high concentration of degradative enzymes (Kornfeld *et al.*, 1989).

The four classes of endocytic organelles EEs, RVs, LEs, and lysosomes, can be distinguished by their cellular localization and pH, but are largely differentiated by their relative kinetics of labeling by endocytic markers (Mellman, 1996). The Ras superfamily of monomeric G proteins (rab proteins) dominate many of the endocytic compartments and regulate numerous steps of membrane trafficking and fusion (Van Der Sluijs *et al.*, 1991, Simons *et al.*, 1993). Vacuoles transiently acquire various Rab proteins, lysosomal glycosylated proteins (Lgps), and lysosome-associated membrane proteins (Lamps) as they traffic through the endocytic pathway, making these proteins useful markers for differentiating EEs from LEs and lysosomes (Nuoffer *et al.*, 1994, Pfeffer, 1994, Zacchi *et al.*, 1998).

2.2 Host and bacterial regulation of compartmentalization

2.2.1 Bacterial invasion of the host cell

Many pathogenic bacteria can enter and survive within a mammalian cell, often utilizing endocytic compartments to establish a replicative niche (Hultgren *et al.*, 1993, O'Riordan *et al.*, 2002). When an invasive bacterium enters a host cell, it uses adhesion molecules or invasins that bind to host cell surface structures. This interaction often dictates tissue specificity and host range of infection (Hultgren *et al.*, 1993, Finlay *et al.*, 1997a). Bacterial adherence triggers the host cell to directly or indirectly facilitate bacterial uptake by one of two mechanisms. The first mechanism is phagocytosis, or

engulfment of a solid particle or bacterium by a cell to form an internal phagosome. Phagocytosis is a host cell driven process that only occurs in specialized cells, such as macrophages or dendritic cells (Finlay *et al.*, 1997a, Haas, 1998). The second mode of entry is through bacterially induced endocytosis, occurring in both phagocytic and non-phagocytic cells. This type of bacterial entrance can occur by either a “zipper” type mechanism involving direct contact between a host cell receptor and bacterium (*L. monocytogenes* and *Yersinia*), or via a “trigger” type mechanism that relies on the secretion of bacterial proteins that causes host cell membrane ruffling, cytoskeletal rearrangement, and bacterial uptake (*Salmonella* and *Shigella*) (Finlay *et al.*, 1997a, Finlay *et al.*, 1997b, Rosenshine *et al.*, 1993, Bliska *et al.*, 1993) In either case, all intracellular bacteria initially enter a membrane-bound vacuole where the fate and character of the pathogen-containing vacuole is determined by both the pathogen and the host (Haas, 1998, O’Riordan *et al.*, 2002). Figure 1.1 displays the distinct replication compartments of different bacterial pathogens within the host cell. These bacteria can be split into two groups based on their intracellular localizations: those that stay within the vacuole (vacuolar bacteria), or those that escape the vacuole and enter into the host cytosol (cytosolic bacteria) (O’Riordan *et al.*, 2002).

2.2.2 Vacuolar bacterial pathogens

Vacuolar bacterial pathogens, such as *S. typhimurium* or *Mycobacterium tuberculosis* (*M. tuberculosis*), stay within a membrane bound vacuole throughout the entire duration of infection of the host cell. However, these and most other vacuolar pathogens, alter the vacuole and its internal environment to avoid fusion with lysosomes

and to establish a replicative environment or niche (Finlay *et al.*, 1997a). Differences in vacuolar pathogens arise based on the bacterial modifications and alterations to their vacuole (O'Riordan *et al.*, 2002, Steele-Mortimer *et al.*, 1999). The *S. typhimurium* and Group A *Streptococcus* (GAS) intracellular lifecycle will be described in further detail, as they are used as model vacuolar pathogens in this thesis.

Salmonella enterica is composed of over 2,000 highly genetically similar serovars that differ based on host range or host specificity and the disease they cause (Steele-Mortimer, 2008). Serovar Typhi causes severe systemic disease in approximately 21 million people/year worldwide, and because it is human-specific, it is an impractical tool to study *Salmonella* infection. However, serovar Typhimurium, which induces gastroenteritis in humans, causes typhoid-like systemic disease in susceptible mice, and is therefore often used as a laboratory model of *Salmonella* infection (Steele-Mortimer, 2008, Mastroeni *et al.*, 2004, Collins *et al.*, 1966).

S. typhimurium virulence is dependent on the ability of the bacterium to invade non-phagocytic host cells and to replicate within a modified endosomes or phagosome, termed the *Salmonella*-containing vacuole (SCV) (Figure 1.3) (Smith *et al.*, 2007, Steele-Mortimer, 2008). The *S. typhimurium* genome encodes two Type Three Secretion Systems (TTSS) or 'needles', that inject bacterial effector proteins from the bacterium into the host cell, mediating invasion and virulence (Lilic *et al.*, 2004, Stebbins *et al.*, 2001). *S. typhimurium* pathogenicity island-1 (SPI-1) encodes the invasion-associated TTSS needle (TTSS-1) that is induced prior to bacterial invasion, and its secreted effectors are essential in host cell membrane ruffling and *S. typhimurium* uptake. The dramatic membrane ruffling that occurs also allows for bystander particle uptake into the

host cell (Galan *et al.*, 2000, Patel *et al.*, 2005). Immediately after invasion, SCV acquires EE markers (EEA1 and Rab5), and by 10-20 minutes after infection these markers quickly are replaced with LAMP-1 (Steele-Mortimer *et al.*, 2002, Steele-Mortimer *et al.*, 1999). At this stage of infection, the SPI-1 genes become down-regulated, and SPI-2 genes involved with intracellular survival (i.e., avoiding fusion with lysosomes) and SCV biogenesis are induced (Steele-Mortimer *et al.*, 2002, Uchiya *et al.*, 1999). In the later stages of infection (>5hpi), *S. typhimurium* begins to replicate, and the SCV localizes near the Golgi where it is hypothesized to intercept trafficking of vesicles containing proteins and/or lipids to be used as bacterial nutrients (Salcedo *et al.*, 2003, Salcedo *et al.*, 2005).

The Gram-positive bacterium GAS is primarily an extracellular pathogen, but can invade and survive within vacuoles of non-phagocytic cells (Hakansson *et al.*, 2005). GAS is a human pathogen that causes a wide spectrum of infections, ranging from the clinically mild conditions pharyngitis ('strep throat') and impetigo, to more severe invasive diseases such as necrotizing fasciitis and streptococcal toxic shock syndrome (Tart *et al.*, 2007, Carapetis *et al.*, 2005). GAS possess a number of known and putative virulence factors such as capsule, toxins, proteases, and the cholesterol-dependent cytolysin (CDC) streptolysin O (SLO) that enable the bacterium to adhere and invade host cells, as well as evade host immune responses (Hakansson *et al.*, 2005, Tart *et al.*, 2007, Bisno *et al.*, 2003, Chhatwal *et al.*, 2005). The persistence of GAS in the population and its broad spectrum of resulting diseases are hypothesized to be due to intracellular GAS that are shielded from the effects of the immune response and antibiotics, resulting in a carrier state (Medina *et al.*, 2003, Tart *et al.*, 2007). The subset

of GAS that enter the host cell appear to secrete lower levels of SLO, and once in the cell, traffic through the host endocytic pathway (Hakansson *et al.*, 2005, Radtke *et al.*, 2007, Rohde *et al.*, 2003). The intracellular lifecycle of GAS only recently has become a subject of investigation in the microbial pathogenesis field, so relatively little is known about how GAS interacts with the host cell.

2.2.3 Cytosolic bacterial pathogens

Cytosolic bacterial pathogens, such as *L. monocytogenes* and *Shigella flexneri*, express multiple gene products that enable them to modulate and escape the vacuole (Portnoy *et al.*, 1988, Tilney *et al.*, 1989, Smith *et al.*, 1995, Barzu *et al.*, 1997). Once in the cytosol, these bacteria take advantage of this nutrient rich environment to replicate. Unlike the vacuole, the cytosol is devoid of reactive oxygen species and harsh acidic pH, making it an advantageous growth environment (Piez *et al.*, 1958). In the rare event that a vacuolar pathogen enters the cytosol of epithelial cells, it is also able to replicate robustly in this biologically favorable environment (Beuzon *et al.*, 2000, Radtke *et al.*, 2007, Brumell *et al.*, 2002). However, this robust cytosolic growth often results in lysis and death of the host cell, in turn potentially exposing them to extracellular immune defenses (Steele-Mortimer *et al.*, 1999).

Chapter 4 of this thesis investigates host mechanisms that contribute to vacuolar escape of the cytosolic pathogen *L. monocytogenes*, a ubiquitous gram-positive, facultative intra-cytosolic, food-borne pathogen with a broad host range. *L. monocytogenes* is the causative agent of listeriosis, a severe systemic illness affecting immunocompromised individuals, newborn babies, elderly or pregnant women (Schlech,

2000). The natural route of infection is through the gastrointestinal (GI) tract, infecting intestinal epithelial cells and disseminating to the bloodstream where the pathogen can encounter and infect macrophages, fibroblasts, in internal organs such as the liver and spleen (Gaillard *et al.*, 1991, Cossart, 2007). *L. monocytogenes* enters cells by either phagocytosis or the ‘zipper’ mechanism, and initially resides within a vacuole unable to replicate (Figure 1.2 and 1.4). Shortly after infection, *L. monocytogenes* secretes the CDC listeriolysin O (LLO) that forms ~25nm pores in the vacuolar membrane (Rossjohn *et al.*, 1997, Heuck *et al.*, 2003). Before membrane integrity is lost, the vacuolar pH acidifies to an average pH of 5.9, an optimal pH for LLO activity (Beauregard *et al.*, 1997, Glomski *et al.*, 2002). The LLO-dependent perforation must occur for *L. monocytogenes* to escape the vacuole, but events that regulate vacuole rupture are poorly understood (Shaughnessy *et al.*, 2006).

Once in the cytosol, the bacteria replicate robustly and nucleate host actin polymerization to form actin tails, a characteristic commonly used to measure entry of *L. monocytogenes* into the cytosol (Figure 1.4). These characteristic “comet” tails allow the bacterium to move throughout the cytosol, eventually forming filopodia-like membrane protrusions that invade neighboring cells (Portnoy *et al.*, 1988, Tilney *et al.*, 1989, Tilney *et al.*, 1990). These pseudopods are pinched off by neighboring cells forming a double membrane bound vacuole. LLO secretion results in the release of *L. monocytogenes* from the secondary vacuole; thus, the bacteria can spread cell-to-cell without being exposed to the extracellular environment (Tilney *et al.*, 1989, Portnoy *et al.*, 1988, Pizarro-Cerda *et al.*, 2006).

2.3 Host defenses against intracellular bacteria

During intracellular bacterial infection, the bacteria utilize host cellular components, processes, and compartments to assist in their survival within the host. Despite host manipulation and exploitation by bacterial pathogens, the cell is able to maintain general physiological function (Bhavsar *et al.*, 2007). Much of what is known about the host contribution during intracellular infection necessary for its survival is through such processes as immune signaling and regulation of the compartmentalization of the bacteria (Meresse *et al.*, 1999, Medzhitov *et al.*, 1999, Medzhitov *et al.*, 2000). However, the work presented in this thesis identifies mechanisms of cellular homeostasis as a critical means host cells utilize to control intracellular infection and maintain physiological function.

2.3.1 Immune signaling and cross-over pathways

The host's first line of defense against invading pathogens is the well-studied innate immune response. Neutrophils, macrophages, and dendritic cells are the major cell types involved in this response to destroy pathogens, and also serve to instruct the adaptive immune response through expression of cytokines, chemokines, and other pro-inflammatory molecules (Esche *et al.*, 2005, Ulevitch *et al.*, 2004, Clark *et al.*, 2005). Intracellular bacteria utilize a host cell as a protective shield to avoid such immune responses; however, the host cell also possesses various intrinsic defenses as well. Such defenses include Toll-like receptors (TLR) present on the plasma membrane that recognize microbial ligands upon the initial bacterial exposure (Takeda *et al.*, 2003, Girardin *et al.*, 2002a, Inohara *et al.*, 2003). Upon stimulation, TLR signaling pathways

can trigger NF κ B activation, in addition to the TANK-binding-kinase-1 (TBK1)-dependent pathway that induces transcription of Type 1 interferon (IFN) genes (Medzhitov *et al.*, 1998, Moynagh, 2005, Perry *et al.*, 2004). The importance of Type 1 (IFN) in anti-bacterial immunity has been well established, but the functional contribution of TBK1-dependent signaling was until recently unknown, and is the major focus of Chapters 2 and 3 (Perry *et al.*, 2005, Radtke *et al.*, 2007, Kopp *et al.*, 2003).

Recently, additional roles for TBK1, and other members of the IKK family, have been discovered that are independent of their well-established roles in the induction of genes involved with immunity and inflammation (Pomerantz *et al.*, 1999). TBK1 was found to be critical during embryonic development in protecting the liver from apoptosis (Bonnard *et al.*, 2000). Other IKK family members have also been associated with protection from apoptosis through their regulation of p53, an anti-apoptotic factor, as well as playing a role in the regulation of numerous genes important in tumor progression and metastasis (Yamaguchi *et al.*, 2007, Agarwal *et al.*, 2005). Additional functions identified for the kinase family members include regulation of smooth muscle adhesion molecules, embryonic skin development, extracellular matrix remodeling, and protection of hepatocytes and myeloid cells from developing insulin resistance (MacKenzie *et al.*, 2007, Liu *et al.*, 2006, Maeda *et al.*, 2007, Olivotto *et al.*, 2008, Arkan *et al.*, 2005). Lastly, the work of this thesis suggests a novel role for TBK1 in maintaining the integrity of pathogen-containing vacuoles (Radtke *et al.*, 2007).

2.3.2 Vacuolar compartmentalization

The role of vacuolar compartmentalization is of key importance in controlling bacterial infection as well, but the cell biology and physiology that regulate the dynamically changing PCV are not well understood (Medzhitov *et al.*, 2000, Meresse *et al.*, 1999). The work in this thesis helps to define the role of host compartmentalization to regulate infection. Previous work has shown that once inside the host cell, an invading pathogen is shielded from many immune effector functions such as neutrophil killing and complement lysis. However, the microorganisms face a formidable battery of cell-intrinsic defenses that occur in the endosomal or phagosomal compartments of the host cell (O'Riordan *et al.*, 2002). First, the physical barrier of the pathogen-containing vacuoles provides an enclosed space within which the host cell can direct potent antimicrobial function such as the oxidative burst. Host enzymes that mediate antibacterial activity might also compromise essential host cell functions if their activity were not confined to the phagosome. Second, the bacteria are contained within an environment that likely has low nutrient availability compared with the host cytosol. Lastly, containment of bacteria within the endocytic pathway facilitates MHC Class II antigen presentation by professional antigen presenting cells that stimulate adaptive immunity (Radtke *et al.*, 2006).

Bacterial compartmentalization by a host cell was a previously underappreciated area of host regulation of infection, and this work will illustrate its importance to the survival of both the host and the pathogen during infection. Compartmentalization is essential to the host by restricting and actively controlling the infection through the confinement of bacteria to a membrane-bounded space. Invasive pathogens, however, have evolved mechanisms to manipulate and utilize this vacuolar space to establish a

fruitful intracellular niche (O'Riordan *et al.*, 2002). The host cell does experience physiological insult and alterations as a result of infection that it must overcome in order to maintain general steady-state function, or cellular homeostasis (Roy *et al.*, 2004). As a result of bacterial components (*S. typhimurium* TTSS) or secreted proteins (GAS SLO), damage to the host plasma and vacuolar membranes occurs. The host cell has multiple response pathways and membrane repair mechanisms to restore membrane function and regain homeostatic balance (Roy *et al.*, 2004, Hakansson *et al.*, 2005, Fein *et al.*, 2005). During bacterial replication within the vacuole, bacteria also secrete metabolites and other by-products that can alter the vacuolar environment (van Baarlen *et al.*, 2007). Overall, intracellular bacteria alter the homeostasis of the host cell and vacuole compartment resulting in changes in pH, osmotic gradients, ion concentrations, and membrane integrity/potential (Yamanaka *et al.*, 1987, Madden *et al.*, 2001, Roy *et al.*, 2004, Shaughnessy *et al.*, 2006). Several homeostatic mechanisms that host cells deploy in response to such perturbations are discussed in the next section.

3. Cellular mechanisms of homeostasis

A mammalian cell is a complex of organelles, where each organelle type plays a distinct role to promote cellular function. Membranes protect the cell from fluctuations in the extracellular environment, but also organize and help define each organelles unique function (Alberts *et al.*, 1994). Cellular membranes are equipped with specific proteins and lipids that enable them to protect the host cell from external stresses, but also serve in cellular molecular transport, signal transduction, anchoring, energy generation,

metabolite synthesis, and homeostatic regulation (Dubyak, 2004, Romero, 2004). This section will discuss the role of membranes and associated proteins in the maintenance of cellular homeostasis and the consequences of perturbation.

3.1 Homeostatic processes

One major role of membranes is to actively maintain homeostasis of a cell, defined as a stable steady-state condition between two different environments. There are four major processes that utilize membranes to maintain cellular homeostasis, 1) ionic homeostasis, 2) generation of membrane potential, 3) volume homeostasis, and 4) pH homeostasis (Romero, 2004). My thesis reveals an important role for ionic homeostasis in host-pathogen interactions, thus is discussed in the greatest detail. However, it is likely that other homeostatic processes also contribute to physiological function of infected host cells.

3.1.1 Ionic homeostasis: channels and transporters

Homeostatic ion regulation refers to the maintenance of highly asymmetric concentrations of cations and anions in two different compartments separated by a membrane. Ionic homeostasis is critical for many functions in the cell, and intracellular ion concentrations are tightly controlled by diverse and specialized components within the membrane (Dubyak, 2004, Alberts *et al.*, 1994). Due to their charged nature, the movement of ions across biological membranes must be facilitated by membrane transport proteins. Ion transport proteins are broadly divided into two classes, channels and transporters (Dubyak, 2004).

Channels

Channels are proteins specifically arranged in the membrane to form pores that permit the regulated movement of ions from one side of the membrane to the other. Extrinsic factors, such as alterations in membrane potential or binding of regulatory molecules, dictate if a channel is in an open or “gated” state allowing ion transport, or in a closed state, incapable of ion movement (Dubyak, 2004). Transmembrane flux of an ion is determined by the electrochemical gradient, the difference in the concentration of ions on each side of the membrane (Fujiyoshi *et al.*, 2002). All channels mediate passive transport, or movement of ions down their respective chemical or electrochemical gradient (Dubyak, 2004).

Aquaporins (AQPs) are examples of membrane channels with a central role in cellular homeostasis through their ability to increase the osmotic permeability of membranes (Agre, 2006). AQPs assemble in the membrane as a tetramer of monomeric units, with each monomeric unit functioning independently as a pore (Reizer *et al.*, 1993). Steric hindrance caused by the pore’s narrow diameter and a fixed positive charge, provides channel specificity and prevents the transport of protons and other small molecules (Hill *et al.*, 2004, Agre, 2006).

There are currently 13 identified members of the AQPs family in mammalian cells that are divided into two groups based on their permeability characteristics and sequence homology. The first group primarily transports water, where the second group, termed aquaglyceroporins, transports water and glycerol (King *et al.*, 2004, Agre *et al.*, 2002, Tajkhorshid *et al.*, 2002). Across the AQP family, differences occur in transcriptional regulation, post-translational modifications, and regulation of activity, where a change in pH, exposure to nitrate, nickel, and cGMP, or phosphorylation, control

the channel's transport capabilities (King *et al.*, 2004). Localization and tissue distribution also differ among AQP family members, potentially reflecting the diverse requirements for cell and organ-specific regulation of water homeostasis (King *et al.*, 2004, Verkman, 2005).

Although the AQPs constitutively reside in the plasma membrane in most cells, the presence of AQPs in secretory granules and vesicles has also been demonstrated (Sugiya *et al.*, 2006). With the ongoing discovery of new AQPs, together with the expression and localization of AQPs in numerous tissues, the list of their physiological and cellular functions is growing. Aside from their role in water and ion transport, AQPs are also thought to contribute to tissue swelling, cell migration, fat metabolism, epidermal biology and neural signal transduction, and may also be important in the pathophysiology of cancer, obesity, immune cell dysfunction and epilepsy (Verkman, 2005).

Observed recently by others, and in the work described in this thesis, AQP channel activity has been found to play a role during infection by invading microorganisms. During *Cryptosporidium parvum* infection, AQP1 was shown to be recruited and assist in parasitic invasion of a host cell (Chen *et al.*, 2005). AQP1 was also found on *Plasmodium falciparum*-containing vacuoles with the predicted function of regulating the environment of the vacuole (Murphy *et al.*, 2004). Upon acute infection of the lungs by adenovirus, AQP1 and 5 expression was downregulated, resulting in abnormal fluid flux and pulmonary inflammation (Towne *et al.*, 2000). During a *Citrobacter rodentium* infection, bacterially secreted effector proteins caused AQP2 and 3 mislocalization, resulting in diarrhoea-like symptoms in mice, whereas AQP4 was found to contribute to brain edema as a result of *Streptococcus pneumoniae* infection of

the cerebrospinal fluid (Guttman *et al.*, 2006, Bloch *et al.*, 2005, Papadopoulos *et al.*, 2005). Lastly, AQP1 was found to accumulate and disrupt cell volume homeostasis upon exposure to *Staphylococcus aureus* alpha-toxin, and as identified in this thesis, has a major function in the regulation of the homeostasis of pathogen-containing vacuoles (Radtke *et al.*, 2008, Schweitzer *et al.*, 2008).

Transporters

Transporters are membrane proteins that are relatively selective for their distinct ions. Upon recognition and binding of the ions, transporters undergo a conformational change that results in the movement of ions across the membrane bilayer. Unlike channels where ion movement and direction is dictated by the electrochemical gradient, transporters actively transport ions against their electrochemical gradients (Alberts *et al.*, 1994, Majumdar *et al.*, 2004). Cellular energy is required for this to occur, and primary active transporters directly couple ion movement to the binding and hydrolysis of ATP, the main energy storage and transfer molecule in the cell. Secondary active transporters such as cotransporters and exchange transporters, couple the movement of one ion against its electrochemical gradient to the movement of another ion down its electrochemical gradient (Alberts *et al.*, 1994, DUBYAK, 2004).

Cystic fibrosis transmembrane conductance regulator (CFTR) transporter is an example of a well studied transporter and also a major player of vacuolar homeostasis are discussed in Chapter 4. CFTR is a multidomain integral membrane glycoprotein and a member of the adenine nucleotide-binding cassette (ABC) transporter family (Li *et al.*, 1996). Its most well characterized function is to maintain ion and fluid homeostasis in the apical membrane of epithelial cells through transport of Cl⁻ ions (Norez *et al.*, 2004,

Gabriel *et al.*, 1993). CFTR mediated Cl⁻ transport can also be induced through phosphorylation by protein kinase A (PKA) resulting in a conformational change of the channel (Chappe *et al.*, 2005).

Cystic fibrosis (CF) is the diagnosed genetic disease that occurs in an individual with deleterious mutations in CFTR. A defect in CFTR function results in failure of ionic and water homeostasis, producing an accumulation of macromolecular secretion in the lung. The viscous mucus that collects causes an increase in microorganism colonization, triggering an inflammatory response that results in severe lung damage. All individuals with CF have mutations in both *cftr* alleles, and ~90% of these cases have a deletion in the F508 codon (Riordan, 2008). Δ F508 CFTR protein is synthesized and glycosylated with only a minor effect on protein structure, but is degraded before it can be transported to the plasma membrane (Lewis *et al.*, 2005, Cheng *et al.*, 1990).

As is apparent from the consequences of the Δ F508 CFTR mutation, appropriate trafficking of the protein is critical to its function in regulating cellular ion homeostasis. The intracellular localization and trafficking of CFTR is described in Figure 1.5 (Bertrand *et al.*, 2003). It has been observed that only ~33% of wildtype CFTR protein is successfully trafficked to the plasma membrane, compared to other ABC transporters exported at 100% efficiency (Riordan, 2008). A substantial percentage of mature functional CFTR is localized within intracellular compartments (Bertrand *et al.*, 2003). Low surface expression combined with CFTR rapid internalization and degradation (10% per minute) could explain its unusually low natural abundance in cells (Lukacs *et al.*, 1997, Loo *et al.*, 1998, Prince *et al.*, 1994). CFTR has also been proposed to regulate other chloride channels, acidify phagosomes in alveolar macrophages, act as a receptor

for *Pseudomonas aeruginosa* internalization, and stimulate cytokine secretion (Gabriel *et al.*, 1993, Pier *et al.*, 1996, Kowalski *et al.*, 2007, Haggie *et al.*, 2007). The multiple locations of CFTR in the cell could help account for the different functions of the transporter based on cellular localization.

3.1.2 Generation of membrane potential

Membrane potential (V_m) is the voltage produced across a membrane when differentially charged environments are separated by a membrane. Gradients of electrical charge are in every cell due to the critical functions they influence, such as the transport of nutrients, movement of salt and water, signaling processes involved in cell movement, and all cognitive processes (Romero, 2004, Wright, 2004). The formation of electrical gradients between membranes arise as a consequence of a Na^+ - K^+ -ATPase ion pump and other Na^+ and K^+ selective channels maintaining a high cytosolic K^+ concentration with a resulting high extracellular and vacuolar Na^+ concentration (Bezanilla, 2005).

Membranes have restricted permeability to all the major inorganic ions, and only K^+ , Na^+ , and Cl^- have an effect on bioelectric processes, with K^+ having the most sizeable effects. Cl^- is often transported as a counter-ion to ‘stabilize’ the resting membrane potential after stimulation, or to restore electrochemical homeostasis (Wright, 2004).

3.1.3 Volume homeostasis

All cells face constant challenges to their volume as a result of changes in their intracellular or extracellular environment solute/solvent concentrations. Under steady-

state conditions, intracellular solute levels are held constant by a balance between solute influx and efflux across a plasma membrane (Haynie, 2001). Upon changes in intracellular or extracellular solute content, an osmotic gradient is generated, resulting in the immediate flow of water into or out of the cells through simple diffusion. Since mammalian cell membranes are unable to generate the resistance needed to stop water flow, cellular swelling or shrinkage occurs (Haynie, 2001, Strange, 2004). To bring a cell back to its original volume after shrinkage/swelling, volume decrease usually occurs through loss of KCl from the cytosol, where increase of cytosolic KCl and NaCl levels results in a volume increase (Strange, 2004). In either case, activation of these ion transport pathways is rapid and extremely sensitive, occurring within seconds after perturbation of a volume change as low as 3% (Sardini *et al.*, 2003, Lang *et al.*, 1998).

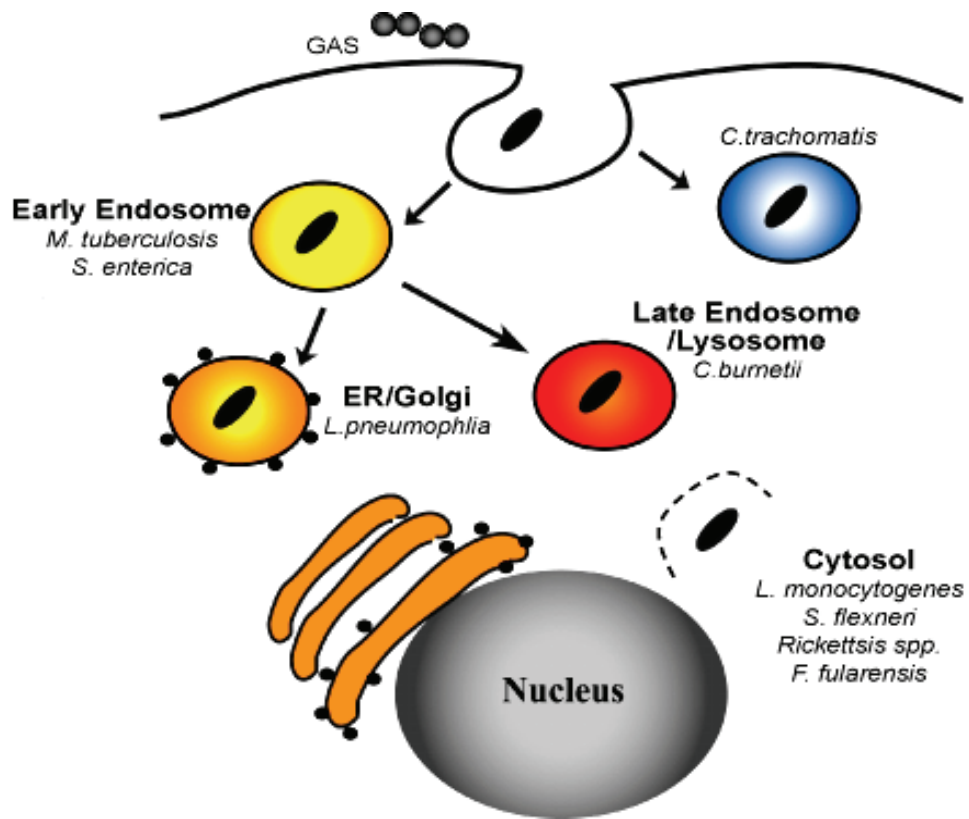
3.1.4 pH homeostasis

pH regulation is necessary in the maintenance of cellular homeostasis in all living cells. Intracellular pH (pH_i) plays a role in many critical cellular steady-state processes ranging from macromolecular transport to intracellular signal transduction for the biological processes of cell morphogenesis, growth, and differentiation (Cosson *et al.*, 1989, Mahnensmith *et al.*, 1985, Heuser, 1989). The pH_i tends to be lower than extracellular pH (pH_o), and, upon acute changes in pH_i , the cell elicits such mechanisms as hydrogen consumption by alternative metabolic pathways and intracellular sequestration by organelles to maintain function (Roos *et al.*, 1981). However these means of regulation are transient, and for long-term or steady-state regulation of pH, a cell utilizes membrane acid-base and ion transport proteins (Lubman *et al.*, 1992).

pH-modulating transporters are ubiquitously expressed in the cell, with the majority found in the plasma membrane regulating cytosolic pH. However, H⁺-ATPase or vacuolar ATPase (vATPase) transporters are present on intracellular organelle membranes to regulate intracompartamental pH (Boron, 1986, Mellman *et al.*, 1986). The vATPase is often a key player in regulating the vacuolar environment of pathogen-containing vacuoles (Haas, 1998, Bidani *et al.*, 2000). vATPase-dependent vacuolar acidification was observed to be necessary for survival and replication of *S. typhimurium* within the macrophage, and is also utilized by *L. monocytogenes* to assist in vacuolar escape (Beauregard *et al.*, 1997, Rathman *et al.*, 1996). Acidification progressively increases from endosomes to lysosomes in the endocytic pathway. These compartmental pH differences are proposed to be due to differing expression and activity levels of transporters, and through a balance between active proton pumping and passive H⁺ efflux or “leak” of the organelle’s membrane (Demaurex, 2002, Mellman *et al.*, 1986).

4. Conclusion

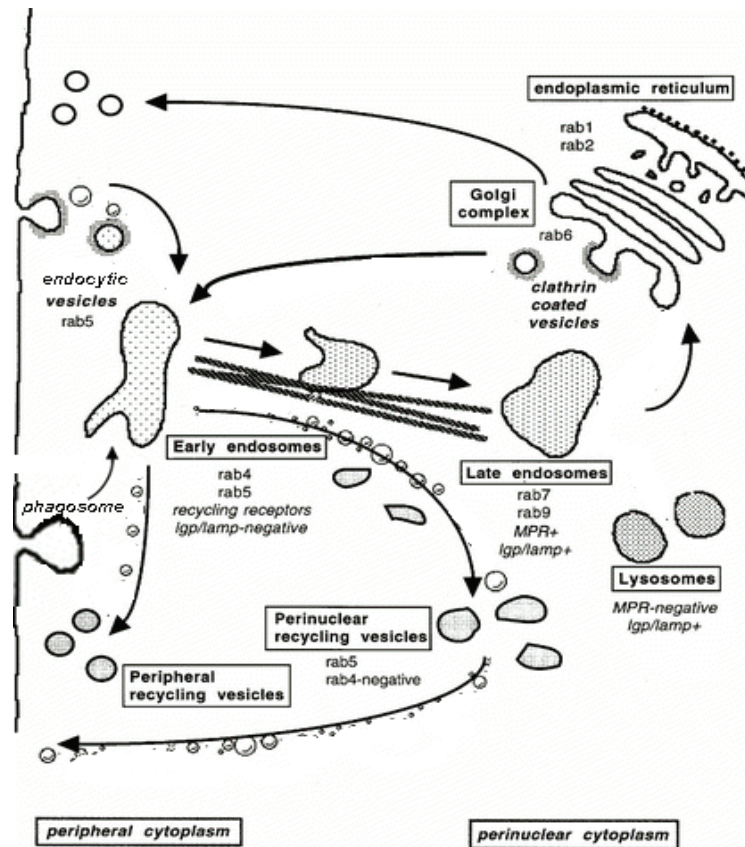
The work presented in this thesis highlights the dynamic interactions that occur between a mammalian host cell and intracellular bacteria during infection. Most of what is understood about this relationship, and host cell regulation of infection, is from mechanisms involving immune signaling pathways and the induction of antimicrobial genes (Medzhitov, 2007, Girardin *et al.*, 2002b). However, an additional and less understood mechanism of homeostatic regulation as means of controlling infection is identified in this thesis. Aside from the previously characterized function of vacuolar membranes compartmentalizing antimicrobial regents to restrict bacterial growth, I have found that the host regulates ionic homeostasis of the vacuole to compartmentalize and constrain infection as well (Karupiah *et al.*, 2000, O'Riordan *et al.*, 2002). This thesis focuses on an under explored aspect of cellular homeostasis during intracellular infection, and provides a potential mechanism by which intracellular pathogens could establish their replicative niche.



(modified from O’Riordan, M.X.D., *et. al.* Trends in Microbiology. 10:361-364)

Figure 1.1 Intracellular niches of bacterial pathogens.

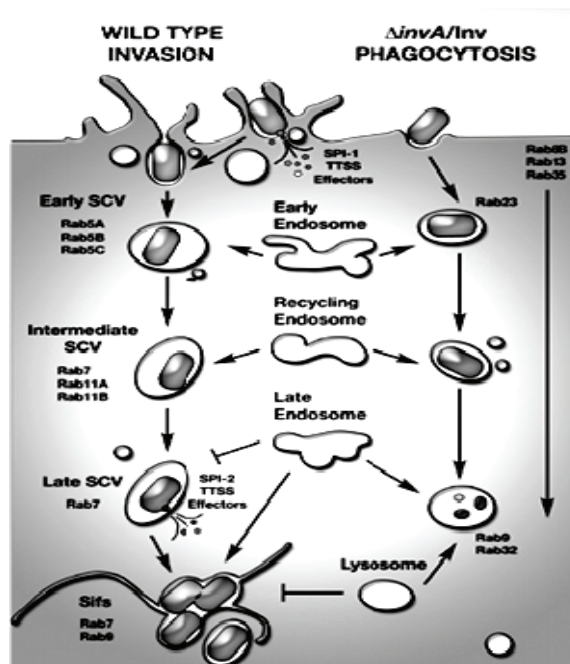
Intracellular bacteria can enter a host cell either through invasion or phagocytosis. Once inside the cell, bacteria can localize to various compartments of the cell such as endosomes (*S. typhimurium enterica* and GAS), lysosomes (*Coxilla burnetii*), the cytosol (*Listeria monocytogenes*), or establish a specialized vacuole (*Mycobacterium tuberculosis*).



(modified: Mellman, I. 1996. *Ann. Rev. Cell Dev. Bio.*)

Figure 1.2 Organization of the endocytic pathway in mammalian cells.

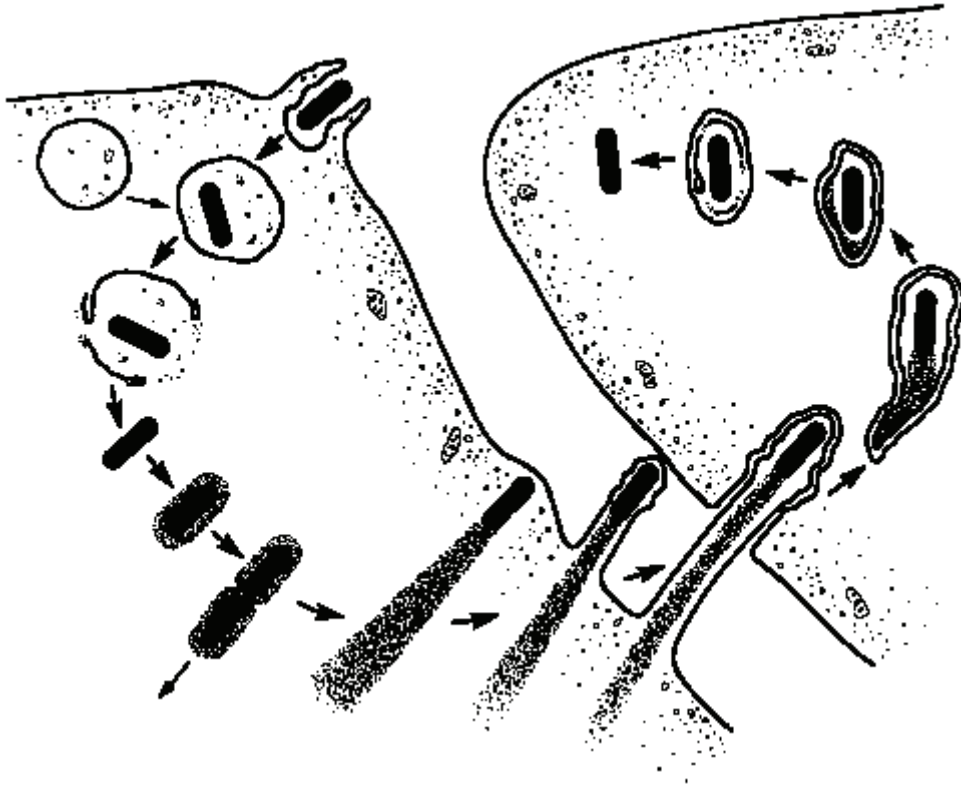
Endocytosis begins with membrane invaginations from the plasma membrane that form either endocytic vesicles or phagosomes. The newly formed vesicles are sorted in the peripheral cytoplasm where they begin to acquire host endocytic markers and fuse with early endosomes. The vesicles continue to traffic to the perinuclear cytoplasm to either fuse with late endosomes and lysosomes, recycle back to the plasma membrane, or acquire membrane components from the ER/Golgi complex.



(Smith A.C., et. al. 2007. J. of Cell Biology, vol. 176)

Figure 1.3 Intracellular lifecycle of *S. typhimurium*.

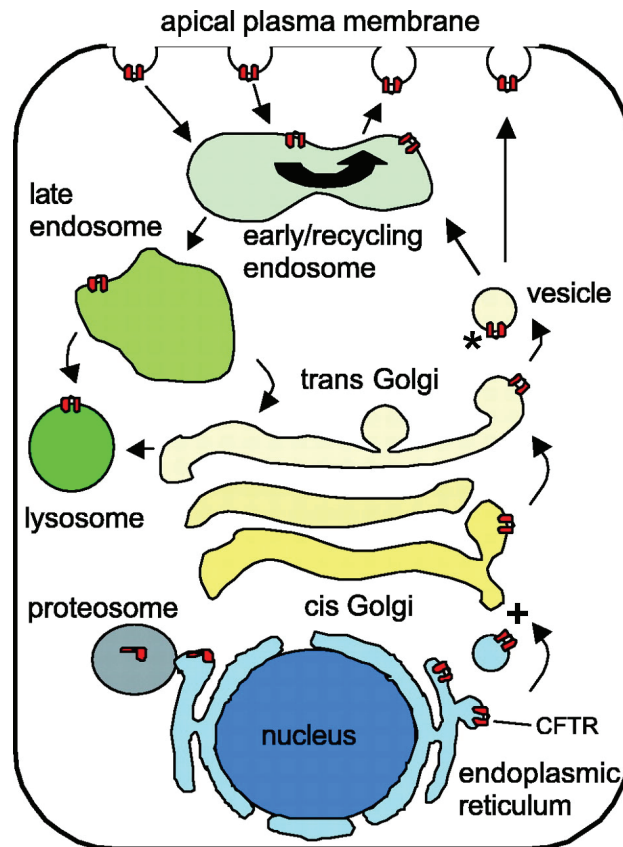
S. typhimurium invades non-phagocytic cells by inducing membrane ruffling through the secretion of TTSS-SP1 effector proteins. The bacterium is internalized into a specialized vacuole or SCV, and begins to acquire host endocytic markers that assist in intracellular trafficking. In the late SCV, *S. typhimurium* TTSS-SP2 injects effectors into the cytosol to establish its replicative niche and avoid fusion with lysosomes.



(Tilney, LG and DA Portnoy. 1998. *J. of Cell Biology*, vol. 109).

Figure 1.4 Intracellular lifecycle of *Listeria monocytogenes*.

L. monocytogenes invades target cells either through phagocytosis or by mediating its own entry through the secretion of invasion proteins. Shortly after infection, the bacterium secretes the hemolysin LLO and two other phospholipases to mediate its escape from the vacuole. Once in the cytosol, *L. monocytogenes* utilizes host actin machinery to form actin tails, propelling itself through the cytosol and into neighboring cells. Escape of *L. monocytogenes* from the secondary vacuole also requires LLO and the two phospholipases.



(modified: Bertrand CA and RA Frizzell. 2003. Am J Physiol Cell Physiol.)

Figure 1.5 Intracellular location of CFTR during maturation, plasma membrane insertion, and degradation.

(+) Properly folded immature CFTR leaves the endoplasmic reticulum (ER) and traffics through the Golgi, where it becomes glycosylated to form the mature CFTR protein (*). Mature CFTR leaves the Golgi in vesicles that either translocate to the apical surface of the plasma membrane or fuse with the recycling endosome (RE). CFTR-containing vesicles continuously traffic between RE and plasma membrane, where they eventually localize to lysosomes for degradation. Misfolded CFTR mutants and wildtype nascent CFTR are degraded by the proteasome.

Chapter 2

TBK1 Protects Vacuolar Integrity during Intracellular Bacterial Infection

1. Abstract

TANK-binding-kinase-1 (TBK1) is an integral component of Type I interferon induction by microbial infection. The importance of TBK1 and Type I interferon in anti-viral immunity is well established, but the function of TBK1 in bacterial infection is unclear. Upon infection of murine embryonic fibroblasts (MEFs) with *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), more extensive bacterial proliferation was observed in *tbk1*^{-/-} than *tbk1*^{+/+} cells. TBK1 kinase activity was required for restriction of bacterial infection, but neither IRF3 nor Type I interferon contributed to this TBK1-dependent function. In *tbk1*^{-/-} cells, *S. typhimurium*, EPEC and *Streptococcus pyogenes* escaped from vacuoles into the cytosol where replication occurred, suggesting that TBK1 regulates the integrity of pathogen-containing vacuoles. Knockdown of *tbk1*

in macrophages and epithelial cells also resulted in increased bacterial localization in the cytosol, indicating that the role of TBK1 in maintaining vacuolar integrity is relevant in different cell types. Taken together, these data demonstrate a requirement for TBK1 in control of bacterial infection distinct from its established role in anti-viral immunity.

2. Introduction

Host organisms employ a multitude of innate defense mechanisms against invading microbial pathogens. Functions of innate immunity include control and destruction of pathogens, and instruction of the developing adaptive immune response through expression of cytokines, chemokines and other proinflammatory molecules (Clark *et al.*, 2005, Esche *et al.*, 2005). Cellular intrinsic defenses against invading pathogens also occur through immune signaling pathways, but can also occur by the less understood mechanism of vacuolar compartmentalization (Kopp *et al.*, 2003, Meresse *et al.*, 1999). Immune signaling can occur through Toll-like receptors (TLRs), which bind to molecules characteristic of microbial organisms like lipopolysaccharide (LPS) (Kopp *et al.*, 2003). Two well characterized signaling pathways associated with TLR stimulation are the MyD88-dependent pathway which primarily results in NF κ B activation, and the TBK1-dependent pathway that induces transcription of Type I interferon genes (Moynagh, 2005). The importance of the MyD88-dependent pathway in anti-bacterial immunity is well established, but the functional contribution of TBK1-dependent signaling in protecting against bacterial infection is unknown (Kopp *et al.*, 2003).

In addition to immune signaling pathways in the protection of a host cell from invading bacteria, microorganisms also face a formidable battery of cell-intrinsic defenses that occur in the endosomal or phagosomal compartments of the host cell (O'Riordan *et al.*, 2002). Pathogen-containing vacuoles provide an enclosed space within which the host cell can direct potent antimicrobial function such as the oxidative burst

(Karupiah *et al.*, 2000). Bacteria localized within in a vacuole are also restricted to a space that likely has low nutrient availability compared with the host cytosol, making it a less replicative environment (Jansen *et al.*, 2006, Radtke *et al.*, 2006). Lastly, containment of bacteria within the endocytic pathway facilitates MHC Class II antigen presentation by professional antigen presenting cells that stimulate adaptive immunity (Sette *et al.*, 1989, Sercarz *et al.*, 2003). In the work presented here, the importance of bacterial compartmentalization can be observed with the loss of vacuolar membrane integrity having damaging effects on the host cell.

To investigate the requirement for TBK1 in response to bacterial infection, we used *Salmonella enterica* serovar Typhimurium as a model Gram-negative bacterium. *S. typhimurium* is a facultative intracellular pathogen that replicates within macrophages and non-phagocytic cells (Wick, 2004, Schlumberger *et al.*, 2006). *S. typhimurium* invades non-phagocytic cells by secreting effector proteins through a syringe-like Type III secretion system (T3SS) encoded on *S. typhimurium* pathogenicity island-1 (SPI-1) to induce membrane ruffling and bacterial uptake (Lostroh *et al.*, 2001). After entry into the host cell, *S. typhimurium* resides in a membrane-bound compartment termed the *Salmonella*-containing vacuole (SCV). Subsequent to invasion, the SCV progresses through early stages of endocytic maturation and acquires the late endosomal marker, lysosomal associated membrane protein-1 (LAMP-1) (Salcedo *et al.*, 2005). At this stage, the SCV can diverge from the endocytic pathway by avoiding lysosome fusion, and localizes to the perinuclear region (Salcedo *et al.*, 2003). While many *S. typhimurium* remain in the SCV and replicate, a small percentage of bacteria escape into the host cytosol where they acquire host-derived ubiquitin (Perrin *et al.*, 2004).

Like viruses, intracellular bacterial pathogens exploit the host cell as a replicative niche. By analogy to known anti-viral immune mechanisms, we hypothesized that TBK1 would also protect host cells from infection by intracellular bacterial pathogens. However, specific effector mechanisms of anti-viral innate immunity, such as Type I interferon-dependent induction of 2-5' oligoadenylate synthase, which facilitates viral RNA degradation, would not necessarily be effective against intracellular bacteria (Samuel, 2001). Since loss of TBK1 results in embryonic lethality, we used *tbk1*^{+/+} and *tbk1*^{-/-} mouse embryonic fibroblasts (MEFs) or RNAi to test the requirement for TBK1 during bacterial infection. Here we show that TBK1 mediates an early cellular response to infection by *S. typhimurium* and other bacteria by maintaining these pathogens in a restrictive vacuolar compartment.

3. Results

3.1 TBK1 restricts intracellular infection by *S. typhimurium*.

To investigate the role of TBK1 in the cellular response to bacterial infection, we infected *tbk1*^{+/+} and *tbk1*^{-/-} MEFs with *S. typhimurium* expressing green fluorescent protein (GFP). *S. typhimurium* invaded the wildtype and mutant MEFs similarly; however, at 8h p.i., *tbk1*^{-/-} MEFs contained approximately ten-fold more bacteria (Figure 2.1A and B). Robust bacterial proliferation was observed in 35-40% of infected *tbk1*^{-/-} MEFs which was consistent with immunofluorescence analysis of individual cells showing a more pronounced phenotype than that observed by measuring net increase in bacterial numbers. By immunofluorescence analysis, the remainder of the *tbk1*^{-/-} infected

MEFs appeared similar to *tbk1*^{+/+} infected MEFs (Figure 2.1A; 40X magnification). Infected *tbk1*^{-/-} MEFs that did not exhibit greater bacterial proliferation might have undergone an unproductive infection, since at 1h p.i., 20-30% of the bacteria in either wildtype or mutant MEFs were found in autophagosomes, and an additional 25-30% in lysosomes, which are likely non-replicative compartments (Figure 2.2 A and B). The increased bacterial growth observed in *tbk1*^{-/-} MEFs, hereafter referred to as hyperproliferation, was not suppressed by addition of exogenous Type 1 interferon, nor was a similar phenotype observed when *S. typhimurium* infection was compared in *irf3*^{+/+} and *irf3*^{-/-} MEFs (Figure 2.1B, C and Figure 2.3A). Inhibition of *de novo* transcription and translation also had no effect on the phenotype (Figure 2.3B and C; data not shown). However, the hyperproliferative phenotype was substantially decreased by transient transfection with a plasmid expressing wildtype TBK1, but not a kinase dead mutant protein (TBK1 KD) (Pomerantz *et al.*, 1999) (Figure 2.1D). Together, these data indicate that TBK1 kinase activity limits intracellular infection of *S. typhimurium* independently of the IRF3-Type I IFN axis.

3.2 *S. typhimurium* escapes from the endocytic pathway in the absence of TBK1

Previous studies have shown that shortly after invasion, SCV co-localize with the late endosomal marker, LAMP-1 (Salcedo *et al.*, 2003). To determine the nature of the *Salmonella*-containing compartment in TBK1-deficient cells, we infected MEFs with *Salmonella*-GFP and analyzed the samples by confocal immunofluorescence microscopy using an anti-LAMP1 antibody (Figure 2.4A and Figure 2.5). SCV in *tbk1*^{+/+} cells were co-localized with LAMP-1 throughout the entire course of infection, with 94.5% co-

localization at 2 h p.i. In contrast, as early as 90 minutes p.i. in *tbk1*^{-/-} cells, individual *S. typhimurium* lost association with LAMP-1, and at 2 h p.i., only 63.0% of bacteria exhibited co-localization. Loss of LAMP-1 co-localization by individual bacteria was commonly observed early in infection, suggesting that replication per se was not required for this abnormal phenotype. Thus, in the absence of TBK1, many SCV lose the late endosomal marker, LAMP-1 and deviate from the characterized *S. typhimurium* endocytic trafficking pathway.

Because *S. typhimurium* in TBK1-deficient cells were found in a LAMP-1 negative compartment dispersed throughout infected cells, we reasoned that the bacteria might be in the host cytosol. It was recently reported that under circumstances when *S. typhimurium* was found in the cytosol, the bacteria associated with host ubiquitin (Perrin *et al.*, 2004). Therefore, we infected MEFs with *Salmonella*-GFP and analyzed the cells by confocal immunofluorescence microscopy to determine whether bacteria were co-localized with ubiquitin (Figure 2.4B and Figure 2.6B). In *tbk1*^{+/+} cells, 0.7% of *S. typhimurium* co-localized with ubiquitin at 4h p.i.; almost all of the bacteria remained in LAMP1⁺ SCV during the course of infection. In contrast, substantial numbers of *S. typhimurium* in *tbk1*^{-/-} MEFs associated with ubiquitin (37.1% by 4h p.i.), suggesting that *S. typhimurium* was released from the SCV into the cytosol. However, the possibility remained that the SCV was perforated; allowing access to cytosolic ubiquitin, but the vacuolar membrane remained around each bacterium. We directly visualized *S. typhimurium* in infected MEFs by transmission electron microscopy, and found that by 1h p.i. in *tbk1*^{-/-} cells, 66.7% of bacteria (n=30) were surrounded by vacuolar space, compared to 87.5% of bacteria (n=24) in *tbk1*^{+/+} cells (Figure 2.4C). From these data, it

was not clear whether TBK1 regulated general integrity of the endocytic pathway, or whether infection specifically triggered a TBK1-dependent process. To test the general function of the endocytic compartment, we measured internalization and degradation of I¹²⁵ labeled epidermal growth factor (EGF) in the absence of infection (Figure 2.4D). We would predict based on earlier studies that general loss of integrity of the endocytic pathway would affect luminal pH and therefore the ability to degrade proteins taken up by endocytosis, such as EGF (Yoshimori *et al.*, 1991). Endocytic uptake and processing of the radiolabeled EGF appeared similar in both *tbk1*^{+/+} and *tbk1*^{-/-} MEFs, demonstrating that escape of *S. typhimurium* into the cytosol of TBK1-deficient cells was not the result of general destabilization of the endocytic compartment. Therefore, TBK1 controls an early response to *S. typhimurium* infection that maintains integrity of the pathogen-containing vacuole.

3.3 TBK1 modulates vacuolar integrity in response to Gram-negative and Gram positive bacterial invasion

Since known mediators of TBK1-dependent signaling were not required to suppress intracellular bacterial growth and endocytic function was not generally compromised, we hypothesized that *S. typhimurium* might be triggering a cellular process that requires TBK1. *S. typhimurium* contains two Type III secretion systems encoded on SPI-1 (termed SPI-1 T3SS) and SPI-2 (SPI-2 T3SS) that enable the bacterium to secrete proteins directly into the host cell cytosol (Lostroh *et al.*, 2001, Kuhle *et al.*, 2004). The SPI-1 T3SS is required for entry into non-phagocytic cells and modulation of endosomal trafficking; later in infection, SPI-2 T3SS-dependent effectors act to regulate membrane

dynamics. We tested the possibility that Type III secretion might contribute to triggering the phenotype observed in TBK1-deficient MEFs. *S. typhimurium* strains deficient in either the SPI-1 (*St* SPI-1⁻) or SPI-2 (*St* SPI-2⁻) encoded T3SS were assessed for their ability to replicate within MEFs and access the host cytosol (Figure 2.6A and B). The SPI-2 deficient bacteria proliferated similarly to wildtype *S. typhimurium* in both *tbk1*^{+/+} and *tbk1*^{-/-} MEFs. We also analyzed a *S. typhimurium* mutant lacking the SPI-2 dependent effector SifA, which exhibits defects in SCV integrity (Beuzon *et al.*, 2000, Brumell *et al.*, 2002, Boucrot *et al.*, 2005). If SifA and TBK1 were acting in concert, we would expect *St*Δ*sifA* to proliferate equally in *tbk1*^{+/+} and *tbk1*^{-/-} cells; however, we still observed increased cytosolic localization and replication by the mutant bacteria in *tbk1*^{-/-} MEFs (Figure 2.7 A and B). In contrast, the SPI-1 deficient bacteria (tet^R), induced to enter independent vacuoles through bystander infection with wildtype *S. typhimurium* (tet^S) as measured by tet^R colony-forming units or immunofluorescence, were unable to replicate in host cells of either genotype, and were never released in the cytosol (Figure 2.6A and B, Figure 2.8) (Steele-Mortimer *et al.*, 2002). A double SPI-1⁻SPI-2⁻ mutant behaved similarly to the SPI-1- single mutant (data not shown). These data suggest that a function associated with the *S. typhimurium* SPI-1 T3SS stimulates TBK1-dependent modulation of the pathogen containing vacuole.

To determine whether vacuolar escape in *tbk1*^{-/-} cells required a *S. typhimurium* specific process, we used an invasive strain of enteropathogenic *E. coli* (EPEC), a related Gram-negative bacterium, to infect MEFs and determined subcellular localization by quantitating co-localization with LAMP-1 (Figure 2.6C)(Miliotis *et al.*, 1989). LAMP-1 co-localization of EPEC was substantially decreased in *tbk1*^{-/-} cells, similar to the

phenotype we previously observed in *S. typhimurium* infection, and increased bacterial replication was observed compared to *tbk1*^{+/+} MEFs (Figure 2.6C and Figure 2.7C). We also investigated subcellular localization of *Streptococcus pyogenes*, an invasive Gram-positive bacterium, and found reproducibly that the streptococci were found less often in a LAMP-1⁺ compartment in TBK1 deficient cells, although the difference between *tbk1*^{-/-} and *tbk1*^{+/+} MEFs was not as striking as that observed for Gram-negative bacteria (Figure 2.6C). In addition, transmission electron microscopy revealed that at 1h p.i., fewer *S. pyogenes* were contained in vacuoles in *tbk1*^{-/-} MEFs (50%; n=30) than in *tbk1*^{+/+} MEFs (91.5%; n=47) (Figure 2.6D). These results demonstrate that TBK1 is necessary for restricting Gram-negative bacteria to the endocytic compartment during infection, and may also play a similar role during cellular invasion by Gram-positive bacteria.

3.4 TBK1 prevents bacterial entry into the cytosol of epithelial cells and macrophages

MEFs represent an amenable genetic system with which to test the role of TBK1 during bacterial infection in the absence of a gene-deficient live animal model; however, they are not a cell type that would be present during a physiological infection. To establish that loss of TBK1 caused a specific defect in response to bacterial infection, we used an RNAi approach to knock down TBK1 expression in HeLa cells, an epithelial cell line commonly used to study *S. typhimurium* pathogenesis, and used the treated cells to examine bacterial growth and compartmentalization. HeLa cells were transfected with *tbk1* siRNA, *gapdh* siRNA, or a non-specific siRNA control for 72h prior to infection; knockdown of TBK1 and GAPDH was confirmed by immunoblot analysis (Figure 2.9A;

data not shown). HeLa cells treated with *tbk1* siRNA supported increased replication of *S. typhimurium* compared to control treated cells, as observed in *tbk1*^{-/-} MEFs (Figure 2.9A). Consistent with our previous observations in MEFs, 27.8% of bacteria in *tbk1* siRNA treated HeLa cells were associated with ubiquitin compared to 2.2% of bacteria in cells transfected with control siRNA (Figure 2.9B and C). No significant increase in ubiquitin-associated bacteria was observed with knockdown of GAPDH. IRF3 was also knocked down by siRNA in HeLa cells; the validated knockdown had no effect on *S. typhimurium* growth or ubiquitin association (data not shown). At 4h p.i., HeLa cells transfected with *tbk1* siRNA had significantly lower numbers of either *S. typhimurium*, EPEC or *S. pyogenes* associated with LAMP-1 compared to the control transfection (Figure 2.9D). These data demonstrate that TBK1-dependent maintenance of vacuolar integrity is not a MEF-specific phenomenon, but also protects epithelial cells during bacterial infection.

We additionally sought to determine if there was a requirement for TBK1 in immune effector cells such as macrophages. We used RNAi to knock down TBK1 expression in the RAW264.7 macrophage cell line (Figure 2.10A). It was previously reported that *S. typhimurium* grow poorly in macrophage cytosol, so LAMP-1 co-localization was assessed to reflect escape of bacteria from the SCV (Beuzon *et al.*, 2000). At 4h p.i., in macrophages treated with *tbk1* siRNA, only 62.0% of *S. typhimurium* co-localized with LAMP-1 compared to 94.7% co-localization in control siRNA treated cells (Figure 2.10B and C). *Tbk1* siRNA treatment of RAW264.7 cells also resulted in decreased LAMP-1 co-localization with bacteria during infection by EPEC or *S. pyogenes*. In contrast, 2 micron beads taken up by phagocytosis remained

completely associated with LAMP-1 in both *tbk1* and control siRNA treated cells. From these observations, we conclude that TBK1 regulates the integrity of pathogen containing vacuoles in multiple cell types.

4. Discussion

We have shown here that the IKK-like kinase, TBK1, mediates an early cellular response to bacterial infection. In the absence of TBK1, *S. typhimurium* replicated rapidly and to high levels in MEFs. The IRF3-Type I interferon axis which contributes to anti-viral immunity was not required for the growth restrictive function of TBK1, nor was de novo transcription or translation. After entry into *tbk1*^{-/-} cells, *S. typhimurium* escaped into the cytosol where proliferation occurred. Loss of vacuolar integrity in TBK1-deficient cells was not specific to *S. typhimurium* infection, but occurred during infection by Gram-negative and Gram-positive bacteria. Thus, TBK1 protects cells during bacterial infection by confining invading pathogens to a membrane-bounded compartment.

The best studied TBK-dependent signaling pathway triggered by bacterial infection is LPS mediated induction of Type I interferons through TLR4, TBK1 and IRF3 (Hiscott *et al.*, 2003, Sharma *et al.*, 2003, Fitzgerald *et al.*, 2003, Palsson-McDermott *et al.*, 2004). However, our data showed that TBK1 did not require IRF3 or Type I interferon to exert a protective effect on host cells during bacterial infection. It is yet unclear whether TLR4 activation contributes to TBK1-dependent maintenance of vacuolar integrity. We still observed a protective effect by TBK1 on pathogen-containing

vacuoles in HeLa cells, which do not express surface TLR4 due to lack of the accessory protein, MD2 (Shimazu *et al.*, 1999). This observation suggests that TLR4 signaling is not absolutely required for the restrictive function of TBK1 in bacterial infection, but further studies will be necessary to definitively determine the role of TLR4 or other TLRs in modulation of vacuolar integrity by TBK1.

The vacuolar compartment is a restrictive anti-microbial environment because of its ability to decrease pH, produce degradative enzymes and in some cell types, to generate harmful reactive oxygen species in a confined environment (O'Riordan *et al.*, 2002, Garin *et al.*, 2001, Hampton *et al.*, 1998). By contrast, there are few cytosolic anti-microbial mechanisms, possibly to minimize damage to cytosolic host machinery. Given the more permissive nature of the host cytosol, it is surprising that relatively few bacterial pathogens exploit this intracellular niche (Bielecki *et al.*, 1990). TBK1-dependent mechanisms may prevent many bacterial pathogens from accessing the cytosol by modulating integrity or function of the endocytic compartment during infection. Our data suggest two non-exclusive models by which TBK1 may contribute to the cellular response to bacterial invasion. First, TBK1 could function at the post-transcriptional level in response to an infection by phosphorylating target proteins. In the case of bacterial infection, there may be TBK1 kinase substrates whose function directly or indirectly modifies the pathogen-containing vacuole. Secondly, TBK1 may act prior to infection to establish a state of immune competence, perhaps by regulating expression of gene products that are important for the immediate response to bacterial invasion. This model requires that TBK1 have some constitutive activity prior to infection. Since there is a known requirement for TBK1 in embryonic development in the absence of infection,

it is likely that TBK1 may function in normal adult animals in the absence of infection as well (Bonnard *et al.*, 2000, Pomerantz *et al.*, 1999, Tojima *et al.*, 2000). Indeed, two recent studies have identified TBK1 as a regulator of angiogenesis and ontogenesis (Chien *et al.*, 2006, Korherr *et al.*, 2006). Furthermore, microarray analysis of uninfected *tbk1*^{+/+} and *tbk1*^{-/-} MEFs identified over 400 genes that were differentially expressed, some of which have known associations with innate immune function (data not shown).

At the molecular level, there are at least two mechanisms by which TBK1 may ultimately regulate vacuolar integrity. In response to bacterial invasion, TBK1 could control initiation of autophagy which can capture bacteria in damaged vacuoles (Birmingham *et al.*, 2006a, Birmingham *et al.*, 2006b). This possibility is less likely, as co-localization with LC3, a marker of autophagosomes, was slightly higher in infected *tbk1*^{-/-} cells than wildtype cells. However, it is notable that all of the bacterial species for which we showed cytosolic localization in *tbk1*^{-/-} cells have mechanisms by which vacuolar membranes are damaged during infection, i.e., *S. typhimurium* and EPEC contain Type III secretion machinery, and *S. pyogenes* encodes the pore-forming toxin Streptolysin O (Sierig *et al.*, 2003, Warawa *et al.*, 1999, Miki *et al.*, 2004). Alternatively, a TBK1-dependent target could modulate influx/efflux of ions or water into the pathogen-containing vacuole to maintain its physical continuity or otherwise alter membrane dynamics in response to infection. Previous studies have demonstrated that host cells activate repair mechanisms in response to membrane damage (Roy *et al.*, 2004, McNeil *et al.*, 2005). Our findings are consistent with damage to the vacuolar membrane as a possible trigger for TBK1-dependent function during bacterial infection.

The innate immune system is required both for controlling pathogen replication and for communicating with other cell types. In viral infections, TBK1 clearly acts as a regulator of innate immunity by communicating with other cells through Type I interferon, which contributes to control of viral replication. However, we have demonstrated that in bacterial infections, TBK1 plays an important role in limiting pathogen replication by protecting the integrity of the pathogen-containing vacuole, independently of Type I interferon. Our findings do not preclude an additional requirement for TBK1 in anti-bacterial immunity through stimulation of cytokine or chemokine expression, but suggest an early TBK1-dependent mechanism by which host cells can achieve innate control of bacterial infection.

5. Materials and Methods

Bacterial strains and cell culture

Strains used in this study are described in Appendix A (Table A). *S. typhimurium* strains expressing GFP were constructed by electroporating the bacteria with pFPV25.1 obtained from C. Detweiler (Valdivia *et al.*, 1996). The *S. typhimurium* strain expressing RFP was constructed by amplifying the RFP gene from pGEM:RFP (J.A. Bauer, University of Michigan Medical School), ligating the sequence into PCR-II-Topo (Invitrogen) and then electroporation into *S. typhimurium*. MEFs were obtained from W.-C. Yeh (*tbk1*^{+/+} and *tbk1*^{-/-}) and K. Mossman and B. Williams (*irf3*^{+/+} and *irf3*^{-/-}). MEFs and RAW264.7 cells (ATCC) were grown in RPMI medium supplemented with 10% fetal bovine serum and 1% L-glutamine at 37°C in 5% CO₂. Experiments in *tbk1*^{+/+} and *tbk1*^{-/-} MEFs were reproduced both with primary MEFs in early passage, and with MEFs that were

immortalized by continuous culture and clonally derived. HeLa cells (ATCC) were grown in MEM α medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids, and 1% sodium pyruvate at 37°C in 5% CO₂.

Infections

All *S. typhimurium* strains were grown overnight in LB medium at 37°C shaking and back-diluted 1:100. When the bacteria reached exponential phase, they were washed twice with Dulbecco's PBS (D-PBS) and used to infect MEFs at a m.o.i. of 10 for 1 hr, whereupon infected cells were washed three times with D-PBS and incubated in medium containing 100 μ g/ml gentamicin for 2h. The cells were then washed 3 times with D-PBS and fresh medium containing 5 μ g/ml gentamicin added. For intracellular growth curves, at indicated times p.i., 3 coverslips containing infected MEFs were removed and individually lysed in 5 mL sterile water; a fraction of the lysate was plated on LB agar to enumerate colony forming units. *St* SPI-1⁻ required bystander infection with wildtype *S. typhimurium*; co-infections were performed with *S. typhimurium* grown and infected under the same conditions as stated for monotypic *S. typhimurium* infection in MEFs except that the *St* SPI-1⁻ mutant was added at an m.o.i. of 100. EPEC was grown statically overnight in LB at 25°C, then backdiluted 1:100 and incubated shaking at 37°C in serum-free DMEM for 1 hr. EPEC were then used to infect MEF cells at an m.o.i. of 25 for 1 hr; washing and gentamicin treatment were carried out as described for *S. typhimurium*. *S. pyogenes* was statically grown overnight at room temperature in BHI. The stationary phase bacteria were washed 2 times with PBS, vigorously vortexed and used to infect MEFs at an m.o.i. of 25. The remainder of the infection was done under

the same conditions as described for *S. typhimurium* infection in MEFs. Bacterial infections in transfected HeLa cells were performed as described for MEFs, except that *S. typhimurium* and *S. pyogenes* were incubated with the host cells for 30 min at an m.o.i. of 100 and EPEC at an m.o.i. of 50 for 1 hr; the HeLa cells were washed 3 times with D-PBS, and incubated with fresh medium for 30 min before gentamicin was added. Infections of RAW264.7 macrophages were performed with *S. typhimurium* at m.o.i. 10 for 30 min, EPEC at m.o.i. 50 for 45 min, and *S. pyogenes* at m.o.i. 25 for 30 min. The macrophages were washed 3 times with D-PBS, and medium with gentamicin added as described for MEFs. Two micron beads were incubated with anti-streptavidin antibody in D-PBS + 1% BSA for 30 minutes, then washed 3 times in D-PBS + 1% BSA, added to the macrophage culture and spun down onto the macrophage monolayer. Beads were incubated with the macrophages for 30 min, then the cells were washed 3 times with D-PBS, before adding fresh medium.

Transfections

HeLa cells or MEFs were resuspended in the appropriate growth medium as described above and plated in a 6 well plate at a concentration of 5×10^5 cells/plate. Cells were transfected for 24 hrs with either 4.2 μ g of plasmid DNA or 40 μ M of the indicated siRNA (Ambion) complexed with Lipofectamine 2000 (Invitrogen). For siRNA experiments, transfected cells were trypsinized, split 1:2 and subjected to a second round of siRNA transfection as above until time of harvest or infection with *S. typhimurium*. For transfection of RAW 264.7 macrophages, cells were resuspended in growth medium

as described above, and plated in a 6 well plate at a concentration of 1×10^6 cells/plate. Macrophages were transfected as described for HeLa and MEF cells.

Microscopy

Cells were grown in appropriate medium in a 6 well plate at a concentration of 7×10^5 cells/plate and infected as described above. At indicated times p.i., coverslips were removed and fixed in 3.7% paraformaldehyde in D-PBS. Cells were then washed 3 times with 0.1% Triton X-100 in PBS and blocked for 10 min with TBS-TX (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton X100, 1% BSA). TBS-TX with primary antibody was added to the cells for 1 hr. Cells were then rinsed 3 times with TBS-TX and then incubated for 30 min with secondary antibody. Coverslips were rinsed with TBS-TX and mounted with Pro-Long Gold Antifade (Invitrogen). Samples were analyzed with the Olympus Fluoview FV-500 Confocal microscope using a 100X objective and Fluoview software. Quantitation of bacteria co-localized with LAMP-1 or ubiquitin was performed by scoring 150 randomly chosen bacteria per experiment for the presence of LAMP-1 or ubiquitin; only bacteria completely surrounded by LAMP-1 or ubiquitin as indicated by antibody staining were scored as co-localized. Transmission electron microscopy was performed on a Philips CM-100 transmission electron microscope equipped with automated compustage and Kodak 1.6 Megaplex high resolution digital camera. Samples were prepared as previously described (Passalacqua *et al.*, 2006).

Antibodies and reagents

The LAMP-1 (1D4B) rat monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc, the anti-ubiquitin monoclonal antibody (FK2) from BIOMOL International, the anti-TBK1 monoclonal antibody from Imgenex and anti-streptavidin antibody from Molecular Probes. TRITC-phalloidin was purchased from Invitrogen, and 4',6-Diamidino-2 phenylindole dihydrochloride (DAPI) from BioChemika. Recombinant mouse interferon- β (PBL Biomedical Laboratories) was used at 100 U/ml to treat cells overnight and throughout the course of infection. LysoTracker Red DND-99 (Invitrogen) was a gift from K. Collins (University of Michigan Medical School). Alpha-amanitin (BioChemika) was used at 50 μ g/ml to pre-treat cells for 1h prior to and during the course of infection. All siRNA reagents were obtained from Ambion. The EGFP-LC3 was obtained from Addgene, Inc. (plasmid 11546) and constructed in the laboratory of K. Kirkegaard. Beads and anti-streptavidin antibody were a gift from J. Swanson (2.01 micron streptavidin silicon oxide microspheres - Corpuscular, Inc.).

Construction of TBK1-KD

The *tbk1* cDNA amplified from *tbk1*^{+/+} MEF RNA was cloned into the expression vector pcDNA3 (Invitrogen). After sequence validation, QuikChange mutagenesis (Wang *et al.*, 1999) was performed to replace lysine-38 with an alanine, which was validated by sequencing. TBK1:pcDNA3 and TBK1-KD:pcDNA3 both encoded a full length TBK1 protein as determined by TNT Quick Coupled Transcription /Translation Systems (Promega) and western blot probed with anti-TBK1 antibody.

Real time quantitative RT-PCR and sequence information

Total RNA was isolated from cells using the RNAeasy kit (Qiagen) and cDNA synthesis carried out using 2.5 µg of total RNA (M-MLV Reverse Transcriptase; Invitrogen). Real time RT-PCR analysis was performed with the MX3000p (Stratagene) and Brilliant SYBR Green MasterMix (Stratagene). Relative amounts of cDNA were normalized to actin cDNA levels in each sample. The following primers were used for amplification: *ip10* (F- 5'ATGAGGGCCATAGG GAAGCTTGAA; R- 5'ACCAAGGGCAATTAGGACTAGCCA), *mx1* (F-5'TTGTCTA CTGCCAGGACCAGGTTT; R-5'TTTCAGGTGCTGGGTCATCTCAGT) *actin* (F- 5'AGGTGTGATGGTGGGAATGG; R-5'GCCTCGTCACCCACATAGGA). Accession numbers for the sequences used for primer design are: *tbk1* (NC_00076.4), *ip10* (NC_00071), *mx1* (NM_010846) and *actin* (NM_007393).

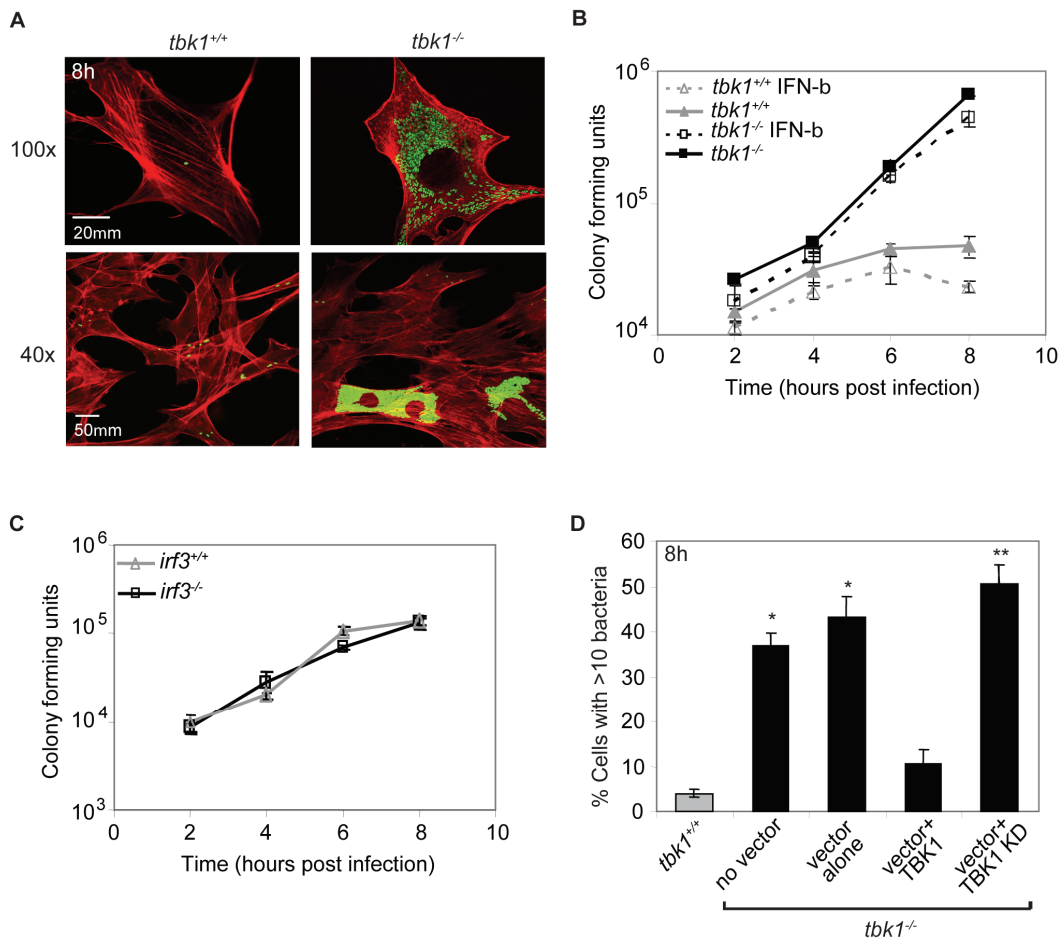


Figure 2.1 TBK1 kinase activity suppresses *S. typhimurium* intracellular growth. (A) MEFs were infected with *Salmonella*-GFP (green), fixed at 8h p.i., counterstained with TRITC-phalloidin to visualize actin (red), and then analyzed by confocal immunofluorescence microscopy. Images were acquired using either a 100X or 40X objective. (B) *S. typhimurium* intracellular growth was measured over time in *tbk1*^{+/+} (grey triangles) and *tbk1*^{-/-} (black squares) MEFs in the absence (solid) or presence (dashed) of Type I interferon. Where indicated, MEFs were treated with 100 U/ml rIFN-β prior to and during infection. The data are representative of 5 independent experiments. (C) *S. typhimurium* intracellular growth in *irf3*^{+/+} (grey triangles) and *irf3*^{-/-} (black squares) MEFs was measured over time. The data are representative of 3 independent experiments. (D) MEFs were transfected with vector alone (pcDNA3), pcDNA3:TBK1, or pcDNA3:TBK1-KD where indicated in combination with pcDNA3:GFP as a marker for transfection. Percent infected cells that contained >10 *S. typhimurium*, out of 150 infected cells counted per experiment at 8h p.i., was determined by fluorescence microscopy using DAPI to identify bacteria (n≥3). Quantitation was limited to GFP-positive (transfected) MEFs. * and ** denote statistical significance of p ≤ 0.05 and 0.001 respectively, according to a one-tailed student's t-test comparing each *tbk1*^{-/-} sample to the *tbk1*^{+/+} control.

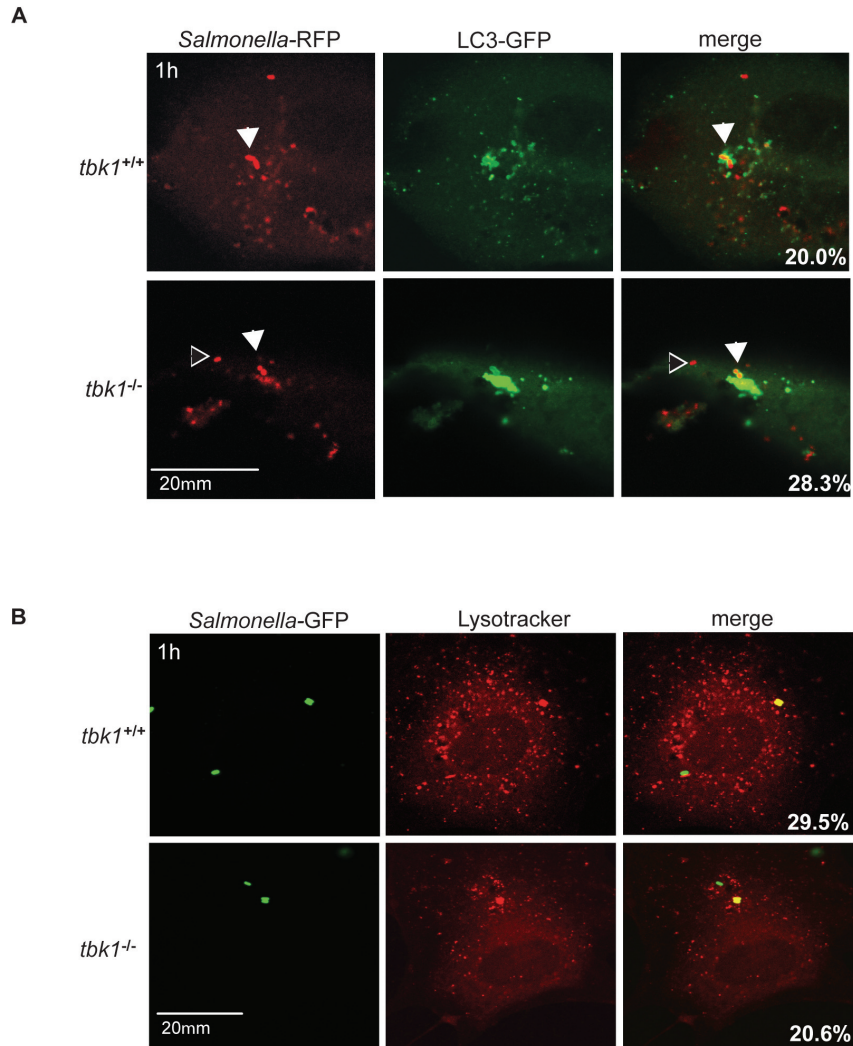


Figure 2.2. Trafficking of *S. typhimurium* populations in *tbk1*^{-/-} MEFs. (A) *Tbk1*^{+/+} and *tbk1*^{-/-} MEFs were transfected with a LC3-GFP expressing plasmid (green), to identify autophagosomes, and infected with *Salmonella*-RFP (red). Infected cells were fixed at 1h p.i. and analyzed by confocal microscopy. Percent co-localization was determined by counting how many bacteria out of 100 per experiment that co-localized with LC3-GFP (n=3). White arrowheads point to *S. typhimurium* co-localized with LC3-GFP, white outlined arrowheads point to bacteria not co-localized with LC3-GFP. (B) *Tbk1*^{+/+} and *tbk1*^{-/-} MEFs were treated with Lysotracker, a dye that fluoresces in acidic compartments, and infected with *Salmonella*-GFP (green). Infected cells were fixed 1h p.i., and analyzed by confocal microscopy. Percent co-localization was determined by counting the number of bacteria out of 150 bacteria that co-localized with bright red acidic compartments.

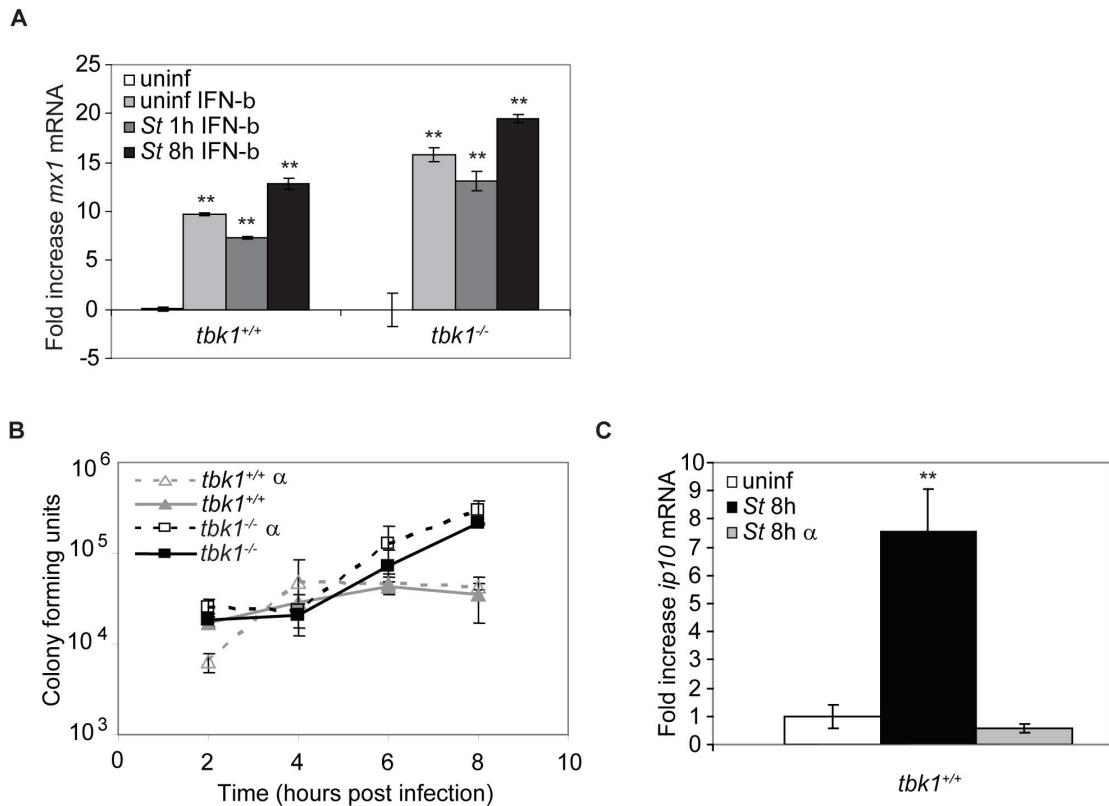


Figure 2.3. Validation of IFN- β stimulation and α -amanitin inhibition in MEFs infected with *S. typhimurium*. (A) Quantitative RT-PCR was performed to determine *mx1* induction in uninfected, 1h and 8h *S. typhimurium* infected MEFs in the presence of 100 U/ml rIFN- β normalized to uninfected untreated cells. ** denotes $p \leq 0.001$, according to a two-tailed student's t-test comparing rIFN- β treated samples to an untreated sample of the same genotype. (B) *S. typhimurium* intracellular growth curve in *tbk1*^{+/+} (grey triangles) and *tbk1*^{-/-} (black squares) MEFs was measured over time in the absence (solid) or presence (dashed) of the RNA polymerase II inhibitor, α -amanitin. Where indicated, MEFs were treated with 50 μ g/ml α -amanitin 1hr prior to and during infection. The data shown are representative of 3 independent experiments. (C) Quantitative RT-PCR was performed to assess *ip10* induction in uninfected and 8h *S. typhimurium* infected *tbk1*^{+/+} MEFs in the presence or absence of α -amanitin (50 μ g/ml). ** denotes $p \leq 0.001$, according to a two-tailed students t-test comparing infected untreated and α -amanitin treated cells to uninfected cells.

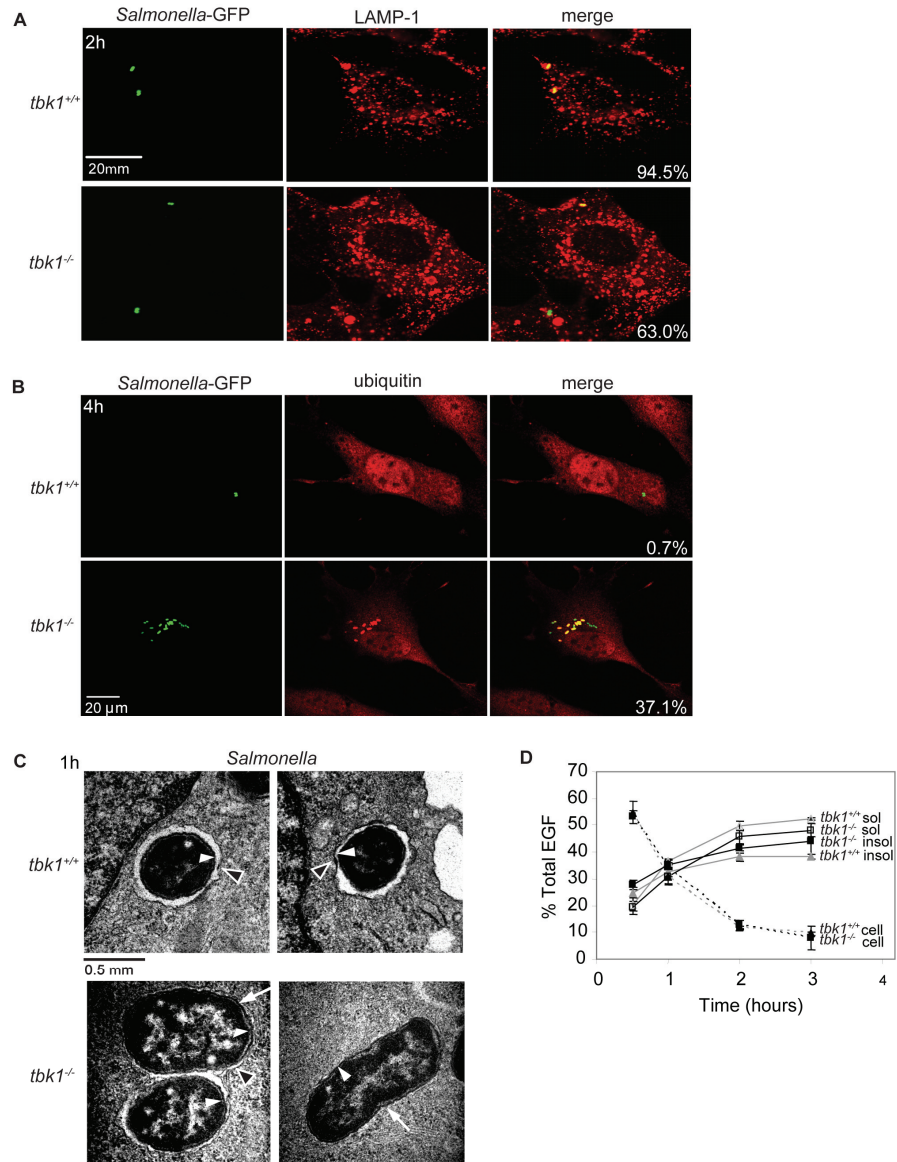


Figure 2.4. In the absence of TBK1, bacteria lose co-localization with LAMP-1 and enter the cytosol. (A) *Tbk1*^{+/+} and *tbk1*^{-/-} MEFs were infected with *Salmonella*-GFP (green). Infected cells were fixed at 2h p.i., stained with anti-LAMP-1 antibody followed by a TRITC-labeled secondary antibody (red) and analyzed by confocal immunofluorescence microscopy. Percent colocalization represents # of bacteria/150 bacteria co-localized with LAMP-1 (n=3). (B) MEFs were infected for 1 hr with the *Salmonella*-GFP (green), fixed at 4h p.i., and stained with an anti-ubiquitin monoclonal antibody followed by a TRITC-labeled secondary antibody (red). Percent ubiquitin associated bacteria represents # bacteria/150 bacteria co-localized with ubiquitin as observed by confocal microscopy (n=3). (C) Transmission electron microscopy was performed on MEFs infected for 1h with *S. typhimurium*, and images acquired at 64,000X magnification. White arrowheads point to bacterial membranes, black arrowheads point to host vacuolar membranes and white black arrows point to bacteria no longer completely surrounded by vacuolar membranes. (D) MEFs were incubated with ¹²⁵I-EGF and the rate of endocytic uptake (cell associated), degradation (soluble) and accumulation of undegraded product (insoluble) was determined at 0.5, 1, 2 and 3 hours after endocytosis (n=4).

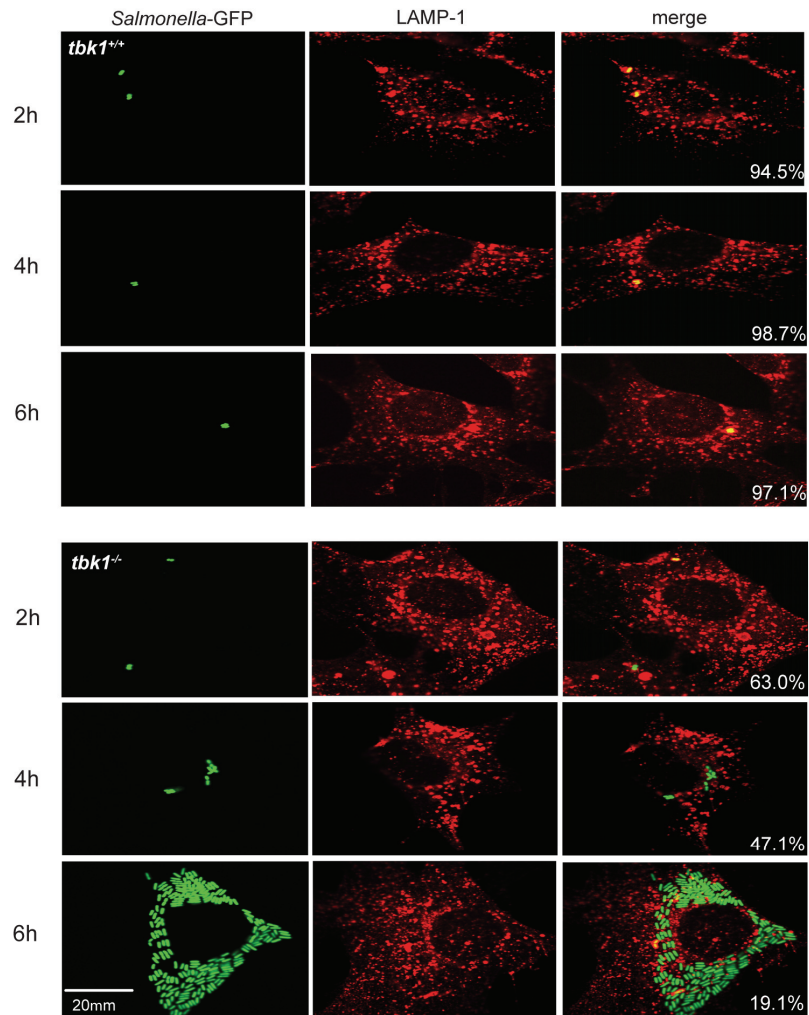


Figure 2.5. Kinetic analysis of loss of LAMP-1 co-localization with *S. typhimurium* in *tbk1*^{+/+} and *tbk1*^{-/-} MEFs. *Tbk1*^{+/+} and *tbk1*^{-/-} MEFs were infected with *Salmonella*-GFP (green). Infected cells were fixed at 2, 4 and 6h p.i., stained with anti-LAMP-1 antibody followed by a TRITC-labeled secondary antibody (red) and analyzed by confocal immunofluorescence microscopy. Percent co-localization was determined by counting the number of bacteria out of 150 bacteria per experiment that co-localized with LAMP-1. The experiment shown is representative of 3 independent experiments

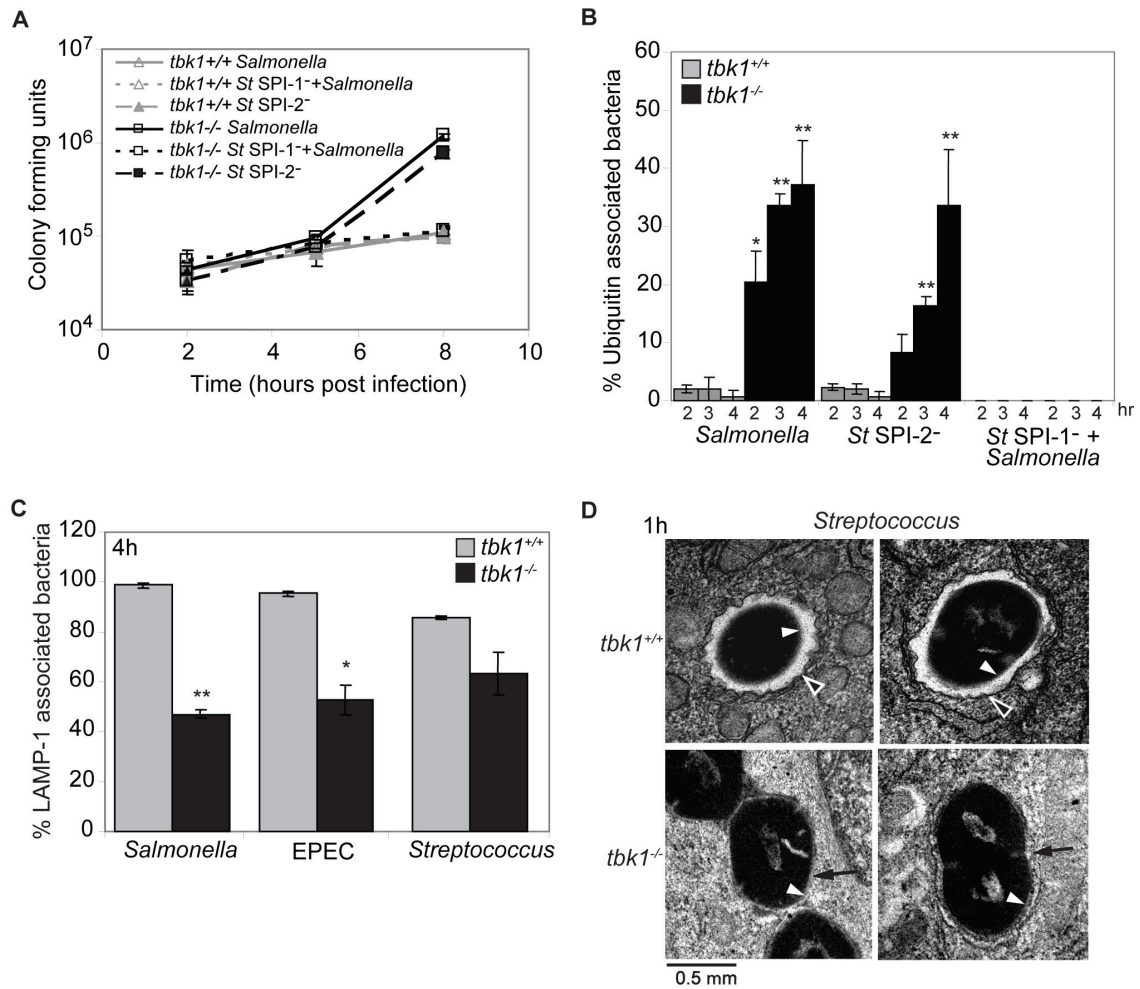


Figure 2.6. TBK1 protects vacuolar integrity in response to bacterial infection. (A) Intracellular growth of wildtype or mutant *S. typhimurium* strains in *tbk1*^{+/+} (triangles) and *tbk1*^{-/-} (squares) MEFs was measured over time. An antibiotic resistant SPI-1 deficient strain (short dashed lines) was co-infected with an antibiotic sensitive wildtype *S. typhimurium* strain to facilitate invasion by the mutant. Only antibiotic resistant colonies are shown for the SPI-1⁻ growth curve. (B) MEFs were infected with *Salmonella*-GFP and fixed at 2, 3 and 4 h p.i. In the case of co-infection (*St SPI-1*⁻ + *Salmonella*), the SPI-1 deficient strain was marked with GFP. The samples were stained as described in Fig. 2B. Percent ubiquitin associated bacteria in *tbk1*^{+/+} MEFs (grey) or *tbk1*^{-/-} MEFs (black) represents # bacteria co-localized with ubiquitin/100 bacteria (n=3, * and ** denote p ≤ 0.05 and 0.001 respectively). (C) MEFs were infected for 1 hr with the indicated bacteria and then fixed at 4h post infection. Samples were subjected to immunofluorescence staining as described in Fig 2.4A. Percent LAMP-1 associated represents # bacteria/ 100 bacteria that co-localized with LAMP-1 (n=3, * and ** denote p ≤ 0.05 and 0.001 respectively). (D) Transmission electron microscopy was performed on MEFs infected for 1h with *Streptococcus*, and images were acquired at 64,000X magnification. White arrowheads point to bacterial membranes, black arrowheads point to host vacuolar membranes and black arrows point to bacteria that are no longer completely surrounded by vacuolar membranes.

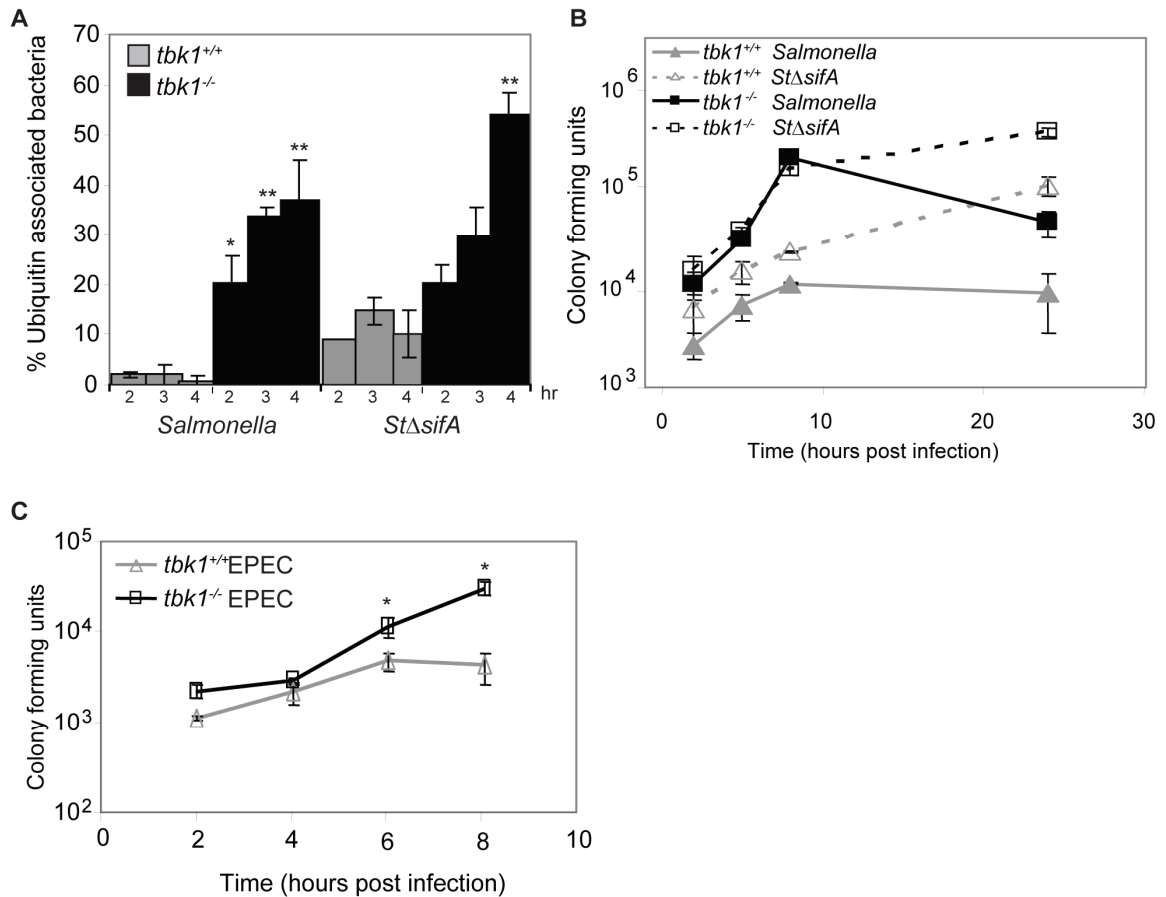


Figure 2.7. A *S. typhimurium sifA* mutant strain and EPEC display similar growth and vacuolar escape as wildtype *S. typhimurium* in *tbk1*^{-/-} MEFs (A) MEFs were infected for 1h with the indicated bacterial strains and then fixed at 2, 3, or 4h post infection. Results from *tbk1*^{+/+} MEFs are shown in grey bars and *tbk1*^{-/-} MEFs are shown in black bars. Samples were stained as in Figure 2.6B. Percent ubiquitin associated represents # bacteria/100 bacteria co-localized with ubiquitin (n=3, * and ** denote p ≤ 0.05 and 0.001 respectively, comparing each time point of *tbk1*^{-/-} to *tbk1*^{+/+} for each bacterial strain). (B) Intracellular growth of wildtype *S. typhimurium* (*St*; solid lines) or a $\Delta sifA$ mutant strain (*StΔsifA*; dashed lines) was measured over time in *tbk1*^{+/+} (triangles) and *tbk1*^{-/-} (squares) MEFs (n=4). (C) Intracellular growth of EPEC was measured over time in *tbk1*^{+/+} (triangles) and *tbk1*^{-/-} (squares) MEFs (n=3, * denotes a p value of ≤ 0.05, comparing *tbk1*^{-/-} to *tbk1*^{+/+} at each time point).

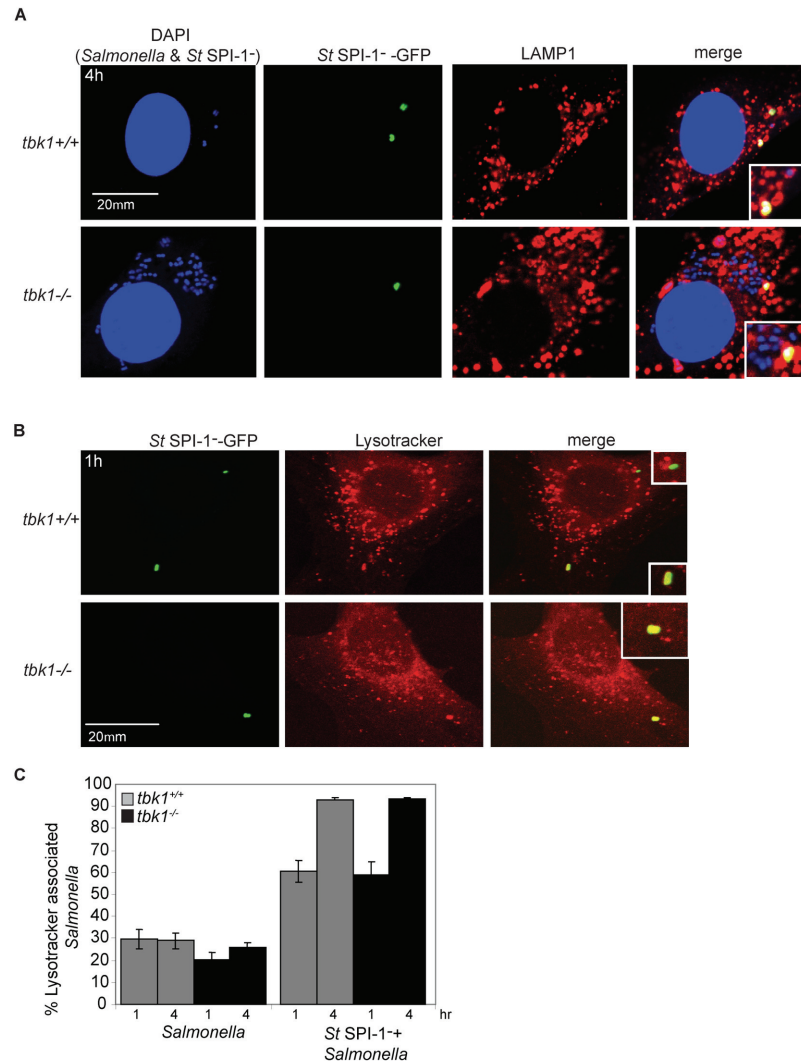


Figure 2.8. A *S. typhimurium* SPI-1 mutant remains in LAMP-1⁺ vacuoles in *tbk1*^{+/+} and *tbk1*^{-/-} MEFs throughout infection. (A) *Tbk1*^{+/+} and *tbk1*^{-/-} MEFs were infected with *St* SPI-1⁻-GFP (green) in the presence of wildtype *S. typhimurium* to induce bystander uptake. Infected cells were fixed at 4h p.i., stained with DAPI (blue), and an anti-LAMP-1 antibody (red) and analyzed by confocal microscopy. (B) *Tbk1*^{+/+} and *tbk1*^{-/-} MEFs were treated with Lysotracker, and subsequently infected with *St* SPI-1⁻-GFP (green) in the presence of wildtype *S. typhimurium* to induce bystander uptake. Infected cells were fixed 1h p.i. and analyzed by confocal microscopy. (C) MEFs were treated with Lysotracker, infected with either wild type *Salmonella*-GFP or *St* SPI-1⁻-GFP in the presence of wildtype unmarked *S. typhimurium* (*Salmonella*) to induce bystander uptake for 1 hr, and fixed at 1 and 4h p.i. Percent Lysotracker associated bacteria represents # GFP⁺ bacteria/100 GFP⁺ bacteria that co-localized with bright red acidic compartments (n=3).

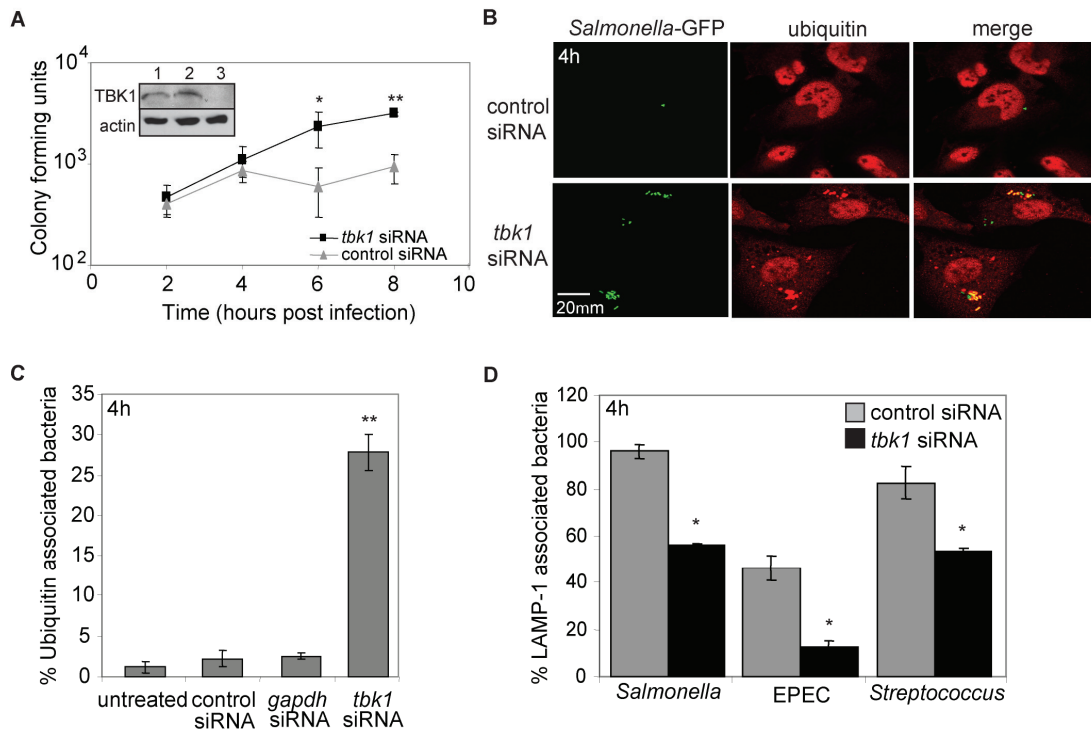


Figure 2.9. TBK1 protect vacuolar integrity in epithelial cells. (A) HeLa cells were transfected with the indicated siRNA and knockdown at 72h post transfection was determined by western blot analysis using an anti-TBK1 antibody: lane 1 (no siRNA), lane 2 (scrambled siRNA), lane 3 (*tbk1* siRNA) (inset). Blots were reprobbed with anti-actin antibody as a loading control. HeLa cells were transfected with *tbk1* or control siRNA and then infected by *S. typhimurium* to measure intracellular growth over time (* and ** denote $p \leq 0.05$ and 0.001 respectively, comparing *tbk1* siRNA with control siRNA treated cells at each time point). (B) HeLa cells transfected with *tbk1* specific or control siRNA were infected with *Salmonella*-GFP. Samples were fixed at 4h p.i., stained with anti-ubiquitin antibody (red) and analyzed by confocal microscopy (n=4). (C) Quantitative analysis of cytosolic GFP-*Salmonella* was performed in infected HeLa cells left untreated or transfected with *tbk1*, *gapdh* or control siRNA. Percent ubiquitin associated represents # bacteria/100 out bacteria that co-localized with ubiquitin at 4h p.i. (n=3, * and ** denote $p \leq 0.05$ and 0.001 respectively, comparing all samples to untreated cells). (D) HeLa cells transfected with *tbk1* specific or control siRNA were infected for 1 hr with the indicated bacteria, fixed at 4h p.i. and stained with anti-LAMP1 antibody. Percent LAMP-1 associated bacteria represents # bacteria/100 bacteria that colocalized with LAMP-1 as observed by confocal microscopy (n=2, * and ** denote $p \leq 0.05$ and 0.001 respectively, comparing *tbk1* siRNA transfected cells to control transfected cells for each bacterial strain).

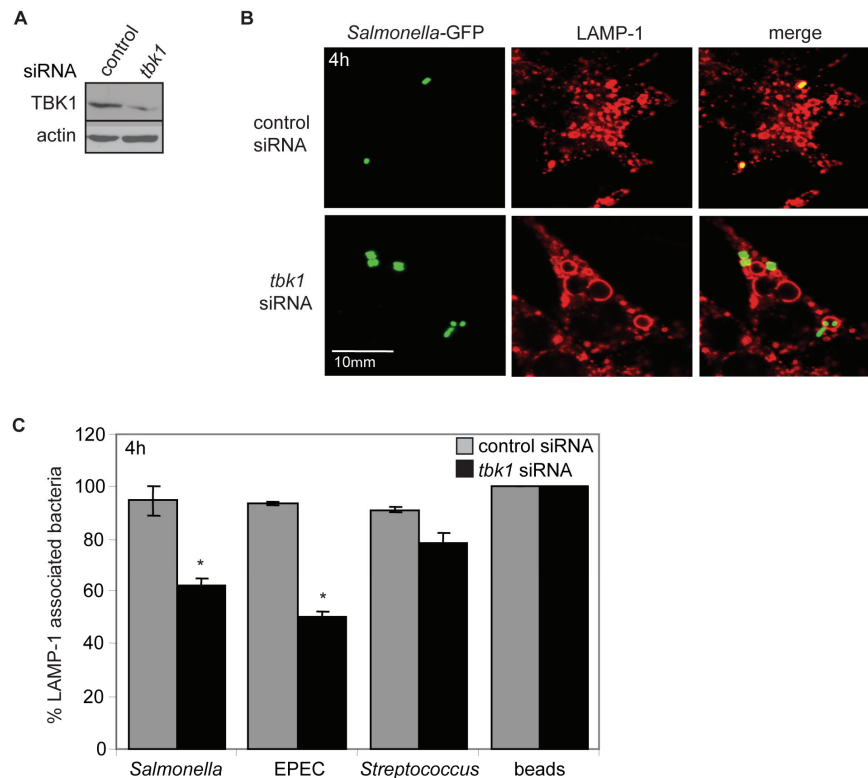


Figure 2.10. Macrophages require TBK1 to efficiently restrict bacteria in phagosomes. (A) RAW264.7 macrophages were transfected with the indicated siRNA, and knockdown at 72h post transfection was determined by western blot analysis using an anti-TBK1 antibody. Blots were re probed with anti-actin antibody as a loading control. (B) RAW264.7 macrophages transfected with *tbk1* specific or control siRNA were infected with *Salmonella*-GFP, fixed at 4h post infection and stained with anti-LAMP1 antibody. (C) RAW264.7 macrophages transfected with *tbk1* specific or control siRNA were infected with the indicated bacterial species. Percent LAMP-1 associated bacteria or beads represents # bacteria or beads/ out 100 that co-localized with LAMP-1 as observed by confocal microscopy (n=2, * denotes $p \leq 0.05$, comparing *tbk1* siRNA transfected cells to control transfected cells).

Chapter 3

Homeostatic maintenance of pathogen-containing vacuoles requires TBK1-dependent regulation of *aquaporin-1*

1. Abstract

Membranes are an integral component of many cellular functions and serve as a barrier to keep pathogenic bacteria from entering the nutrient rich host cytosol. TANK-binding-kinase-1 (TBK1), a kinase of the I κ B kinase family, is required for maintaining integrity of pathogen-containing vacuoles (PCV) upon bacterial invasion of host cells. Here we investigate how vacuolar integrity is maintained during bacterial infection, even in the presence of bacterial membrane-damaging agents. We found that Aquaporin-1 (AQP1), a water channel that regulates swelling of secretory vesicles, associated with PCV. AQP1 levels were elevated in TBK1-deficient cells, and overexpression of AQP1 in wildtype cells led to PCV destabilization, similar to that observed in *tbk1*^{-/-} cells. Inhibition of physiological levels of AQP1 in multiple cell types also led to increased instability of PCV, demonstrating a need for tightly regulated AQP1 function to maintain vacuole homeostasis during bacterial infection. AQP1-dependent modulation of PCV

was triggered by bacterially induced membrane damage and ion flux. These results highlight the contribution of water channels to promoting PCV membrane integrity, and reveal an unexpected role for TBK1 and AQP1 in restricting bacterial pathogens to the vacuolar compartment.

2. Introduction

Infection or invasion by bacterial pathogens results in compartmentalization of the bacteria within pathogen-containing vacuoles (PCV). Restriction of bacteria to PCV can benefit host cells by limiting nutrients and preventing collateral host damage by antimicrobial mechanisms (Appelberg, 2006). However, many intracellular pathogens have evolved mechanisms by which they can survive and replicate within the vacuolar environment. During infection, bacterial factors that mediate virulence, including pore-forming proteins, can damage vacuolar membranes (Aroian *et al.*, 2007, Gonzalez *et al.*, 2008). Damage of cellular membranes may temporarily perturb homeostasis, defined as the physiological regulation by which cells maintain internal equilibrium. The mechanisms by which host cells modulate integrity of subcellular compartments in response to bacterially induced membrane damage are not completely understood. Such homeostatic mechanisms likely include resealing of pores and regulation of ion channels that respond to compartment-specific changes in electrochemical gradients (Roy *et al.*, 2004, King *et al.*, 2004).

We previously reported that TANK-binding kinase-1 (TBK1), promotes bacterial compartmentalization within PCV, restricting replication of some intracellular pathogens (Radtko *et al.*, 2007). Upon infection of TBK1-deficient cells, PCV containing the Gram-negative pathogen *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) or the Gram-positive Group A Streptococcus (GAS) ruptured, releasing bacteria into the cytosol between 1 and 2h post infection (pi). In contrast, invading bacteria were largely maintained in vacuoles in *tbk1*^{+/+} cells. *S. typhimurium* replicated robustly after exit from a

LAMP-1+ endosomal compartment into the cytosol of *tbk1*^{-/-} cells, a phenomenon that we term hyperproliferation, similar to the phenotype observed for *S. typhimurium* mutants that escape into the cytosol (Beuzon *et al.*, 2000, Brumell *et al.*, 2002). Although a deficiency in TBK1 led to disruption of PCV in infected cells, steady state endocytosis remained intact, as did vacuoles containing latex beads (Radtke *et al.*, 2007). Together, these observations led us to hypothesize that the integrity of pathogen-containing vacuoles is dynamically regulated by specific host cell factors, and that TBK1 contributes to vacuolar homeostasis.

The function of TBK1 and other I κ B kinase (IKK) family members in innate immune signaling has been well studied, however, these kinases also play key roles in mammalian physiology (Hacker *et al.*, 2006). Here we show that cells deficient in TBK1 exhibit highly elevated levels of the water channel, Aquaporin-1 (AQP1), which modulates the integrity of pathogen containing vacuoles (PCV). Aquaporins are channels that permit movement of water across membranes to regulate osmotic homeostasis (King *et al.*, 2004). Despite the importance of host membranes as a key host-pathogen interface, relatively little is known about how aquaporins function in bacterial infection. We found that AQP1 co-localized with PCV and promoted PCV integrity in different cell types. Dysregulated AQP1 expression resulted in increased instability of the PCV in response to bacterial membrane damaging agents and ion flux. Our data suggest a model in which bacterially induced membrane damage triggers electrochemical disequilibrium across the PCV membrane, requiring water and ion transport to minimize vacuole disruption. We therefore propose that TBK1 and aquaporins can play a role in host-bacterial interactions

by regulating the homeostatic response to vacuolar membrane damage caused by bacterial pathogens.

3. Results

3.1 AQP1 expression is elevated in the absence of TBK1.

The vacuolar membrane surrounding invading bacterial pathogens loses integrity between 1 and 2 hours post infection (h pi) in the absence of TBK1 (Radtke *et al.*, 2007). We performed live cell imaging of infected *tbk1*^{+/+} and *tbk1*^{-/-} MEFs to observe kinetic or physiological differences in PCV. MEFs were infected with Group A *Streptococcus* (GAS) in the presence of a fluid phase fluorescent dye, Texas Red (TR)-dextran (70kDa), as an indicator of vacuolar integrity (Figure 3.1A). As early as 45 min pi, GAS in *tbk1*^{-/-} MEFs were found in vacuoles that appeared to enlarge over time, compared to GAS in wildtype cells, which were contained in tight vacuoles. The apparent difference in vacuolar volume between *tbk1*^{-/-} and *tbk1*^{+/+} MEFs was maintained until approximately 90 min pi, when the fluorescent TR-dextran associated with GAS was abruptly lost in the *tbk1*^{-/-} MEFs. However, vacuoles without bacteria retained the dye in *tbk1*^{-/-} MEFs, suggesting that loss of membrane integrity was specific to PCV. Although GAS can secrete the pore-forming toxin, Streptolysin O (SLO), TR-dextran remained associated with GAS-containing vacuoles in *tbk1*^{+/+} cells throughout the 2.5h infection. These data are consistent with previous reports suggesting that intracellular GAS secrete lower levels of SLO, preventing efficient vacuolar escape (Hakansson *et al.*, 2005). The larger volume of PCV in *tbk1*^{-/-} MEFs prior to vacuole rupture led us to hypothesize that physiological

mechanisms of vacuole volume regulation, or homeostasis, might be dysregulated in TBK1-deficient cells.

The study of cell and vesicle volume regulation has revealed that homeostasis is maintained through the concerted action of ion channels and water transporters, such as the aquaporins (Lang *et al.*, 1998). Specifically, AQP1 regulates swelling of exocytic vesicles and is found on the parasitophorous vacuole in *Plasmodium falciparum* infections (Cho *et al.*, 2006, Cho *et al.*, 2002, Murphy *et al.*, 2004). Quantitative analysis of *aqp1* mRNA levels and AQP protein expression in wildtype and *tbk1*^{-/-} MEFs, revealed substantially increased levels of AQP1 in TBK1-deficient cells compared to wildtype cells (Figure 3.1B and C). Conversely, quantitative analysis of other AQP family members mRNA levels in wildtype and *tbk1*^{-/-} MEFs displayed a decrease in levels in the absence of TBK1 (Figure 3.2). In wildtype mouse tissues, AQP1 levels were inversely proportional to the amount of TBK1, with some tissues such as kidney, expressing low levels of TBK1 and high levels of AQP1 (Figure 3.1D). These data suggested that *tbk1*^{+/+} and *tbk1*^{-/-} MEFs might be utilized as a model to explore how vacuolar homeostasis is regulated during bacterial infection in different cell or tissue types with varying levels of AQP1. Taken together with our previous studies, these data indicate that TBK1 modulates PCV integrity, and indirectly or directly leads to suppression of *aqp1* gene expression.

3.2 AQP1 is present on pathogen-containing vacuoles

If AQP1 contributed to regulation of PCV homeostasis, the protein should be present on vacuoles containing *S. typhimurium* or GAS. We performed confocal

immunofluorescence microscopy to determine the subcellular localization of AQP1 in infected *tbk1*^{-/-} cells (Figure 3.3A). At 1h pi, AQP1 exhibited marked localization around intracellular bacteria (*S. typhimurium* 79.7% ±3.2%; GAS 85% ±3.6%), consistent with the possibility that AQP1 functions at the PCV membrane. We further investigated if AQP1 colocalized with PCV in wildtype cells. As AQP1 levels could not reliably be detected by immunofluorescence in *tbk1*^{+/+} cells (Figure 3.4), we utilized a mouse intestinal epithelial cell line, m-IC_{c12} that has easily detectable AQP1 expression (Figure 3.3B). In m-IC_{c12} cells, AQP1 displayed marked colocalization with *S. typhimurium* PCV (73% ±3.0%). GAS infected m-IC_{c12} cells poorly; thus an example of AQP1 colocalization with GAS is shown, but colocalization was not quantitated. Previous studies have shown that AQP1 is present in cholesterol-rich microdomains, sites where many bacterial pathogens preferentially invade (Kobayashi *et al.*, 2004). Consistent with these reports, we found that cholesterol depletion of *tbk1*^{-/-} cells by methyl-β-cyclodextrin substantially decreased AQP1 co-localization with intracellular bacteria (Figure 3.5). We conclude from these data that AQP1 can be found associated with pathogen-containing vacuoles during intracellular bacterial infection.

3.3 Dysregulation of AQP1 expression or function destabilizes PCV

We hypothesized that increased AQP1 in *tbk1*^{-/-} MEFs contributed to impaired water homeostasis between the PCV and the cytosol, causing vacuolar membrane instability. If this were the case, perturbation of AQP1 levels or function would result in changes in vacuolar membrane integrity. To test this hypothesis, we transiently overexpressed AQP1 in MEFs, HeLa cells, and m-IC_{c12}, and infected cells with *S.*

typhimurium to quantitate loss of PCV integrity by hyperproliferation, as this phenotype is associated with PCV rupture (Radtke *et al.*, 2007). AQP1 overexpression increased the number of wildtype host cells exhibiting hyperproliferation to a level similar to that observed in *tbk1*^{-/-} MEFs (Figure 3.6A and B). Our results suggest that increased expression of *aqp1* in *tbk1*^{-/-} cells is largely responsible for instability of the PCV associated with loss of TBK1. Thus, elevated levels of AQP1 impair the ability of host cells to restrict bacteria to the vacuolar compartment.

If AQP1 function contributes to vacuolar homeostasis in wildtype cells infected with *S. typhimurium*, then inhibition of AQP1 would increase PCV instability, resulting in more host cells exhibiting the hyperproliferation phenotype. We first determined the relative levels of AQP1 in MEFs, HeLa, and m-ICc₁₂ cells by immunoblot analysis using an anti-AQP1 antibody (Figure 3.7A insert). Prior to infection with *Salmonella*-GFP, cells were left untreated or treated with the AQP1 inhibitor mercuric chloride (HgCl₂), followed by quantitation of bacterial hyperproliferation (Figure 3.7A and Figure 3.8) (Yang *et al.*, 2006). Upon HgCl₂ inhibition of AQP1 in wildtype MEFs, HeLa, and m-ICc₁₂, we observed a significant increase in *S. typhimurium* hyperproliferation. In contrast, HgCl₂ treatment suppressed the *S. typhimurium* hyperproliferative phenotype in *tbk1*^{-/-} MEFs. We interpret these data to suggest HgCl₂ restores PCV integrity in *tbk1*^{-/-} MEFs because AQP1 is overexpressed, but exacerbates hyperproliferation in wildtype cells because AQP1 cannot function at all. HgCl₂ is a commonly used AQP1 inhibitor, but can also inhibit other aquaporins (Yang *et al.*, 2006, Sugiya *et al.*, 2006). To test the specific role of AQP1 in modulating PCV integrity, we performed siRNA knockdown of *aqp1*, validating the efficiency of knockdown by immunoblot (Figure 3.7B insert), and

infected the treated cells with *Salmonella*-GFP to score hyperproliferation. As AQP1 protein expression was low in wildtype MEFs, we validated *aqp1* knockdown by qRT-PCR, and observed a 4.2-fold decrease in mRNA expression compared to control treated cells (data not shown). Wildtype infected cells treated with *aqp1* siRNA exhibited more hyperproliferation than control treated cells, while infected *tbk1*^{-/-} MEFs subjected to *aqp1* knockdown supported less hyperproliferation, consistent with HgCl₂ treatment (Figure 3.7B). These results suggest that tightly regulated AQP1 expression and function are necessary to modulate homeostasis and membrane stability of the pathogen-containing vacuole.

3.4 AQP1 controls membrane integrity in response to bacterially induced membrane damage

We reasoned that AQP1 function could be stimulated by perturbations to the PCV membrane, resulting in regulated water flux to restore vacuolar integrity in wildtype cells but dysregulated water flux in AQP1-overexpressing cells leading to vacuole rupture. In support of this idea, high levels of AQP1 in *tbk1*^{-/-} cells destabilize PCV, but uninfected vacuoles or bead-containing vacuoles remain intact (Radtke et al., 2007). We therefore tested whether a bacterial component triggered AQP1-dependent modulation of vacuolar integrity, using destabilization of the PCV in cells overexpressing AQP1 levels as a readout. *S. typhimurium* and GAS can damage host membranes through the Type III secretion system (TTSS) needle-like apparatus or SLO, respectively (Aroian et al., 2007). We first confirmed that AQP1 was still able to co-localize with SLO⁻ GAS, or with SPI-1-*Salmonella*-GFP (Figure 3.9A and B). Co-localization was observed, but to a lesser degree than with the wildtype parental bacteria, possibly due to decreased association of

the mutants with cholesterol rich microdomains (Garner *et al.*, 2002, Rohde *et al.*, 2003). We then asked whether SLO⁻ GAS could mediate disruption of PCV in *tbk1*^{-/-} MEFs, where AQP1 levels are elevated. Wildtype and TBK1-deficient MEFs were infected by GAS or SLO⁻ GAS, and the number of bacteria colocalized with LAMP-1 at 1h pi was determined by confocal immunofluorescence microscopy. Greater than 90% of the SLO⁻ bacteria remained in LAMP-1⁺ vacuoles throughout infection, compared to approximately 50% localization with wildtype bacteria at 4h pi (Figure 3.9 C-E). We previously demonstrated that SPI-1 deficient *S. typhimurium* did not escape from PCV in either *tbk1*^{+/+} or *tbk1*^{-/-} MEFs (Radtke *et al.*, 2007). Thus, AQP1-dependent modulation of the PCV is responsive to bacterially induced membrane damage.

3.5 AQP1-dependent regulation of PCV homeostasis involves ion flux

In models of cell volume homeostasis, potassium flux alters the electrochemical gradient across the membrane leading to increased transport of other ions, such as Na⁺ or Cl⁻ (Faundez *et al.*, 2004). SLO and the *S. typhimurium* SPI-1 TTSS mediate secretion of bacterial effectors into the cytosol by creating pores in host membranes, which can result in potassium flux (Yamanaka *et al.*, 1987, Madden *et al.*, 2001, Galan *et al.*, 2006). We therefore hypothesized that temporally limited pore formation by bacterial factors would cause potassium flux, altering the electrochemical gradients across the vacuolar membrane and resulting in water transport and ion flux to re-establish the gradient (Figure 3.10A) (Kelly *et al.*, 2005). We tested the role of potassium flux in AQP1-dependent PCV modulation by treating cells with Kryptofix, a potassium chelator, and tetraethylammonium (TEA), an inhibitor of AQP1 and structurally related potassium

channels (Figure 3.10B) (Yellen *et al.*, 1991, Detmers *et al.*, 2006, Beny *et al.*, 2000). Both treatments decreased the number of *tbk1*^{-/-} cells exhibiting *S. typhimurium* hyperproliferation from 42.7% in untreated cells to approximately 10% in the treated cells, but did not affect extracellular bacterial growth (data not shown). EGTA, a chelator of extracellular calcium, had no effect on hyperproliferation in *tbk1*^{-/-} cells, indicating specificity for potassium flux in triggering vacuole rupture. Perturbation of vacuolar homeostasis by potassium flux involved AQP1 but not other TBK1-dependent targets, since TEA and Kryptofix also suppressed *S. typhimurium* hyperproliferation in AQP1-overexpressing *tbk1*^{+/+} MEFs from 60% in untreated cells to approximately 10% in Kryptofix treated cells (Figure 3.10C). We interpret these data to suggest that potassium flux due to pore formation in vacuolar membranes can trigger AQP1 function.

To determine if active ion transport plays a role in regulating homeostasis of PCV, we treated *S. typhimurium* infected cells with inhibitors of different classes of ion channels, DPC (blocks non-specific inwardly rectifying Cl⁻ channels), IAA-94 (blocks Ca²⁺ activated Cl⁻ channels), and Tamoxifen (blocks volume activated outwardly rectifying Cl⁻ channels) (Duan *et al.*, 1997, Cremaschi D, 2001, Parai *et al.*, 2002). These inhibitors did not affect extracellular bacterial growth (data not shown). Tamoxifen substantially increased hyperproliferation in wildtype and *tbk1*^{-/-} MEFs, and in AQP1-overexpressing cells, indicating loss of PCV integrity in the absence of regulated chloride transport (Figure 3.10D and E). These results lead us to propose that AQP1 modulates stability of PCV in response to changes in electrochemical gradients across the vacuolar membrane, caused by bacterially induced membrane damage.

4. Discussion

In this study, we show that AQP1 modulates membrane integrity of pathogen-containing vacuoles. During intracellular bacterial infection, AQP1 could be found associated with PCV. Upon perturbation of AQP1 by overexpression, inhibition, or TBK1 deficiency, infected cells were unable to maintain the homeostatic balance between the vacuolar environment and the cytosol, eventually leading to membrane disruption upon bacterially induced membrane stress and subsequent ion flux. Overall, our data suggest that regulated AQP1 function enhances the ability of host cells to keep invading pathogens in the restrictive vacuolar compartment.

The function of aquaporins in cellular processes such as water transport and cell migration has been well documented; however the role of aquaporins during infection is not well understood. Recently, several studies have indicated that aquaporins may be an integral part of infection and pathology caused by some microbial pathogens. The murine attaching/effacing bacterial pathogen, *Citrobacter rodentium*, causes mislocalization of aquaporins that contribute to diarrhoeal-like disease in mice (Guttman et al., 2006). *Cryptosporidium parvum*, a protozoan parasite, recruits AQP1 and a Na⁺/glucose transporter to facilitate invasion (Chen et al., 2005). AQP9, the major glycerol transporter in mouse erythrocytes contributes to *Plasmodium berghei* infection in a mouse model of malaria (Liu et al., 2007). Altogether, these data and ours suggest that regulation of aquaporin expression and function is a key aspect of host-pathogen interactions.

The physical properties of vacuoles are tightly regulated to maintain vesicle volume and osmotic homeostasis between the vacuole and the cytosol (Bone et al., 1998).

Aquaporins are thought to be important for control of vacuolar homeostasis to neutralize osmotic and hydrostatic pressure gradients under conditions of stress or appropriate physiological stimuli (Hill et al., 2004). AQP1 is found on secretory granules in pancreatic acinar cells, and regulates granule swelling and exocytosis in association with chloride and potassium ion channels (Cho *et al.*, 2006, Cho *et al.*, 2002, Kelly *et al.*, 2005). By analogy, we propose that during bacterial infection, AQP1 acts to equilibrate electrochemical gradients created across the PCV membrane caused by bacterial factors (Figure 3.10A). In this manner, infected cells maintain vacuolar volume and integrity, restricting bacteria to the vacuolar compartment. Aquaporins, and host factors such as TBK1 that affect aquaporin expression and function, may impact many aspects of cellular physiology during microbial infection, from establishment of a microbial niche to more complex aspects of disease such as intestinal inflammation and diarrhea.

5. Materials and Methods

Reagents

Antibodies against specified antigens were obtained from the following sources: LAMP-1 (1D4B; Santa Cruz Biotechnology) used for immunofluorescence and LAMP-1 (ab24170; Abcam) used for immunoblot, TBK1 (IMG-139A; Imgenex), AQP1 (AB3065; Chemicon International), Calnexin (SPA-860; Stressgen Bioreagents Corp.), Caveolin-1 (610406; BD Transduction Laboratories), TR-phalloidin (T-7471; Molecular Probes), and actin (sc-1615; Sigma-Aldrich). 4',6-Diamidino-2 phenylindole dihydrochloride (DAPI) was purchased from BioChemika, and 70,000mw fluorescent Texas-Red dextran was

purchased from Invitrogen. Chemical reagents were obtained from Sigma-Aldrich except where otherwise specified. Methyl- β -cyclodextrin (MBCD) (Sigma-Aldrich) was used at 7.5 mM in medium without fetal bovine serum (FBS). After 1h, cells were washed twice and medium without FBS was replaced. Where indicated, MEFs were pretreated with 10 μ M HgCl₂, 1.75 mM Kryptofix 2.2.2, 20 mM tetraethylammonium chloride (TEA), 0.5 mM ethylene glycol tetraacetic acid (EGTA), 25 μ M R(+)-IAA-94, 15 μ M Tamoxifen (MP Biomedicals, LLC), and 0.75 mM *N*-phenylanthranilic acid (DPC). All siRNA reagents were obtained from Ambion and transfected using Lipofectamine 2000 (Invitrogen) as reported (Radtke et al., 2007).

Plasmids

The AQP1 expression vector was constructed by amplifying *aqp1* cDNA from *tbk1*^{-/-} MEF total RNA, which was subcloned into pcDNA3 (Invitrogen) (*aqp1*:pcDNA3) (Table 3.1). pcDNA-CMV-EGFP or mCherry-N1 were used as controls for transfection efficiency. *Tbk1*:pcDNA3 was constructed as previously described (Radtke et al., 2007).

Strains

The *Salmonella enterica* serovar Typhimurium strains used were wildtype SL1344, wildtype SL1344 expressing green fluorescent protein (GFP), or an SL1344 *orgA::tet* mutant expressing an *rpsM* promoter driven eGFP (Valdivia et al., 1996). The Group A *Streptococcus* (GAS) strains used were wildtype GAS188 or GAS188SLO⁻ (Sierig et al., 2003, Hakansson et al., 2005). Also see Appendix A Table A.

Cell culture and infections

Tbkl^{+/+} and *tbkl*^{-/-} MEFs (G. Barber, University of Miami) were immortalized by continuous culture and clonally derived as previously described (Radtke et al., 2007). HeLa cells and RAW264.7 cells (American Type Culture Collection) were derived and cultured and infected as previously described (Radtke et al., 2007). HEK cells were a kind gift from Dr. H. Mobley. MEFs and RAW26.7 cells were grown in RPMI medium supplemented with 10% fetal bovine serum and 1% L-glutamine, HEK were grown in DMEM media supplemented with 10% FBS, and HeLa cells were grown in MEM α supplemented with 1% L-glutamine, 1% essential amino acids, 1% sodium pyruvate, and 10% fetal bovine serum. M-IC_{c12} cells were grown under the conditions previously reported (Bens et al., 1996). M-IC_{c12}, HeLa, and HEK cells were infected similarly to MEFs at an m.o.i. of 10. RAW 264.7 cells were infected at a m.o.i. of 1 for 30 min. All cells were grown at 37°C in 5% CO₂.

Microscopy

Olympus BX60 Microscope with Epi-Fluorescence was used to quantitate the % cells with >10 *S. typhimurium*, as previously reported (Radtke *et al.*, 2007). Briefly, cells were infected with *S. typhimurium* expressing GFP, fixed at 8h pi, host cells stained with TR-phalloidin, and the number of viable host cells (with normal nuclear morphology as observed by DAPI staining) containing > 10 *Salmonella*-GFP per 100 infected cells was counted in at least 3 independent experiments. *Tbkl*^{-/-} MEFs displayed a bimodal *S. typhimurium* population at 8h pi; *Salmonella*-GFP growth appeared either as in Figure 3.6A or as in *tbkl*^{+/+} MEFs, and as previously described (Radtke et al., 2007). All other immunofluorescence images, analysis, and quantitation other than determining % cells

with >10 *S. typhimurium* were analyzed by confocal microscopy, performed as previously reported (Radtke et al., 2007). Colocalization of bacteria with AQP1 or LAMP-1 was determined by fixing cells at indicated times, staining the cells with anti-AQP1 antibody or anti-LAMP-1 antibody and DAPI, then counting # bacteria colocalized with AQP1 or LAMP-1 per 100 in 3 independent experiments. For transfection experiments, only bacteria in cells expressing co-transfected eGFP or mCherry expressing control plasmids were counted. Live cell imaging was performed by infecting MEFs on 25 mm coverslide with medium containing GAS and 5 mg/ml TR-dextran for 30 min. The cells were washed once, followed by addition of Ringer's Buffer with 100µg/ml gentamicin to the live-cell imaging chamber. Cells were visualized on an Olympus IX70 inverted microscope; frames were taken every 2 min. Movies were generated using Metamorph Premier software (Universal Imaging Corporation).

Tissue Homogenization

Liver, brain, kidney, and spleen from a wildtype C57Bl/6 mouse were placed in 5mL of PBS and homogenized with a 7mmX75mm Generator and tissue homogenizer from Pro Scientific Inc. A fraction of the homogenate was added to 2X SDS-PAGE buffer and analyzed by SDS-PAGE followed by immunoblot with anti-AQP1 and anti-actin antibodies.

Isolation of vacuoles and cholesterol rich microdomains

Uninfected and *S. typhimurium* containing vacuoles was performed with modifications as described; in brief, 1.2×10^7 MEF cells were either harvested or infected with *S. typhimurium* for 0.5 h and then harvested and resuspended in cold homogenization buffer

(Anes *et al.*, 2003). The cells were lysed using a 22-gauge needle; nuclei and intact cells were removed by centrifugation at 500xg for 5 minutes. The supernatant was layered on top of a discontinuous sucrose gradient of 60%, 40%, and 25% sucrose, which was centrifuged at 100,000xg for 60 minutes at 4°C. For isolation of CRM, MEFs were infected with *S. typhimurium* at a m.o.i. of 50 for 1h, followed by purification of CRM fractions using a non-detergent based method of discontinuous gradient centrifugation (Vetrivel *et al.*, 2004, Waugh *et al.*, 1999).

Quantitative RT-PCR

Total RNA isolation, cDNA synthesis, and qRT-PCR analysis was performed as previously described (Radtke *et al.*, 2007). The fold change between samples was determined by the $\Delta\Delta C_t$ method and normalized to actin levels (Pfaffl, 2001). The sequences of the primers used are listed in Table 3.1.

Statistical analysis

Data sets in triplicate were analyzed using Microsoft Excel and SPSS software to calculate the Student's unpaired *t*-test for independent samples. *p*-values of <0.05(*) and <0.001(**) were considered significant and highly significant respectively.

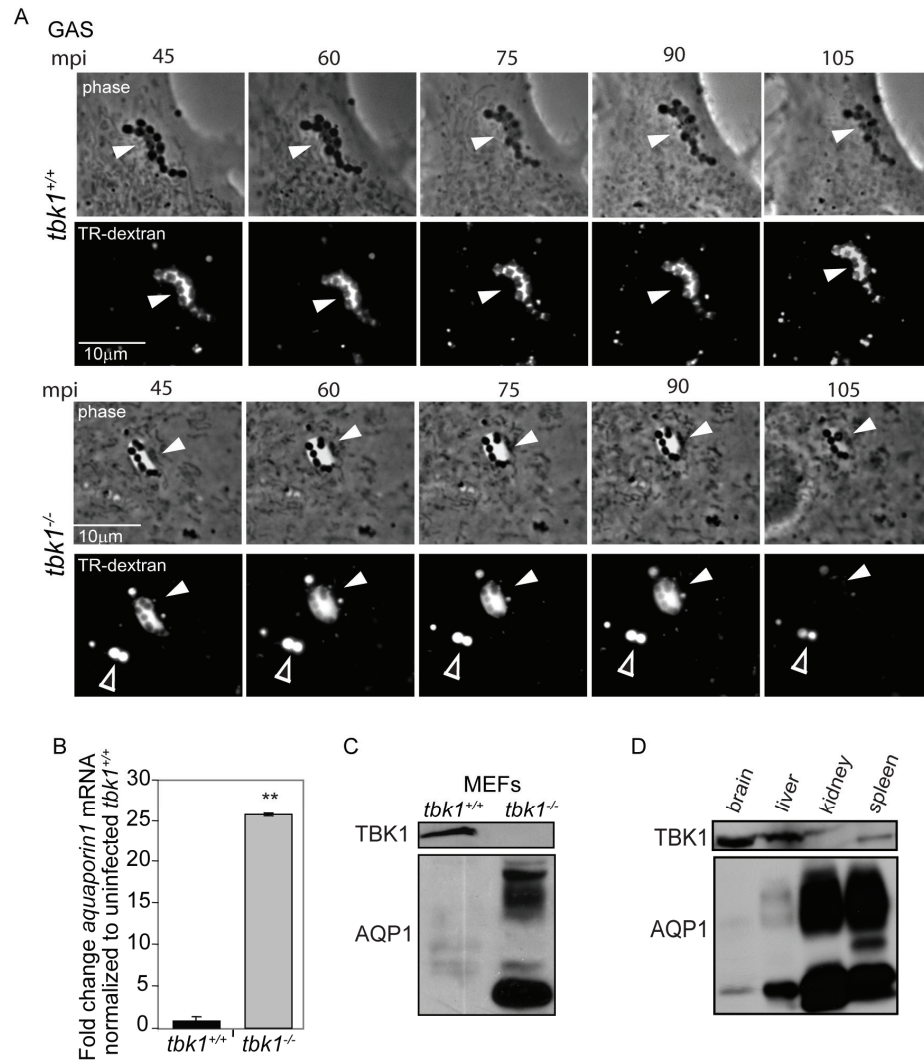


Figure 3.1. AQP1 levels are elevated in the absence of TBK1. (A) Live-cell imaging of *tbk1*^{+/+} and *tbk1*^{-/-} MEFs infected with GAS (white arrowhead) at a m.o.i. of 25 for 30 min in medium containing 70kDa Texas Red-dextran to label intact vacuoles. GAS is indicated by white arrowhead at 15mpi intervals. A TR-dextran labeled vacuole with no bacterium is indicated by the white open arrowhead for comparison. **(B)** Fold change in *aqp1* mRNA expression in MEFs quantified by qRT-PCR, setting the value for *tbk1*^{+/+} MEFs = 1 (n=3; ***p*<0.001). Values for *aqp1* expression were normalized to levels of *actin* expression. **(C)** Immunoblot analysis of 20μg whole cells lysate from *tbk1*^{+/+} and *tbk1*^{-/-} MEFs and probed with anti-TBK1 antibody, then stripped and re-probed with anti-AQP1 antibody. **(D)** Immunoblot analysis of 50μg of brain, liver, kidney, and spleen tissue homogenate. The immunoblot was probed with anti-TBK1 antibody, then stripped, and re-probed with anti-AQP1 antibody.

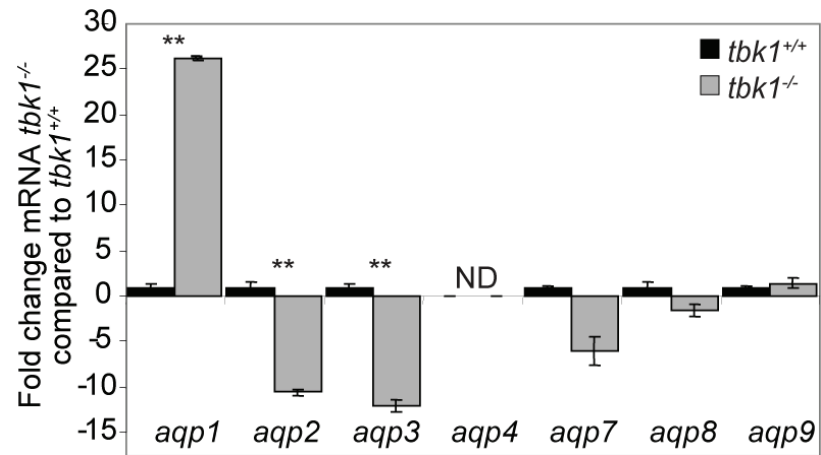


Figure 3.2. Influence of TBK1 on AQPs mRNA expression levels. Fold changes in *aqp* mRNA expression in MEFs quantified by qRT-PCR, setting the value for *tbk1*^{+/+} MEFs as 1 (n=3; ***p*<0.001). ND: not detected.

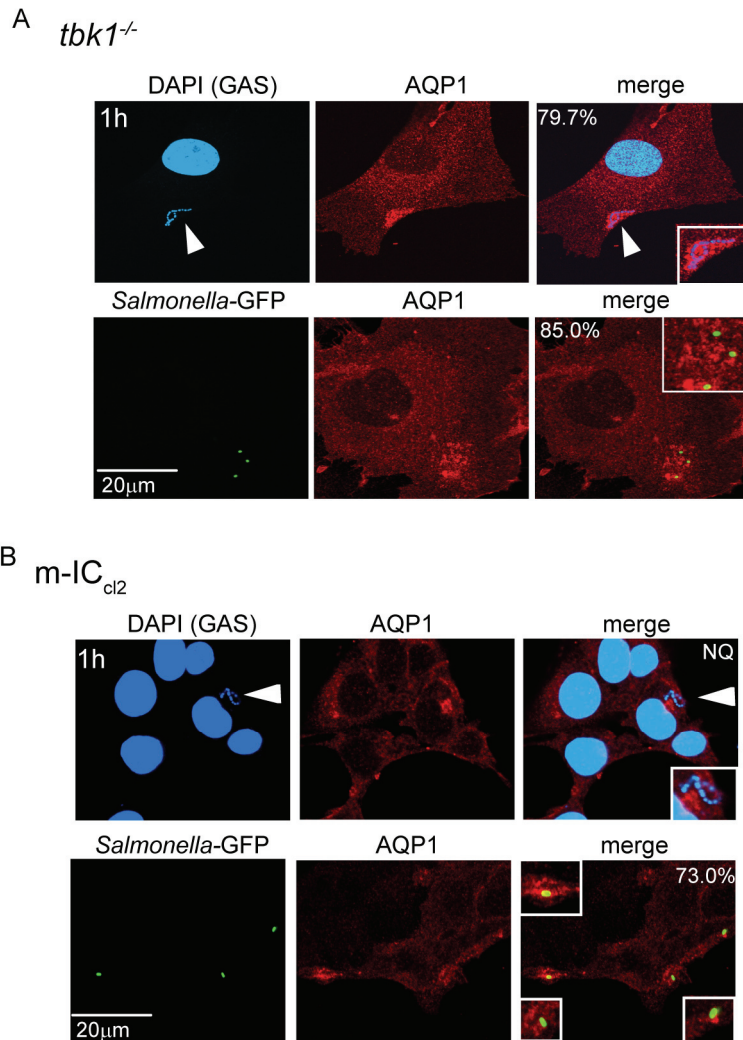


Figure 3.3. AQP1 colocalizes with pathogen-containing vacuoles.

Confocal immunofluorescence microscopy of *tbk1*^{-/-} MEFs (**A**) and m-IC-cl2 (**B**) infected for 1h pi with GAS (arrowheads) as observed by DAPI staining, or *Salmonella*-GFP, and stained with anti-AQP1 antibody, followed by a rhodamine-conjugated secondary antibody. Percentages represent # bacteria/100 bacteria colocalized with AQP1 as quantified by confocal imaging from 3 independent experiments (NQ=not quantitated).

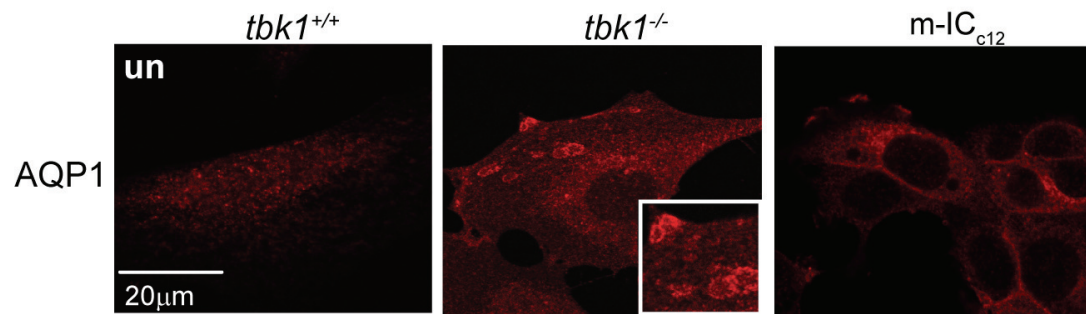


Figure 3.4. Subcellular localization of AQP1 in uninfected cells. Confocal immunofluorescence microscopy of uninfected *tbk1*^{+/+} MEFs, *tbk1*^{-/-} MEFs, and m-IC_{c12} stained with anti-AQP1 antibody.

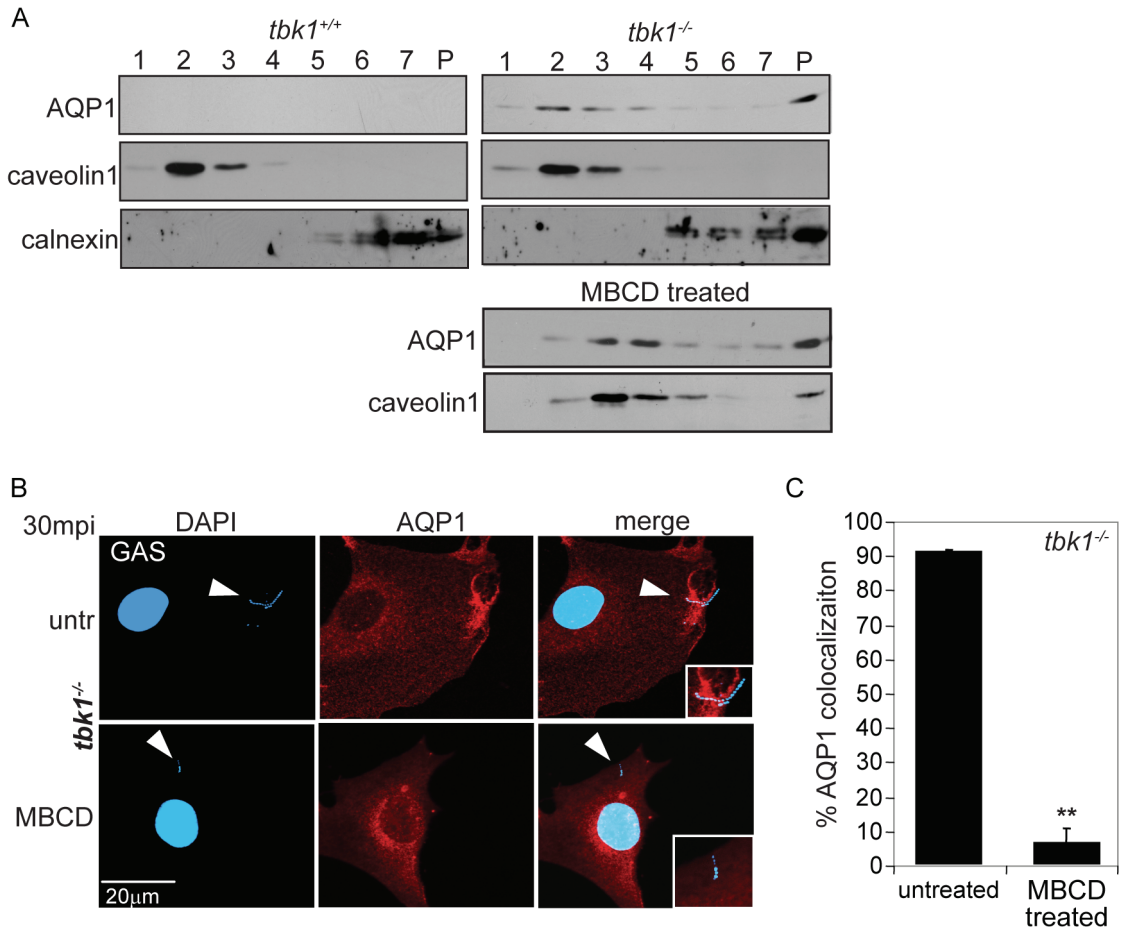


Figure 3.5. PCV can acquire AQP1 upon pathogen entry at cholesterol rich microdomains (CRM). (A) Immunoblot analysis of non-detergent based isolation CRM from *tbk1*^{+/+} and *tbk1*^{-/-} MEFs. Numbers represent sequential fractions taken from a discontinuous sucrose gradient containing cell lysate; 1-top fraction, P-pellet. CRM located in fractions 1-3, non-CRM fractions located in fractions 4-7, and the pellet contains whole cells. The immunoblots were probed for AQP1, caveolin-1; control for CRM localized proteins, and calnexin; control for non-CRM protein localization. *Tbk1*^{-/-} MEFs were treated for 1hr with MBCD to deplete cholesterol and probed with anti-AQP1 and anti-caveolin-1 antibody. (B) Confocal immunofluorescence microscopy of *tbk1*^{-/-} MEFs untreated or treated with MBCD for 1hr to deplete membrane cholesterol, washed, and replaced with medium without FBS. Cells were infected for 1h pi with GAS (arrowheads) and stained with anti-AQP1 antibody and DAPI to visualize bacteria. (C) *Tbk1*^{-/-} MEFs treated as in (B); percentages represent # of GAS that colocalized with AQP1/100 bacteria from 3 independent experiments (** $p < 0.001$).

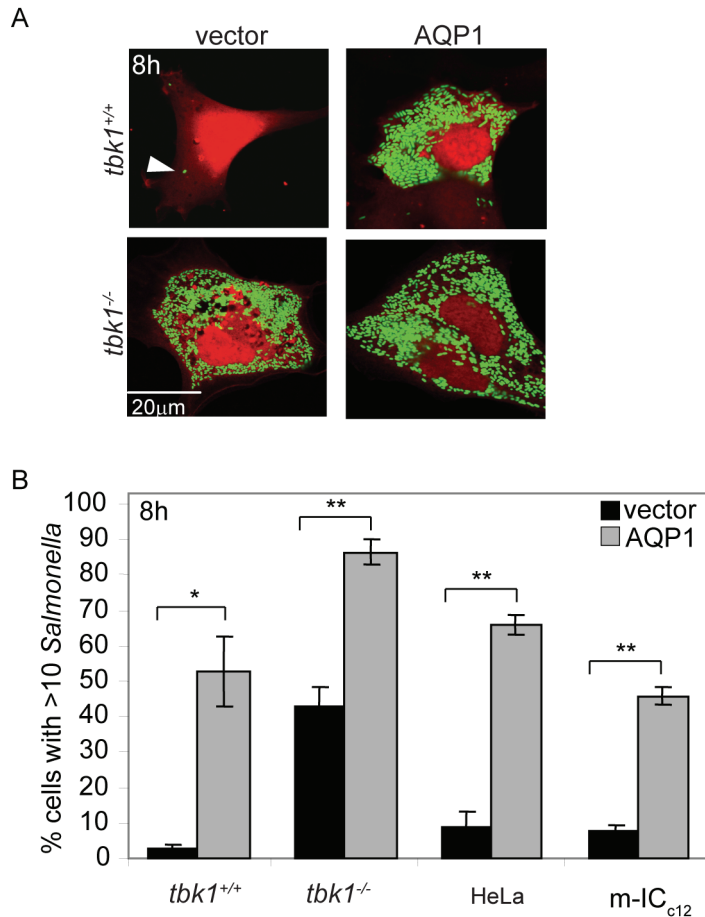


Figure 3.6. Dysregulated AQP1 expression allows bacterial escape into the host cytosol. (A) Merged confocal immunofluorescence images representative of MEFs transfected with mCherry-N1 (transfection control) and expression vector or *aqp1*:pcDNA3, infected at a m.o.i. of 10 with *Salmonella*-GFP, and fixed at 8h pi. **(B)** MEFs, HeLa, and m-IC_{c12} cells transfected with *aqp1*:pcDNA3 or vector alone, infected with *Salmonella*-GFP at a m.o.i. of 10, and fixed at 8h pi. Percentages represent # of cells with >10 *Salmonella*/100 infected+transfected cells from 3 independent experiments (* $p < 0.05$ and ** $p < 0.001$).

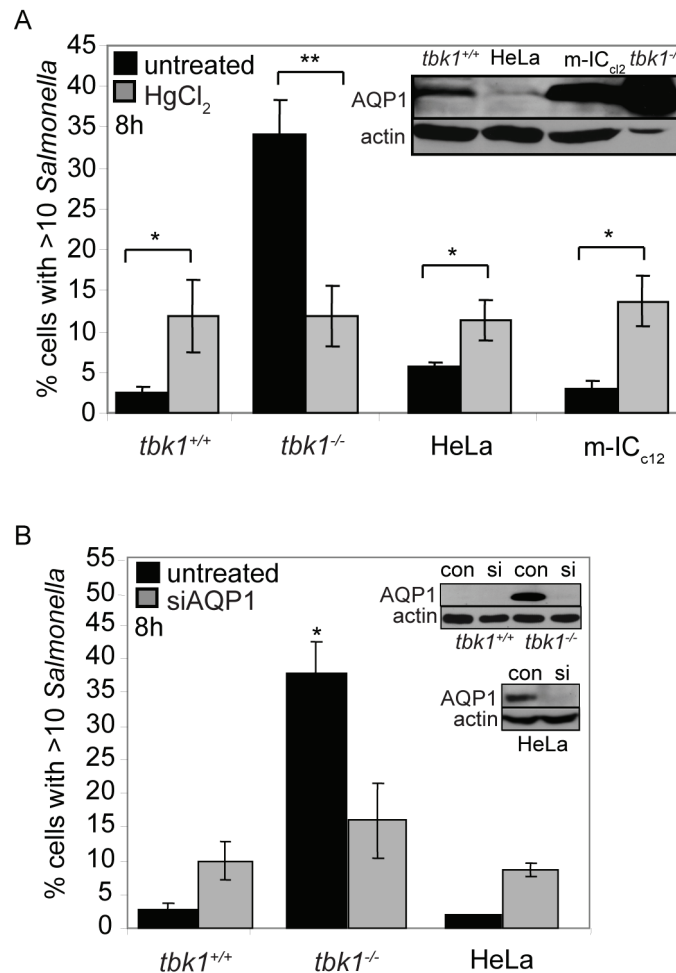


Figure 3.7. AQP1 enhances integrity of pathogen-containing vacuoles.

(A) MEFs, HeLa, and m-IC_{c12} cells untreated or treated with HgCl₂, infected with *Salmonella*-GFP, and fixed at 8h pi. Fixed cells were stained with Texas Red-phalloidin and analyzed by standard epifluorescence microscopy. Percentages reflect # of cells with >10 *Salmonella*/100 infected cells from 3 independent experiments (**p*<0.05 and ***p*<0.001). Insert: Anti-AQP1 immunoblot of *tbk1*^{+/+} MEFs, HeLa, m-IC_{c12}, and *tbk1*^{-/-} MEF cells. Lysate from *tbk1*^{-/-} MEFs was deliberately underloaded to allow visualization of AQP1 in wildtype cells. The blot was reprobbed with anti-actin antibody as a loading control. **(B)** MEF and HeLa cells transfected with control siRNA or *aqp1* siRNA, infected with *S. typhimurium*, and fixed 8h pi. Fixed cells were stained with Texas Red-phalloidin and analyzed by standard epifluorescence microscopy. Percentages represent # of cells with >10 *Salmonella*/100 infected cells from 3 independent experiments (**p*<0.05 and ***p*<0.001). Insert: Anti-AQP1 immunoblot of MEFs and HeLa cells treated with control (con) or *aqp1* (si) siRNA. Blots were reprobbed with anti-actin antibody as a loading control.

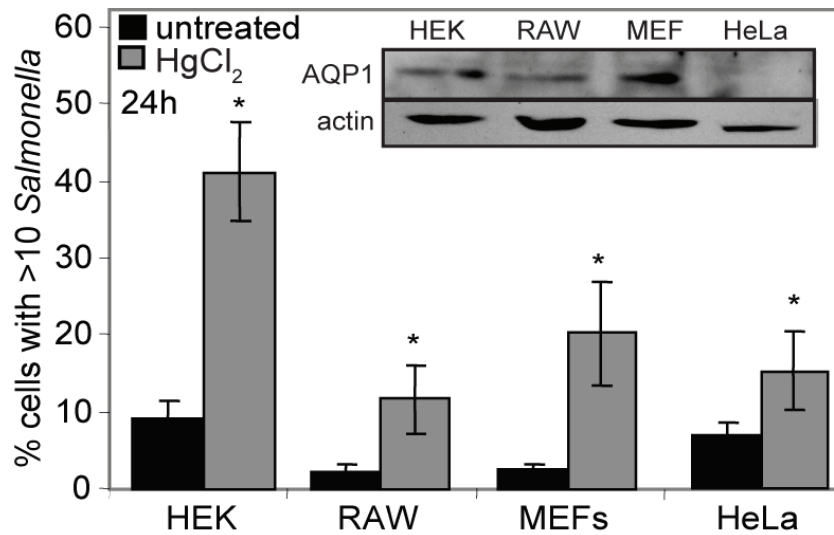


Figure 3.8. Dysregulated AQP1 function leads to loss of PCV integrity. MEFs, HeLa, HEK, and RAW264.7 cells were left untreated or treated with HgCl₂ and infected with *S. typhimurium* and fixed at 24h pi. Percentages reflect # cells with >10 *Salmonella*/100 cells (n=3); mean of triplicates ± SD is shown. * indicate p < 0.05 comparing treated to untreated cells for each cell type. Insert: Immunoblot of AQP1 expression in cells using anti-AQP1 antibody. Blots were loaded with ~3 times more protein than in Figure 3.1C to permit visualization of AQP1, and re-probed with anti-actin antibody as a loading control.

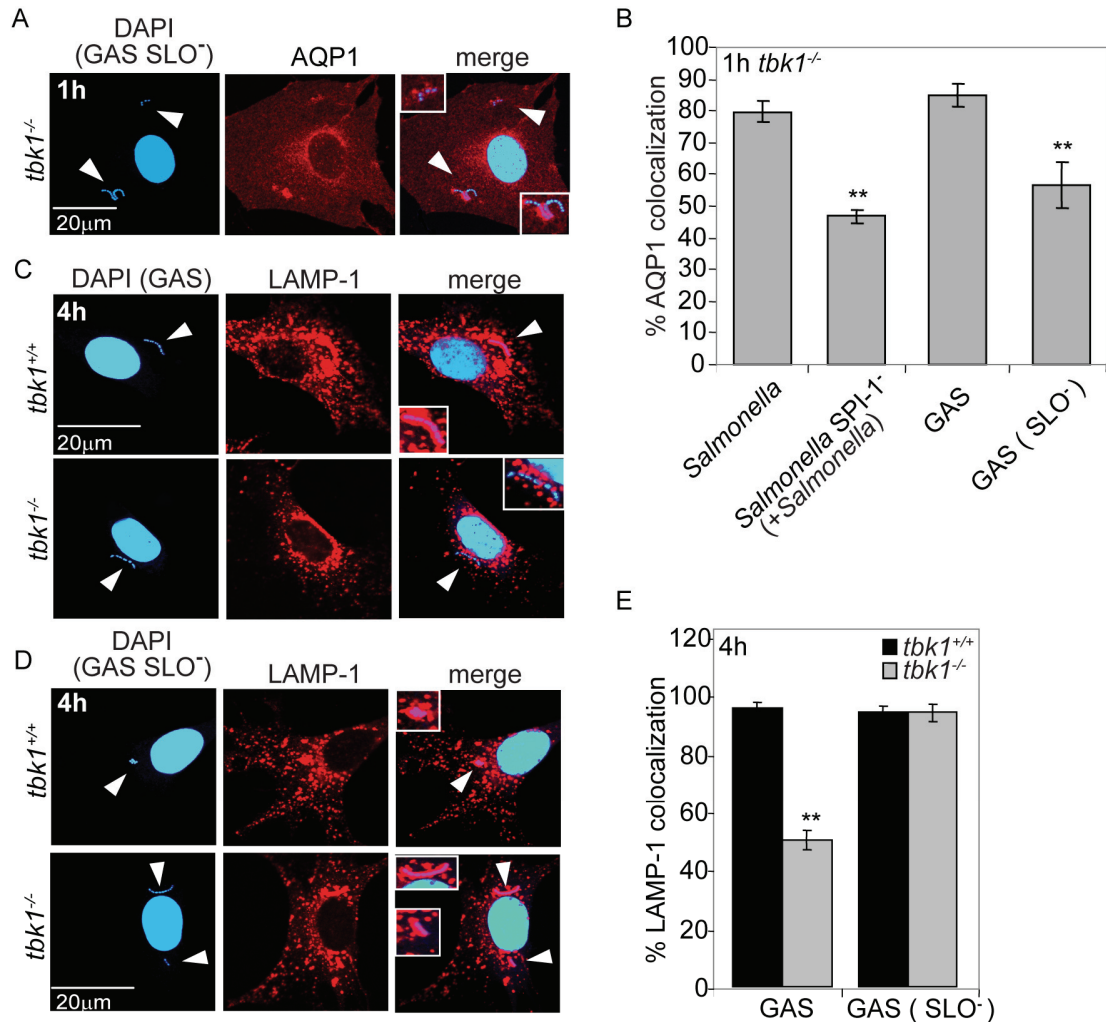
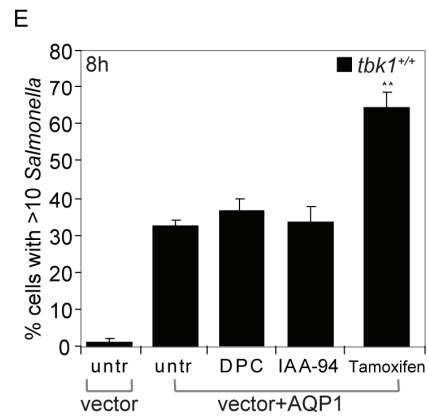
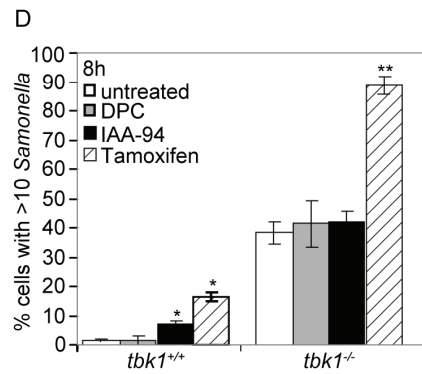
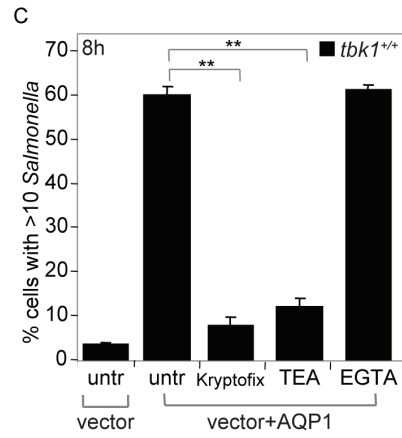
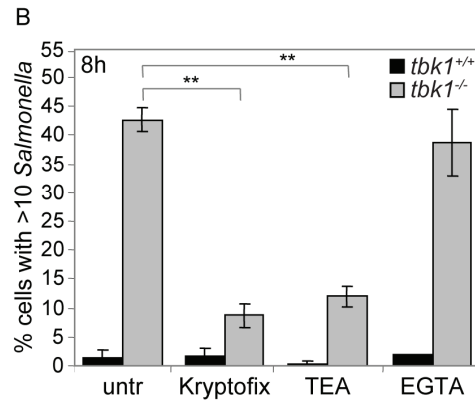
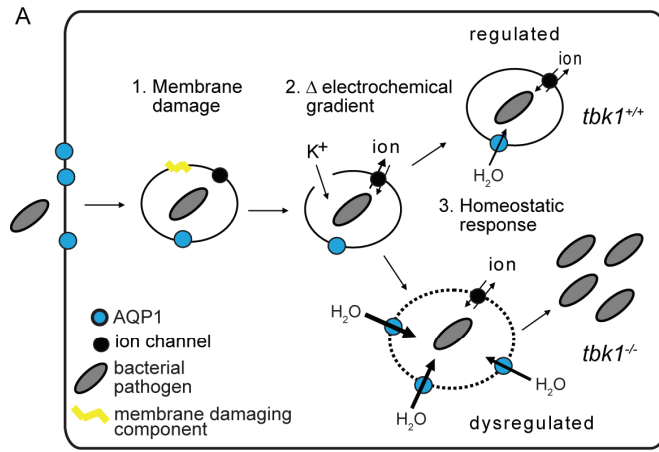


Figure 3.9. Bacterially induced membrane damage triggers PCV rupture in cells with a dysregulated homeostatic response. (A) Confocal immunofluorescence microscopy of *tbk1*^{-/-} cells infected with SLO⁻ GAS (arrowheads), fixed at 1h pi, and stained with anti-AQP1 antibody and DAPI to visualize bacteria. (B) *Tbk1*^{-/-} MEFs infected with wildtype *S. typhimurium*, *S. typhimurium* SPI-1⁻ (co-infected with wildtype *S. typhimurium* to induce uptake of the non-invasive mutant strain into independent vacuoles), wildtype GAS, or SLO⁻ GAS, fixed 1h pi, stained with anti-AQP1 antibody and DAPI to visualize bacteria, and analyzed by confocal microscopy. Percentages represent # bacteria colocalized with AQP1/100 bacteria from 3 independent experiments (***p*<0.001). (C-D). Confocal immunofluorescence microscopy of MEFs infected with either wildtype GAS (C) or a GAS SLO⁻ mutant (D) (white arrowheads), fixed at 4h pi, and stained with anti-LAMP-1 antibody and DAPI to visualize bacteria. (E) MEFs treated as in (C-D); percentages represent # bacteria colocalized with LAMP-1 /100 bacteria as analyzed by confocal microscopy from 3 independent experiments (***p*<0.001).

Figure 3.10. Ion flux modulates AQP1-dependent vacuole homeostasis. (A) A model of AQP1 function in modulating vacuolar homeostasis to control membrane integrity during bacterial infection. (B) MEFs were treated for 1h with Kryptofix (potassium chelator), TEA (potassium channel inhibitor), or EGTA (calcium chelator), infected with *Salmonella*-GFP, fixed at 8h pi, stained with TR-Phalloidin, and analyzed by standard epifluorescence microscopy. Percentages represent # cells with >10 *Salmonella*-GFP/100 cells at 8h pi from 3 independent experiments (** $p < 0.001$). (C) *Tbk1*^{+/+} MEFs were transfected with expression vector alone or *aqp1*:pcDNA3 and treated with Kryptofix, TEA or EGTA for 1h. Cells were infected with *Salmonella*-GFP and fixed at 8h pi. The percentages represent # of transfected cells with >10 *Salmonella*/100 cells at 8h pi from 3 independent experiments (** $p < 0.001$). (D) MEFs were left untreated or treated for 1h with the chloride channel inhibitors DPC (broad specificity inhibitor of inwardly rectifying Cl⁻ channels), IAA-94 (blocks Ca²⁺ activated Cl⁻ channels), and Tamoxifen (inhibitor of volume activated outwardly rectifying Cl⁻ channels). Cells were then infected with *Salmonella*-GFP in the presence of inhibitor, fixed 8h pi, stained with TR-Phalloidin, and analyzed by standard epifluorescence microscopy. Percentages represent # cells with >10 *Salmonella*-GFP/100 cells at 8h pi from 3 independent experiments (* $p < 0.05$ and ** $p < 0.001$). (E) *Tbk1*^{+/+} MEFs were transfected with *aqp1*:pcDNA3 or vector alone, and left untreated or treated with DPC, IAA-94 or Tamoxifen for 1h. Cells were then infected with *Salmonella*-GFP in the presence of inhibitor and fixed at 8h pi. The percentages represent # transfected cells with >10 *Salmonella*-GFP/100 cells at 8h pi from 3 independent experiments (* $p < 0.05$ and ** $p < 0.001$).



| gene | primer sequence |
|----------------|---|
| qRT-PCR | |
| <i>actin</i> | F-5' AGGTGTGATGGTGGGAATGG R-5' GCCTCGTCACCCACATAGGA |
| <i>aqp1</i> | F-5' AATGCTACAGCTTGTGTTGCAGCC R-5' ACCTGAAGAATGTGGCTCTCGGTT |
| <i>aqp2</i> | F-5' TGCTCTCCACAACAATGCAACAGC R-5' TCATGGAGCAGCCGGTCAAATAGA |
| <i>aqp3</i> | F-5' ATCTGGGCCTTTGCCAACAATGAG R-5' TCAACGATGGCCAGTACACACACA |
| <i>aqp4</i> | F-5' TGCCAGCTGTGATTCCAAACGAAC R-5' GTGGTTTGCCCAGTTTCCCATGAT |
| | F-5' TGCCAGCTGTGATTCCAAACGAAC R-5' GTGGTTTGCCCAGTTTCCCATGAT |
| <i>aqp7</i> | F-5' ATGATGGTGTGGCCTTGGTTCC R-5' AAAGTCACTGCGGCATTCATGTGG |
| <i>aqp8</i> | F-5' CCCAACACAAAGCTCTTCGTGGAA R-5' TGAAGTGTCCACCGCTACAACCAA |
| <i>aqp9</i> | F-5' ATACCTGTCGTGGGCCCTATGATT R-5' TGCCACTACATGATGACGCTGAGT |
| cloning | |
| <i>aqp1</i> | F-5' GGGGTACCGGAGTTGAGCACCAGGCATCCAGCG R-5' GAATTCCGTTGGGCCAATGCAGAGAGAAGACT |

Table 3.1. Primer sequences used in Chapter 3.

Chapter 4

***Listeria monocytogenes* exploits CFTR-mediated chloride transport to escape the pathogen-containing vacuole**

1. Abstract

Listeria monocytogenes is a cytosolic bacterial pathogen whose virulence is dependent on vacuolar escape mediated by the pore-forming toxin listeriolysin O (LLO). Previous studies have suggested a multistep process of vacuole perforation followed by rupture, however the role of the host cell in *L. monocytogenes* escape from the vacuole is ill-defined. Here we show that *L. monocytogenes* cytosolic entry is dependent on vacuolar ion transport and concentration. *L. monocytogenes* vacuolar escape was also suppressed upon pharmacological inhibition of the cystic fibrosis transmembrane conductance regulator (CFTR) or in CFTR Δ F508 mutant peritoneal macrophages. Treatment of wildtype mice with a CFTR inhibitor markedly decreased dissemination of *L. monocytogenes* across the gastrointestinal barrier. We show that high sodium chloride concentration enhances LLO activity and provide evidence for a model where CFTR-mediated chloride transport potentiates LLO pore formation and vacuole rupture.

2. Introduction

Listeria monocytogenes (*L. monocytogenes*) is a ubiquitous food-borne pathogen that can infect intestinal epithelial cells of the gastrointestinal (GI) tract, as well as disseminate across the intestinal barrier to the bloodstream of most mammals (Gaillard et al., 1991, Cossart, 2007). *L. monocytogenes* is an intracellular cytosolic pathogen that enters host cells through phagocytosis or by internalin-mediated uptake, resulting in bacterial confinement to a membrane bound vacuole that does not support replication. Within the vacuole, *L. monocytogenes* secretes a cholesterol-dependent cytolysin (CDC), listeriolysin O (LLO) that forms small pores in the phagosomal membrane leading to vacuolar rupture and *L. monocytogenes* entry into the cytosol where replication occurs. In the cytosol, *L. monocytogenes* nucleates host actin polymerization to form actin tails that propel the bacterium from one cell to another (Tilney et al., 1990). *L. monocytogenes* cytosolic entry is primarily LLO dependent, but the host contribution in mediating escape is poorly understood (Cossart et al., 1989). The initial ~25 nm pore formed by LLO oligomers would not be large enough for bacterial escape, and events preceding the LLO-induced membrane perforation resulting in vacuole rupture are not known (Heuck et al., 2003, Shaughnessy et al., 2006, Rossjohn et al., 1997, Portnoy et al., 1988).

In previous work, we demonstrated the contribution of water and ion channels in the regulation of vacuolar homeostasis to control intracellular bacterial infection (Radtke et al., 2008). Phagosomes containing the vacuolar pathogen *Salmonella enterica* serovar

Typhimurium ruptured upon perturbation of host homeostatic regulation, resulting in release of the bacteria into the cytosol. These data led us to hypothesize that similar host homeostatic mechanisms might also be exploited by *L. monocytogenes* to escape the vacuole. Vacuolar homeostasis can be defined as a steady-state equilibrium between the cytosol and the vacuolar luminal environment (Alberts *et al.*, 1994). The vacuolar membrane functions to separate these two cellular compartments, and membrane transporters are integral in establishing pH, osmotic/volume, or ionic homeostatic gradients necessary for cellular function (Romero, 2004). Vacuolar homeostasis dynamically is maintained by membrane channels and transporters that respond to perturbations in the phagosomal environment by acting to restore equilibrium (Dubyak, 2004). Transporters play a critical role in controlling vacuolar ion concentrations by facilitating directed ion movement across the vacuolar membrane to maintain the physiological function of this compartment.

The vacuolar compartment is often subject to membrane damage as a result of external and internal environmental sampling, with uptake of pathogens being a common cause of vacuolar membrane wounding (Reddy *et al.*, 2001). For example, *Salmonella enterica* serovar Typhimurium and *Yersinia pseudotuberculosis* transiently puncture membranes using the type III secretion system, and *L. monocytogenes* and *Streptococcus pyogenes* secrete membrane pore-forming toxins that perforate the membrane, causing pH and ion gradient dysregulation (Shaughnessy *et al.*, 2006, Viboud *et al.*, 2001, Schoehn *et al.*, 2003, Bhakdi *et al.*, 1995). However, vacuolar rupture is generally avoided due to triggering of calcium-dependent membrane repair mechanisms and

restoration of disrupted gradients by membrane ion transporters (Shaughnessy *et al.*, 2006, Reddy *et al.*, 2001, Roy *et al.*, 2004).

The phagosome is rich in ion transporters that can assist in the maintenance of vacuolar homeostasis (Mellman, 1996, Haas, 1998). One such transporter, the cystic fibrosis transmembrane conductance regulator (CFTR), is a well characterized chloride transporter that maintains ion and fluid homeostasis in membranes of epithelial cells and alveolar macrophages (Li *et al.*, 1996, Norez *et al.*, 2004, Gabriel *et al.*, 1993, Di *et al.*, 2006). CFTR is present on the plasma membrane, endosomes, and other intracellular compartments and may contribute to phagosomal acidification in alveolar macrophages and hypochlorous acid production in polymorphonuclear neutrophils (Bertrand *et al.*, 2003, Gabriel *et al.*, 1993, Painter *et al.*, 2008, Biwersi *et al.*, 1994). CFTR is hypothesized to aid in the clearance of *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* from the lungs (Jarry *et al.*, 2006, Di *et al.*, 2006, Painter *et al.*, 2008). Additionally, CFTR was also proposed to function as a receptor mediating *P. aeruginosa* and *Salmonella* Typhi epithelial invasion (Schroeder *et al.*, 2002, Pier *et al.*, 1998, Pier, 2002, Pier *et al.*, 1996). These reports demonstrated a previously unrecognized role for CFTR in promoting pathogenesis and, depending upon the bacterium, either controls or enhances infection. Here we show that CFTR-mediated chloride transport is exploited by *L. monocytogenes* to escape the vacuole.

LLO is an essential virulence factor of *L. monocytogenes* that is required for vacuolar escape (Portnoy *et al.*, 1988). *L. monocytogenes* mutants lacking LLO are sequestered in the phagosome, which eventually fuses with LAMP-1⁺ lysosomes (Gaillard *et al.*, 1986, Marquis *et al.*, 1997). When the non-pathogenic bacterium

Bacillus subtilis was engineered to produce LLO, some of the organisms could enter the macrophage cytosol. These observations suggest LLO is the only bacterial factor required for escape, although the contribution of the host remains largely unknown (Bielecki *et al.*, 1990). Much of what is known about LLO structure and function has been modeled after the well characterized CDC family member, perfringolysin O (PFO) (Heuck *et al.*, 2003, Rossjohn *et al.*, 1997, Shaughnessy *et al.*, 2006). CDCs bind to cholesterol in mammalian membranes, followed by oligomerization and insertion into the membrane to form a pore composed of 35-50 monomers and ~25 nm in diameter (Figure 4.1) (Tveten, 2005, Olofsson *et al.*, 1993). To maintain the integrity of the intracellular niche, it is critical that LLO pore formation be maximized within the vacuole, but minimized in the cytosol. Both the host and *L. monocytogenes* have mechanisms regulating LLO expression or function (Kreft *et al.*, 2001, Schnupf *et al.*, 2007). Host cells regulate LLO function through cellular compartmentalization. In the cytosol, LLO can be phosphorylated, ubiquitylated, and degraded, minimizing LLO activity, and protecting the plasma membrane from LLO-mediated lysis (Schnupf *et al.*, 2006, Schnupf *et al.*, 2007). When LLO is compartmentalized within the phagosome, several mechanisms contribute to increase pore formation. **First**, LLO activity is optimal at pH 5.5, a pH characteristic of late endosomes and lysosomes generated by the activity of the host vacuolar ATPase (Glomski *et al.*, 2002, Haas, 1998, Beauregard *et al.*, 1997). **Second**, the phagosome houses the vacuolar enzyme γ -interferon-inducible lysosomal thiol reductase (GILT) that reduces the single cysteine of LLO, necessary for oligomerization and pore formation (Singh *et al.*, 2008). High sodium chloride is hypothesized to increase LLO stability and hemolytic activity, but the effect of high

sodium chloride on LLO activity has not been definitively demonstrated experimentally. High sodium chloride is predicted to neutralize charges on critical residues of LLO, a necessary step for oligomerization and insertion of LLO into membranes (Figure 4.1) (Schuerch *et al.*, 2005, Saito *et al.*, 2003, Walton *et al.*, 1999). Since we previously found that pathogen-induced membrane damage altered host ion flux, we hypothesize that host-mediated ion transport in response to *L. monocytogenes* perturbation of vacuolar homeostasis potentiates LLO activity, resulting in vacuole lysis.

Here we provide evidence for a novel mechanism exploited by *L. monocytogenes* to escape the vacuole. We found that CFTR-mediated chloride transport was required for *L. monocytogenes* to efficiently escape the vacuole in peritoneal macrophages and epithelial cells. CFTR was critical for *L. monocytogenes* dissemination across the intestinal epithelial barrier to cause systemic infection in a murine model of oral listeriosis. These data suggest that CFTR activity could be acting to enhance LLO activity within the vacuole. Overall, these data suggest a previously uncharacterized mechanism of bacterial exploitation of a host homeostatic process necessary for the establishment of disease.

3. Results

3.1 Ion flux is required for *L. monocytogenes* vacuolar escape

Previous work has shown that LLO-dependent perforation results in disruption of vacuolar ion concentrations, but this perforation does not result in immediate phagosome

rupture, and subsequent ill-defined event must occur for rupture and bacterial release (Shaughnessy *et al.*, 2006). To test the hypothesis that host ion flux is required for the rupture of the vacuole and release of *L. monocytogenes* into the cytosol, we utilized chelators of potassium, chloride, and calcium, the major ions that regulate electrochemical gradients in the cell, during *L. monocytogenes* infection (Figure 4.2A). The murine peritoneal macrophage cell line RAW264.7 (RAW) and human epithelial colorectal cell line Caco-2, representative of cell types infected during listeriosis, were treated with MQAE (chloride chelator), Kryptofix (potassium chelator), and EGTA (calcium chelator) prior to infection. One hour after chelator treatment, cells were infected with *L. monocytogenes*, fixed at 2 hours post infection (hpi), and the number of bacteria out of 100 bacteria that colocalized with actin, a marker of cytosolic localization, was quantitated. In the presence of potassium and chloride chelators, *L. monocytogenes* exhibited significantly less actin colocalization compared to untreated cells, whereas chelation of calcium had no effect on *L. monocytogenes* escape. These data suggest that potassium and chloride, but not calcium, are needed to allow *L. monocytogenes* escape from the vacuole. To test that the chelators were not differentially affecting actin polymerization of the host cell, we treated RAW cells with MQAE and Kryptofix at the indicated times post-infection, fixed cells at 2hpi, and determined *L. monocytogenes* actin colocalization (Figure 4.2B). In cells treated with chelators starting at 30, 60, or 90 mpi, actin polymerization around the bacteria at 2hpi was not affected by the ion chelators, with observable actin colocalization with *L. monocytogenes*. These data demonstrate that potassium and chloride ions must be present and accessible between 0-30 mpi for *L. monocytogenes* to escape the vacuole.

Cellular ion regulation can play a role in vesicular trafficking in different physiological contexts; thus, we tested if host cell trafficking was altered in the presence of Kryptofix and MQAE (Figure 4.2C) (Mellman *et al.*, 1986, Johnson *et al.*, 1993). RAW cells, in the presence or absence of ion chelators, were infected with the *L. monocytogenes* LLO-deficient mutant that is unable to escape the vacuole, and the percent of bacteria colocalized with the late endosomal marker LAMP-1 were quantitated at the indicated times pi (Gaillard *et al.*, 1986). The wildtype *L. monocytogenes* strain was not used because the bacterium modulates trafficking of the phagosome to avoid fusion with lysosomes, and also escapes in an asynchronous manner (Gaillard *et al.*, 1986, Shaughnessy *et al.*, 2006). There was no significant difference in *L. monocytogenes* LLO⁻ mutant LAMP-1 acquisition upon treatment of cells with the chelators compared to untreated cells, suggesting that kinetics of phagolysosomal fusion were largely unaffected in the presence of MQAE or Kryptofix. The chelators also did not affect *L. monocytogenes* growth in broth culture at the same concentrations used during intracellular infection (data not shown). These data show that ion concentrations are critical for *L. monocytogenes* escape from the vacuole, but do not appear to alter overall kinetics of phagolysosome fusion.

3.2 *L. monocytogenes* vacuolar escape is mediated by CFTR

To identify the host regulators responsible for the ion movement between the cytosol and vacuole necessary for *L. monocytogenes* escape, we utilized broad spectrum inhibitors that blocked the function of ion transporter families and monitored the effect on *L. monocytogenes* escape. The anion channel inhibitors Diphenylamine-2-carboxylic

acid (DPC), natriuretic peptide precursor type B (NPPB), glyburide, and (Dihydroindenyl)oxy]acetic acid (DIOA) resulted in significant inhibition of *L. monocytogenes* escape, and all are commonly used to inhibit the chloride transporter CFTR (Figure 4.3A and data not shown) (Zhang *et al.*, 2000, Ito *et al.*, 2001). To test if CFTR-mediated chloride transport was playing a role in *L. monocytogenes* vacuolar escape, we treated RAW cells at the times indicated pre- and post-infection with CFTR(inh)-172, a specific CFTR inhibitor, and DPC, a less selective chloride channel inhibitor (Caci *et al.*, 2008, Ma *et al.*, 2002, McDonough *et al.*, 1994, Schultz *et al.*, 1999). RAW cells were fixed at 2 hpi and *L. monocytogenes* colocalization with actin was quantitated (Figure 4.3A). RAW cells pretreated or treated at the time of infection with DPC and CFTR(inh)-172 significantly inhibited *L. monocytogenes* escape compared to untreated cells. When CFTR(inh)-172 was added after 30mpi, actin polymerization around the bacteria was observed, demonstrating that CFTR(inh)-172 does not affect actin polymerization *per se* of the cell and CFTR-mediated chloride transport must occur between 0-30 mpi for *L. monocytogenes* to escape the vacuole. In contrast, in cells treated at different times with DPC, *L. monocytogenes* colocalization with actin was not detected until DPC was added at 60mpi. These data suggest that ion flux through a DPC-sensitive transporter must occur between 30-60 mpi in order for *L. monocytogenes* vacuolar escape to occur. These results indicate two stages of chloride transport are necessary for *L. monocytogenes* escape, the first requiring CFTR (0-30 mpi), and the second requiring a distinct DPC-sensitive transporter (30-60 mpi). The work presented here will focus on the role of CFTR in mediating *L. monocytogenes* escape.

Pharmacological inhibitors can have non-specific effects on a cell; thus, to more directly test if CFTR was specifically contributing to *L. monocytogenes* vacuolar escape, we infected primary peritoneal macrophages (PMac) from wildtype mice or littermates with a F508 deletion allele of CFTR (Δ F508) (Figure 4.3). The F508 mutation is the most common mutation in patients with cystic fibrosis (CF) that results in inappropriate CFTR trafficking and dysregulated ionic and water homeostasis across membranes (Riordan, 2008). *L. monocytogenes* escape was significantly suppressed in the Δ F508 PMacs compared with wildtype, clearly demonstrating a role for CFTR in promoting *L. monocytogenes* vacuolar escape in this cell type. Treatment of wildtype PMacs with MQAE, DPC, and CFTR(inh)-172, inhibited *L. monocytogenes* escape to the same level as untreated Δ F508 PMac. Treatment of Δ F508 PMac with CFTR(inh)-172 had no further inhibition of *L. monocytogenes* escape compared to untreated Δ F508 PMac, demonstrating the specificity of this inhibitor in blocking CFTR-specific chloride transport. From these data we show that CFTR is necessary for *L. monocytogenes* vacuolar escape in physiologically relevant cell types.

3.3 Sodium chloride enhances LLO pore-forming activity

Previous studies hypothesized that high concentrations of sodium chloride stabilize LLO oligomerization and increase hemolytic activity (Schuerch *et al.*, 2005, Walton *et al.*, 1999, Myers *et al.*, 1993). We therefore hypothesized that CFTR-mediated chloride transport into the *L. monocytogenes* vacuole could result in increased LLO oligomerization and pore-forming activity. The increase in vacuolar pores would result in further homeostatic perturbation to the vacuole, leading to phagosomal rupture. To

validate that high concentrations of sodium chloride increased LLO activity, we treated both bacterial lysates from an *E. coli* strain expressing LLO upon IPTG induction (*E. coli* iLLO) or *L. monocytogenes* stationary phase supernatants, with 428mM sodium chloride, a concentration previously identified to significantly increase hemolytic activity of *L. monocytogenes* during stationary growth phase, and measured hemolytic activity (Figure 4.4A and B). Bacterial lysates and supernatants treated with 428mM sodium chloride resulted in a significant increase in hemolytic activity compared to control treated samples, suggesting high levels of sodium chloride induce LLO hemolytic activity. Lysates from *E. coli* not expressing LLO, or supernatants from *L. monocytogenes* LLO⁻ mutant, treated with either PBS or 428mM sodium chloride did not affect hemolysis of sheep red blood cells (SRBC). SRBC were also treated with 0.2% Triton X-100 detergent in the presence or absence of 428 mM sodium chloride that resulted in no significant RBC hemolytic difference between samples. These data corroborated that high concentration of sodium chloride does not alter hemolytic properties of RBC membranes. From these results, we demonstrate that high sodium chloride concentrations significantly increase LLO hemolytic activity.

To investigate if the increase in LLO hemolytic function was a result of an increase in LLO oligomerization, we analyzed the *E. coli* lysates from Figure 4.4B treated with either 428 mM sodium chloride or PBS by non-denaturing gel electrophoresis and examined LLO by immunoblot (Figure 4.4C). Using an anti-LLO antibody, we observed an increase in higher molecular weight forms of LLO upon the treatment of lysates with high sodium chloride, suggesting that enhanced LLO activity in the presence of high sodium chloride might be a result of increased LLO oligomerization,

a prerequisite step for pore formation. From these data, we hypothesize that host chloride transport enhances LLO oligomerization and activity resulting in vacuolar rupture.

3.4 CFTR promotes *L. monocytogenes* dissemination from the gastrointestinal tract

CFTR expression is most abundant in epithelial cells of the lungs and gastrointestinal (GI) tract of mammals, as well as in alveolar and peritoneal macrophages as compared to other macrophage types where CFTR is almost undetectable (Riordan, 2008, Di *et al.*, 2006) (data not shown). As a food-borne pathogen, the natural route of infection of *L. monocytogenes* is through the GI tract, where *L. monocytogenes* could exploit CFTR to invade cells, and disseminate to the liver and spleen (Cossart, 2007, Schleich, 2000). CFTR knock-out mice would be an ideal model to investigate the role of CFTR during a *L. monocytogenes* infection, however, these mice already suffer from intestinal dysfunction, low body weight, and altered expression of innate immune response genes, complicating interpretation of such an experiment (Davidson *et al.*, 2001, Norkina *et al.*, 2004, Canale-Zambrano *et al.*, 2007). Alternatively, we treated wildtype mice with the CFTR(inh)-172 analog, CFTR Inhibitor II (GlyH-101) that has superior pharmacokinetics in mice compared to CFTR(inh)-172, to determine the effect of CFTR inhibition on *L. monocytogenes* infection in mice (Muanprasat *et al.*, 2004). Thirty minutes after intraperitoneal injection of mice with one dose of inhibitor or DMSO control, the mice were infected orally with 10^9 *L. monocytogenes*, and bacterial dissemination into the liver and spleen was measured at ~18 hpi (Figure 4.5). There was approximately a 100-fold reduction of *L. monocytogenes* growth in the liver and spleen in the presence of the CFTR inhibitor compared to control treated mice. These observations

suggest that functional CFTR is necessary for *L. monocytogenes* to cross the gastrointestinal tract and establish infection in a murine model of listeriosis.

4. Discussion

Our studies have revealed an unanticipated role for CFTR in mediating the escape of *L. monocytogenes* from the vacuole, a process critical for infection. Previous work has shown that for *L. monocytogenes* to establish a successful infection, the bacteria must secrete the pore forming protein LLO to escape the vacuole and avoid fusion with lysosomes (Gaillard *et al.*, 1986, Portnoy *et al.*, 1988). However, the host contribution in the LLO-dependent rupture of the *L. monocytogenes* phagosome had been unclear. We demonstrated that chloride transporter is critical in mediating *L. monocytogenes* vacuolar escape. We identified CFTR as a host chloride transporter necessary for *L. monocytogenes* cytosolic entry and dissemination across the intestinal barrier of mice to establish infection.

From these data we propose a mechanism for CFTR-dependent escape of *L. monocytogenes* from the vacuole (Figure 4.6). We hypothesize that *L. monocytogenes* stimulates CFTR chloride transport in response to secreted LLO or by direct bacterial contact upon infection, consistent with reports of enteroinvasive bacteria inducing chloride and water secretion upon infection (Resta-Lenert *et al.*, 2002, Epple *et al.*, 2004). The presence of high chloride during *L. monocytogenes* uptake potentiated LLO oligomerization and pore formation in the vacuole. Vacuole homeostasis is disrupted as a result of LLO-mediated vacuolar perforation, and in response a second round of ion

transport occurs through DPC-sensitive channel(s) (30-60 mpi). Compounding LLO-mediated pores and subsequent ion and water transport, overwhelm mechanisms of repair and homeostasis. As a result, catastrophic membrane rupture occurs, releasing *L. monocytogenes* into the cytosol. This model puts forth a mechanism by which *L. monocytogenes* exploits host cell homeostatic processes to enhance infection, and identifies host chloride transport as a key parameter of *L. monocytogenes* virulence.

For many decades, *L. monocytogenes* has been used as a model to investigate such bacterial determinants of pathogenesis and has also served as a tool to elucidate fundamental mechanisms of host cell biology (Mackness, 1962). After many years of study, it was still not completely understood how *L. monocytogenes* escaped the vacuole and reached its replicative niche. Overall, there are still many questions to how intracellular pathogens acquire nutrients, replicate, and successfully establish their niche within the host cell, and this is especially true for less studied invasive pathogens (Garcia-del Portillo *et al.*, 1995). Our work, along with other recent studies, has identified host proteins and general cellular functions utilized by invasive pathogens to cause infection that previously had few if any connections with pathogenesis. Our studies identified a novel role for CFTR chloride transport in mediating *L. monocytogenes* escape from the vacuole. In other work, the host vacuolar ATPase that functions to acidify vacuoles was identified to be essential in the establishment of *Salmonella*, *L. monocytogenes*, and *Coxiella burnetii* infection (Beauregard *et al.*, 1997, Haas, 1998, Alpuche Aranda *et al.*, 1992, Maurin *et al.*, 1992). As previously described in this chapter, the host vacuolar protein GILT reduces LLO to allow pore formation that is necessary for *L. monocytogenes* to establish infection (Singh *et al.*, 2008). For a

successful *Legionella pneumophila* intracellular infection, recruitment of host mitochondria and ribosomes to the pathogen-containing vacuole correlates with *Legionella* survival, however the function of these proteins are not completely understood (Berger *et al.*, 1993). These few examples, demonstrate how several host homeostatic processes are exploited by pathogens to establish infection, and brings to light new mechanisms pathogens could utilize to establish their replicative niche. This work also displays the potential of using pathogens, such as *L. monocytogenes*, as probes to understand cellular homeostatic sensors and responses.

In summary, this work gives evidence for a model by which *L. monocytogenes* exploits CFTR-mediated chloride transport to escape the vacuole, and this event is essential for the establishment of infection by the oral route in mice. The exact mechanism of *L. monocytogenes* LLO-dependent escape from the vacuole was previously unknown, and we have identified a host chloride transporter as a key parameter in this escape. We found that vacuolar escape of *L. monocytogenes* is also dependent on host chloride transport that occurs in two stages. The first stage occurs in the initial 30 minutes of infection that involves CFTR-mediated chloride transport into the vacuole, presumably to increase LLO oligomerization and activity. The second stage includes DPC-sensitive transporter(s) that exacerbate vacuolar ionic disequilibrium in response to the increase in LLO-mediated vacuolar pores, resulting in the final catastrophic rupture of the phagosomal membrane. Only the first stage of CFTR-mediated chloride transport was investigated here, and studies are ongoing to identify DPC-sensitive transporter(s) causing the second stage of ion transport necessary for *L. monocytogenes* escape. Overall, this work identifies a mechanism of host homeostatic exploitation by a cytosolic

pathogen to establish its intracellular niche, and raises the potential that other membrane-damaging pathogens exploiting similar host mechanisms to cause infection as well.

5. Materials and Methods

Antibodies and reagents

Antibodies against specified antigens were obtained from the following sources: LAMP-1 (1D4B; Santa Cruz Biotechnology), *L. monocytogenes* (223021; BD Difco), LLO (ANT0006; Diatheva), TR-phalloidin (T-7471; Molecular Probes), TRITC-donkey anti-rat (64982; Jackson Immuno Research Laboratories), and FITC-goat anti-rabbit secondary (56894; Jackson Immuno Research Laboratories). Chemical reagents were obtained from Sigma-Aldrich except where otherwise specified. Where indicated, RAW cells were either pretreated for 1hr or at the times indicated post infection with 1.25 mM Kryptofix 2.2.2, 0.5mM ethylene glycol tetraacetic acid (EGTA), 0.25 mM *N*-phenylanthranilic acid (DPC), 5 mM MQAE (Invitrogen), 10 μ M CFTR(inh)-172. CFTR Inhibitor II (GlyH-101) was obtained from Calbiochem.

Cell culture, bacterial strains, and infections

RAW264.7 cells (ATCC) were grown in RPMI medium supplemented with 10% fetal bovine serum and 1% L-glutamine at 37°C in 5% CO₂. Caco-2 cells (ATCC) were grown in DMEM medium supplemented with 10% fetal bovine serum, 1% essential amino acids, 1% sodium pyruvate, and 1% L-glutamine at 37°C in 5% CO₂.

For cell culture infections, *Listeria monocytogenes* strains 10403S (wildtype) and DP-L2161 *hly*⁻(Δ LL0) were inoculated into liquid brain-heart infusion (BHI) broth and incubated at 30°C overnight without shaking (Freitag *et al.*, 1993) (Appendix A, Table A). Prior to infection, *L. monocytogenes* was washed and resuspended in PBS, and used to infect RAW264.7 and Caco-2 cells at a m.o.i. of 1 for 30 minutes. Cells were washed 3 times and corresponding medium with 50 μ g/ml gentamicin was added to cells.

Isolation and culture of peritoneal macrophages

Mice were sacrificed by administration of carbon dioxide, and 5 ml of ice-cold 20% sucrose solution was injected i.p. After gentle massaging of anterior and lateral walls of the abdomen, the peritoneal cavity was opened and the peritoneal lavage was collected, added to 40 ml PBS, and placed on ice. Cells were washed twice with PBS and resuspended in DMEM medium supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1% β -mercaptoethanol, and 30% L929 conditioned medium.

Microscopy

An Olympus BX60 Microscope with epi-fluorescence was used to quantitate the percentage of *L. monocytogenes* colocalized with actin or LAMP-1. Briefly, cells were infected with *L. monocytogenes*, fixed at the indicated times pi, permeabilized and stained with TR-phalloidin and *L. monocytogenes* stained with an anti-*Listeria* antibody followed by a FITC labeled goat-anti-rabbit secondary (Radtke *et al.*, 2007). The number of *L. monocytogenes* colocalized with actin per 100 bacteria was counted in at least 3

independent experiments. Colocalization of bacteria with LAMP-1 was determined by fixing cells at indicated times, staining the cells with anti-LAMP-1 antibody (TRITC conjugated to a donkey-anti-rat secondary) and anti-*Listeria* antibody (FITC conjugated to a goat-anti-rabbit secondary), then counting number of bacteria colocalized with LAMP-1 per 100 bacteria in 3 independent experiments.

Oral Infections

Twelve- to 16-week-old female C57BL/6J (#000664) mice were obtained from Jackson Laboratory. Mice were starved for 4 hours then fed 50 μ l 10% sodium bicarbonate orally and injected i.p. with 8 mg/kg CFTR Inhibitor II in 1.5% DMSO or 1.5% DMSO alone 30 min prior to infection. *L. monocytogenes* were grown to an OD₆₀₀ of 0.5 in BHI, washed with PBS, and resuspended at a concentration of 1.5×10^9 bacteria/0.1mL in PBS. Thirty minutes after inhibitor treatment, mice were infected orally with 100 μ l of 1.5×10^9 bacteria. At 16- to 18-hours pi, mice were sacrificed, and liver and spleens were removed. The organs were placed in a tube containing 1ml of 0.2% NP40 in PBS with beads, homogenized for 1 min at 6000 RPM in a MagNa Lyser (Roche), and the homogenate was plated on Luria Broth (LB) agar to quantitate colony forming units.

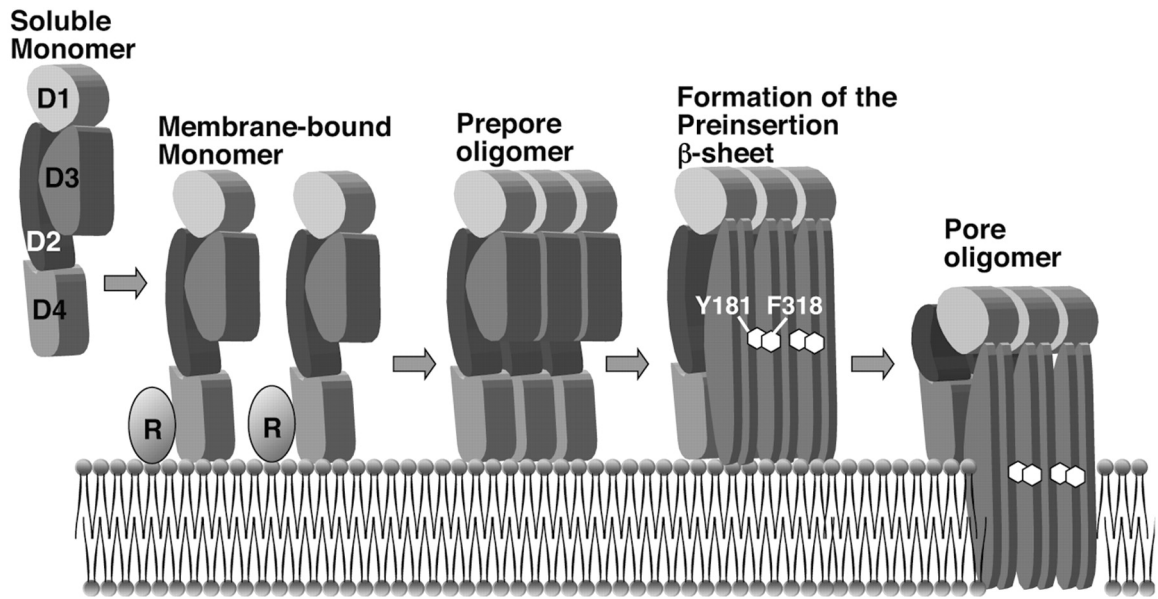
Hemolytic Assay

Stationary phase overnight culture of *L. monocytogenes* grown in Tryptic Soy Broth (TSB) was pelleted, 428mM NaCl or PBS (control) was added to supernatants for 5 minutes, and supernatants were incubated with 5 mM DTT for 1 hour at 37°C. A hemolytic assay was performed as described (Portnoy *et al.*, 1988). In short, two-fold

serial dilutions of bacterial supernatants were added to sheep red blood cells (SRBC) that were washed and resuspended in hemolysis assay buffer (HAB) adjusted to a pH 5.5 with glacial acetic acid. Samples were incubated at 37°C for 30 minutes, centrifuged, and optical density of the supernatant was analyzed at a wavelength of 545 nm. Hemolytic units were expressed as the reciprocal of the highest dilution showing complete hemolysis. Complete hemolysis was observed by adding 0.10% Triton X-100 (1/100 volume) to the sample.

Statistical analysis

Data sets in triplicate were analyzed using Microsoft Excel to calculate the Student's unpaired *t*-test for independent samples. Outliers were determined and removed according to IQRx1.5 rule (Xu *et al.*, 2003). *p*-values of <0.05(*) and <0.001(**) were considered significant and highly significant respectively.



(Tweten R, *Infection and Immunity* (2005) 10, 6199-205)

Figure 4.1. Proposed model of CDC insertion into membranes.

Soluble CDC monomers interact with host membrane (either by direct binding to cholesterol-rich membranes or via a specific receptor (R)), or can be exposed to high sodium chloride or low pH that neutralizes the charges on domains (D) 2 and 3, triggering structural changes in the monomer. These changes in D2 and D3 of the protein initiate oligomerization of the monomers into the prepore structure. The final prepore-to-pore conversion occurs upon monomers forming transmembrane β -barrel and inserting into the membrane.

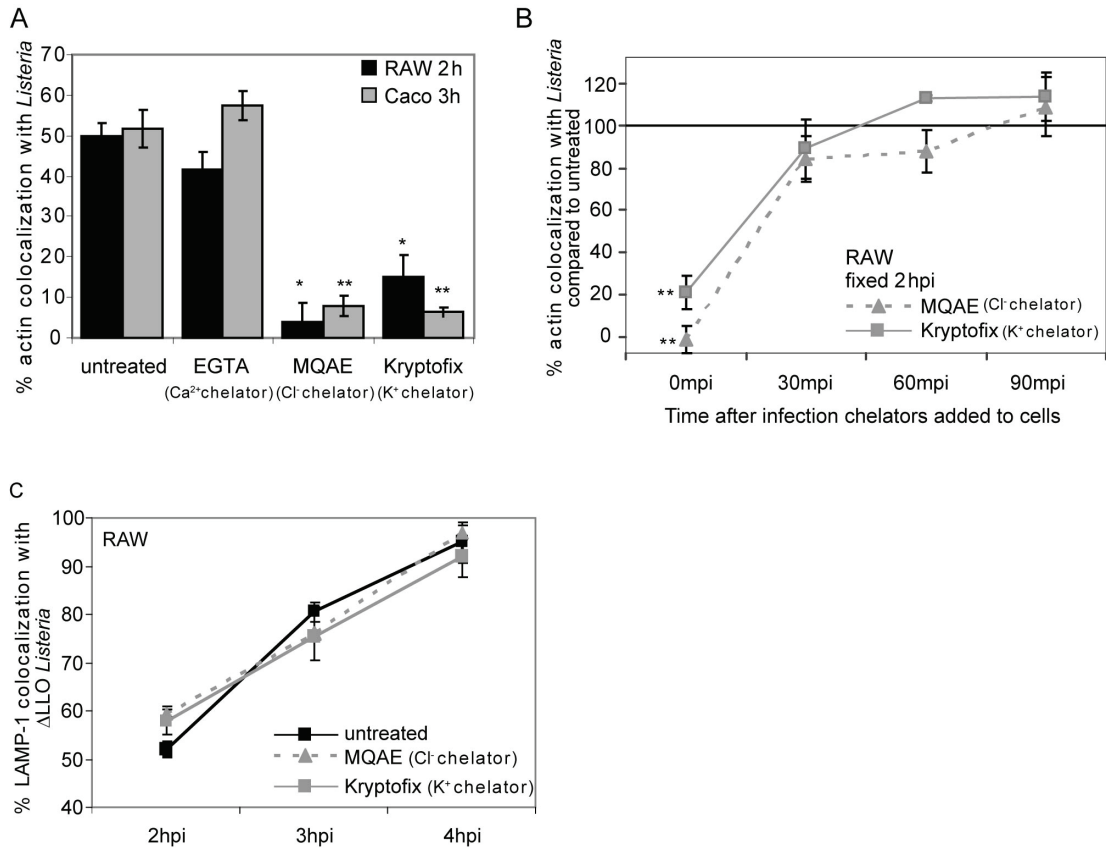


Figure 4.2. Host mediated ion flux is required for *L. monocytogenes* vacuolar escape. (A) RAW and Caco cells were treated for 1h with EGTA (calcium chelator), MQAE (chloride chelator), or Kryptofix (potassium chelator), infected with *L. monocytogenes*, fixed at 2h pi (RAW) or 3h pi (Caco), stained with TR-Phalloidin and anti-*Listeria* antibody, and analyzed by standard epifluorescence microscopy. Percentages represent # of bacterium colocalized with actin (marker of cytosolic entry) at indicated times for each cell (n=3, * $p < 0.05$ and ** $p < 0.001$, comparing treated with untreated cells for each cell type). (B) RAW cells were infected with *L. monocytogenes* and at the time of infection (0mpi), 30mpi, 60mpi, or 90mpi, were treated with MQAE or Kryptofix. The cells were fixed at 2hpi, stained with TR-Phalloidin and anti-*Listeria* antibody, and analyzed by standard epifluorescence microscopy. Black line represents untreated RAW cells infected with *L. monocytogenes* and fixed at 2hpi. Percentages represent # of bacterium/ 100 bacteria colocalized with actin compared to untreated, setting untreated at 100% (n=3, ** $p < 0.001$, comparing untreated with treated cells for each timepoint). (C) RAW cells were left untreated or treated for 1h with MQAE or Kryptofix, infected with *L. monocytogenes* LLO deficient mutant, fixed at 2, 3 and 4hpi, stained with anti-LAMP-1 and anti-*Listeria* antibodies, and analyzed by standard epifluorescence microscopy. Percentages represent # of bacterium colocalized with LAMP-1/100 bacteria (n=3).

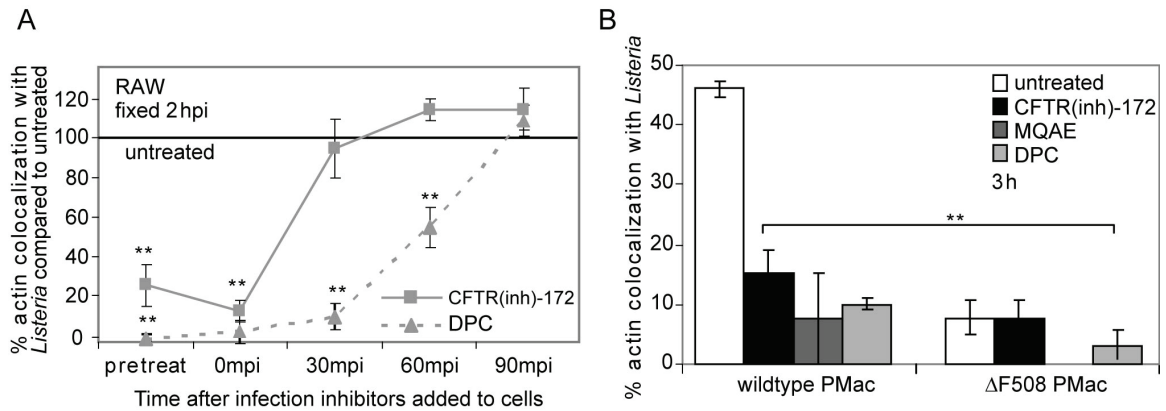


Figure 4.3. CFTR facilitates *L. monocytogenes* escape in RAW cells and PMac. (A) RAW cells were infected with *L. monocytogenes* and left untreated or treated with CFTR(inh)-172 or DPC (anion transporter inhibitor) 1hr prior to infection, at the time of infection (0mpi), 30mpi, 60mpi, or 90mpi, cells were fixed at 2hpi, stained with TR-Phalloidin and anti-*L. monocytogenes* antibody, and analyzed by standard epifluorescence microscopy. Black line represents untreated RAW cells infected with *L. monocytogenes* and fixed at 2hpi. Percentages represent # of bacteria/ 100 bacteria colocalized with actin compared to untreated, setting untreated at 100% (n=3, **p<0.001, comparing untreated and treated cells for each time point). (B) Wildtype or ΔF508 primary PMac were treated for 1h with CFTR(inh)-172, MQAE (chloride chelator), DPC (anion channel inhibitor), or left untreated, infected with *L. monocytogenes*, fixed at 3hpi, stained with TR-Phalloidin and anti-*Listeria* antibody, and analyzed by standard epifluorescence microscopy. Percentages represent # of bacterium colocalized with actin/100 bacteria (n=3, **p<0.001, compared to untreated wildtype PMac).

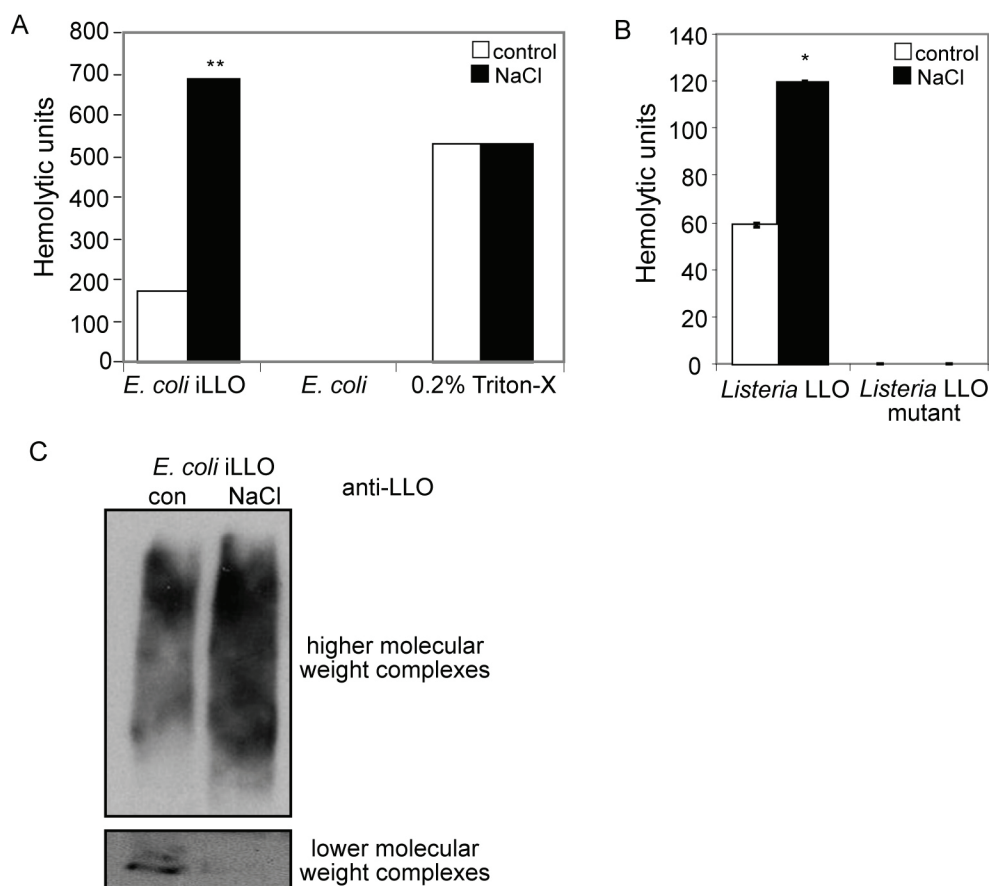


Figure 4.4. Increased sodium chloride concentration potentiates LLO pore forming activity. (A) Hemolytic activity of bacterial lysate prepared from an *E. coli* strain expressing LLO upon IPTG induction (*E. coli* iLLO), the wildtype parental *E. coli* strain, and SRBC treated with 0.2% Triton-X detergent, treated with 428mM NaCl or PBS for 5 min. Hemolytic units=1/average dilution factor of 100% red blood cell lysis as measured by OD 545nm (** $p < 0.001$, comparing control treated and NaCl treated lysate). (B) Hemolytic activity of supernatants obtained from stationary phase broth cultures of wildtype *L. monocytogenes* and *L. monocytogenes* LLO mutant and treated with 428 mM NaCl or PBS for 5 min. Hemolytic units=1/average dilution factor of 100% red blood cell lysis as measured by OD 545nm (* $p < 0.05$, comparing control treated and NaCl treated supernatants). (C) Immunoblot analysis of lysates taken from (A) and analyzed on a non-denaturing gel, and probed with anti-LLO antibody.

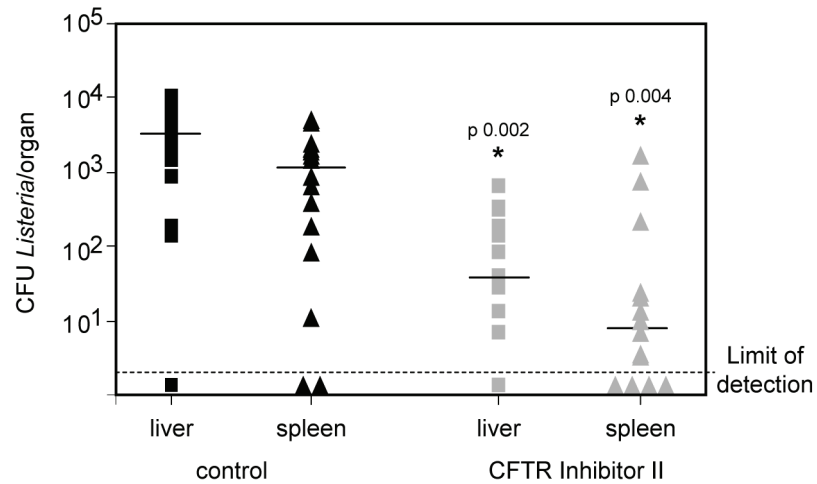


Figure 4.5. CFTR aids dissemination of *L. monocytogenes* across the intestinal barrier. C57BL/6 wildtype mice were injected intraperitoneally with CFTR Inhibitor II (6mg/kg) or 1.5% DMSO, infected orally with 1.5×10^9 *L. monocytogenes* / .1mL 30min post inhibitor treatment, ~18h pi mice spleens and livers were removed, homogenized, and *L. monocytogenes* CFU (colony forming units) were quantitated per organ. Data represents the pooling of two independent experiments. Limit of detection represents 10 CFU *L. monocytogenes* /organ (*p*-values are indicated comparing control and CFTR Inhibitor II treated mice for corresponding organs after the removal of outliers, **p*<0.05, n=3).

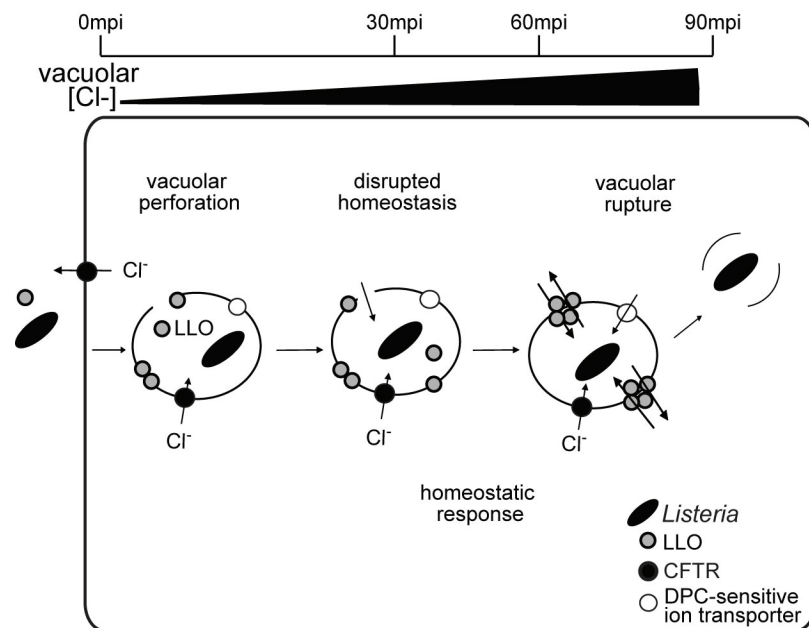


Figure 4.6. Model: CFTR-mediated chloride transport necessary for *L. monocytogenes* vacuolar escape.

L. monocytogenes stimulates CFTR chloride transport in response to secreted LLO or by direct bacterial contact upon infection. High chloride during *L. monocytogenes* uptake, potentiates LLO oligomerization, causing LLO-mediated vacuolar perforation and disruption of vacuolar homeostasis. DPC-sensitive channel(s) respond to vacuolar perturbation, further inducing LLO-mediated pore formation and ion and water transport. Compounding effects of this homeostatic response, results in catastrophic membrane rupture, and *L. monocytogenes* release into the cytosol.

Chapter 5

Perspective

The body of work presented in this thesis highlights the dynamic interactions that occur at the vacuolar interface between intracellular bacteria and the mammalian host cell, focusing on the phagosome, a specialized compartment of the endocytic pathway. The endocytic compartment is subject to complex regulation by the host and pathogen that influence the outcome of infection. Chapters 2 and 3 focused on homeostatic processes utilized by the host to control infection of vacuolar bacterial pathogen *S. typhimurium*. In contrast, Chapter 4 provides evidence for a mechanism by which *L. monocytogenes*, a cytosolic pathogen, exploits a cellular homeostatic process to escape the vacuole. Thus, regulation of vacuolar homeostasis can be advantageous to either the pathogen or the host depending upon the pathogenic context. Although mechanisms of cellular homeostasis have been well studied, their contribution to host-pathogen interactions has not been extensively explored. Therefore, this thesis brings together the fields of cellular homeostasis and microbial pathogenesis to elucidate key parameters of infection. This chapter will discuss advances in the field of phagosomal biology, and the contextual framework these advances provide in our understanding of how perturbation of homeostasis contributes to disease.

Over 100 years ago Eli Metchnikov first demonstrated that cells could take up material from the extracellular environment and degrade it in intracellular compartments (Underhill *et al.*, 2002, Stossel, 1999). However, the complexity of the spatial organization and function of the endocytic compartment could not be appreciated with the limited tools available at that time. Advances in molecular and cellular biology led to biochemical, functional, and genetic means of probing the endocytic pathway, leading to a model of progressive maturation and fusion of the phagosome with endolysosomal vesicles (Mellman, 1996). In the past decade, the development of highly sensitive and quantitative proteomic technology has enabled characterization of the phagosome proteome (Desjardins *et al.*, 1994). Combining proteomics with kinetic analysis, it was demonstrated that the profile of approximately 150 phagosomal proteins changed in a complex manner over a 24 hour period following phagocytosis (Garin *et al.*, 2001). These data suggested that the model of linear maturation of the phagosome was oversimplified, and additional pathways were most likely involved in phagosome maturation (Gotthardt *et al.*, 2006, Rogers *et al.*, 2008). Recent advances in quantitative proteomic techniques have resulted in the identification of 382 predicted proteins present on phagosomes isolated from murine macrophages (Rogers *et al.*, 2007). Using a systems biology approach to study the *Drosophila melanogaster* phagosome, 617 proteins were identified and validated as a part of the phagosome proteome, of which 70% had mammalian orthologs (Stuart *et al.*, 2008). These studies revealed novel regulators of phagocytosis and vacuole function, leading to a more detailed model of the phagosome and its dynamic functions within the cell. However, there is still much to be

learned about the mechanisms of regulation of the phagosome in different physiological contexts.

Regulators of ion transport and homeostasis were found to be key components of the *Drosophila melanogaster* phagosome proteome, with up to 15 clearly identifiable transporters (Stuart *et al.*, 2008). While it is likely that some of these transporters would act to preserve vacuolar function during infection, others might paradoxically enhance infection. A high-throughput siRNA screen using a *Drosophila* phagocytic cell line, revealed multiple host ion transporters that promoted *L. monocytogenes* infection (Agaisse *et al.*, 2005). Although the involvement of these transporters was not validated in mammalian cells, the data are consistent with studies that performed proteomic analysis of *Francisella tularensis*-containing vacuoles, which identified Nramp1 as one of two proteins preferentially associated with the pathogen-containing vacuole (Rogers *et al.*, 2008, Kovarova *et al.*, 2002). Nramp1, a divalent cation transporter, has a well established role in the innate control of *Mycobacterium tuberculosis* and *S. typhimurium*. In contrast, *F. tularensis* exploits Nramp1-mediated ion transport to establish infection in mice (Vidal *et al.*, 1995, Kovarova *et al.*, 2002, Blackwell *et al.*, 1991, Baker *et al.*, 2000, Jabado *et al.*, 2000, Zwilling *et al.*, 1999). My thesis also showed that *L. monocytogenes* depends on the host ion transporter, CFTR, to promote infection, suggesting that utilization of host ion transport may be a common virulence mechanism in intracellular pathogenesis. Orthologs of mouse and human CFTR were found on the *Drosophila* phagosome, supporting a role for CFTR in vacuolar homeostasis, in addition to its role in fluid and ion homeostasis across apical membranes (Stuart *et al.*, 2008, Riordan, 2008). As many more ion transporters were identified as part of the phagosome proteome,

further study of these transporters may elucidate mechanisms utilized by other intracellular pathogens to establish infection.

In addition to the complexity of the protein profile of the phagosome population, the proteome may vary on individual phagosomes. I observed heterogeneity between pathogen-containing vacuoles at a single timepoint during infection, and I hypothesize that this heterogeneity may have profound physiological effects on vacuolar function. In Chapters 2 and 3, we found that in cells with overall high AQP1 expression, only some pathogen-containing vacuoles within a cell were strongly associated with AQP1 while others were not. I observed that only half of the infected AQP1-expressing host cells supported vacuolar rupture and subsequent *S. typhimurium* cytosolic replication, whereas the other half did not. I speculate that the difference in these two outcomes of infection might arise from heterogeneous levels of AQP1 on individual SCV, where vacuoles with high AQP1 levels are more likely to rupture. SCV heterogeneity was also identified in wildtype macrophages with the observation that SCV within the same cell displayed different vacuolar markers and two different *S. typhimurium* growth states (Buchmeier *et al.*, 1997). Early *Listeria*-containing vacuoles also display heterogeneity of tight versus spacious vacuoles in both macrophages and epithelial cells (Shaughnessy *et al.*, 2006, A.L. Radtke unpublished observations). As demonstrated in this thesis and in other published reports, *L. monocytogenes* escapes from 50-70% of vacuoles, as measured by actin colocalization. The observed heterogeneity in vacuolar morphology was predicted to influence the efficiency and kinetics of *L. monocytogenes* escape (Shaughnessy *et al.*, 2006). Thus, in addition to sophisticated proteomic analysis of PCV, analysis of

individual phagosomes by live cell imaging may reveal important principles of vacuole function that govern intracellular bacterial infection.

L. monocytogenes has been used as a model to investigate bacterial determinants of pathogenesis, and has also served as a tool to elucidate fundamental mechanisms of host cell biology and immunology (Mackanness, 1962, Pamer, 2004, Welch *et al.*, 1998, Cossart, 2007). Although many years of study yielded key insights into host mechanisms, such as actin nucleation, that promote *L. monocytogenes* infection, the host factors that contributed to the essential process of escape into the cytosol are largely uncharacterized. The work presented in this thesis identified multiple mediators of cellular homeostasis as modulators of intracellular infection, including AQP1, CFTR, and unidentified Tamoxifen- and DPC-sensitive transporters. However, the complexity of the ion transport regulators on the phagosome hints at the rich biology that remains to be explored in the context of infection. Identification of vacuole regulatory mechanisms will not only set the foundation for elucidating key aspects of infection, but could unveil homeostatic processes that play a role in non-infectious pathologies. Several diseases and conditions have been associated with defective cellular homeostasis, such as Parkinson's disease, epilepsy, pancreatic diseases, diabetes mellitus, dehydration, renal failure, sickle cell anemia, cerebral edema, and cystic fibrosis (Strange, 2004, Wright, 2004, Bezanilla, 2005, Sardini *et al.*, 2003). The use of infectious disease models to study cellular homeostasis may provide new avenues for therapeutic discovery, development, and implementation.

Appendices

Appendix A: Bacterial strains

Table A. Bacterial strains used in this thesis.

| Name | Genotype or Phenotype | Strain | Reference | Stock # |
|---|--|-------------------------|--|----------------|
| <i>Salmonella enterica</i> serovar Typhimurium | wildtype – virulent in mice, vacuolar intracellular localization | SL1344 | (Wray <i>et al.</i> , 1978) | MOR23 |
| <i>Salmonella</i> -GFP | wildtype + pFPV25.1 | SL1344 | (Valdivia <i>et al.</i> , 1996) | MOR36 |
| <i>Salmonella</i> -RFP | wildtype + pCR-BluntII-Topo-RFP | SL1344 | This study | MOR149 |
| <i>S. typhimurium</i> SPI-1 ⁻ -GFP | <i>orgA::Tn5lacZY</i> + pFPV25.1 | SL1344 | (Jones <i>et al.</i> , 1994);this study | MOR37 |
| <i>S. typhimurium</i> SPI-2 ⁻ | <i>ssaV::MudJ</i> | SL1344 | (Suvarnapunya <i>et al.</i> , 2005) | MOR25 |
| <i>S. typhimurium</i> SPI-2 ⁻ -GFP | <i>ssaV::MudJ</i> + pFPV25.1 | SL1344 | (Suvarnapunya <i>et al.</i> , 2005);this study | MOR38 |
| <i>S. typhimurium</i> Δ <i>sifA</i> | <i>ΔsifA</i> | SL1344 | (Stein <i>et al.</i> , 1996) | MOR26 |
| <i>S. typhimurium</i> Δ <i>sifA</i> -GFP | <i>ΔsifA</i> + pFPV25.1 | SL1344 | (Stein <i>et al.</i> , 1996);this study | MOR38 |
| EPEC | invasive, vacuolar intracellular localization | E2348/69 | (Donnenberg <i>et al.</i> , 1990) | MOR180 |
| <i>Streptococcus pyogenes</i> Group A | invasive, vacuolar intracellular localization | MGAS 166 | (Musser <i>et al.</i> , 1995) | MOR113 |
| <i>Streptococcus</i> Group A | invasive, vacuolar intracellular localization | GAS188 | (Sierig <i>et al.</i> , 2003) | MOR202 |
| <i>Streptococcus</i> SLO ⁻ | invasive, vacuolar intracellular localization Δ SLO | GAS188 SLO ⁻ | (Hakansson <i>et al.</i> , 2005) | MOR203 |
| <i>Listeria monocytogenes</i> | CDC family member, cytosolic intracellular localization | 10403S | (Freitag <i>et al.</i> , 1993) | MOR1 |
| <i>Listeria monocytogenes</i> LLO ⁻ | <i>hly⁻</i> (Δ LLO), unable to escape vacuole | 10403S | (Ikonomidis <i>et al.</i> , 1994) | MOR31 |

Appendix B: TBK1-dependent regulation of *aqp1* expression

Chapter 3 showed in the absence of the IKK kinase TBK1, expression of the membrane water channel AQP1 was dramatically increased. As a result, the pathogen-containing vacuoles (PCV) were destabilized and bacteria were released into the host cytosol in a manner dependent on bacterially-induced membrane damage and ion flux. Although the phenotypic mechanism of TBK1 suppression of AQP1 to regulate PCV integrity was identified, the genetic mechanism by which TBK1 regulates AQP1 expression to control vacuolar integrity still remains unclear.

AQP1 was first identified in 1991 by Peter Agre, and he was later granted a Nobel Prize in Chemistry for its discovery (Preston *et al.*, 1991). Since AQP1 discovery, 13 other AQPs have been identified (Agre, 2006). Although it has been almost two decades since the discovery of AQP1, it is not entirely understood how AQP1 expression is regulated, but is proposed to be through transcriptional regulation (Umenishi *et al.*, 2002, King *et al.*, 1996). Upstream of the *aqp1* start site is an untranslated 4.4kb region containing an extensive amount of predicted transcription factor binding sites. Studies have identified *aqp1* to be transcriptionally regulated by a butyrate-response element, hypertonicity, dexamethasone, and corticosteroids. Thus far, all transcription factor response elements identified to regulate *aqp1* expression have resided within the first 1.8kb immediately upstream of *aqp1* transcriptional start site (Umenishi *et al.*, 2003,

Umenishi *et al.*, 2002, Umenishi *et al.*, 1998, Moon *et al.*, 1997). More recent studies have focused on post-translational modifications and functional regulation of AQP1 (Umenishi *et al.*, 2002). It was observed that cellular exposure to hypertonic stress resulted in a decrease of AQP1 ubiquitination that increased the protein's stability and expression levels. Only one study has proposed a mechanism of AQP1 suppression, in which AQP1 was observed to be recruited to lipid rafts with the hypothesis this event eventually results in AQP1 degradation (Kobayashi *et al.*, 2006). In all of the studies, AQP1 protein and RNA levels were monitored in response to environmental stimuli, and in most cases, AQP1 levels were induced. However, in Chapter 3 we observed a significant increase in *aqp1* expression in the absence of an environmental stimulus.

AQP1 expression levels are substantially different based upon cell type or tissue, and these levels often reflect the function carried out by that cell or tissue (Umenishi *et al.*, 1998, Borgnia *et al.*, 1999). After analyzing several cells lines and mouse tissues, we observed an inverse relationship between TBK1 and AQP1 protein expression. This appendix section will described several observations and preliminary experiments conducted in attempt to understand the inverse expression relationship between TBK1 and AQP1.

Utilizing wildtype and TBK1-deficient mouse embryo fibroblasts (MEFs) as a tool to investigate the regulation of AQP1 expression, we first examined if AQP1 protein degradation was the primary means of its regulation. We observed by immunoblot analysis that the proteasome inhibitor LLnL had no effect on AQP1 expression levels in wildtype MEFs, suggesting the undetectable protein levels of AQP1 in wildtype MEFs is not a result of AQP1 degradation by the proteasome. These data suggested a possible

regulatory relationship between TBK1 and *aqp1* at the level of transcription or mRNA stability. To investigate if the low *aqp1* expression levels in MEFs were a result of mRNA stability, wildtype and TBK1-deficient MEFs were treated with Actinomycin D, a potent inhibitor of RNA chain propagation of DNA-directed RNA transcription, and mRNA levels were monitored by qRT-PCR (Figure BI). TBK1 did not appear to impact *aqp1* mRNA stability, since the half-life of *aqp1* transcripts was longer than 8h in both *tbk1*^{+/+} and *tbk1*^{-/-} MEFs. To determine if *aqp1* transcript levels were responsive to TBK1, we transfected MEFs with a plasmid expressing TBK1 (Figure BII). After 48 hr, endogenous *aqp1* transcripts decreased markedly in *tbk1*^{-/-} cells transiently expressing TBK1, but not control cells. We then tested whether TBK1 controlled *aqp1* promoter activity, by subcloning the entire 4.4 kb untranslated region upstream of the *aqp1* gene into a luciferase reporter plasmid. Luciferase activity from the reporter plasmid was higher in transiently transfected *tbk1*^{-/-} than *tbk1*^{+/+} MEFs (Figure BIII). Taken together, these data suggest the predominant means of *aqp1* regulation is through transcriptional repression and identifies TBK1 as a modulator of *aqp1* transcription.

To identify the region in the *aqp1* promoter that is required for TBK1-dependent suppression of *aqp1*, segments of the promoter were deleted in various sized increments, transfected into wildtype MEFs, and luciferase expression was measured. However, after removing 4kb of the *aqp1* untranslated region, we were unable to observe an increase in luciferase activity in wildtype type MEFs that was comparable to TBK1 deficient MEFs (data not shown). We hypothesize that the *aqp1* regulatory region could be located elsewhere on the chromosome and not immediately upstream of the gene, making the identification of *aqp1* response element more difficult. These studies investigating

TBK1-dependent regulation of *aqp1* were not carried out any further, but this work serves as a good foundation for future studies in the identification of the mechanism of TBK1-dependent regulation of *aqp1*.

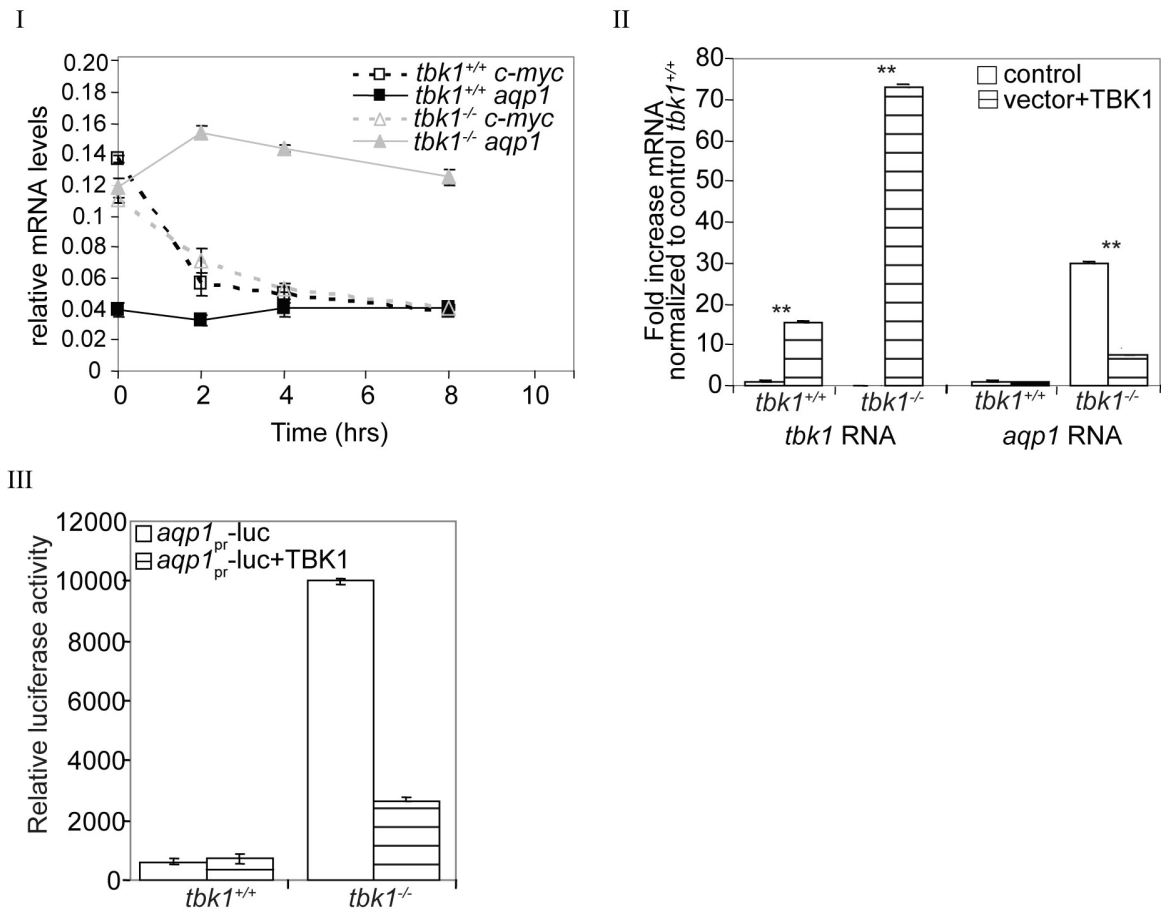


Figure B. TBK1-dependent regulation of *aqp1* transcript levels.

(I) MEFs were treated with 5 μ g/ml Actinomycin D for the indicated times, and *aqp1* and *c-myc* (positive control for mRNA degradation) mRNA levels determined by qRT-PCR (Bernstein *et al.*, 1992). **(II)** MEFs were transiently transfected with *tbk1*-pcDNA3 or pcDNA3 alone. *Tbk1* and *aqp1* mRNA levels were quantified by qRT-PCR. Values for each gene were determined by comparison to control transfected *tbk1*^{+/+} MEFs (n=3; ***p*<0.001). **(III)** MEFs were transfected for 36h with either *aqp1* promoter-driven luciferase reporter plasmid (*aqp1*_{pr}-luc) alone or transfected with *aqp1*_{pr}-luc and *tbk1*-pcDNA3, and both conditions were also transfected with β -gal expression plasmid used to normalize each transfected sample. The Enhanced Luciferase Assay Kite (BD Biosciences) was used to measure luciferase expression of transfected cells (n=3).

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