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Characterization of Listeria monocytogenes Peptidoglycan N-Deacetylase and O-Acetylase Mutations and the Role of Lysozyme Resistance During Infection

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Characterization of *Listeria monocytogenes* Peptidoglycan N-Deacetylase and O-Acetylase Mutations and the Role of Lysozyme Resistance During Infection

by

Chris Steven Rae

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Committee in charge:

Professor Dan Portnoy, Chair

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Abstract

Characterization of *Listeria monocytogenes* Peptidoglycan N-Deacetylase and O-Acetylase Mutations and the Role of Lysozyme Resistance During Infection

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Listeria monocytogenes is a Gram-positive intracellular pathogen that is naturally resistant to lysozyme. Recently, it was shown that peptidoglycan modification by N-deacetylation or O-acetylation confers resistance to lysozyme in various Gram-positive bacteria including *L. monocytogenes*. *L. monocytogenes* peptidoglycan is deacetylated by the action of N-acetylglucosamine deacetylase (Pgd) and acetylated by an O-acetylmuramic acid transferase (Oat). We characterized Pgd-minus, Oat-minus and double mutants to determine the specific role of *L. monocytogenes* peptidoglycan acetylation in conferring lysozyme sensitivity and during infection of macrophages and mice. Pgd-minus and Pgd-minus/Oat-minus double mutants were attenuated approximately 2 and 3.5 logs respectively, in vivo. In bone-marrow derived macrophages, the mutants demonstrated intracellular growth defects and increased induction of cytokine transcriptional responses that emanated from a phagosome and the cytosol. Lysozyme-sensitive mutants underwent bacteriolysis in the macrophage cytosol resulting in AIM-2-dependent pyroptosis. Each of the *in vitro* phenotypes was rescued upon infection of LysM-minus macrophages. The addition of extracellular lysozyme to LysM-minus macrophages restored cytokine induction, host-cell death and *L. monocytogenes* growth inhibition. This surprising observation suggested that extracellular lysozyme can access the macrophage cytosol and act on intracellular, lysozyme-sensitive, bacteria.

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List of Abbreviations

BMM	bone marrow derived macrophage
TEPM	thioglycolate elicited peritoneal macrophage
LZ	lysozyme
LysM	lysozyme M
PGN	peptidoglycan
PAMP	pathogen associated molecular pathogen
TLR	toll like receptor
LLO	listeriolysin O
mCRAMP	mouse cathelicidin-related antimicrobial peptide
HNP-1	human nuetrophil peptide-1
LTA	lipoteichoic acid
LPS	lipopolysaccharide
CMV	cytomegalovirus
LDH	lactate dehydrogenase
DC	dendritic cell
MLN	mesenteric lymph node
NOD	nucleotide oligomerization domain
MDP	muramyl dipeptide
Wt	wild-type

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Chapter 1: A general background on the pathogenesis of *Listeria monocytogenes*

I. General introduction to *L. monocytogenes*

A model organism

L. monocytogenes is a Gram-positive, facultative intracellular pathogen that has been used for decades as a model organism to study immunity and pathogenesis in tissue culture and mouse models of infection. *L. monocytogenes*' broad host range, ease of handling, rapid growth rate, and high degree of genetically tractability are characteristics that have lent well to experimentation among a wide range of investigators. It is one of the most well characterized bacterial pathogens and has significantly contributed to the fields of microbiology, innate and acquired immunology, cell biology, and bacterial pathogenesis. *L. monocytogenes* has also been a useful investigative tool for clinicians, medical microbiologists and food safety microbiologists. *L. monocytogenes* is an extraordinarily valuable model system that provides insight on aspects of other intracellular infections such as *Mycobacterium tuberculosis,* Malaria, and HIV, which account for a large majority of the global health burden of infectious disease.

Microbiology

L. monocytogenes is a small (.4 by 1-1.5 um), rod-shaped, facultatively anaerobic, nonsporulating bacillus with low G+C content that is closely related to *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus*, and *Staphylococcus* species. *L. monocytogenes* have no capsule and express polar flagella and exhibit tumbling motility under tight temperature-dependent regulation. Optimal growth is 30°-37°C, but *L. monocytogenes* are capable of growth at refrigerator temperatures (as low as 3°C). There are 6 listerial species, but only *L. monocytogenes* is pathogenic to humans. There are at least 13 serotypes based on O and H flagellar antigens but almost all disease is due to types 4b, 1/2a, and 1/2b. *L. monocytogenes* is ubiquitous in nature and has been isolated from a wide range and variety of environmental and animal sources. *Listeria* was discovered in the 1920s and was first isolated in 1921 from a patient with meningitis. It was later isolated as the causative agent of a disease affecting rabbits and guinea pigs in an English laboratory in 1924. The first case of human infection by *L. monocytogenes* was documented in 1929 in Denmark (Vazquez-Boland, Kuhn et al. 2001). However, it was not until 1983 that *L. monocytogenes* was declared a public health threat after the first documented human outbreak, which resulted in a high case-fatality rate (Schlech, Lavigne et al. 1983).

Listeriosis

Listeriosis is the infectious disease caused by *L. monocytogenes*, typically as a result of ingestion of contaminated food. It is a severe disease and one of the most deadly bacterial infections, however, it typically infects immunocompromised, elderly or pregnant individuals. Listeriosis manifests as septicemia, meningitis, encephalitis, or intrauterine or cervical infections in pregnant women, which can cause spontaneous abortion or stillbirth. The onset of listeriosis is usually preceded by influenza-like symptoms including persistent fever.

L. monocytogenes resists the effects of freezing, drying, and heat remarkably well for a bacterium that does not form spores. Its ability to grow at temperatures as low as 3°C permits multiplication in refrigerated foods. These qualities lend well to surviving food safety processing techniques and *L. monocytogenes* is an increasing public health threat (Swaminathan and Gerner-Smidt 2007). *L. monocytogenes* has been associated with foods such as raw milk, supposedly pasteurized fluid milk, soft cheeses, ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry, raw and smoked fish, and cantaloupe.

Models of Infection in vivo

L. monocytogenes has been a tremendously useful tool to study infection and immunity, primarily in mice. The natural route of infection is oral and is dependent on *L. monocytogenes* InlA interaction with E-cadherin for uptake in the gut (Mengaud, Ohayon et al. 1996). Upon ingestion and crossing of the intestinal barrier, *L. monocytogenes* traffics to the mesenteric lymph nodes (MLN), liver, and spleen. However, the route and kinetics are not well understood in an oral infection largely because mice have a mutation in E-cadherin that prevents interaction with InlA and a reliable oral model of listeriosis in mice does not yet exist (Lecuit, Dramsi et al. 1999; Luo, Lee et al. 2003; Ireton 2007; Bonazzi, Veiga et al. 2008). Efforts to use humanized E-cadherin expressing mice are underway (Luo, Lee et al. 2003). The most common model used to study *L. monocytogenes* infection in the mouse is by iv injection. George Mackaness developed the murine model of listeriosis in classic work that demonstrated resistance and immunity to *L. monocytogenes* infection was mediated by activated macrophages (Mackaness 1962) and that acquired immunity was highly specific (Mackaness 1964) due to antigen specific T-cells (Lane and Unanue 1972; North 1973; Unanue 1997; Pamer 2004). By iv injection, *L. monocytogenes* traffics through the blood and the majority is delivered to the liver (approximately 90%) and is taken up by Kuppfer cells (liver resident macrophages) and later spreads to hepatocytes, which is the principle site of replication in the liver (Vazquez-Boland, Kuhn et al. 2001). During early liver colonization, neutrophils are recruited at the sites of infection and have been shown to play an important roll in controlling the acute phase of infection. A fraction of the injected bacteria is also trafficked to the spleen and interacts with DCs, then resides in infectious foci of macrophages and recruited neutrophils (Waite, Leiner et al. 2011).

Early host resistance to infection requires the production of IFNγ, primarily made by NK cells (in response to IL-12 and TNFα produced by infected macrophages), and most *L. monocytogenes* are killed by activated resident macrophages (Vazquez-Boland, Kuhn et al. 2001). IFNγ-deficient mice are highly susceptible to *L. monocytogenes* infection (Seki, Tsutsui et al. 2002). *L. monocytogenes* reside in vivo and increase in number for 2-5 days post infection. *L. monocytogenes* begin to disappear from mouse tissues and by day 5-7 the few remaining bacteria are cleared by a strong antigen-specific CD8 T-cell response, which peaks near 8 days post infection (Pamer 2004).

Following initial infection, strong CD8-T-cell-dependent long-term protective immunity is acquired. Upon challenge of immunized mice, memory CD8 T-cells undergo rapid

clonal expansion and clear the infection, providing logs of protection (Pamer 2004). Induction of protective immunity requires entry into the cytosol (Berche, Gaillard et al. 1987; Bahjat, Liu et al. 2006). *L. monocytogenes* that lack LLO or heat-killed bacteria fail to elicit a protective response. However, some virulence-attenuated strains, such as ActA-minus *L. monocytogenes* still induce strong protective immunity (Goossens and Milon 1992).

II. Cell Biology of Infection: the intracellular infectious cycle

Invasion: Internalins

L. monocytogenes is able to invade and survive in both phagocytic and non-phagocytic cells. *L. monocytogenes* is internalized by most adherent cells, although the efficiency of uptake is highly variable. *L. monocytogenes* can induce its internalization in non-phagocytic cells such as epithelial cells, fibroblasts, hepatocytes and endothelial cells through internalins that induce a zipper like mechanism of engulfment distinct from membrane ruffle process characteristic of *Salmonella* and *Shigella* (Vazquez-Boland, Kuhn et al. 2001) (Fig 1.1). InternalinA is required for invasion of epithelial cells and InlB is required for uptake of hepatocytes (Dramsi, Kocks et al. 1993; Dramsi, Biswas et al. 1995; Lingnau, Domann et al. 1995). InlB mutants show less liver toxicity during infection (Brockstedt, Giedlin et al. 2004).

Vacuolar escape: Listeriolysin O and Phospholipase C's

Following uptake by a phagocytic cell, the *L. monocytogenes*-containing vacuole begins to mature and acidify. *L. monocytogenes* express *hly*, the primary virulence determinant, which encodes listeriolysin O (LLO), a pore-forming cholesterol-dependent cytolysin that facilitates vacuolar escape and grants access to the host cytosol, where *L. monocytogenes* is able to replicate to high number (>200 bacteria without causing host cell toxicity) (Portnoy, Jacks et al. 1988; Schnupf and Portnoy 2007) (Fig 1.1). LLO is secreted as a soluble monomer, binds the phagosomal membrane, and oligomerizes to form a pre-pore. LLO then inserts into the membrane, undergoing dramatic structural changes, and forms pores that consist of 33-50 monomers and may be as large as 350 angstroms in diameter. Interestingly, these pores are not sufficient for *L. monocytogenes* to pass through (Henry, Shaughnessy et al. 2006; Shaughnessy, Hoppe et al. 2006) and it has been hypothesized that they facilitate dissolution of the vacuole and may allow translocation for phospholipase C's, which are implicated in escape from a secondary vacuole following cell spread (Fig 1.1). LLO is essential for escape from both a primary and secondary vacuole and *L. monocytogenes* that lack *hly* are confined to the acidified phagosome and fail to enter the cytosol and replicate. LLO is necessary and sufficient for escape and *B. subtilis* that express LLO allowed escape and subsequent cytosolic access (Bielecki, Youngman et al. 1990). LLO-minus *L. monocytogenes* are extremely attenuated in vivo (Vazquez-Boland, Kuhn et al. 2001).

The pore-forming activity of LLO is confined to the phagosome and is tightly regulated. Misregulation results in severely attenuated virulence (Glomski, Decatur et al. 2003). LLO expression is largely limited to the phagosome and pore-forming activity is optimal

at pH 5.5 (Glomski, Gedde et al. 2002). In fact, phagosomal acidification is required for *L. monocytogenes* to escape. *hly* is transcriptionally regulated, primarily by PrfA, the major transcriptional regulator of virulence genes, and a very stable transcript is increasingly expressed during exposure to high temperature (37C), nutrient limitation, and intracellular growth. Although transcript is abundant during intracellular growth, *hly* is translationally repressed in the cytosol and very little LLO is produced . Various mutants in the N-terminal region, such as S44A, result in increased LLO synthesis in the cytosol and subsequent host cell toxicity, presumably by formation of pores in the plasma membrane (Glomski, Decatur et al. 2003). Conversely, a mutation, G486D, results in LLO that facilitates phagosomal escape, but is catalytically dead in the cytosol (unpublished observation). LLO is also post-translationally regulated by phosphorylation, ubiquitination, and targeting to the proteosome, which results in rapid cytosolic degradation (Schnupf, Portnoy et al. 2006; Schnupf, Zhou et al. 2007).

Actin-based motility and cell spread: ActA

Intracellular *L. monocytogenes* are able to initiate host actin polymerization through the action of ActA, an actin nucleator localized to the bacterial surface (Kocks, Gouin et al. 1992; Brundage, Smith et al. 1993; Welch, Rosenblatt et al. 1998; Skoble, Portnoy et al. 2000; Skoble, Auerbuch et al. 2001). Cytosolic *L. monocytogenes* are initially surrounded by an actin cloud, which rearranges to form a polarized actin tail at 2hrs post infection. Polar assembly of actin filaments propels the bacteria in the cytoplasm and eventually into adjacent cells, whereby *L. monocytogenes* enters a secondary vacuole (Tilney and Portnoy 1989) (Fig 1.1). ActA mutants replicate in the cytosol to high number but fail to spread and eventually become toxic to host cells. *actA* expression is restricted to the cytosol and is regulated by PrfA, the central virulence transcription factor (Moors, Levitt et al. 1999; Shetron-Rama, Marquis et al. 2002).

III. Host Innate immune detection of *L. monocytogenes*

Three primary cellular pathways of innate immunity are triggered during infection by *L. monocytogenes*. The first represents a generic, MyD88-dependent, response that emanates from cell surface and phagosomal recognition and leads to the transcription of inflammatory cytokines including IL-12 and IL-1β (O'Riordan, Yi et al. 2002; Leber, Crimmins et al. 2008) (Fig 1.2). A second pathway is triggered by listerial c-di-AMP and stimulates an IRF3/Sting-dependent, My-D88 independent response leading to the expression of IFNβ and co-regulated genes (Sauer, Sotelo-Troha et al. ; Sauer, Witte et al. ; Woodward, Iavarone et al. ; O'Riordan, Yi et al. 2002; Ishikawa, Ma et al. 2009) (Fig 1.2). A third pathway triggered by *L. monocytogenes* infection, but only to a small extent, is activation of the DNA-dependent, AIM2 inflammasome and the consequent activation of Caspase-1 and cell death by pyroptosis (Kim, Bauernfeind et al.) (Fig 1.3).

Vacuolar Pathway

TLRs are pathogen recognition receptors that signal in response to various distinct conserved microbial ligands, termed pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA),

lipoproteins, flagellin, CpG DNA, dsRNA and ssRNA. TLR signaling occurs through the cytosolic adaptor molecule MyD88, or in the case of TLR3 or vacuolar TLR4, TRIF, and results in the activation of NFkB and subsequent transcription of pro-inflammatory cytokines (Barton and Medzhitov 2002; Takeda, Kaisho et al. 2003; Kawai and Akira 2007; Kumar, Kawai et al. 2009).

L. monocytogenes infection activates two distinct transcriptional cytokine responses in mouse bone marrow macrophages (BMM). This was demonstrated in experiments using mouse cDNA microarrays to determine gene expression induced by cytosolic and vacuolar localized *L. monocytogenes* in infected primary mouse BMM (McCaffrey, Fawcett et al. 2004). Wild-type (wt), LLO-minus, and killed *L. monocytogenes* each triggered early and persistent activation of a gene cluster enriched with pro-inflammatory cytokines, chemokines, receptors and signal transducers associated with TLR signaling pathways. However, only wt *L. monocytogenes*, which access the cytosol, induced activation of a second cluster comprised of IFN-responsive genes. The vacuolar transcriptional response was further characterized in a later microarray study in which 252 genes were identified as significantly upregulated following infection with LLO-minus *L. monocytogenes* (Leber, Crimmins et al. 2008). LLO-minus mutants failed to significantly induce transcription of any of these genes in BMM deficient in MyD88, a primary TLR signaling adapter, indicating the early response is entirely facilitated by TLR detection (Leber, Crimmins et al. 2008). Presumably, TLR2, which recognizes components of the bacterial cell wall, contributes to this response (Torres, Barrier et al. 2004). However, the precise ligand(s) that stimulate this pathway have yet to be identified. TLRs 3, 7, 8, and 9 are localized to the endosome and detect nucleic acid. TLR3 detects viral dsRNA and polyIC and signals through TRIF, while TLRs 7 and 8 detect ssRNA and TLR 9 detects CpG DNA (Kumar, Kawai et al. 2009) and are therefore potentially involved in *L. monocytogenes* detection.

Cytosolic surveillance pathway

The cytosolic surveillance pathway detects bacterial, viral and protozoan pathogens, leading to the activation of IRF3 and NF-κB, resulting in the induction of IFN-β and co-regulated genes. Viral and bacterial nucleic acids are known ligands that activate this pathway and recently it has been shown that bacterial cyclic di nucleotides stimulate the cytosolic surveillance pathway through the cytosolic receptor, STING. *L. monocytogenes* stimulates an IRF3/Sting-dependent, My-D88 independent response. *L. monocytogenes* DNA and muramyl dipeptide (MDP) were shown to reconstitute the cytosolic surveillance response in vitro and this pathway was amplified by NOD2, a cytosolic PGN receptor (Leber, Crimmins et al. 2008). *L. monocytogenes* mutants that lack negative transcriptional regulators of multidrug resistance transporters were identified that hyperinduce IFNβ during infection. Later, it was shown that secreted listerial c-di-AMP is the ligand that activates this response (Woodward, Iavarone et al.).

Inflammasome activation

The inflammasomes are a family of cytosolic signaling molecules that form multi-molecular signaling complexes and activate pro-inflammatory caspases and interleukin (IL) cytokines (Martinon and Tschopp 2007; Schroder and Tschopp 2010; Gross, Thomas

et al. 2011). They are typically composed of a receptor protein, the adapter, apoptosis-related speck-like protein (ASC), and the effecter, pro-Caspase-1. Inflammasome activation entails ligand binding, formation of a multi-protein complex, proteolytic cleavage and activation of Caspase1 and interleukin 1β or 18 (IL-1β, IL-18), and subsequent pyroptotic cell death and secretion of active proinflammatory cytokines. Pyroptosis is a programmed cell death pathway dependent on Caspase-1 and associated with inflammation, in contrast with other cell death pathways such as apoptosis and necrosis (Martinon, Mayor et al. 2009). To date, the NLRP1, NLRP3, NLRP4, and AIM2 inflammasomes have been described and are activated by various microbial ligands (Horvath, Schrum et al. 2011). NLRP1 is a cytosolic sensor for *Bacillus anthraxis* lethal toxin and activation results in proteolytic cleavage of pro-Caspase 1 and subsequent pyroptosis and secretion of processed IL-1β (Faustin, Lartigue et al. 2007). Muramyl dipeptide has also been shown to activate NLRP1 in reconstituted systems in vitro (Hsu, Ali et al. 2008). The NRLP3 inflammasome is activated by various ligands including, lysozyme-derived peptidoglycan fragments, reactive oxygen species, uric acid, and potassium efflux and results in secretion of IL-1β, but not pyroptotic cell death (Mariathasan, Weiss et al. 2006; Horvath, Schrum et al. 2011). The NLRC4 inflammasome is activated by flagelin in a Naip5 dependent manner or by rod proteins of type three secretion system apparatus of various bacteria in a Naip2 dependent manner (Kofoed and Vance 2011). Activation of NLRC4 can result in induction of pyroptosis and secretion of IL-1β and IL-18 (Martinon, Mayor et al. 2009; Schroder and Tschopp 2010; Gross, Thomas et al. 2011; Horvath, Schrum et al. 2011). AIM2 is activated by cytosolic viral or bacterial DNA and results in activation of Caspase-1 and subsequent pyroptosis and secretion of IL-1β (Mariathasan, Weiss et al. 2006; Ozoren, Masumoto et al. 2006; Franchi, Kanneganti et al. 2007). *L. monocytogenes* infection triggers activation of the AIM2 inflammasome and the consequent activation of Caspase-1 and cell death by pyroptosis, but only to a small extent (Kim, Bauernfeind et al.). AIM2-dependent induction of pyroptosis is caused by the infrequent lysis of intracellular *L. monocytogenes* (Sauer, Witte et al.).

IV. Bacterial cell wall

Cell wall structure

The Gram-positive cell wall surrounds the inner membrane and is composed of thick repeating units of peptidoglycan (PGN) interlaced with cell wall proteins and additional polysaccharide structures such as teichoic acid. The cell wall provides maintenance of cell shape, osmotic stability and allows resistance to high turgor pressure. PGN is the major scaffolding structure of the cell wall and is composed of alternating units of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) that are linked through a β1-4 glycosidic bond (Vollmer 2008) (Fig 1.4). Peptide side chains are attached to the N-acetylmuramic acid residues at their carboxyl group and are typically composed of five amino acids: L-Ala-D-Glu-X-D-Ala-D-Ala, where X is either L-Lys or diaminopimelic acid (Dap). *L. monocytogenes* are one of only several Gram-positives whose PGN peptide side chains contain a Dap residue (Popowska, Kloszewska et al. 1999; Vazquez-Boland, Kuhn et al. 2001; Popowska 2004; Vollmer 2008). Layers of *L.*

monocytogenes PGN are cross linked by peptide bridges that form among the amino groups of the Dap residues and carboxyl group of terminal D-Ala residues of the peptide side chains.

Bacterial autolysins

Bacteria must tightly regulate the structure of their cell wall, particularly during cell division. Careful disassembly and restructuring of the cell wall during growth, septation, and PGN turnover is carried out by the coordinate activity of a group of cell wall associated bacterial enzymes known as autolysins or PGN hydrolases (Vollmer, Joris et al. 2008). Among bacteria, there are autolysins with activity for every glycosidic and amide bond in PGN and most species express multiple autolysins. There are three types of autolysins that act on the glycan strand: N-acetylglucosaminidases, lysozymes, and lytic transglycosylases. Lysozymes, and lytic transglycosylases cleave the glycosidic bond between MurNAc and GlcNAc residues, but lytic transglycosylase activity results in formation of a 1,6-anhydro ring on the MurNAc residue, which has immunostimulatory activity. Amidases cleave the amide bond between MurNAc and the N-terminal alanine residue of the peptide side chain, while endopeptidases and carboxypeptidases cleave amide bonds in the peptide side chains or at the C-terminal, respectively. Autolysin activity is often specific for a species type of PGN, high-molecular weight PGN or small fragments, or for the presence or absence of secondary modifications (Vollmer, Joris et al. 2008).

The most well characterized listerial autolysins are NamA, p60, Ami, Auto, IspC, and p45, although additional putative autolysins have been identified (Popowska, Kloszewska et al. 1999; Popowska 2004; Calvo, Pucciarelli et al. 2005; Wang and Lin 2008). p45 is an endopeptidase, Ami is a secreted amidase involved in adherence, Auto is an N-acetylmuramidase, p60 is an endopeptidase, and NamA is a N-acetylmuramidase that is dependent on SecA2, an auxiliary secretion system. Mutations in these autolysins result in various degrees of virulence defects, rough colony formation, and chaining (Lenz, Mohammadi et al. 2003; Popowska 2004).

Peptidoglycan modifications and host lysozyme resistance

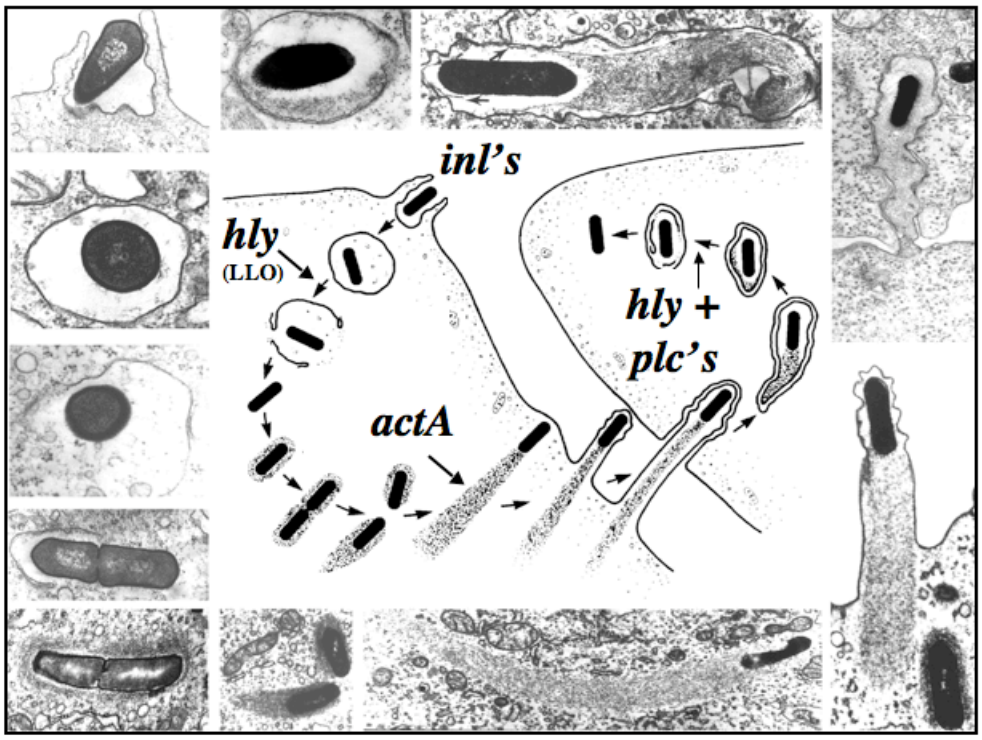
Lysozyme is an abundant and conserved host antimicrobial peptide that cleaves PGN at the β-1,4 glycosidic bond between MurNAc and GlcNAc residues breaking down the structural integrity of the cell wall and resulting in bacteriolysis (Callewaert and Michiels) (Fig 1.4). Lysozyme is present in epithelial cell secretions such as mucous and tears and is produced and secreted by neutrophils and macrophages. Due to the abundance of lysozyme expressed in hosts, pathogens avoid lysozyme activity by modifying their PGN. In fact, many bacteria modify their PGN after synthesis and insertion into the cell wall. The most common PGN modifications are N-deacetylation, N-glycolylation, and O-acetylation of the glycan strands and often confer resistance to host lysozyme (Davis and Weiser ; Vollmer 2008). Deacetylated glycan residues (both MurNAc and GlcNAc) are formed by peptidoglycan deacetylases and the enzymatic step occurs after PGN is polymerized since deacetylated precursors are not detected in species with deacetylases. PGN deacetylase genes have been identified in *Bacillus, Lactobacilus, Micrococcus, Streptococcus, Heliobacter, Enterococcus* and *Listeria* species (Davis and

Weiser ; Vollmer 2008). Many bacteria modify their PGN by O-acetylation (only MurNAc residues) through the action of O-acetylase activity, which contributes to lysozyme resistance in some species. O-acetylase PGN modification has been identified in both Gram-positive and Gram-negatives including *Staphylococcus*, *Enterococcus, Lactococcus, Staphylococcus, Streoptococcus, Campylobacter, Heliobacter, Neisseria, Bacillus,* and *Listeria (Davis and Weiser ; Vollmer 2008)*. O-acetylation has been shown to regulate endogenous autolysin activity in some species (Moynihan and Clarke 2011). A third PGN modification of the glycan strand is N-glycolylation of MurNAc residues by *Mycobacterium* species. This modification is performed by NamH, a UDP-MurNAc-pentapeptide monooxygenase, and is introduced during synthesis to the last soluble cytoplasmic precursor and only a fraction of the PGN is glycolylated (Raymond, Mahapatra et al. 2005; Mahapatra, Crick et al. 2008; Vollmer 2008; Coulombe, Divangahi et al. 2009). *L. monocytogenes* N-deacetylates GlcNAc residues through the action of Pgd and O-acetylates MurNAc residues through the action of Oat (Fig 1.4). Approximately 50% of its PGN is N-deacetylated and 23% is O-acetylated (Boneca, Dussurget et al. 2007; Aubry, Goulard et al. 2011).

Bacteriolysis, peptidoglycan fragments, and host sensors

Bacteria contain numerous ligands that are sensed by host innate immune receptors. PGN is an exceptionally good candidate ligand for host detection since it is unique to bacteria, essential, and abundant. TLR2 detects cell wall associated teichoic acids and lipoproteins. It has been reported that TLR2 also senses PGN, however, this remains controversial (Travassos, Girardin et al. 2004). PGN is detected by NOD1 and NOD2, which are cytosolic NLR family receptors that signal through RIP2 and activate NF-κB, resulting in inflammatory cytokine production (Girardin, Boneca et al. 2003; Girardin, Travassos et al. 2003; Philpott and Girardin 2004; Boneca 2005). NOD1 is expressed in all cell types and detects PGN fragments that contain the peptide side chain terminating in a Dap residue. NOD2 is expressed in monocytes, macrophages, and nuetrophils and detects fragments containing muramyl dipeptide (MDP), which consists of a MurNAc residue and a two amino acid peptide side chain. TLR2, NOD1 and NOD2 have been implicated in the innate immune response to various bacterial infections, however, their contribution to detection of *L. monocytogenes* is unclear (Edelson and Unanue 2002; Torres, Barrier et al. 2004; Boneca, Dussurget et al. 2007). Peptidoglycan recognition proteins (PGRPs) are host proteins that interact with peptidoglycan. Mammals have four PGRPs, however, none of them has been directly linked to the sensing of peptidoglycan. PGRPs have direct bactericidal activities that occur by binding and disruption of PGN synthesis, which may contribute to bacteriolysis (Davis and Weiser ; Dziarski 2004; Dziarski and Gupta 2006).

The aim of this work was to characterize *L. monocytogenes* mutations in the PGN N-deacetylase, Pgd, and PGN O-acetyltransferase, Oat, and to determine the role of host lysozyme during *L. monocytogenes* infection.



adapted from Portnoy et al. J Cell Biol. 2002.

Figure 1.1- The intracellular infectious lifecycle of *L. monocytogenes.*
L. monocytogenes enter cells by phagocytosis or induced uptake through the action of internalins (*inl*) and enter a vacuole. As the vacuole begins to mature, *L. monocytogenes* express *hly*, which encodes LLO, a pore forming cholesterol-dependent cytolysin that facilitates dissolution of the vacuole and grants access to the cytosol. There, *L. monocytogenes* replicate and express *actA* to polymerize host cell actin, which is used for cell spread. Following cell spread, LLO and phospholipase C's (*plc's*) are required for escape from a secondary vacuole.

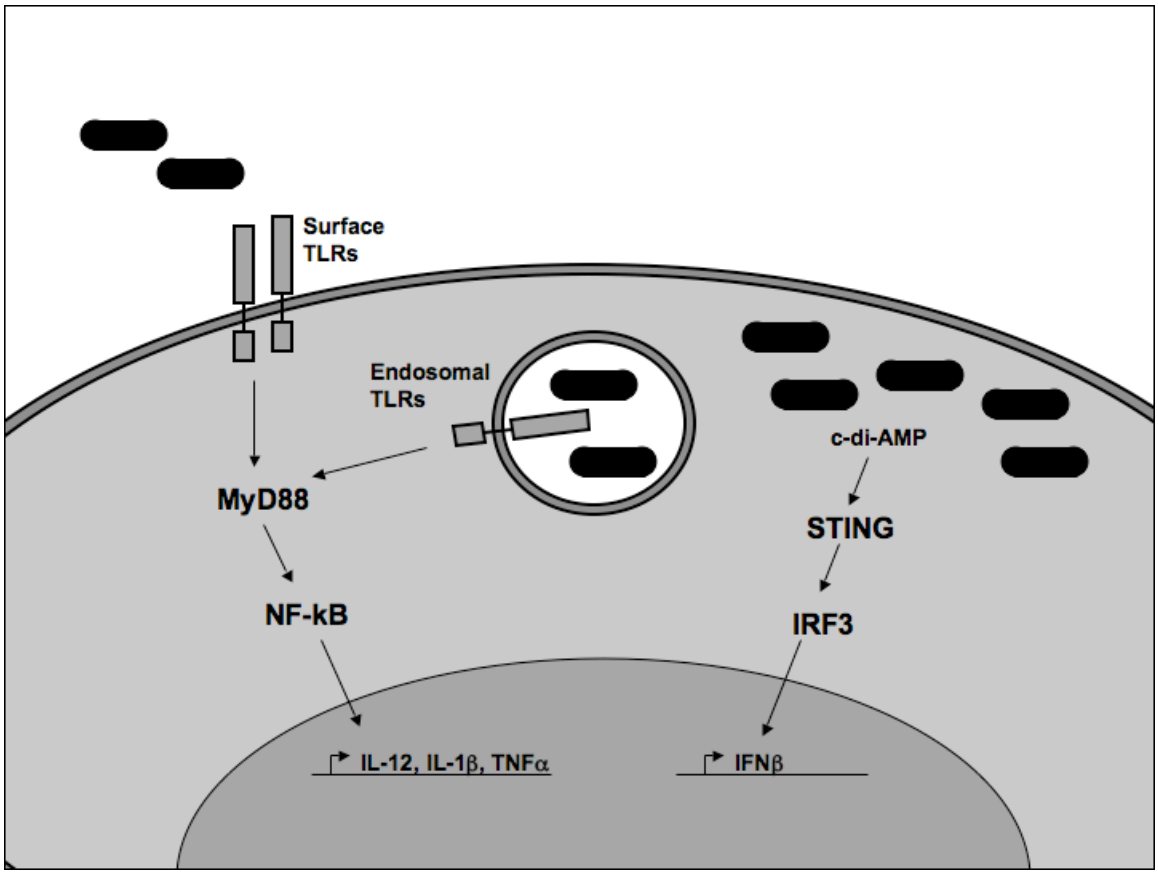


Figure 1.2- The host cytosolic and vacuolar cytokine responses to *L. monocytogenes.* During infection of macrophages, *L. monocytogenes* is activates two distinct cytokine responses. The first is entirely MyD88 dependent, emanates from a vacuole, and results in transcription of inflammatory cytokines. The second is a STING/IRF3 dependent, MyD88 independent, response that emanates from the cytosol and results in transcription of IFNβ and co-regulated genes.

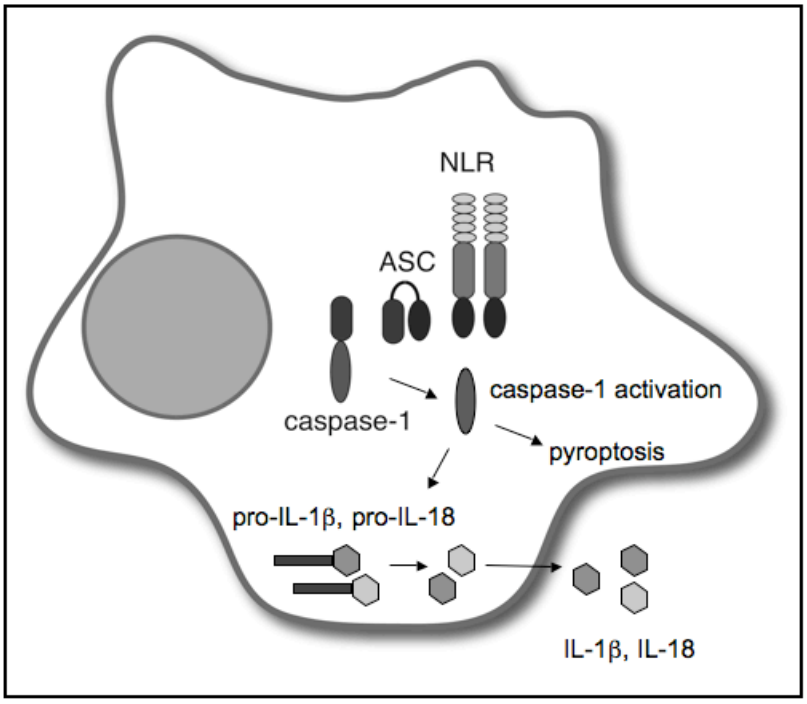
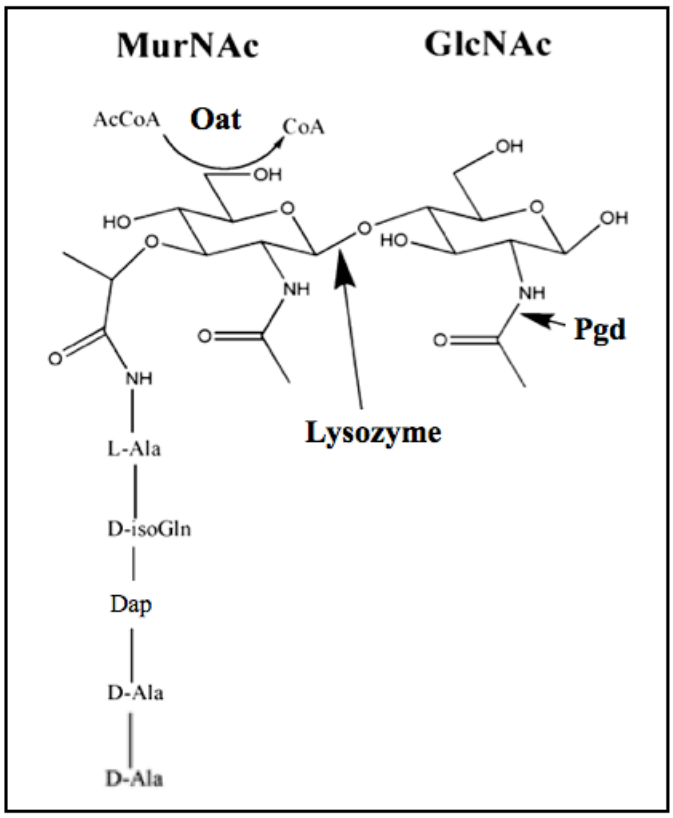


Figure 1.3- *L. monocytogenes* activate the AIM2 inflammasome.
A third pathway triggered by *L. monocytogenes* infection, but only to a small extent, is activation of the DNA-dependent, AIM2 inflammasome, and is caused by infrequent bacteriolysis. Listerial DNA is detected by AIM2, a cytosolic NOD receptor, and dependent on the adaptor, ASC, results in activation of Caspase-1 and subsequent pyroptosis (programmed cell death distinct from apoptosis and necrosis) and proteolytic processing and secretion of IL-1β and IL-18.



adapted from Davis et al. PLoS Pathog. 2008

Figure 1.4- *L. monocytogenes* peptidoglycan modifications.
L. monocytogenes PGN is composed of repeating units of MurNAc and GlcNAc residues and contains a Dap type peptide side chain. *L. monocytogenes* modifies its PGN through the action of Pgd, a N-deacetylase that acts on GlcNAc, and Oat, an O-acetyltransferase that acts on MurNAc. Lysozyme acts on the 1-4 β glycosidic bond linking MurNAc and GlcNAc residues.

Chapter 2: Mutations of the *Listeria monocytogenes* Peptidoglycan N-Deacetylase and O-Acetylase Result in Enhanced Lysozyme Sensitivity, Bacteriolysis, and Hyper-induction of Innate Immune Pathways

Introduction

Lysozyme

Lysozyme is a potent antimicrobial peptide identified in all major taxa of living organisms that represents an ancient host defense mechanism against invading microbes (Callewaert and Michiels ; Medzhitov and Janeway 2000). Lysozyme was discovered in 1922 by Alexander Fleming and has been studied extensively as a model enzyme (Fleming 1922). Lysozyme is typically found in host fluids including tears, saliva, airway fluid, serum, mucus, and in lysozomal granules of neutrophils and macrophages (Callewaert and Michiels). Lysozyme activity results in bacteriolysis and potential release of microbial ligands that may be detected by host innate immune pathways (Ginsburg 2002). The ubiquitous presence of lysozyme has undoubtedly selected pathogens that avoid its effects.

Lysozyme displays muramidase activity that hydrolyzes the β1-4 glycosidic linkage between N-acetyl muramic acid and N-acetyl glucosamine residues, which make up the glycan backbone of peptidoglycan (Bera, Herbert et al. 2005). Lysozyme also displays cationic antimicrobial activity independent of muramidase action (Nash, Ballard et al. 2006). Two lysozyme genes, lysozyme M and P, are expressed in mice (Cross, Mangelsdorf et al. 1988), while only a single lysozyme is expressed in humans. Lysozyme M (LysM) is the predicted ortholog of human lysozyme and is the predominant form expressed in most cells including bone marrow derived macrophages (BMM) (Cross, Mangelsdorf et al. 1988). Previous studies have demonstrated that disruption of LysM in mice resulted in increased susceptibility to some bacterial infections. For instance, LysM-minus (LysM-) mice had increased bacterial burden when infected with *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* (Markart, Korfhagen et al. 2004; Cole, Thapa et al. 2005). Similarly, Shimada et al showed increased susceptibility to middle ear infection in LysM- mice infected with *Streptococcus pneumoniae* (Shimada, Moon et al. 2008). Although lysozyme has been studied for decades, its precise role during bacterial infection is not yet fully understood.

Cell Wall Modifications confer resistance to Lysozyme

Many pathogens modify their peptidoglycan to resist host lysozyme (Davis and Weiser). Common modifications of peptidoglycan include N-deacetylation, N-glycolylation, and O-acetylation (Vollmer 2008). *Bacillus anthrasis, S. pneumoniae, and L. monocytogenes* are Gram-positive pathogens that N-deacetylate the N-acetylglucosamine residues of their peptidoglycan and are consequently resistant to lysozyme (Zipperle, Ezzell et al. 1984; Vollmer and Tomasz 2000; Boneca, Dussurget et al. 2007). For example Vollmer et al identified *pgdA* in *S. pneumoniae*, which encodes a N-acetylglucosamine deacetylase and contributes to lysozyme resistance (Vollmer and Tomasz 2000). Boneca

et al identified *pgdA* in *L. monocytogenes* and showed that it provides resistance to lysozyme in broth culture and contributes to virulence (Boneca, Dussurget et al. 2007). *Staphylococcus aureus* is resistant to lysozyme due to modification of its peptidoglycan by O-acetylation. Bera et al identified *oatA* in *S. aureus* and showed that its action mediated resistance to lysozyme and that its presence in some *Staphylococcal* species correlated with lysozyme resistance (Bera, Herbert et al. 2005; Bera, Biswas et al. 2006). *S. pneumoniae* also encodes an O-acetyl transferase, Adr, and Davis et al showed that both *adr* and *pgdA* contribute to lysozyme sensitivity and pathogenesis (Crisostomo, Vollmer et al. 2006; Davis, Akinbi et al. 2008). Interestingly, the double mutants were hypersensitive to lysozyme and attenuated *in vivo* but were able to out-compete wild-type *S. pneumoniae* in LysM- mice, demonstrating a fitness cost for expression of *pgdA* and *adr* (Davis, Akinbi et al. 2008).

In this study, we addressed the role of *L. monocytogenes* Pgd (lmo 0415) and Oat (lmo 1291) during infection in bone marrow derived macrophages (BMM). Pgd-minus and Pgd/Oat-minus mutants were lysozyme sensitive, had intracellular growth defects, increased induction of cytokine transcriptional responses and increased bacteriolysis and subsequent pyroptosis. Each of these phenotypes was rescued in the absence of lysozyme.

Methods

Bacterial Strains

All *L. monocytogenes* strains used and generated in this study were in the 10403S background. In-frame deletion mutants were constructed by splice-overlap extension and introduced by allelic exchange (Camilli, Tilney et al. 1993). Strains were grown in Brain Heart Infusion media at 30°C overnight without shaking to stationary phase for macrophage infections.

Broth growth curves

Stationary phase cultures were back diluted 1:40 in BHI and grown to mid-log phase at 37°C with shaking. Titrations of hen egg white lysozyme in phosphate-buffered saline (PBS) were added in constant volumes at mid-log phase (OD 600nm = 0.3 at 240 min) and growth was measured by OD 600nm at 15 min intervals over the course of 8h. CFUs were measured by plating culture dilutions on LB agar plates. Plates were incubated overnight at 37°C and colonies were counted the following day.

Disc diffusion assay

$3x10^8$ stationary phase *L. monocytogenes* were plated in 250uL BHI on LB agar plates. 1mg hen egg white lysozyme was added to Whatman paper absorbent discs on the plate in 10uL volume. Plates were incubated overnight at 37°C and the diameter of the zone of growth inhibition around each disc was measured the following day.

Macrophages

Bone-marrow derived macrophages were prepared from 6-8 week old female mice as previously described (Jones and Portnoy 1994). All mice were in the C57BL/6 genetic background. LysM- mice were a gift from Tomas Ganz. AIM2 shRNA knockdown vectors were a gift from Katherine Fitzgerald and immortalized C57BL/6 macrophages were a gift from Russell Vance.

Intracellular growth curves

$2x10^6$ BMM were plated overnight and infected with LLO expressing strains at a MOI of 0.1 and LLO-minus *L. monocytogenes* at a MOI of 1. Thirty minutes after infection, macrophage monolayers were washed with PBS and fresh media was added. At 1h post infection, 50μg/ml gentamicin was added to kill extracellular bacteria. Replication was quantified as previously described (Portnoy, Jacks et al. 1988). Briefly, three coverslips at each time point were washed with water to lyse macrophages. Bacteria recovered from each coverslip were plated on LB agar plates and CFUs were determined.

Macrophage gene expression by quantitative RT-PCR

$2x10^6$ BMM were infected at a MOI of 1 for 4h. Thirty minutes after infection, macrophage monolayers were washed with PBS and fresh media was added. At 1h post infection, 50μg/ml gentamicin was added to kill extracellular bacteria. RNA was purified using the RNAqueous kit according to the manufacturer's instructions (Ambion; Austin, TX). RNA was DNase treated, processed, and analyzed by real-time quantitative reverse transcription-PCR as previously described (Leber, Crimmins et al. 2008).

Luciferase Reporter Delivery System

$5x10^5$ IFNR- BMM were infected at an MOI of 5 with *L. monocytogenes* strains bearing a plasmid reporter, pBHE573, that encodes luciferase under a CMV promoter as previously described (Sauer, Witte et al.). 1 mg/ml extracellular hen egg white lysozyme was added at 1h post infection where indicated. IFNα/βR- BMM were used to prevent *L. monocytogenes* induced IFN signaling and host protein synthesis inhibition.

LDH release and IL-1β ELISA

$5x10^5$ BMM were pretreated 12-16h with 100ng/ml Pam3CSK4 (Invivogen; San Diego, CA) and infected at MOI of 5 as previously described (Sauer, Witte et al.). Extracellular hen egg white lysozyme (Sigma) was added at 1mg/ml at 1h post infection where indicated. Supernatants were collected 6h post infection and analyzed for LDH release and IL-1β secretion. To measure LDH release, 60uL supernatant was added to 60uL LDH detection reagent as previously described in duplicate in 96-well plates (Sauer, Witte et al.). IL-1β secretion was determined using mouse IL-1β ELISA Ready-SET-Go! according to the manufacturer's instructions (eBioscience; San Diego, CA).

Immunofluorescence Microscopy

Infected BMM were fixed with 4% paraformaldehyde. Samples were stained using a rabbit polyclonal anti-*L. monocytogenes* primary antibody (Difco) and an Alexa-Fluor 488 conjugated goat anti-rabbit IgG (Invitrogen) secondary. Cells were stained with Rhodamine-Phalloidin for actin and Dapi for nucleic acid. Images were acquired with an Olympus IX81 epifluorescence microscope using a 60X objective. Images were digitally

overlayed using Metamorph software (Universal Imaging). BMM were scored positive for bacterial degradation based on the presence of multiple fluorescent specks smaller than *L. monocytogenes*.

PGN preparation and delivery to RAW-κB reporter cells
PGN was prepared from 15 mL wt, Pgd-, Pgd/Oat-minus *L. monocytogenes* stationary phase cultures. Cultures were spun down, resuspended in 2% SDS, pelleted, washed with PBS and acetone. Washed pellets were bead-beat and resuspended in PBS. PGN concentration was quantified using a ninhydrin assay and normalized by Dap standard curve. 5x104 RAW-κB reporter cells were plated in 96 well plates and PGN mixed with 10X digitonin permeablization solution was delivered to the cytosol by, aspirating media and incubating for 30 minutes. Wells were aspirated, fresh media was added and cells were incubated for an additional 2h. Media was aspirated and 40uL TNT lysis solution and 40 μL of luciferase substrate solution were added to each well and luminescence was measured.

Results

Pgd-minus *L. monocytogenes* are sensitive to lysozyme.
To determine the relative lysozyme sensitivity of Pgd-minus, Oat-minus and Pgd/Oat-minus *L. monocytogenes*, we assayed growth in broth culture and on LB agar in the presence or absence of hen egg white lysozyme. Bacteria were grown for 4h to mid-log phase followed by the addition of titrated concentrations of lysozyme within the physiological range (Hansen and Karle 1971; Gordon, Todd et al. 1974; Hankiewicz and Swierczek 1974; Prixova 1975; Cohn 1978; Mendiondo, Suit et al. 1978; Vinding, Eriksen et al. 1987; Cole, Dewan et al. 1999), and growth was monitored for an additional 4h (Fig 2.1A-1D). Wild-type and Oat-minus bacteria grew similarly in the presence or absence of lysozyme at all concentrations tested. In contrast, the growth of Pgd-minus was inhibited by 100μg/ml lysozyme, while growth of Pgd/Oat-minus was inhibited by a concentration of 50μg/ml. In addition, optical density of the cultures decreased in the presence of 100μg/ml lysozyme indicating bacteriolysis. Similarly, bacterial CFUs of Pgd-minus and Pgd/Oat-minus decreased greater than 2 and 3 logs respectively in the presence of lysozyme while wild-type were unaffected (data not shown). Pgd- and Pgd/Oat-minus stationary phase cultures were also sensitive to lysozyme (data not shown). Similar results were seen using a disc diffusion assay. Bacterial lawns, grown on LB plates with Whatman paper discs containing 1mg lysozyme were measured for zones of clearance of each strain of *L. monocytogenes* (Fig 2.1E-1H). As expected, no clearance was observed for wild-type or Oat-minus *L. monocytogenes*, while the average zone of clearance for Pgd-minus was 16.8mm diameter and 18.75mm for the Pgd/Oat-minus strain. These data indicated that wild-type and Oat-minus *L. monocytogenes* were lysozyme resistant whereas the double Pgd/Oat-minus mutant were more sensitive to lysozyme than the Pgd-minus mutation alone.

Lysozyme-sensitive *L. monocytogenes* demonstrate intracellular growth defects that are rescued in the absence of LysM.

To determine the role of Pgd and Oat during the intracellular lifecycle, bone marrow derived macrophages (BMM) from C57BL/6 (wild-type) mice were infected with either wild-type, Pgd-minus, Oat-minus or Pgd/Oat-minus *L. monocytogenes*. Oat-minus *L. monocytogenes* had no detectable growth defect (data not shown), Pgd-minus had a small defect, and Pgd/Oat-minus *L. monocytogenes* demonstrated a more significant defect at early time points (Fig 2.2A). CFUs between 0.5h to 2h increased 1.5-fold during wt infection and 1.1-fold during infection with Pgd-minus. In contrast Pgd/Oat-minus CFUs decreased 2.4-fold during this time and the difference between wt and Pgd/Oat-minus CFUs was statistically significant at 2h by students t test ($p=0.0163$). An LLO-minus mutation was introduced into each of these strains to directly examine the fate of bacteria trapped in phagosomes. LLO/Pgd- and LLO/Pgd/Oat-minus had approximately a half-log and 2-log reduction in CFUs respectively at 8h in wild-type BMM (Fig 2.2C). These data suggested that the primary observed defect occurred within phagosomes, while cytosolic bacteria grew nearly as well as wild-type. The growth defect of the Pgd-minus and Pgd/Oat-minus was rescued in LysM- BMM (Fig 2.2B). Similarly, the loss of CFUs in LLO/Pgd- and LLO/Pgd/Oat-minus infections was rescued in LysM- BMM (Fig 2.2D). These data suggested that lysozyme sensitivity of Pgd- and Pgd/Oat-minus *L. monocytogenes* accounted for the defects in intracellular growth in BMM.

Lysozyme-sensitive *L. monocytogenes* induce increased vacuolar and cytosolic cytokine signaling.

To determine if lysozyme susceptibility results in a differential host innate immune response, we analyzed vacuolar and cytosolic cytokine expression in C57BL/6 (wild-type) or LysM- BMM infected with lysozyme-sensitive *L. monocytogenes*. *L. monocytogenes* that secrete LLO, a pore forming hemolysin, access the cytosol and induce strong MyD88 and TRIF-independent IFNβ expression (O'Riordan, Yi et al. 2002). *L. monocytogenes* that lack LLO are unable to escape from the phagosome and fail to induce IFNβ, but instead stimulate a specific MyD88-dependent cytokine expression program that emanates from the vacuole that includes IL-12 and IL-1β transcription (O'Riordan, Yi et al. 2002).

Expression of IL-1β (Fig 2.3A) and IL-12 (Fig 2.3B) increased during infection by LLO/Pgd- and LLO/Pgd/Oat-minus *L. monocytogenes* compared to wild-type. Increased IL-1β and IL-12 expression by lysozyme-sensitive strains was not observed in LysM- BMM (Fig 2.3A, 2.3B). Surprisingly, Pgd- and Pgd/Oat-minus *L. monocytogenes,* that expressed LLO, also stimulated increased IL-1β and IL-12 expression in a lysozyme-dependent manner (Fig 2.3A, 2.3B).

Pgd-minus and Pgd/Oat-minus *L. monocytogenes* stimulated increased IFNβ transcription in wild-type BMM but not LysM- (Fig 2.3C). Pgd- and Pgd/Oat-minus induced 2.75- and 1.70-fold more IFNβ transcription respectively compared to wt infection. Increased IFNβ expression was not observed in LysM- BMM infected with lysozyme-sensitive strains. As expected, LLO/Pgd- and LLO/Pgd/Oat-minus *L. monocytogenes* did not induce IFNβ (data not shown). These data suggested that lysozyme-sensitive *L. monocytogenes* that have escaped the phagosome, are more readily sensed by a cytosolic surveillance pathway leading to increased IFNβ expression. Similarly, lysozyme-

sensitive *L. monocytogenes* are more readily sensed by TLRs, leading to enhanced transcription of IL-1β and IL-12.

Lysozyme-sensitive *L. monocytogenes* undergo increased bacteriolysis in the macrophage cytosol

We sought to determine if lysozyme-sensitive strains lyse during infection. Wild-type or LysM- BMM were infected with wild-type, Pgd-minus or Pgd/Oat-minus *L. monocytogenes* and were visualized by fluorescence microscopy for evidence of bacteriolysis. After 2h, degradation of Pgd/Oat-minus *L. monocytogenes* was clearly observed in wild-type BMM (Fig 2.4A) but not in LysM- BMM (Fig 2.4B). Evidence of bacterial degradation was observed in 20.7% wild-type BMM infected with Pgd/Oat-minus *L. monocytogenes* and 4.0% Pgd-minus *L. monocytogenes*. Less than 1% of LysM- BMM infected with Pgd or Pgd/Oat *L. monocytogenes* showed evidence of bacterial degradation. None of the BMM infected with wild-type *L. monocytogenes* showed evidence of degradation.

To indirectly measure cytosolic bacteriolysis, we used a reporter plasmid bearing a luciferase gene under the control of a CMV promoter (Sauer, Witte et al.). Lysis of cytosolic bacteria bearing the reporter plasmid results in delivery of the plasmid to the cytosol and host expression of luciferase. A positive control for release of plasmid into the cytosol was a *L. monocytogenes* strain expressing PSA bacteriophage holin and lysin under the control of the *actA* promoter (holin/lysin) designed to lyse in the cytosol. Pgd-minus infection stimulated approximately 2.5-fold increase in luciferase expression compared to wild-type (Fig 2.4C), while Pgd/Oat-minus stimulated 11-fold more, establishing that Pgd-minus and Pgd/Oat-minus underwent cytosolic bacteriolysis and released DNA to the cytosol. This led us to hypothesize that additional cytosolic receptors could be activated by Pgd- and Pgd/Oat-minus *L. monocytogenes*.

Lysozyme-sensitive *L. monocytogenes* induce AIM2-dependent pyroptosis.

To determine the role of lysozyme activity in induction of pyroptosis, we assayed wild-type and LysM- BMM infected with lysozyme-sensitive strains for release of lactate dehydrogenase (LDH) and secretion of IL-1β. To determine the pathway of activation, we assayed BMM lacking ASC or Nlrp3. Holin/lysin and *L. monocytogenes* Δ2473 were recently identified as hyperinducers of pyroptosis and were therefore used as positive controls (Sauer, Witte et al.).

Infection of wild-type BMM with Pgd- and Pgd/Oat-minus *L. monocytogenes* stimulated 2 and 2.5-fold more LDH release than wild-type respectively (Fig 2.5A). Pgd- and Pgd/Oat-minus *L. monocytogenes* stimulated 3 and 5-fold more IL-1β secretion than wild-type respectively (Fig 2.5B). As expected, lysozyme-sensitive strains, in the LLO-minus background, stimulated no cell death or IL-1β secretion (Fig 2.5A, 2.5B). The increase in LDH release and IL-1β secretion induced by Pgd- and Pgd/Oat-minus *L. monocytogenes* in wild-type BMM was not observed in LysM- BMM (Fig 2.5A, 2.5B), indicating a lysozyme dependent mechanism of inflammasome induction. Pgd- and Pgd/Oat-minus *L. monocytogenes* induced increased LDH release and IL-1β secretion in Nlrp3- BMM, but no increase was observed in ASC- BMM. This indicated that

lysozyme-sensitive *L. monocytogenes* activate an ASC-dependent pathway of inflammasome activation independent of Nlrp3.

L. monocytogenes strains that lyse in the cytosol activate AIM2 (Sauer, Witte et al.), a cytosolic DNA receptor that stimulates inflammasome activation in an ASC dependent manner (Warren, Armstrong et al. ; Hornung, Ablasser et al. 2009). To test the hypothesis that AIM2 detects lysozyme-sensitive *L. monocytogenes*, we measured cell-death and IL-1β release in immortalized BMM with shRNA mediated AIM2 stably knocked down. LDH and IL-1β secretion were completely dependent on the presence of AIM2 (Fig 2.6A, 2.6B). A control strain ectopically expressing *Legionella pneumophila* flagellin (JD Sauer & DAP unpublished observation) was used to stimulate AIM2-independent cell death mediated by Nlrc4 (IPAF) activation. These data indicated that lysozyme sensitive strains stimulated the AIM2 inflammasome following lysozyme-induced bacteriolysis and subsequent release of bacterial DNA into the host cell cytosol.

Host innate immune response to lysozyme-sensitive *L. monocytogenes* is independent of TLR2, NOD1, and NOD2 receptors

TLR2 deficient mice have been reported as more sensitive to *L. monocytogenes* infection. TLR2 detects cell wall-associated ligands. Therefore, we hypothesized that TLR2 may be increasingly activated by lysozyme-sensitive *L. monocytogenes*. To determine whether TLR2 contributed to detection and increased vacuolar response elicited by Pgd- and Pgd/Oat-minus *L. monocytogenes*, we infected wt or TLR2- BMM and measured the vacuolar cytokine response. Expression of IL-12 increased during infection by Pgd-minus compared to wild-type *L. monocytogenes* in both wt and TLR2- BMM (2.7A). *L. monocytogenes* PGN is predicted to contain ligands of both NOD1 and NOD2. Previously, it was shown that activation of NOD2 was required for full induction of IFNβ during infection with wild-type *L. monocytogenes* when BMM were tolerized with a TLR2 ligand to reduce NF-κB activation by TLRs (Leber, Crimmins et al. 2008). To determine whether NOD1 or NOD2 contributed to hyper induction of IFNβ by lysozyme-sensitive *L. monocytogenes*, we infected MyD88 deficient BMM that also lacked NOD1, NOD2, or both NOD receptors and measured IFNβ transcription by RT-qPCR (Fig 2.7B). Interestingly, there was no significant difference in IFNβ elicited by wild-type in each of the infections. Similarly, Pgd-minus induced increased IFNβ to the same extent in the absence of MyD88 and either NOD1, NOD2 or both NOD receptors. The NOD2 ligand, MDP, has previously been reported to activate the NLRP1 inflammasome and NOD2 has been reported to interact with the NLRP1 inflammasome (Faustin, Lartigue et al. 2007; Hsu, Ali et al. 2008). To determine whether NOD2 or NOD1 contribute to hyper-induction of the inflammasome by lysozyme-sensitive *L. monocytogenes*, we infected wt, NOD2-, or NOD1/NOD2-minus BMM and measured LDH release. No significant difference in induction of cell death was observed in NOD2- or NOD1/NOD2-minus BMM (Fig 2.7C). TLR2, NOD1, and NOD2 signaling activate the pro-inflammatory transcription factor, NF-κB. To determine whether acetylated *L. monocytogenes* PGN stimulates increased innate signaling, we delivered wt, Pgd-, or Pgd/Oat-minus PGN to the cytosol of RAW-κB cells, which express luciferase under the control of iκκ. PGN was mixed with digitonin permeablization solution or added directly to RAW-κB cells and NF-κB activation was measured (Fig 2.7D). No significant

difference in NF-κB activation by wt, Pgd-, or Pgd/Oat-minus PGN was observed when delivered by digitonin. There appeared to a slight increase in NF-κB activation by Pgd/Oat-minus PGN, however, the increase was not statistically significant. Taken together, these results suggested that TLR2, NOD1 and NOD2 do not significantly contribute to the innate immune response to lysozyme-sensitive *L. monocytogenes*.

Discussion

The precise role of lysozyme during innate immunity to microbial infection is yet to be fully appreciated. The aim of this study was to assess the role of host lysozyme resistance by comparing *L. monocytogenes* strains that were either resistant or sensitive to lysozyme. The results of this study showed that lysozyme acts extracellularly, within phagocytic vacuoles, and surprisingly, in the cytosol of infected cells. In phagosomes, lysozyme activity led to the release of bacterial ligands that activated a vacuole-specific program of cytokine induction. In the cytosol, lysozyme led to bacteriolysis, thereby activating two distinct cytosolic innate immune pathways, one leading to the expression of IFNβ and another leading to DNA-dependent, AIM2-dependent pyroptosis. All of the phenotypes associated with lysozyme sensitivity were reversed during infection of macrophages lacking LysM. Surprisingly, all of the above phenotypes were restored by simply adding lysozyme to the extracellular media.

IL-12 and IL-1β transcription is stimulated by *L. monocytogenes* trapped in phagosomes (O'Riordan, Yi et al. 2002). Lysozyme-sensitive *L. monocytogenes* stimulated increased expression of these cytokines in LLO-minus and LLO-expressing backgrounds. TLR2 is a candidate receptor involved in this pathway since it is both localized at the cell surface and recruited to phagosomes upon internalization of microbes (Ozinsky, Smith et al. 2000). TLR2 has been reported to detect various cell wall associated ligands including peptidoglycan (although there is controversy whether it is a bona fide ligand) and has been shown to form heterodimers with TLR1 or TLR6 to detect lipopeptides and LTA (Travassos, Girardin et al. 2004; Kumar, Kawai et al. 2009). It is likely that lysozyme activity generates increased cell wall fragments that are detected by TLR2. However, although vacuolar cytokines were dependent on MyD88, infection with Pgd-minus *L. monocytogenes* induced similar levels of IL-1β and IL-12 in TLR2- and wild-type BMM (Fig 2.7 A). Therefore the precise nature of the bacterial ligand(s) or host receptor(s) is not known.

Cytosolic *L. monocytogenes* induce a MyD88-independent response that is monitored by IFNβ expression. Here, IFNβ expression is elevated approximately 2-fold by lysozyme-sensitive mutants. One possible explanation is that cytosolic bacteria are lysed resulting in release of DNA and c-di-AMP, two ligands that activate IFNβ expression. A second possibility is that N-deacetylated peptidoglycan shed from growing bacteria has enhanced stimulatory activity. Indeed, Boneca et al reported that N-deacetylated peptidoglycan had increased NOD1 stimulatory activity on peritoneal elicited macrophages and that digested Pgd-minus PGN induced increased NFκB activity in both NOD1 and NOD2 dependent manner (Boneca, Dussurget et al. 2007). However, using NOD1-, NOD2-, or NOD1/NOD2-minus BMM, we still observed an approximate 3-fold difference in IFNβ

expression between wild-type and Pgd-minus bacteria (Fig 2.7B). While it is possible that NOD1 and NOD2 expression was different, our data favor the hypothesis that increased bacteriolysis and release of nucleic acids led to enhanced IFNβ expression.

This study also revealed that lysozyme acts in the cell cytosol causing bacteriolysis and subsequent host cell death. These results support the recently emerging concept that cytosolic bacteriolysis is linked to activation of the AIM2-dependent inflammasome (Sauer, Witte et al.). Indeed, using strains differentially susceptible to lysozyme we showed that increased lysozyme-sensitivity correlates with increased bacteriolysis and AIM2-dependent pyroptosis. This is the first study to show a link between lysozyme and AIM2 stimulation. However, a previous study has linked lysozyme activity to the Nlrp3 inflammasome. Shimada et al have shown that vacuolar digestion of *S. aureus* PGN by lysozyme is required for Nlrp3 stimulation (Shimada, Park et al.). However, we found no significant role for Nlrp3 in response to lysozyme-sensitive *L. monocytogenes* strains despite distinct lysozyme activity in the vacuole, suggesting *L. monocytogenes* PGN is not sensed via the same pathway. It is unknown whether *S. aureus* lysozyme-digested PGN is the ligand detected directly by Nlrp3 or if activation is stimulated by its feature as a small crystalline molecule (Lamkanfi and Kanneganti). Nevertheless, similar to *S. aureus*, *L. monocytogenes* specifically subvert inflammasome activation by modification of its peptidoglycan.

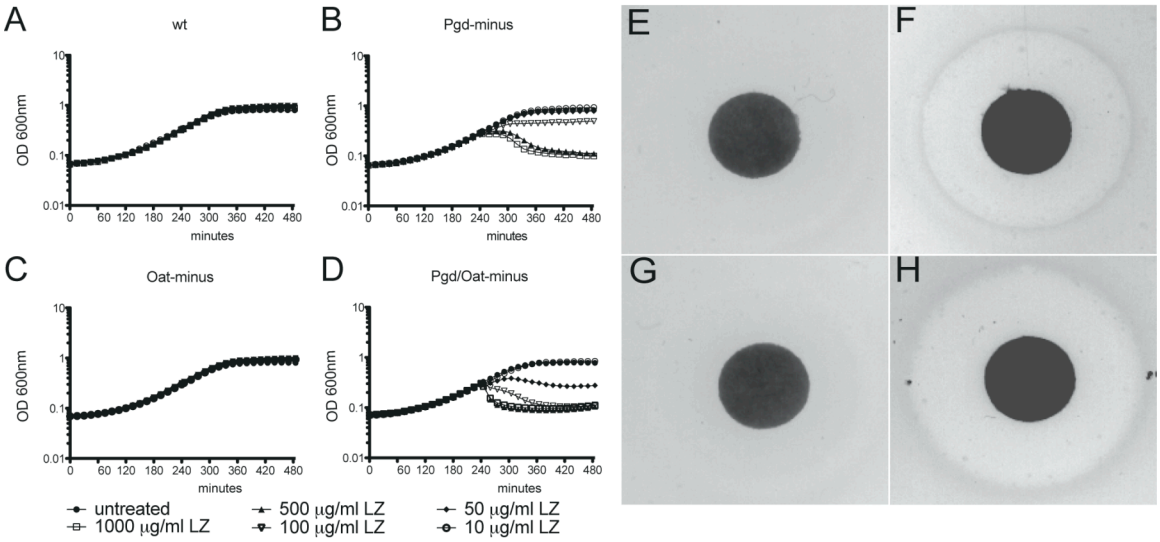


Figure 2.1- Sensitivity of *L. monocytogenes* strains to lysozyme.
wt (A), Pgd- (B), Oat- (C), and Pgd/Oat-minus (D) *L. monocytogenes* growth in BHI in the presence of titrated HEW lysozyme **(**LZ). Lysozyme was added to cultures in equal volumes at 240 minutes after bacterial inoculation and OD 600nm was monitored at 15 minute intervals. wt (E), Pgd- (F), Oat- (G), Pgd/Oat-minus (H) *L. monocytogenes* growth on LB plates containing 1mg lysozyme on a Whatman paper disc. Bacterial lawns were grown overnight and the zone of clearance was measured the next day. Data is representative of more than 3 independent experiments with similar results.

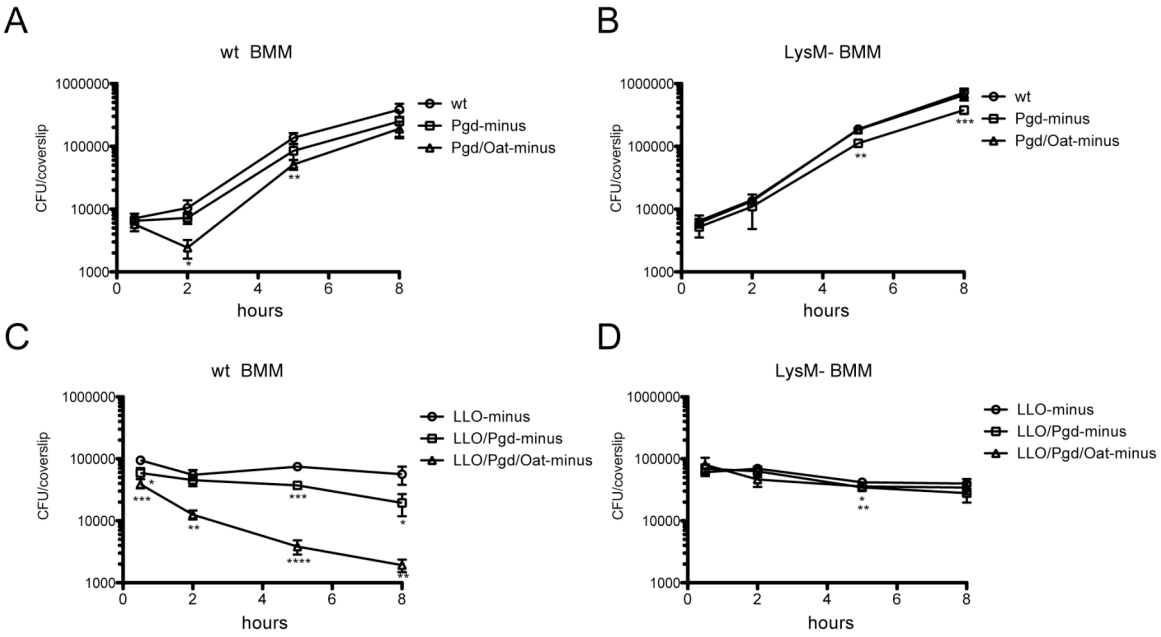


Figure 2.2- Growth of *L. monocytogenes* strains in BMM.
$2x10^6$ wt (A) or LysM- (B) BMM were infected with wt, Pgd- or Pgd/Oat-minus *L. monocytogenes* at a MOI = 0.1. $2x10^6$ wt (C) or LysM- (D) BMM were infected with LLO-minus *L. monocytogenes* strains at MOI = 1. BMM were lysed and bacterial CFUs were quantified. Error bars represent the standard deviation of the means of technical triplicates and data is representative of more than 3 independent experiments with similar results. Student's t test was used to analyze statistical significance compared to wt *L. monocytogenes* infection at each time point where * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$, and **** indicates $P<0.0001$.

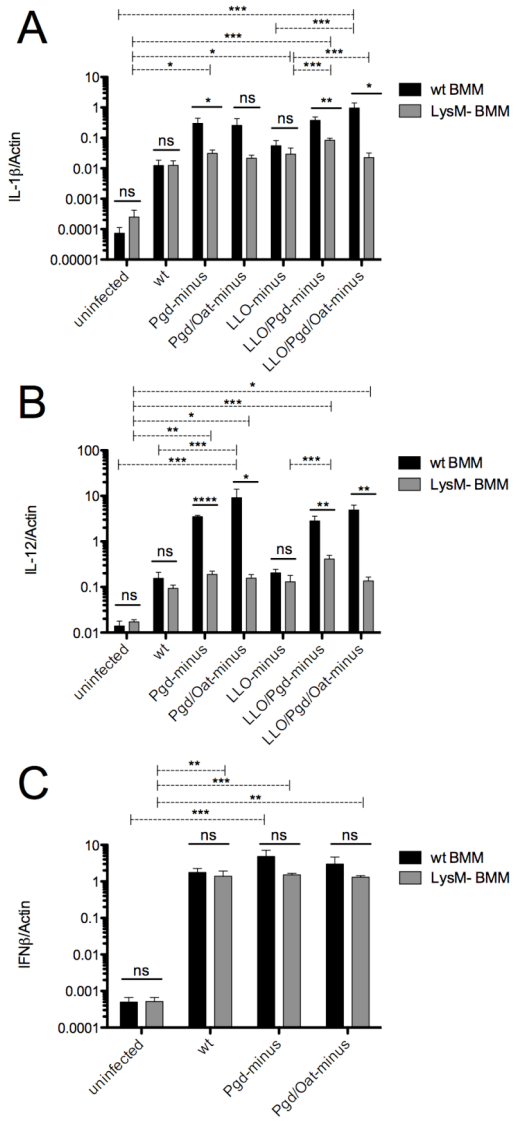


Figure 2.3- Pgd-minus and Pgd/Oat-minus *L. monocytogenes* induce increased cytosolic and vacuolar cytokine responses dependent on the presence of host lysozyme.

10^6 wt (black bars) or LysM- (grey bars) BMM were infected with wt or lysozyme-sensitive *L. monocytogenes* at MOI = 1 for 4h. LLO-minus infections were also performed at MOI = 1. RNA was harvested and IL-1β (A), IL-12 (B) and (C) IFNβ transcripts were measured relative to β-actin by qPCR. Error bars represent the standard deviation of the means determined in triplicate. Data is representative of at least three independent experiments with similar results. One-way ANOVA followed by post hoc Tukey test was used to analyze significance of infections in either wt or LysM- BMM (dashed lines). Student's t test was used to analyze significance for each bacterial strain. * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$, and **** indicates $P<0.0001$.

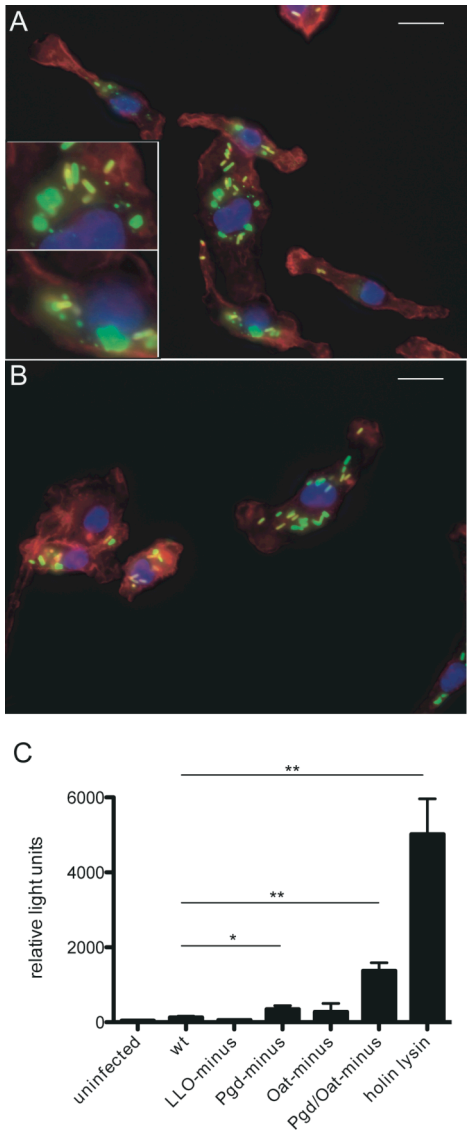


Figure 2.4- Intracellular bacteriolysis of lysozyme-sensitive *L. monocytogenes.* Immunofluorescence microscopy of wt (A) or LysM- (B) BMM infected with Pgd/Oat-minus *L. monocytogenes* (green) imaged at 90 minutes post infection (Actin and nuclei were stained red and blue, respectively). Insets depict bacterial degradation. Size bars represent 10μm. Cytosolic DNA delivery is increased in Pgd-minus and Pgd/Oat-minus *L. monocytogenes* **(**C**)**. IFNα/βR- BMM were infected with *L. monocytogenes* bearing a plasmid containing luciferase under a CMV promoter at MOI = 5. Luciferase expression was detected at 6h post infection. Error bars represent standard deviation of the means determined in triplicate. Statistical significance was evaluated using a Student's t test where * indicates $P<0.01$ and ** indicates $P<0.0001$. Data is representative of at least 3 separate experiments with similar results.

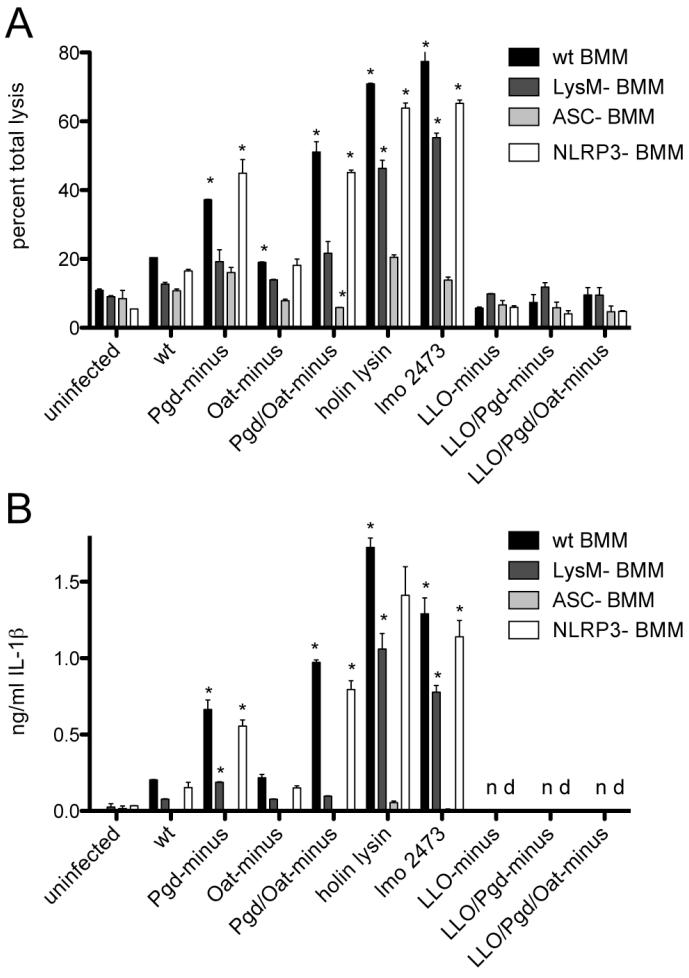


Figure 2.5- Pgd-minus and Pgd/Oat-minus *L. monocytogenes* induce increased inflammasome activation.

LDH release in wt, LysM-, ASC-, or NLRP3-minus Pam3CSK4-stimulated BMM (A). $5x10^5$ BMM were infected for 6h at MOI= 5. IL-1β secretion detected by ELISA in wt, LysM-, ASC-, or NLRP3-minus Pam3CSK4 stimulated BMM at 6h post infection at MOI= 5 (B). Error bars represent the standard deviation of the means. Statistical significance was evaluated compared to wt *L. monocytogenes* infection using a Student's t test where * indicates $P<0.01$. Data are representative of more than three independent experiments with similar results.

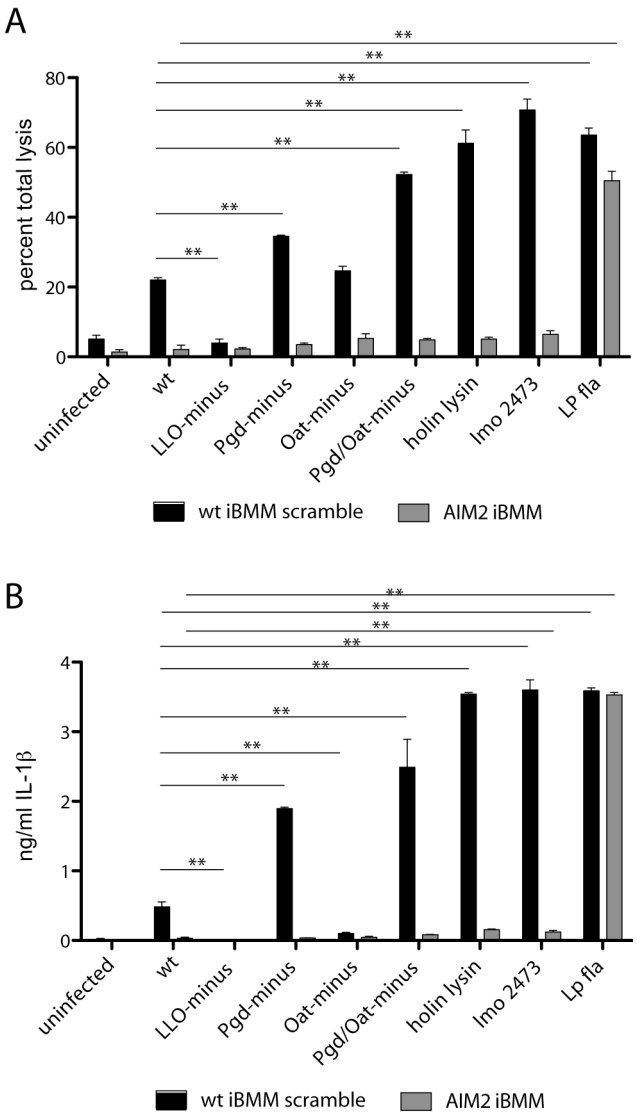


Figure 2.6- Pgd-minus and Pgd/Oat-minus *L. monocytogenes* activate the AIM2 inflammasome.

Pam3CSK4-stimulated immortalized shRNA AIM2 knockdown or shRNA scrambled macrophages were infected at MOI=5 and LDH was measured at 6h (A). IL-1β secretion was measured by ELISA in Pam3CSK4 stimulated immortalized shRNA AIM2 knockdown or shRNA scrambled immortalized macrophages infected for 6h (B). Error bars represent the standard deviation of the means determined in triplicate. Statistical significance was evaluated using a Student's t test where ** indicates $P<0.0001$. Data are representative of more than three independent experiments with similar results.

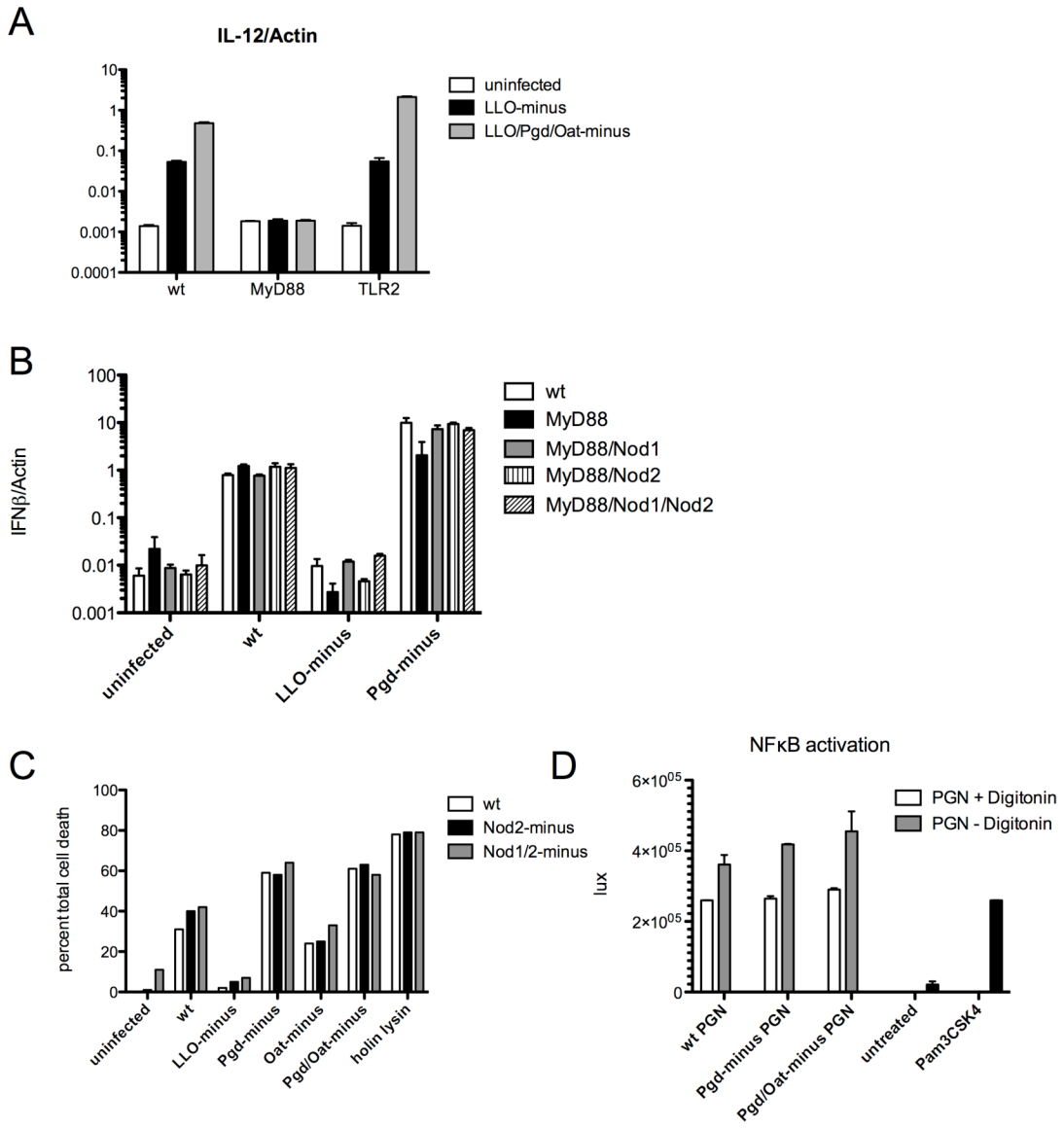


Figure 2.7- Pgd-minus and Pgd/Oat-minus *L. monocytogenes* hyper-induction of innate immune pathways is independent of TLR2, NOD1, and NOD2.
10^6 wt, MyD88-minus, or TLR2-minus BMM were infected with wt or lysozyme-sensitive *L. monocytogenes* at MOI = 1 for 4h. RNA was harvested and IL-12 transcripts were measured relative to β-actin by qPCR (A). 10^6 wt, MyD88-minus, MyD88/NOD1-minus, MyD88/NOD2-minus, or MyD88/NOD1/NOD2 BMM were infected with wt or lysozyme-sensitive *L. monocytogenes* at MOI = 1 for 4h. RNA was harvested and IFNβ transcripts were measured relative to β-actin by qPCR (B). RAWκB cells were treated with wt, Pgd-, or Pgd/Oat-minus PGN in the presence or absence of digitonin permeablization solution for 4h. Cells were lysed with TNT lysis buffer, luciferase substrate solution was added and luminescence was measured (C). LDH release in wt, NOD2-minus, or NOD1/NOD2-minus Pam3CSK4-stimulated BMM (D). $5x10^5$ BMM were infected for 6h at MOI= 5 and LDH release was measured at 6h post infection.

Chapter 3: Extracellular Lysozyme Affects Cytosolic *Listeria monocytogenes* and Characterization of Lysozyme-sensitive *Listeria monocytogenes* in vivo

Introduction

In mammals, lysozyme is one of the most abundant proteins in both epithelial secretions (including mucous, tears, saliva, and milk) and phagocytes, however, its specific localized and temporal interactions with invading microbes are not fully characterized (Callewaert and Michiels). Lysozyme activity is readily detected in blood and has a ubiquitous tissue distribution (Callewaert and Michiels ; Hansen and Karle 1971; Hankiewicz and Swierczek 1974; Prixova 1975; Mendiondo, Suit et al. 1978; Vinding, Eriksen et al. 1987). Lysozyme is the primary component of neutrophil granules and the major secretory product of macrophages (Callewaert and Michiels ; Gordon, Todd et al. 1974; Cohn 1978). To our knowledge, the precise concentration of lysozyme in a macrophage vacuole is unknown. It is also unknown whether lysozyme is present in the cytosol. Reports have shown immunogold staining of cytosolic lysozyme (Resnitzky, Shaft et al. 1994), however, to our knowledge there has been no additional evidence of cytosolic lysozyme.

Lysozyme has been studied extensively since its discovery in the 1920's by Alexander Fleming and has greatly contributed to the fields of biochemistry and enzymology, however, the biological role of lysozyme in vivo have is yet to be determined. Mice expressing rat lysozyme under a lung-specific promoter had increased concentrations of lysozyme in airway fluid, which resulted in increased killing of group B *Streptococcus* and *Psuedomonas aeruginosa*, suggesting a role as a bactericidal effector (Cole, Thapa et al. 2005). Lysozyme deficient mice have only recently been engineered. Humans express a single lysozyme gene, while mice express two: LysM and LysP. LysM is the human homolog and is found in leukocytes and epithelial secretions, and LysP is expressed in intestinal paneth cells. LysM and LysP differ by only six amino acid substitutions and are located within 5kb of each other and have similar muramidase activity (Cross, Mangelsdorf et al. 1988; Markart, Faust et al. 2004). LysM- disruption in mice results in up-regulation of LysP and partial compensation of muramidase activity (Ganz, Gabayan et al. 2003; Markart, Faust et al. 2004). However, LysM- mice showed increased bacterial burden and mortality during *Klebsiella pneumoniae* or *P. aeruginosa* infection (Markart, Korfhagen et al. 2004; Cole, Thapa et al. 2005). Similarly, LysM- mice showed increased susceptibility to middle ear infection by *Streptococcus pneumoniae* (Shimada, Moon et al. 2008) and lysozyme-sensitive mutants displayed a competitive disadvantage in respiratory tract colonization that was rescued in LysM- mice (Davis, Akinbi et al. 2008). These results have strengthened the notion of lysozyme function as a bactericidal effector. However, LysM-mice have shown increased inflammation caused by infection by non-pathogenic *Micrococcus luteus*, suggesting lysozyme is important for breaking down PGN fragments to reduced prolonged, inappropriate innate immune signaling by non-pathogens (Ganz, Gabayan et al. 2003).

The in vivo role of lysozyme during *L. monocytogenes* infection is unknown. The most common in vivo model used to study *L. monocytogenes* infection in the mouse is by iv injection. *L. monocytogenes* traffic through the blood and are delivered to liver and spleen and establish their primary replicative niche in resident macrophages. CFUs increase for 2-5 days post infection and begin to disappear by days 5-7 (Vazquez-Boland, Kuhn et al. 2001; Portnoy, Auerbuch et al. 2002). Early host resistance to infection requires the production of IFNγ, primarily made by NK cells in response to IL-12 and TNFα produced by infected macrophages. Most *L. monocytogenes* are killed by activated resident macrophages, however, early recruitment of neutrophils also seems to contribute. IFNγ-deficient mice are highly susceptible to *L. monocytogenes* infection, as are MyD88-deficient mice (Tripp, Wolf et al. 1993; Tripp, Kanagawa et al. 1995; Edelson and Unanue 2002; Seki, Tsutsui et al. 2002). Following the acute phase of infection, the few remaining *L. monocytogenes* are cleared by a strong antigen-specific CD8 T-cell response, which peaks near 8 days post infection, and provides robust protective immunity (Pamer 2004).

The precise aspects of *L. monocytogenes* infection that contribute to such a robust protective immune response are under investigation, however, the role of lysozyme is yet to be determined. It is assumed that prior to development of immunity, innate immune detection occurs and is the basis adjuvanticity. A critical feature of *L. monocytogenes* infection required for the generation of protective immunity is that bacteria escape from the vacuole and access the cytosol (Berche, Gaillard et al. 1987; Bahjat, Liu et al. 2006). LLO-minus *L. monocytogenes* fail to stimulate protective immunity, whereas ActA-minus *L. monocytogenes*, which are severely attenuated but still access the cytosol, induce strong immunity. Innate immune detection of cytosolic *L. monocytogenes* influences the generation of acquired immunity. MyD88 is critical for acute protection, but is not required for generation of protective immunity by ActA-minus *L. monocytogenes (Edelson and Unanue 2002; Serbina, Kuziel et al. 2003; Kursar, Mittrucker et al. 2004; Bahjat, Meyer-Morse et al. 2009; Tam and Wick 2009)*. In fact, activation of the vacuolar response suppresses generation of immunity. In experiments to determine whether induction of immunity by ActA-minus was dominant over LLO-minus *L. monocytogenes*' failure to induce immunity, Bahjat et al showed that LLO-minus suppress immunity by induction of MyD88-dependent IL-10 (Bahjat, Meyer-Morse et al. 2009). STING-minus mice also develop immunity to *L. monocytogenes*, suggesting activation of the cytosolic surveillance pathway is not required for immunity. Similarly, Caspase1-minus mice develop immunity to wild-type *L. monocytogenes*. However, a strain that ectopically expresses *L. pneumophila* flagelin (Lp fla) and activates the NLRC4 inflammasome was deficient in inducing protective immunity (Sauer, Pereyre et al. 2011).

It is unknown whether N-deacetylation by Pgd serves additional roles besides to confer resistance to lysozyme activity. Pgd-minus *L. monocytogenes* are more sensitive to autolysis inducing agents, triton-X-100 and EDTA, and various antibiotics (Popowska, Kusio et al. 2009), suggesting a role in protection from additional antimicrobial stresses. It is predicted that Pgd-minus *L. monocytogenes* bearing N-acetylated glucosamine residues would have a more negative surface charge due to lack of an exposed primary

amine residue and would thus be more susceptible to cationic peptides (Vollmer 2008). Production of cationic antimicrobial peptides is an innate host immune defense that many Gram-positive bacteria avoid by increasing cell surface electronegativity (Hancock and Scott 2000; Peschel 2002). One mechanism to increase cell surface charge is modification of teichoic acids by d-ala esterification, which is carried out through the action of the *dlt* operon in a variety of bacteria including *L. monocytogenes* (Abachin, Poyart et al. 2002; Collins, Kristian et al. 2002; Peschel 2002; Neuhaus and Baddiley 2003; Kristian, Datta et al. 2005). Inactivation of *dltA* has been shown to inhibit d-ala esterification resulting in susceptibility to cationic peptides in many Gram-positives including *L. monocytogenes (Abachin, Poyart et al. 2002; Collins, Kristian et al. 2002; Peschel 2002; Neuhaus and Baddiley 2003; Vadyvaloo, Arous et al. 2004; Kristian, Datta et al. 2005; Mandin, Fsihi et al. 2005)*. Another mechanism to increase cell surface charge is lysinylation of membrane phosphatidyl glycerol, which is carried out through the action of *mprF* in several Gram-positives, including *L. monocytogenes* (Peschel, Jack et al. 2001; Thedieck, Hain et al. 2006). Interestingly, lysozyme displays cationic antimicrobial activity independent of muramidase action and the role of its cationic activity during *L. monocytogenes* infection remains unknown (Masschalck, Deckers et al. 2002; Nash, Ballard et al. 2006).

In this section, we investigated the surprising observation that lysozyme-sensitive *L. monocytogenes* lysed in the host cytosol and hyper-induced the cytosolic surveillance response and the AIM2-dependent inflammasome. We discovered that cytosolic replication was required for inflammasome activation and, surprisingly, that exogenous lysozyme added after infection could act on cytosolic lysozyme-sensitive bacteria. We examined the consequences of lysozyme sensitivity in vivo during acute infection and generation of protective immunity and discovered that virulence defects in Pgd- and Pgd/Oat-minus *L. monocytogenes* were not rescued in LysM- mice. We hypothesized the Pgd may have additional roles besides lysozyme resistance and discovered that it may also function to regulate autolysin activity.

Methods

Bacterial Strains

All *L. monocytogenes* strains used and generated in this study were in the 10403S background. In-frame deletion mutants were constructed by splice-overlap extension and introduced by allelic exchange (Camilli, Tilney et al. 1993). Strains were grown in Brain Heart Infusion media at 30°C overnight without shaking to stationary phase for macrophage infections.

Macrophages

Bone-marrow derived macrophages were prepared from 6-8 week old female mice as previously described (Jones and Portnoy 1994). All mice were in the C57BL/6 genetic background. LysM- mice were a gift from Tomas Ganz.

Intracellular growth curves

$2x10^6$ BMM were plated overnight and infected with LLO expressing strains at a MOI of 0.1 and LLO-minus *L. monocytogenes* at a MOI of 1. Thirty minutes after infection, macrophage monolayers were washed with PBS and fresh media was added. At 1h post infection, 50μg/ml gentamicin was added to kill extracellular bacteria. Replication was quantified as previously described (Portnoy, Jacks et al. 1988). Briefly, three coverslips at each time point were washed with water to lyse macrophages. Bacteria recovered from each coverslip were plated on LB agar plates and CFUs were determined.

Luciferase Reporter Delivery System

$5x10^5$ IFNR- BMM were infected at an MOI of 5 with *L. monocytogenes* strains bearing a plasmid reporter, pBHE573, that encodes luciferase under a CMV promoter as previously described (Sauer, Witte et al.). 1 mg/ml extracellular hen egg white lysozyme was added at 1h post infection where indicated. IFNα/βR- BMM were used to prevent *L. monocytogenes* induced IFN signaling and host protein synthesis inhibition.

LDH release and IL-1β ELISA

$5x10^5$ BMM were pretreated 12-16h with 100ng/ml Pam3CSK4 (Invivogen; San Diego, CA) and infected at MOI of 5 as previously described (Sauer, Witte et al.). Extracellular hen egg white lysozyme (Sigma) was added at 1mg/ml at 1h post infection where indicated. Supernatants were collected 6h post infection and analyzed for LDH release and IL-1β secretion. To measure LDH release, 60uL supernatant was added to 60uL LDH detection reagent as previously described in duplicate in 96-well plates (Sauer, Witte et al.). IL-1β secretion was determined using mouse IL-1β ELISA Ready-SET-Go! according to the manufacturer's instructions (eBioscience; San Diego, CA).

Immunofluorescence Microscopy

Infected BMM or HeLa cells were fixed with 4% paraformaldehyde. Samples were stained using a rabbit polyclonal anti-*L. monocytogenes* primary antibody (Difco) and an Alexa-Fluor 488 conjugated goat anti-rabbit IgG (Invitrogen) secondary. Cells were stained with Rhodamine-Phalloidin for actin and Dapi for nucleic acid. Images were acquired with an Olympus IX81 epifluorescence microscope using a 60X objective. Images were digitally overlayed using Metamorph software (Universal Imaging). BMM were scored positive for bacterial degradation based on the presence of multiple fluorescent specks smaller than *L. monocytogenes*.

IFNβ bioassay.

Supernatant from wt or LysM-minus BMM that had been infected with *L. monocytogenes* for 6h was overlayed on interferon responsive ISRE-L929 cells for 4h. Media was aspirated and 40uL TNT lysis solution and 40 μL of luciferase substrate solution were added to each well and luminescence was measured.

In vivo infections

C57BL/6 (The Jackson Laboratory) or LysM- mice were infected with 1 LD_{50} ($1x10^5$ CFU) of wild-type, Pgd- or Pgd/Oat-minus *L. monocytogenes* and spleen and liver were harvested 48h later. 1mg TriNAG (MP Biomedicals) was injected in 200 μL iv into C57BL/6 mice approximately 30 minutes before *L. monocytogenes* infection. C57BL/6

(The Jackson Laboratory) or LysM- mice were immunized with $1x10^5$ wild-type, Pgd-, Pgd/Oat-minus, or $1x10^5$ActA-minus, $1x10^5$ ActA-minus +$1x10^8$ LLO-minus, or $1x10^5$ ActA-minus + $1x10^8$ LLO/Pgd/Oat-minus *L. monocytogenes* and subsequently challenged with 2 LD_{50} ($1x10^5$ CFU) of wild-type *L. monocytogenes*. Spleen and liver were harvested 72h later and analyzed for CFU.

Lysozyme detection assay

Serum was collected from C57BL/6 or LysM- mice using BD Microtainer Serum Separator Tubes (Becton, Dickinson and Company; Franklin Lakes, NJ) and was analyzed for the presence of lysozyme activity using an EnzChek Lysozyme Assay Kit according to the manufacturer's instructions (Invitrogen).

Broth growth curves

Stationary phase cultures were back diluted 1:40 in BHI and grown to mid-log phase at 37°C with shaking.). 2000μg/mL Lysozyme, 50μg/mL ly9, 50μg/mL mCRAMP, 50μg/mL HNP-1, or 50μg/mL Rectrocylin were added to cultures in equal volumes at 240 minutes after bacterial inoculation and OD 600nm was monitored at 15 minute intervals over the course of 8h. Alternatively, stationary phase cultures were back diluted 1:40 in BHI with titrated pH or concentrations of hydrogen peroxide and growth was measured by OD 600nm at 15 min intervals over the course of 12h.

Zymography

PGN was prepared from 15 mL wt, Pgd-, Pgd/Oat-minus *L. monocytogenes* stationary phase cultures. Cultures were spun down, resuspended in 2% SDS, pelleted, washed with PBS and acetone. Washed pellets were bead-beat and resuspended in PBS. PGN was embedded into 10% polyacrylamide gels. Secreted proteins from bacterial culture supernatant were precipitated with 10% trichloroacetic acid, washed with acetone, and resuspended in SDS-PAGE sample buffer. 20 μL precipitate was run on wt, Pgd-, or Pgd/Oat-minus PGN embedded 10% polyacrylamide gels. Gels were incubated in the presence of Triton X-100 to remove SDS and permit protein refolding. Gels were stained with 0.1% Methylene Blue + 0.01% KOH and incubated in buffer for several days until zones of clearance were visible indicating autolytic activity.

Results

Bacterial replication is required for induction of Pgd-minus and Pgd/Oat-minus mediated cell death.

We hypothesized that during infection with Pgd- and Pgd/Oat-minus *L. monocytogenes*, vacuolar lysozyme could generate increased amounts of bacterial ligands, potentially released to the cytosol following dissolution of the phagosome. An alternative hypothesis was that phagosomal lysozyme might compromise cell wall integrity of lysozyme-sensitive strains, resulting in bacteriolysis in the cytosol. If either hypothesis were correct, we would observe rapid induction of pyroptosis. However, we were unable to detect significant LDH release induced by Pgd-minus or Pgd/Oat-minus *L. monocytogenes* in wild-type BMM until 4.5h post infection (Fig 3.1A).

An alternative hypothesis was that lysozyme, encountered in secondary vacuoles following cell-to-cell spread, could generate ligands that resulted in pyroptosis. However, inhibition of cell-to-cell spread by treatment of macrophages with cytochalasin D, an inhibitor of actin polymerization, at 4h post infection, resulted in a slight drop in LDH in all samples (Fig 3.1B) (Tilney and Portnoy 1989). Pgd-minus and Pgd/Oat-minus *L. monocytogenes* induced increased overall pyroptosis, suggesting cell-to-cell spread was not required and that increased bacterial ligands were not likely generated by lysozyme in secondary vacuoles.

We then considered that cytosolic bacterial replication might be required to induce pyroptosis. Bacterial translation was blocked at 1, 2, 3, 4, or 5h after infection using 100μg/ml chloramphenicol (cm), a bacteriostatic antibiotic, and LDH release was measured at 6h (Fig 3.1C). No significant LDH release was detected in macrophages treated with cm at 1h. However, a strain ectopically expressing *Legionella* flagellin (Lp fla) that stimulates the Nlrc4 inflammasome induced rapid LDH release. An *L. monocytogenes* strain, expressing holin/lysin, which expresses phage holin and lysin under the *actA* promoter, did not induce LDH release when treated with cm at 1 or 2h, presumably because the *actA* promoter was not activated until 2h post infection. When allowed to replicate for 2h, Pgd-minus *L. monocytogenes* stimulated 6% total LDH release and Pgd/Oat-minus *L. monocytogenes* stimulated 16% total LDH release while wild-type bacteria stimulated only 5% total LDH release, indicating that increased bacteriolysis of the lysozyme-sensitive strains occurs soon after escape. However, LDH release increased with the amount of time that Pgd- and Pgd/Oat-minus *L. monocytogenes* replicated in the cytosol. These data suggested that bacterial intracellular replication was required to stimulate lysozyme dependent cell death and that *L. monocytogenes* were subject to lysozyme activity in the cytosol.

Extracellular lysozyme acts on Pgd-minus and Pgd/Oat-minus cytosolic bacteria.
To determine whether lysozyme could gain access to the macrophage cytosolic compartment, we assayed for bacteriolysis using the delivery of plasmid DNA as described above. In the presence of extracellular lysozyme, lysozyme-sensitive strains delivered plasmid, while delivery by wild-type was not significantly affected (Fig 3.2A).

To visualize whether extracellular lysozyme could induce bacteriolysis, LysM- BMM were infected with wild-type or Pgd/Oat-minus *L. monocytogenes* and 1mg/ml lysozyme was added extracellularly after 1h, which is within the biological range (Hansen and Karle 1971; Gordon, Todd et al. 1974; Hankiewicz and Swierczek 1974; Prixova 1975; Cohn 1978; Mendiondo, Suit et al. 1978; Vinding, Eriksen et al. 1987; Cole, Dewan et al. 1999). Infected LysM- BMM were visualized at 2h post infection for bacterial degradation (Fig 3.2E- 3.2H). 19.2% of Pgd/Oat-minus infected LysM- BMM treated with extracellular lysozyme showed evidence of bacterial degradation (Fig 3.2H) whereas no degradation was observed in untreated Pgd/Oat-minus infected LysM- BMM (Fig 3.2G). Similarly, LysM- BMM infected with LLO/Pgd/Oat-minus *L. monocytogenes* and treated with extracellular lysozyme had evidence of bacterial degradation, while

untreated BMM did not (data not shown), suggesting extracellular lysozyme accesses *L. monocytogenes*-containing vacuoles.

Extracellular lysozyme also resulted in induction of increased LDH release, but did not affect lysozyme resistant strains (Fig 3.2B). Addition of extracellular lysozyme 1h post infection in LysM- BMM also resulted in increased IFNβ by lysozyme-sensitive *L. monocytogenes*, but did not affect wild-type infections. Similarly, addition of extracellular lysozyme inhibited intracellular growth of lysozyme-sensitive *L. monocytogenes* in LysM- BMM, but not wild-type (Fig 3.2 I). Likewise, addition of extracellular lysozyme restored increased IFNβ production by lysozyme-sensitive *L. monocytogenes* in LysM- BMM, but not wild-type and holin lysine (Fig 3.2 J). Neither the addition of extracellular mutanolysin or the transfer of supernatant from Pam3CSK4-treated uninfected wild-type BMM (Fig 3.2D) had any affect on LDH release. As a control to verify that extracellular lysozyme was not activating host cells, Pam3CSK4-stimulated LysM- BMM were treated with cycloheximide to prevent host translation and were infected with *L. monocytogenes* following treatment with extracellular lysozyme. No significant difference was observed in the presence of cycloheximide, suggesting that lysozyme did not activate a pathway that induced cytosolic bacteriolysis (Fig 3.2C). These data suggested that extracellular lysozyme accessed host cytoplasm and can act on lysozyme-sensitive *L. monocytogenes* resulting in bacteriolysis and generation of cytosolic ligands, which stimulate cell death.

LLO-induced pores in a plasma membrane do not grant significant cytosolic access to extracellular lysozyme

To determine if LLO-induced pores in the plasma membrane allowed the transport of lysozyme into the cytosol we constructed strains LLO G486D/Pgd-minus, LLO G486D/Pgd/Oat-minus, LLO S44A/Pgd-minus, and LLO S44A/Pgd/Oat-minus, which have misregulated LLO activity. The LLO S44A mutation is active in the cytosol and forms pores in the plasma membrane, whereas the LLO G486D mutation allows escape from a vacuole, but is catalytically dead in the cytosol. We hypothesized that if LLO induced pores in the plasma membrane facilitated lysozyme transport to the cytosol, infection of LysM- BMM with lysozyme-sensitive strains in the LLO S44A background would result in strongly increased LDH release compared to those strains in the wild-type background following addition of extracellular lysozyme. Similarly, we hypothesized that strains in the LLO G486D background would result in no increase in LDH release upon the addition of extracellular lysozyme. Infection with Pgd- and Pgd/Oat-minus strains in the LLO S44A or LLO G486D backgrounds resulted in no significant difference in LDH release in the presence or absence of extracellular lysozyme (Fig 3.3A-3.3C). LLO is not required for vacuolar escape in HeLa cells. Therefore we characterized LLO-minus infection in HeLa cells in the presence or absence of extracellular lysozyme. HeLa cells were infected with LLO-minus (Fig 3.3 G, H) or LLO/Pgd/Oat-minus (Fig 3.3D, E, F) *L. monocytogenes* and extracellular lysozyme was added at 1h post infection. Infected cells were visualized and *L. monocytogenes* actin associated tails were clearly observed, indicating vacuolar escape. Increased bacteriolysis was observed following addition of 1mg extracellular lysozyme to LLO/Pgd/Oat-minus infections (Fig 3.3D), but not to LLO-minus infection (Fig 3.3 G).

The addition of increased concentrations of lysozyme resulted in decreased number of LLO/Pgd/Oat-minus *L. monocytogenes* observed (Fig 3.3E, F), indicating killing. Taken together, these data suggested the primary route of access to the cytosol by lysozyme is independent of LLO induced pores in the plasma membrane.

Lysozyme-sensitive *L. monocytogenes* demonstrate in vivo defects that are not rescued in the absence of LysM.

To determine if the in vitro phenotype of lysozyme-sensitive strains affected virulence in vivo, we infected B6 and LysM- mice by iv injection and quantified CFUs at 48h. Pgd-minus *L. monocytogenes* had an approximate 2-log defect in both spleen and liver and Pgd/Oat minus had a 3.5-log defect in the spleen and liver (Fig 3.4). Experiments testing for rescue in LysM- mice were initially inconsistent, however, we did not observe a significant rescue in LysM- mice. This could be due to the presence of residual lysozyme activity detected in the serum of LysM- mice (Fig 3.5C). To determine if the affect of serum lysozyme in LysM- mice could be reduced, TriNAG, a competitive inhibitor of lysozyme, was injected approximately 30 minutes before inoculation with either wild-type or Pgd/Oat-minus *L. monocytogenes* (Fig 3.5 A, B). TriNAG injection slightly increase Pgd/Oat-minus CFUs in spleen and liver, however, statistical significance was only observed in spleen. Taken together, this data suggested that lysozyme is present in LysM- mice.

Lysozyme-sensitive vacuolar *L. monocytogenes* hyper suppress generation of protective immunity

L. monocytogenes immunization leads to logs of protective immunity that is largely due to antigen-specific CD8 T-cells (Pamer 2004). Generation of immunity requires live, replicating bacteria that reach the cytosol. Interestingly, ActA-minus *L. monocytogenes* are severely attenuated in vivo, yet still grow in host cytosol and are potent inducers of immunity (Goossens and Milon 1992). It is presumed that generation of acquired immunity requires prior induction of innate immunity. Therefore, we hypothesized that hyper-induction of innate immunity by lysozyme-sensitive *L. monocytogenes* could influence the induction of acquired immunity.

To determine if lysozyme-sensitive strains could induce protective immunity, B6 mice were immunized with 10^3 wild-type, Pgd-, or Pgd/Oat-minus *L. monocytogenes* by iv injection and were challenged 30 days later with $2x10^5$ wild-type *L. monocytogenes*. Pgd-minus displayed an approximate log reduction in protection in spleen and liver (Fig 3.6A, 3.6B), while Pgd/Oat-minus immunization resulted in a 3 log reduction in spleen (Fig 3.6A) and a log reduction in liver (Fig 3.6B). This result was unsurprising since Pgd- and Pgd/Oat-minus have in vivo defects and resulting in logs fewer CFUs, which influences immunity. Similar protective immunity was observed in LysM- mice (data not shown). To more directly address whether innate immunity could influence the induction of acquired immunity, we used a system of co-infection with ActA-minus and either LLO- or LLO/Pgd/Oat-minus *L. monocytogenes*. Previously, it was shown that generation of immunity by ActA-minus could be suppressed by co-infection with LLO-minus dependent on the presence of MyD88 and induction of IL-10. Increasing the number of LLO-minus bacteria in co-infection with a constant number of ActA-minus

increasingly suppressed immunity. Therefore we hypothesized that hyper-induction of the vacuolar response by LLO/Pgd/Oat-minus *L. monocytogenes* would result in increased suppression of immunity. B6 mice were immunized with 10^5 ActA-, 10^5 ActA- and 10^5 LLO-minus, or 10^5 ActA- and 10^5 LLO/Pgd/Oat-minus *L. monocytogenes* by iv injection and were challenged 30 days later with $2x10^5$ wild-type *L. monocytogenes*. Immunization with 10^5 ActA- and 10^5 LLO-minus resulted in an approximate log reduction in protection in liver compared to immunization with ActA-minus only (Fig 3.6D). In contrast, immunization with 10^5 ActA- and 10^5 LLO/Pgd/Oat-minus resulted in a 1.5 log reduction in spleen (Fig 3.6C) and a 2 log reduction in liver (Fig 3.6D) compared to immunization with ActA-minus only. Interestingly, this phenotype was not rescued in LysM- mice (data not shown), suggesting the presence of lysP affects induction of the vacuolar response in vivo. Taken together, these results suggested that increased vacuolar lysis and subsequent hyper-induction of the vacuolar cytokine response results in increased suppression of acquired protective immunity.

Intracellular survival of lysozyme-sensitive *L. monocytogenes* is not completely rescued in LysM- minus IFNγ-activated peritoneal macrophages

The lack of rescue of lysozyme-sensitive *L. monocytogenes* phenotypes in LysM- mice could be attributed to the presence of lysozyme in serum, presumably due to compensation by *lysP* expression. However, we hypothesized that addition antimicrobial defenses not present in BMM could affect Pgd- and Pgd/Oat-minus *L. monocytogenes* and contribute to the defects in vivo. Peritoneal macrophages are much more restrictive to infection with *L. monocytogenes* infection than BMM and highly responsive to IFNγ. To determine whether lysozyme-sensitive *L. monocytogenes* are susceptible to antimicrobial activity besides lysozyme, IFNγ-activated wt or LysM- thioglycolate elicited peritoneal macrophages (TEPMs) were infected with wt, Pgd-, or Pgd/Oat-minus *L. monocytogenes*. As expected, Pgd- and Pgd/Oat-minus had a survival defect in wt TEPMs (Fig 3.7A), however, the defect was not completely rescued in LysM- TEPMs (Fig 3.7B), suggesting lysozyme-sensitive *L. monocytogenes* are sensitive to host antimicrobial defenses in addition to lysozyme.

Lysozyme-sensitive *L. monocytogenes* are not susceptible to cationic peptide treatment, low pH, or hydrogen peroxide

N-deacetylation of GlcNAc by Pgd results in the presence of a primary amine group with a positive charge. Thus, it is predicted that Pgd-minus PGN is less electronegative than wt PGN *L. monocytogenes* and thus Pgd-minus *L. monocytogenes* could be increasingly sensitive to cationic peptide activity (Vollmer 2008). Therefore, we assayed growth of wt, Pgd-, and Pgd/Oat-minus *L. monocytogenes* in broth in the presence several classes of cationic peptides (mCRAMP is a cathelicidin, HNP-1 is a defensin, and retrocyclin is a theta-defensin) (Scott and Hancock 2000; Lehrer and Ganz 2002; Ganz 2004). DltA- and MprF-minus *L. monocytogenes* are sensitive to cationic peptides and were used as a positive control (Abachin, Poyart et al. 2002; Mandin, Fsihi et al. 2005; Thedieck, Hain et al. 2006). lmo 2473 is slightly sensitive to cationic peptides and was also included as a control. As expected, DltA- and MprF-, were highly sensitive to each cationic peptide tested, especially mCRAMP and lmo 2473 was moderately sensitive to each. Wild-type was not sensitive to ly9 or mCRAMP, but HNP-1 and retrocyclin treatment inhibited

growth. Pgd- and Pgd/Oat-minus showed similar phenotype to wt in the presence of each cationic peptide tested. Pgd- and Pgd/Oat-minus showed very moderate decrease in growth kinetics in the presence of mCRAMP, however, in repeated experiments this difference did not seem consistent. We hypothesized that surface charge could affect sensitivity to pH or hydrogen peroxide treatment. Pgd- and Pgd/Oat-minus grew in the presence of low pH with similar kinetics to wild-type. Pgd- and Pgd/Oat-minus showed very slight sensitivity to broth growth in the presence of hydrogen peroxide compared to wt (Fig 3.8C) and this result was confirmed using a disc diffusion assay (not shown). Taken together, this data suggested that N-deacetylation by Pgd did not provide significant resistance to cationic peptides, low pH, or peroxide and thus likely serves primarily to resist the catalytic activity of lysozyme.

***L. monocytogenes* autolysins display increased activity on N-acetylated peptidoglycan**

O-acetylation precludes lytic transglycosylase activity in numerous Gram-negative bacteria (Moynihan and Clarke 2011). We hypothesized that N-deacetylation or O-acetylation by Pgd and Oat respectively could regulate autolysin activity. Zymography was used to analyze the autolytic activities of *L. monocytogenes* secreted proteins. Briefly, wild-type or Pgd/Oat-minus *L. monocytogenes* PGN was used as a substrate and was incorporated into polyacrylamide gels. Secreted proteins from wild-type, Pgd-, Pgd/Oat, SecA2-, NamA-, Ami-, p60-, and lmo717/Oat-minus *L. monocytogenes* supernatant were electrophoresed through the wild-type or Pgd/Oat-minus PGN zymography gels and were incubated in buffer containing a detergent, allowing the autolysins to renature (the autolysin-minus mutants were included as controls). The renatured autolysins degraded the embedded PGN and gels were assayed for autolytic activity. Interestingly, *L. monocytogenes* autolysins were much more active on Pgd/Oat-minus PGN and much greater zones of clearance were observed (Fig 3.9). As expected, lysozyme showed greatly enhanced activity on Pgd/Oat-minus PGN embedded gels. Surprisingly, the autolytic profiles of the secreted autolysins was much different on wild-type or Pgd/Oat-minus PGN. NamA activity was greatly increased on Pgd/Oat-minus PGN. The secreted autolysin profiles of Pgd- and Pgd/Oat-minus were similar to wild-type on wt PGN gels (Fig 3.9A). However, Pgd- and Pgd/Oat-minus seemed to have significantly less NamA activity than wild-type on Pgd/Oat-minus PGN (Fig 3.9B). Pgd/Oat-minus PGN embedded zymographic gels appeared identical to Pgd-minus PGN gels and wt PGN embedded gels appeared identical to Oat PGN gels (not shown). Similar results were observed when whole bacterial lysates were electrophoresed on zymographic gels (not shown). Zymographic analysis suggested Pgd N-deacetylation plays a role in regulation of autolysin activity.

Discussion

All of the phenotypes associated with lysozyme sensitivity could be restored simply by adding lysozyme to the culture media. In our experiment we used 1mg/ml HEWL, which is within the biological range (Hansen and Karle 1971; Gordon, Todd et al. 1974; Hankiewicz and Swierczek 1974; Prixova 1975; Cohn 1978; Mendiondo, Suit et al. 1978; Vinding, Eriksen et al. 1987; Cole, Dewan et al. 1999). Although HEWL has the same

enzymatic activity as mammalian lysozyme it may have properties that differ in specificity for de-acetylated PGN or access to BMM cytosol compared to endogenous mouse lysozyme M. There is evidence that macrophages internalize other extracellular peptides in addition to lysozyme. For example, Tan et al have reported that macrophages can acquire antimicrobial peptide defensins from apoptotic neutrophil granules to become increasingly resistant to *M. tuberculosis* (Tan, Meinken et al. 2006). The Lehrer lab has shown that macrophages can take up alpha-defensins (personal communication) (Hazrati, Galen et al. 2006). It has also been shown that anti-LLO antibodies can act in a phagosome to inhibit *L. monocytogenes* escape (Edelson and Unanue 2001). Therefore soluble innate immune factors including secreted lysozyme may also concentrate in macrophages.

How lysozyme enters the cytosol is less obvious. However, several other small cationic peptides have been reported to translocate from the extracellular milieu to the cytosol. HIV-1 Tat, HSV type 1 VP22 transcription factor, and a Drosophila protein, Antp, have been shown to enter cells from the culture media and localize in the cytoplasm. Of these translocated proteins, HIV-1 Tat has been the most well studied. Tat is secreted from infected cells and subsequently enters adjacent cells and accumulates in the cytosol (Frankel and Pabo 1988; Mann and Frankel 1991). Tat is a 101 amino acid cationic peptide with a calculated isoelectric point of 9.61, and has been used as a fusion protein to deliver ovalbumin, β-galactosidase, peroxidase, and GFP into cells (Hauber, Malim et al. 1989; Ruben, Perkins et al. 1989; Fawell, Seery et al. 1994). A basic domain rich in arginine and lysine residues has been identified in Tat as the region required for translocation (Vives, Brodin et al. 1997; Nagahara, Vocero-Akbani et al. 1998). Although lysozyme does not share a homologous domain, it too is a small cationic peptide of 14kd with a calculated isoelectric point of 9.11. Although speculative, its similar features in size and cationic quality are properties that could suggest a mechanism for its access to the cytosol.

Lysozyme-sensitive *L. monocytogenes* were severely attenuated in the mouse model of listeriosis. However, the loss of virulence was not rescued in LysM- mice. The most likely explanation was a compensatory and redundant mechanism of LysP. LysP compensation has been reported in LysM- mice separately by Markart and Ganz (Ganz, Gabayan et al. 2003; Markart, Faust et al. 2004). Markart et al showed that LysP mRNA is typically not expressed in the alveolar space in wild-type mice, but was upregulated significantly in LysM- mice. Ganz et al showed that lysozyme was detected with an antibody that recognized both M and P isoforms in alveolar and peritoneal macrophages. Certainly, LysP compensatory expression in relative tissues could explain the lack of rescue. Lysozyme P circulating in the blood may also be a contributing factor. Pgd/Oat-minus *L. monocytogenes* had a log decrease in CFUs in liver by 30 min (data not shown) suggesting an early encounter with lysozyme. Also, based on our results circulating lysozyme P may enter macrophages and act intracellularly in a vacuole and the host cell cytosol.

In addition, lysozyme sensitive *L. monocytogenes* may be susceptible to other host cell hydrolytic enzymes or stresses not present in BMM. This was supported by the

observation that Pgd- and Pgd/Oat-minus *L. monocytogenes* were not fully rescued in LysM- IFNγ-activated peritoneal macrophages (Fig 3.7B). Popowska has shown the Pgd-minus *L. monocytogenes* are more sensitive to autolysis inducing agents, triton-X-100 and EDTA, and various antibiotics (Popowska, Kusio et al. 2009). Indeed, it is predicted that Pgd-minus *L. monocytogenes* bearing N-acetylated glucosamine residues would have a more negative surface charge due to lack of an exposed primary amine residue and would thus be more susceptible to cationic peptides (Vollmer 2008). However, Pgd- and Pgd/Oat-minus *L. monocytogenes* were not sensitive to cationic peptides including ly9, a human lysozyme-derived 9mer, in comparison to a strains lacking DltA or MprF, which are known to be sensitive to cationic agents (Thedieck, Hain et al. 2006; Herbert, Bera et al. 2007; Zemansky, Kline et al. 2009). Similarly, Pgd- and Pgd/Oat-minus showed no increased susceptibility to low pH or hydrogen peroxide treatment. In the future it will be of interest to determine whether it is the lytic activity of mouse lysozyme or its cationic properties that act on Pgd- and Pgd/Oat-minus *L. monocytogenes*. Interestingly, *L. monocytogenes* secreted autolysins showed enhanced activity on Pgd- and Pgd/Oat-minus PGN compared to wt and Oat-minus PGN, suggesting a role in the regulation of autolysins. How Pgd is regulated is unknown, however, it has been reported that it is upregulated in vivo (Camejo, Buchrieser et al. 2009). Zymography of *L. monocytogenes* proteins collected from infected macrophages shows increased autolytic activity compared to *L. monocytogenes* whole bug lysates isolated from culture (Aimee Geissler and Dan Portnoy unpublished observation), suggesting increased autolysin activity in vivo. Perhaps Pgd-minus *L. monocytogenes* endure an increased defect in autolysin regulation in vivo, which contributes to the lack of rescue in LysM- mice.

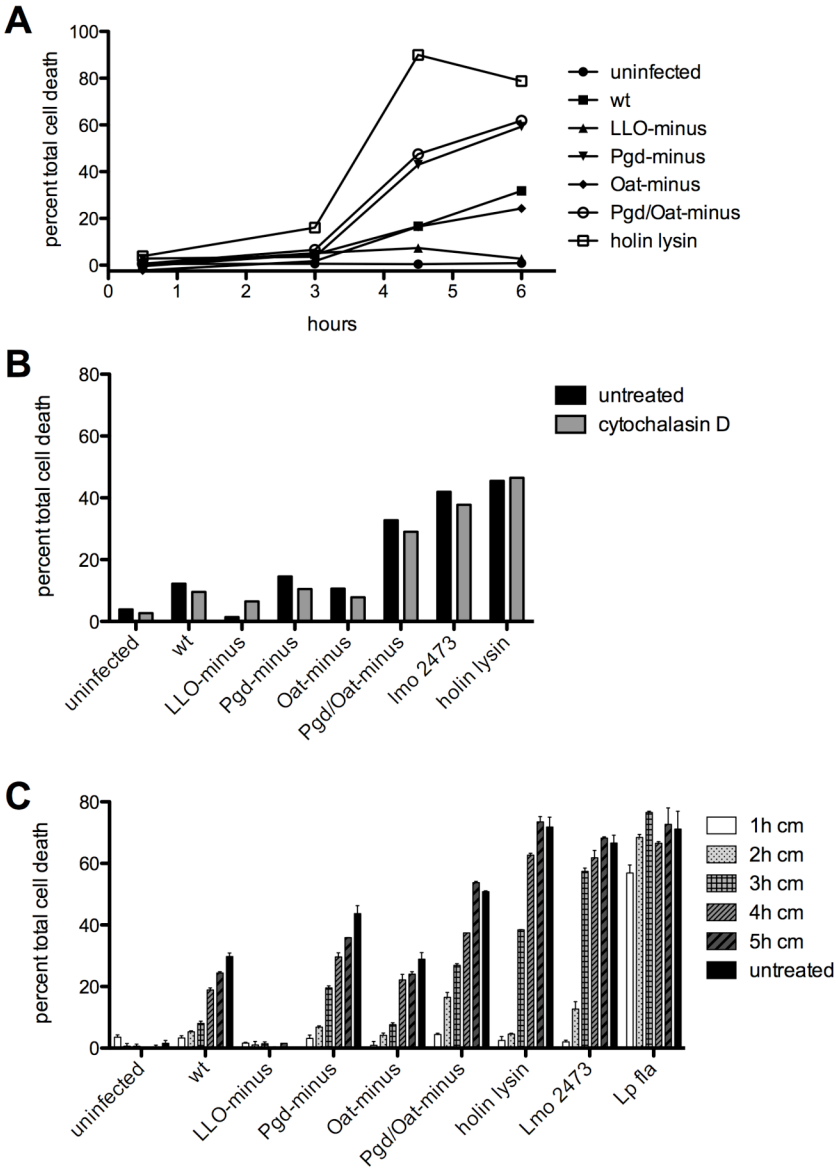
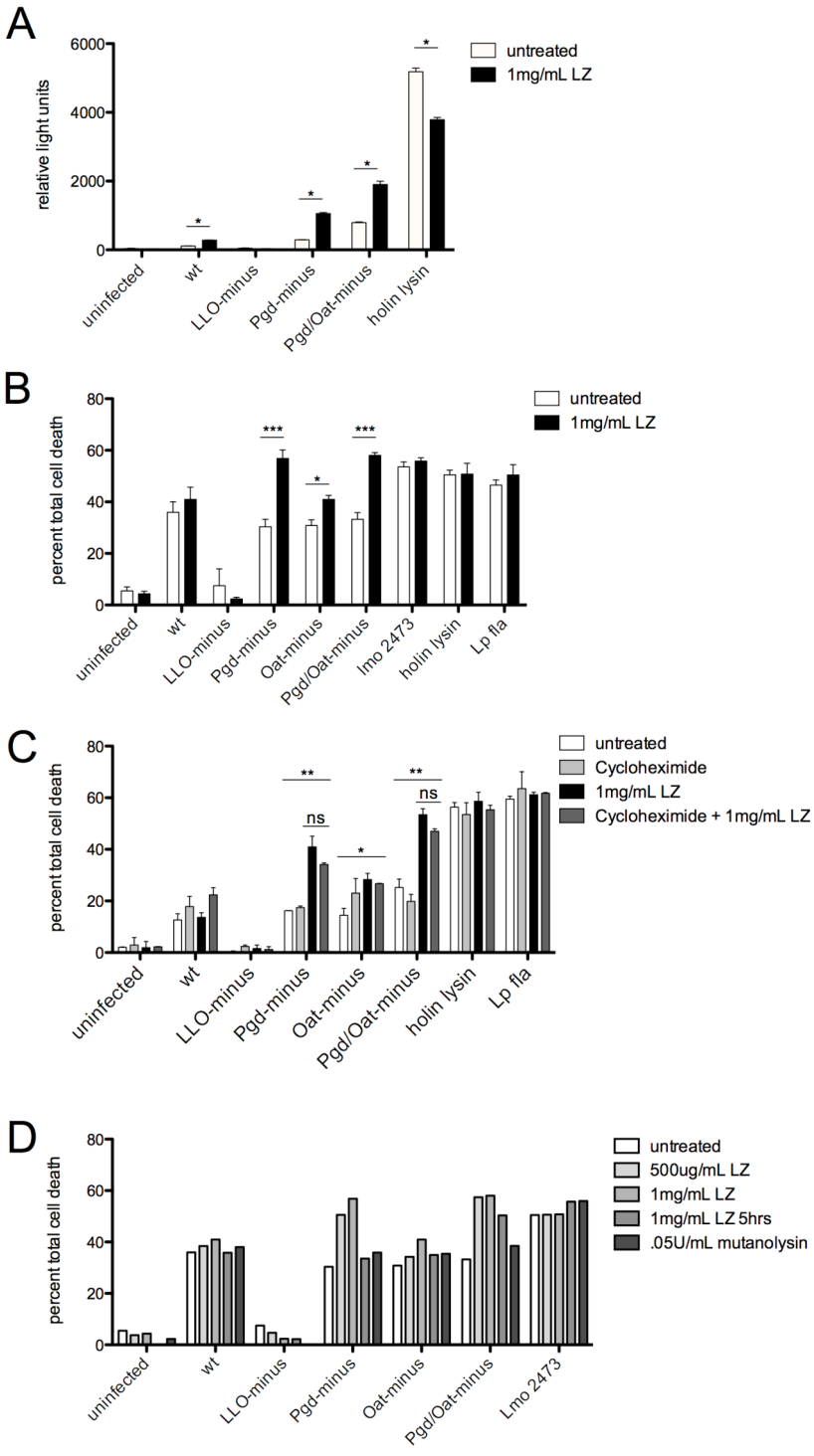


Figure 3.1- Bacterial replication but not cell-to-cell spread is required for release of LDH.

LDH release over time. Pam3CSK4 stimulated wt BMM were infected at MOI=5 and LDH release was assayed at 3, 4.5 and 6h post infection (A). Pam3CSK4 stimulated wt BMM were treated with cytochalasin D 30 minutes before infection at MOI=5 and LDH release was assayed 6h post infection (B). 0.1mg/ml chloramphenicol (cm) was added at 1, 2, 3, 4 or 5h post infection and LDH release was measured at 6h post infection (C). Error bars represent the standard deviation of the means. Data are representative of more than three independent experiments with similar results.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relative light units
6000
4000
2000
0
untreated
1mg/mL LZ
*
uninfected
wt
LLO-minus
Pgd-minus
Pgd/Oat-minus
holin lysin
B
percent total cell death
80
60
40
20
0
untreated
1mg/mL LZ
***
*
***
uninfected
wt
LLO-minus
Pgd-minus
Oat-minus
Pgd/Oat-minus
lmo 2473
holin lysin
Lp fla
C
percent total cell death
80
60
40
20
0
untreated
Cycloheximide
1mg/mL LZ
Cycloheximide + 1mg/mL LZ
**
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ns
ns
*
uninfected
wt
LLO-minus
Pgd-minus
Oat-minus
Pgd/Oat-minus
holin lysin
Lp fla
D
percent total cell death
80
60
40
20
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untreated
500ug/mL LZ
1mg/mL LZ
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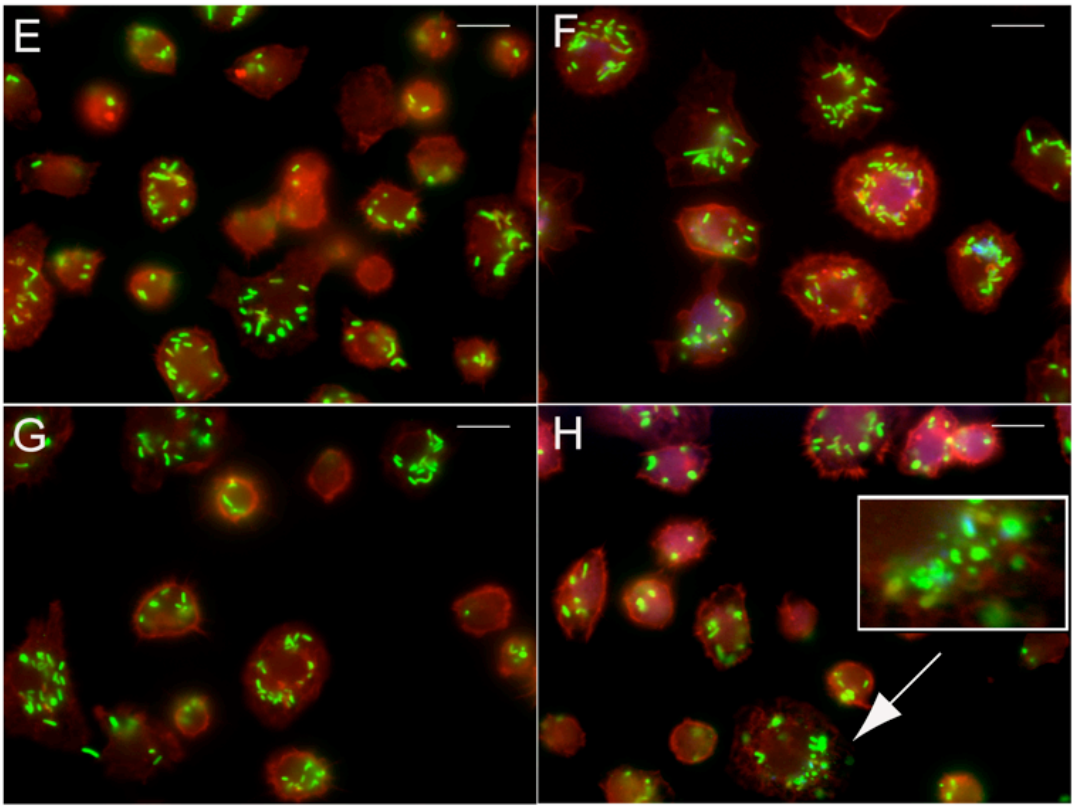
.05U/mL mutanolysin
uninfected
wt
LLO-minus
Pgd-minus
Oat-minus
Pgd/Oat-minus
Lmo 2473


E
F
G
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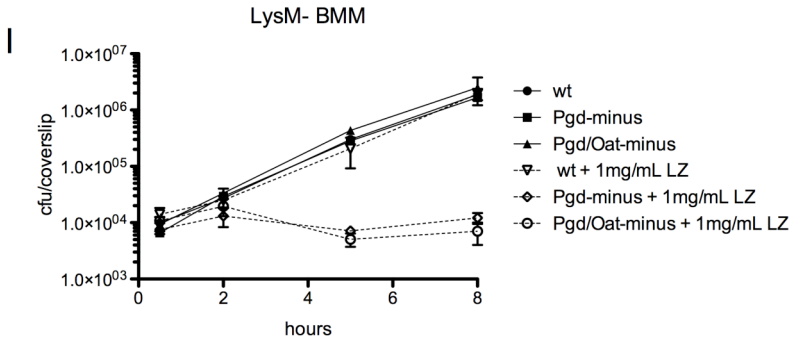
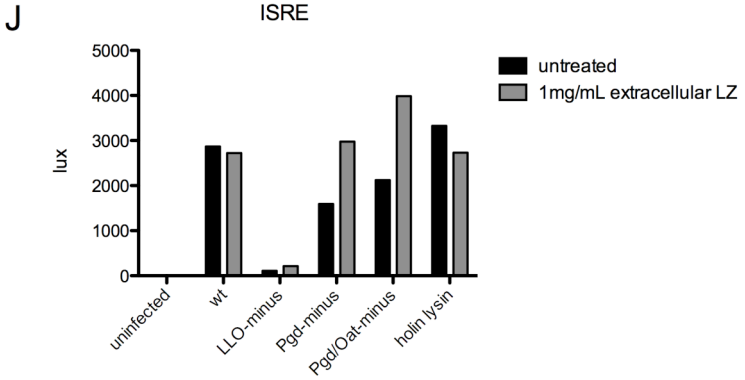

I
LysM- BMM
cfu/coverslip
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04
1.0×10^03
0
2
4
6
8
hours
wt
Pgd-minus
Pgd/Oat-minus
wt + 1mg/mL LZ
Pgd-minus + 1mg/mL LZ
Pgd/Oat-minus + 1mg/mL LZ


J
ISRE
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5000
4000
3000
2000
1000
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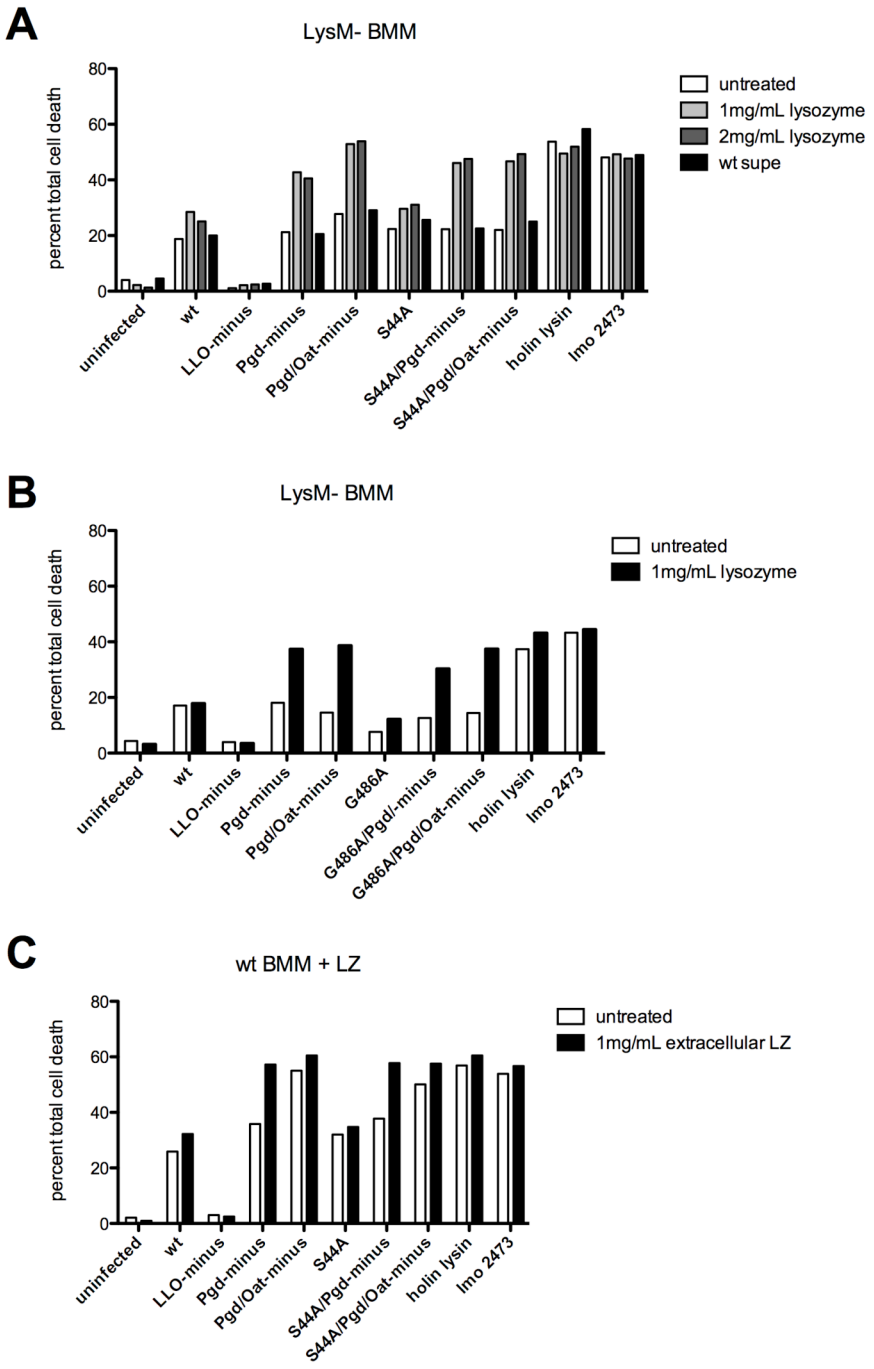
uninfected
wt
LLO-minus
Pgd-minus
Pgd/Oat-minus
holin lysin
untreated
1mg/mL extracellular LZ

Figure 3.2- Extracellular lysozyme accesses BMM cytosol and results in increased degradation of lysozyme-sensitive *L. monocytogenes* strains and subsequent host cell death.

IFNα/βR- BMM were infected with *L. monocytogenes* bearing a plasmid containing luciferase under a CMV promoter and 1mg/ml extracellular lysozyme was added at 1h post infection. Luciferase expression was detected at 6r post infection (A). Pam3CSK4 stimulated LysM- BMM were infected at MOI=5 and 1mg/ml extracellular lysozyme was added at 1h post infection. LDH release was measured at 6h (B). Pam3CSK4 stimulated LysM- BMM were treated with cycloheximide, infected at MOI=5 and LDH release was measured at 6h (C). Pam3CSK4 stimulated LysM- BMM were infected at MOI=5 and either 0.5mg/ml, 1mg/ml extracellular lysozyme, or mutanolysin was added at 1h post infection or 1mg/ml was added at 5 h post infection. LDH release was measured at 6h (D). Error bars represent the standard deviation of the means. Statistical significance was evaluated in A and B using a Student's t test where * indicates $P<0.05$ and *** indicates $P<0.0001$. One Way ANOVA test was performed in C to analyze statistical significance of LDH release following various treatment of BMM infected by individual *L. monocytogenes* strains where * indicates $P<0.05$ and ** indicates $P<0.001$. The means of LDH values from infections treated with lysozyme compared to those treated with lysozyme plus cycloheximide were not determined significant using a Student's t test (ns). The means of LDH values from infections with Pgd-, Oat-, or Pgd/Oat-minus *L. monocytogenes* were significant by One Way ANOVA test. Data are representative of at least three independent experiments with similar results. LysM- BMM were infected with either wt (E, F) or Pgd/Oat-minus *L. monocytogenes* (G, H) for 2h. 1mg/ml extracellular lysozyme was added at 1h post infection (F, H) and cells were scored for evidence of bacterial degradation (depicted in inset). *L. monocytogenes* were stained green, host cell actin was stained red, and nuclei appear blue. Size bars represent 10μm. Data are representative of more than three independent experiments with similar results. Pam3CSK4 stimulated LysM- BMM were infected at MOI=5 and 1mg/ml extracellular lysozyme was added at 1h post infection. Supernatant was overlayed on interferon responsive ISRE-L929 cells for 4h. ISRE-L929 cells were lysed and mixed with luciferase substrate and luminescence was measured (I). $2x10^6$ LysM- BMM were infected with wt, Pgd- or Pgd/Oat-minus *L. monocytogenes* at a MOI = 0.1 and BMM were untreated (solid lines) or 1mg/ml extracellular lysozyme was added at 1 h post infection (dashed lines). BMM were lysed and bacterial CFUs were quantified.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LysM- BMM
percent total cell death
0
20
40
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80
untreated
1mg/mL lysozyme
2mg/mL lysozyme
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LLO-minus
Pgd-minus
Pgd/Oat-minus
S44A
S44A/Pgd-minus
S44A/Pgd/Oat-minus
holin lysin
lmo 2473
B
LysM- BMM
percent total cell death
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1mg/mL lysozyme
uninfected
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LLO-minus
Pgd-minus
Pgd/Oat-minus
G486A
G486A/Pgd/-minus
G486A/Pgd/Oat-minus
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wt BMM + LZ
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1mg/mL extracellular LZ
uninfected
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LLO-minus
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holin lysin
lmo 2473

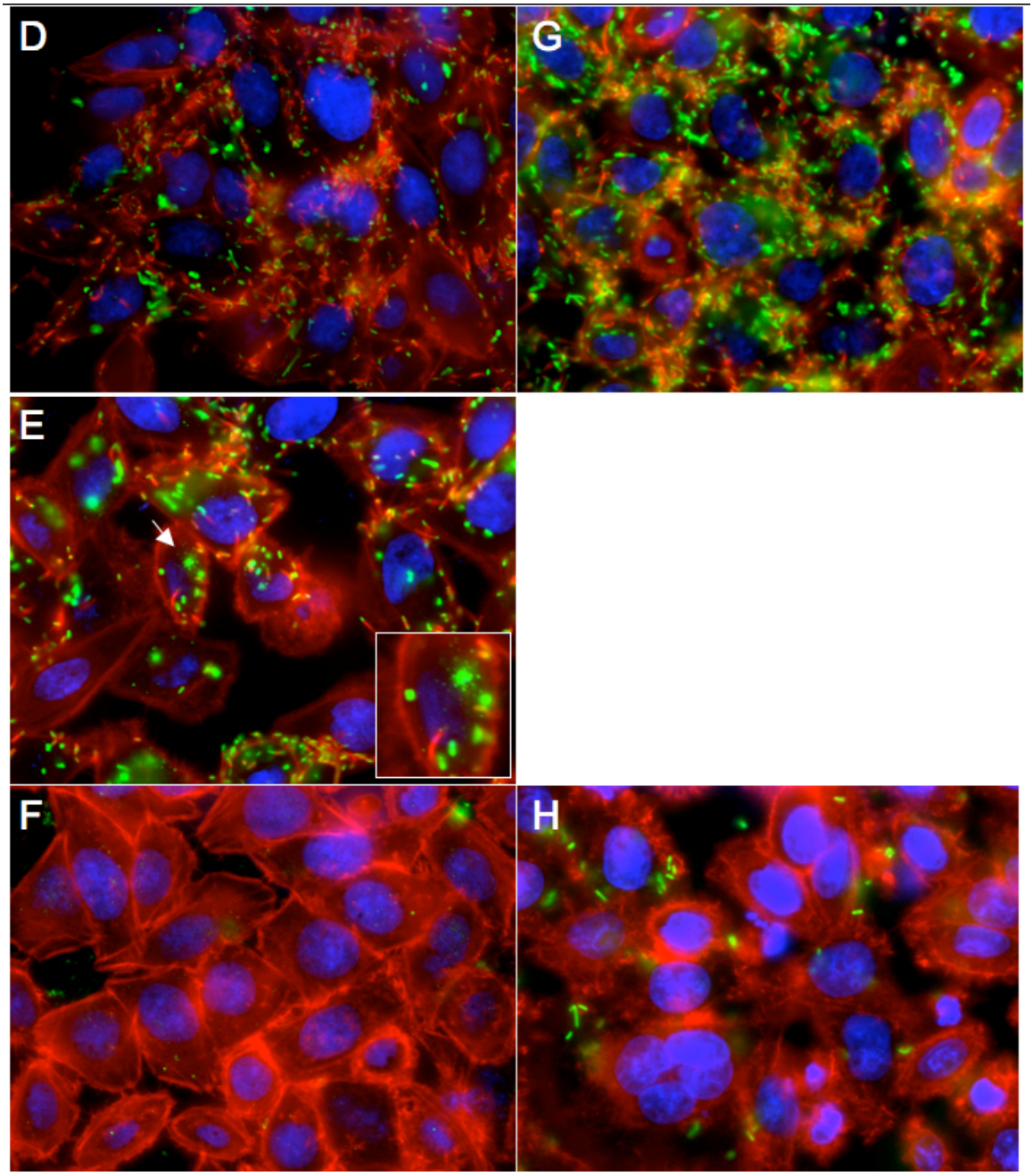


Figure 3.3- LLO-induced plasma membrane pores do not influence extracellular lysozyme access to BMM cytosol and subsequent host cell death of lysozyme-sensitive *L. monocytogenes* strains.
Pam3CSK4 stimulated LysM- (A, B) or wt (C) BMM were infected at MOI=5 and 1mg/ml extracellular lysozyme was added at 1h post infection. LDH release was measured at 6h (A-C). HeLa cells were infected with LLO/Pgd/Oat-minus (D, E, F) or LLO-minus (G, H) *L. monocytogenes* at MOI=20 and either 1 (D, G), 3 (E), or 20 (F, H) mg/ml extracellular lysozyme was added at 1h post infection. Infections were visualized at 3 h post infection. *L. monocytogenes* were stained green, host cell actin was stained red, and nuclei appear blue. Inset depicts bacterial degradation.

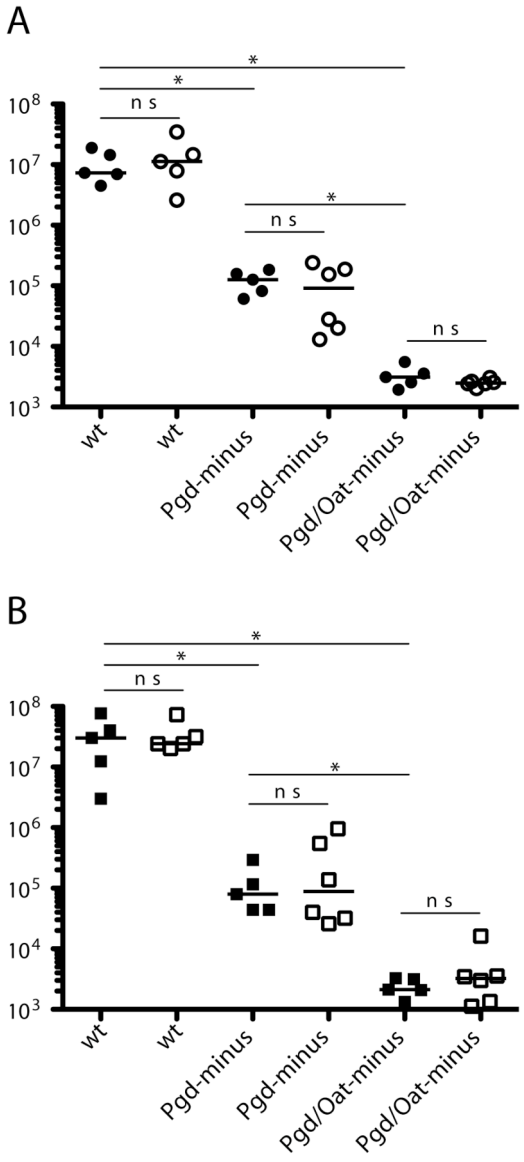


Figure 3.4- Pgd-minus and Pgd/Oat-minus *L. monocytogenes* are defective in vivo but are not rescued in LysM- mice.
C57BL/6 or LysM- mice were infected by iv injection with $1x10^5$ bacteria and CFUs were quantified in spleen (A) and liver (B) at 48h post infection. Spleen and liver are represented by circles and squares respectively. Black and open shapes represent C57BL/6 and LysM- mice respectively. Statistical significance was evaluated using a Mann Whitney test where * indicates $P<0.02$ and ns indicates not significant. Data is representative of more than 3 independent experiments with similar results.

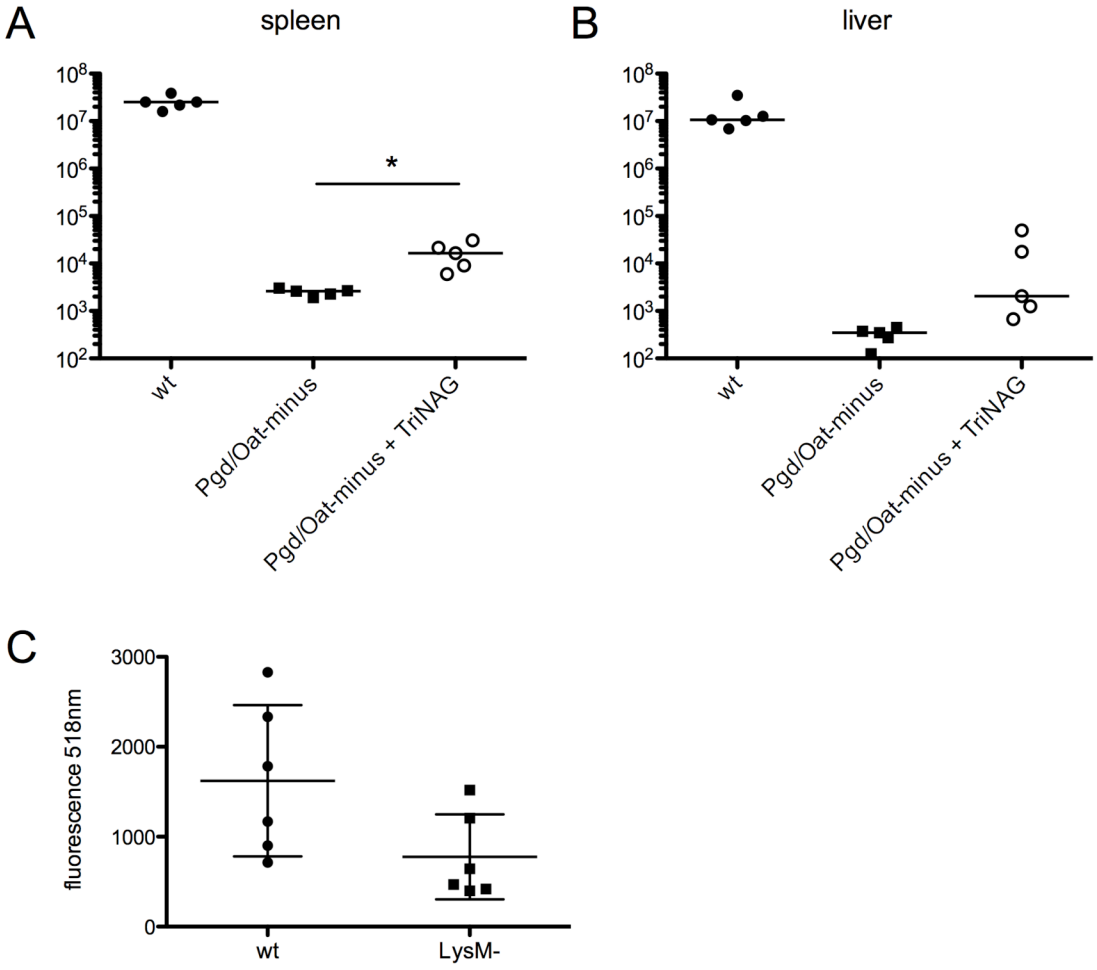


Figure 3.5- Lysozyme is present in serum of LysM- mice.
LysM- mice were injected with 1mg TriNAG (open circles), a competitive inhibitor of lysozyme, or were untreated (black shapes) and then infected by iv injection with $1x10^5$ wild-type or Pgd/Oat-minus bacteria. CFUs were quantified in spleen (A) and liver (B) at 48h post infection. Serum was collected from C57BL/6 or LysM- mice (n=6) and assayed for the presence of lysozyme activity. Lysozyme activity correlates with fluorescence detected at 518nm. Error bars represent standard deviation of the means. Student's t test showed the means were not statistically significant. Data is representative of at least 3 separate experiments with similar results.

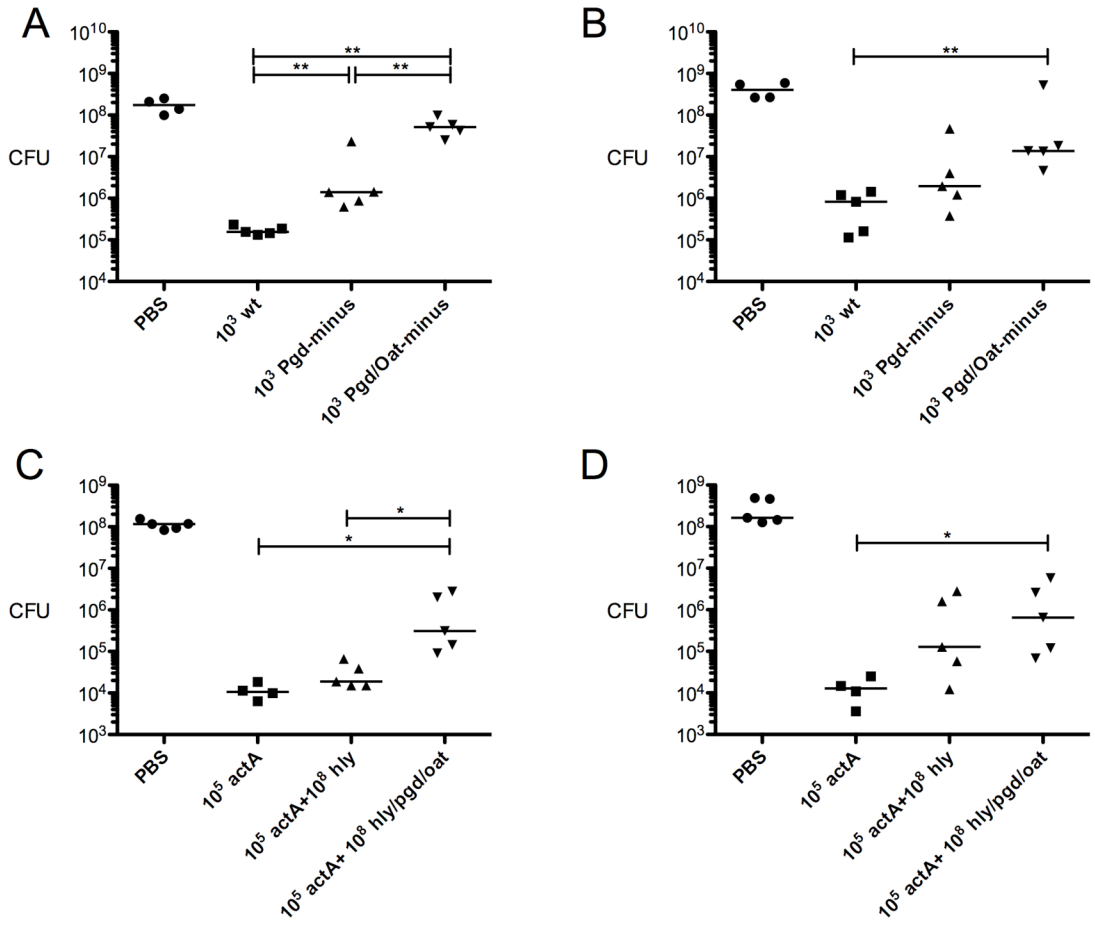


Figure 3.6- Pgd-minus and Pgd/Oat-minus *L. monocytogenes* are defective in generating protective immunity and LLO/Pgd/Oat-minus hyper-suppress immunity compared to LLO-minus *L. monocytogenes*.

C57BL/6 mice were immunized by iv injection with $1x10^3$ Pgd-minus, Pgd/Oat-minus or wild-type *L. monocytogenes* and challenged 30 days later with $1x10^5$ wild-type *L. monocytogenes*. CFUs were quantified in spleen (A) and liver (B) at 72h post infection. C57BL/6 mice were immunized by iv injection with $1x10^5$ ActA-minus, or coinfection with $1x10^5$ ActA-minus and $1x10^8$ LLO/Pgd-minus, or $1x10^5$ ActA-minus and $1x10^8$ LLO/ Pgd/Oat-minus *L. monocytogenes*. Mice were challenged 30 days later with $1x10^5$ wild-type *L. monocytogenes* and CFUs were quantified in spleen (C) and liver (D) at 72h post infection. These experiments were done with Thomas Burke.

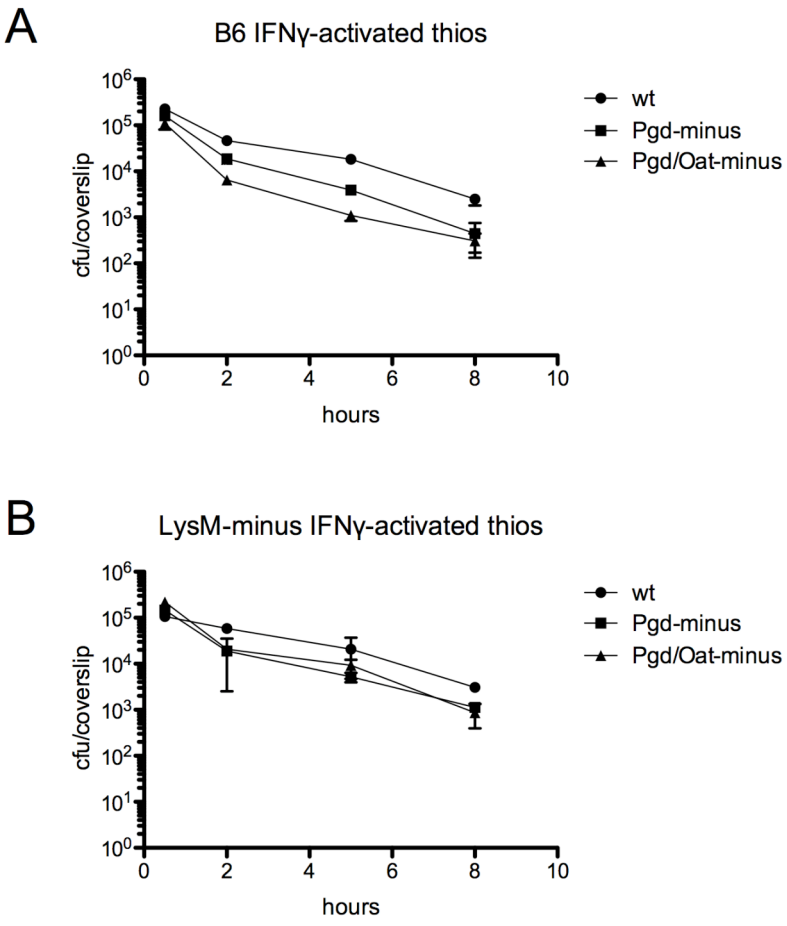
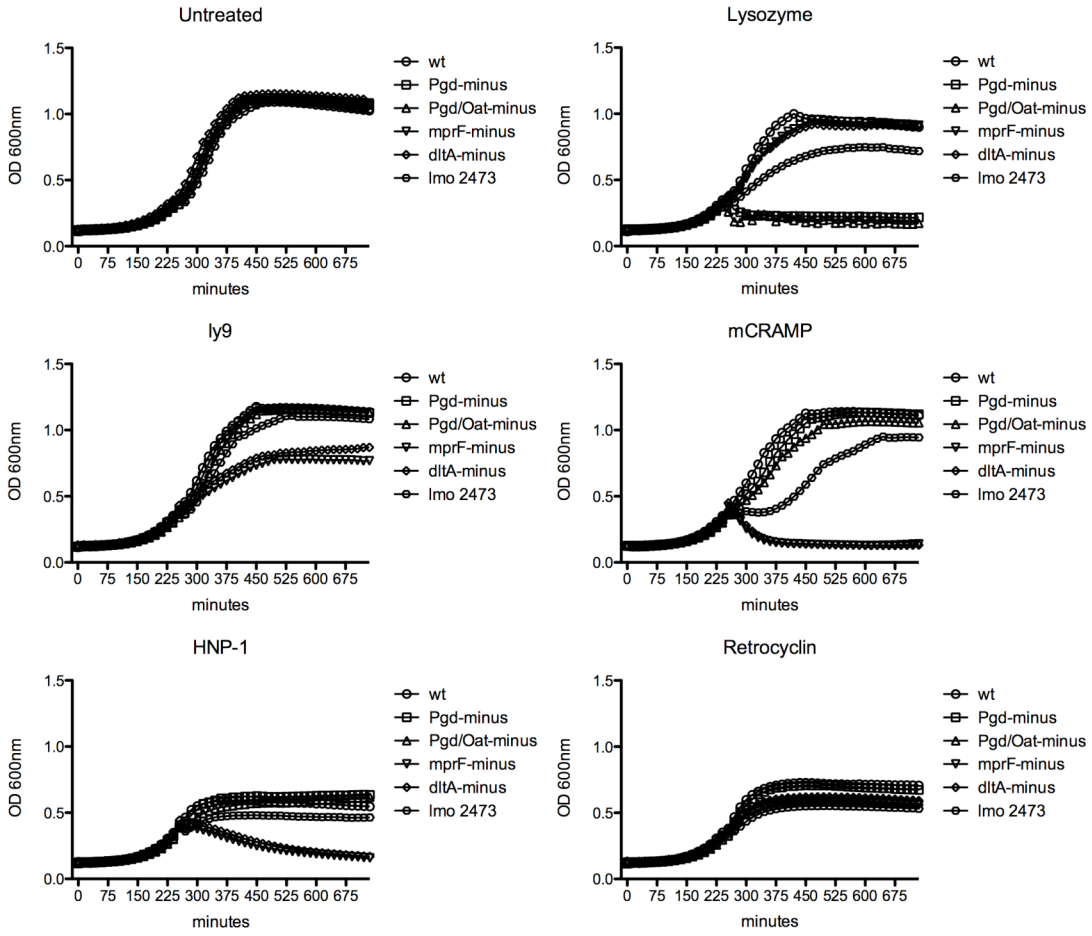
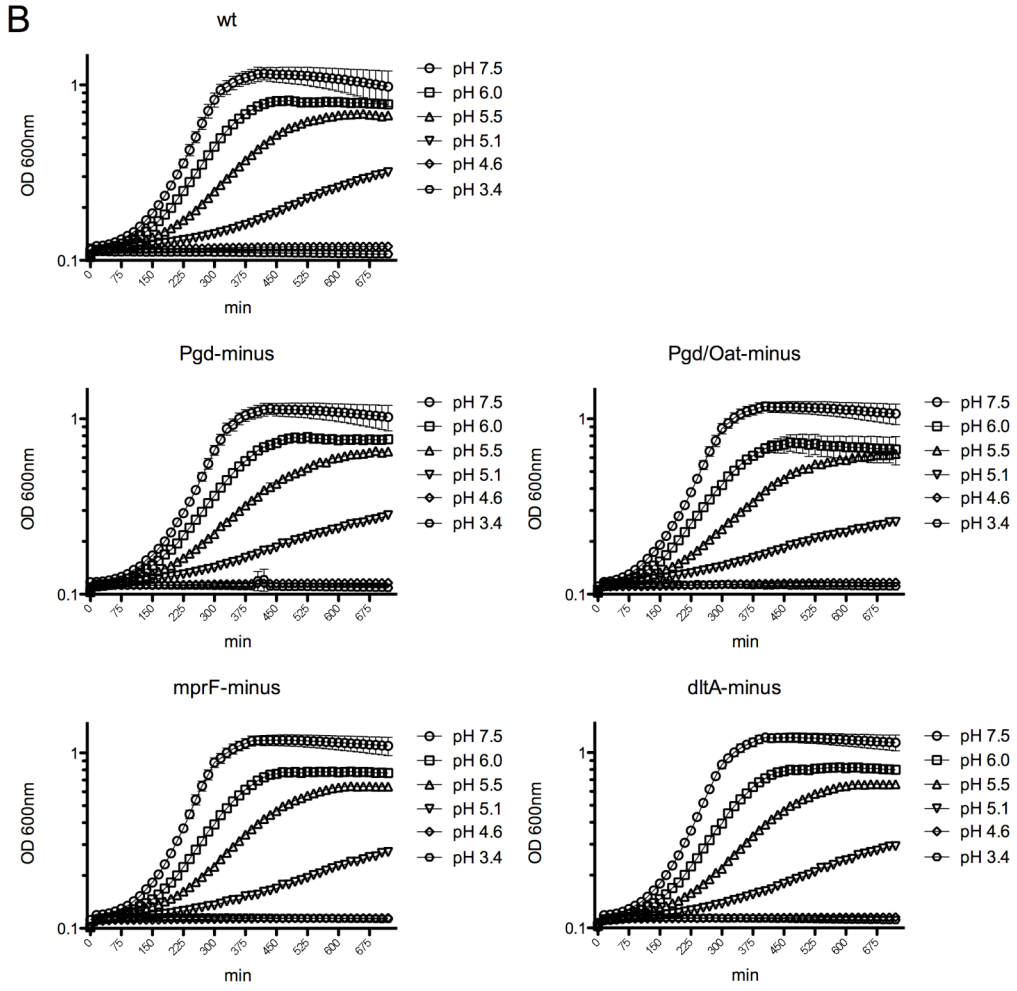


Figure 3.7- Pgd-minus and Pgd/Oat-minus *L. monocytogenes* intracellular survival defects in LysM deficient IFNγ-activated thioglycolate elicited peritoneal macrophages are not fully rescued.
2x106 wt (A) or LysM- (B) IFNγ-activated thioglycolate elicited peritoneal macrophages were infected with wild-type, Pgd- or Pgd/Oat-minus *L. monocytogenes* at MOI=10. BMM were lysed and bacterial CFUs were quantified.

A


Untreated
Lysozyme
ly9
mCRAMP
HNP-1
Retrocyclin
OD 600nm
minutes
0 75 150 225 300 375 450 525 600 675
0.0 0.5 1.0 1.5
wt
Pgd-minus
Pgd/Oat-minus
mprF-minus
dltA-minus
lmo 2473


B
wt
Pgd-minus
Pgd/Oat-minus
mprF-minus
dltA-minus
OD 600nm
min
pH 7.5
pH 6.0
pH 5.5
pH 5.1
pH 4.6
pH 3.4

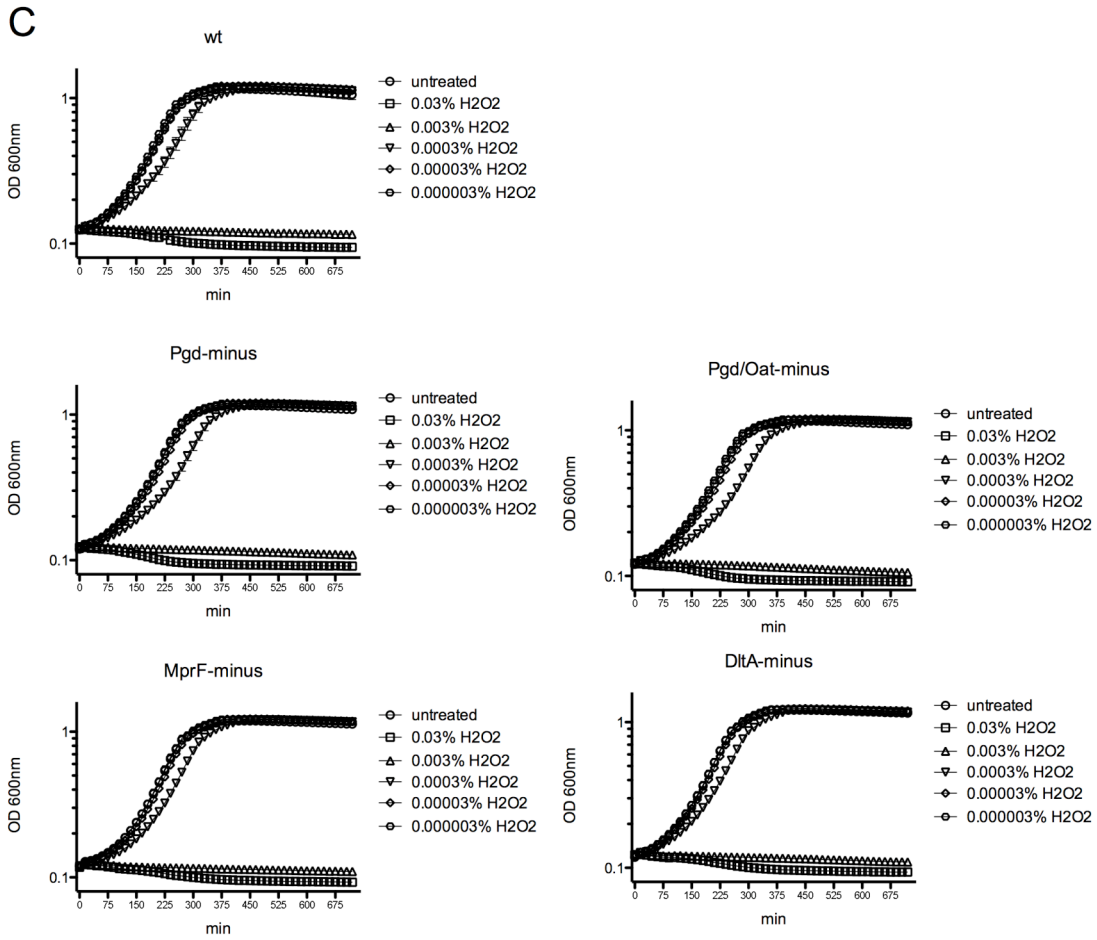


Figure 3.8- Pgd-minus and Pgd/Oat-minus *L. monocytogenes* are not sensitive to cationic peptides, pH, or hydrogen peroxide stress.
wt, Pgd-, Pgd/Oat-minus, lmo 2473, DltA- and MprF-minus *L. monocytogenes* growth in BHI in the presence of HEW lysozyme or cationic peptides (A). 2000µg/mL Lysozyme, 50µg/mL ly9, 50µg/mL mCRAMP, 50µg/mL HNP-1, or 50µg/mL Rectrocylin were added to cultures in equal volumes at 240 minutes after bacterial inoculation and OD 600nm was monitored at 15 minute intervals. wt, Pgd-, Pgd/Oat-minus, DltA- and MprF-minus *L. monocytogenes* growth in BHI at pH 7.5, 6.0, 5.5, 5.1, 4.6, or 3.4 and OD 600nm was monitored at 15 minute intervals for 12h (B). wt, Pgd-, Pgd/Oat-minus, DltA- and MprF-minus *L. monocytogenes* growth in BHI in the presence of titrated concentrations of hydrogen peroxide (H2O2) (C). OD 600nm was monitored at 15 minute intervals for 12h.

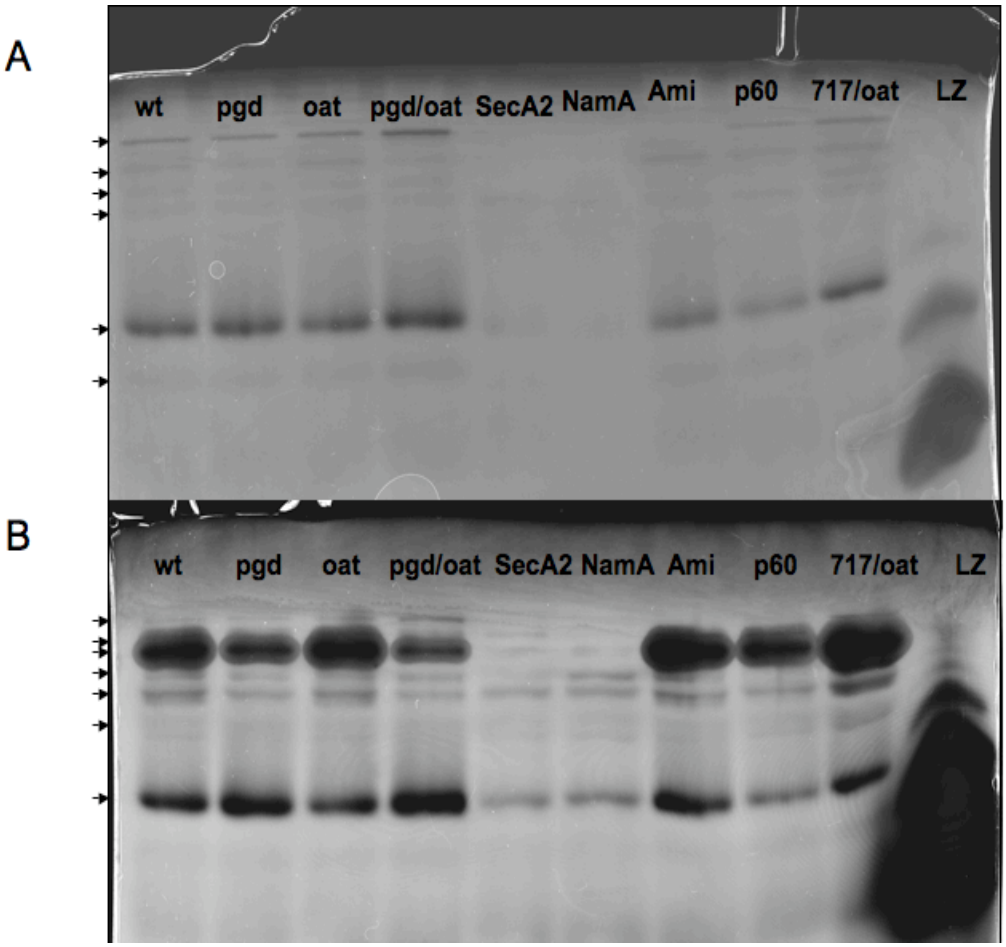


Figure 3.9 - *L. monocytogenes* autolysins have increased activity on acetylated PGN, Pgd-minus and Pgd/Oat-minus *L. monocytogenes* display differential secreted autolysin activity.
Wt (A) or Pgd/Oat-minus (B) PGN was embedded into polyacrylamide gels. Bacterial culture supernatant was precipitated and SDS-PAGE was performed. Gels were incubated in buffer containing TritonX-100 and stained with brilliant blue solution.

Chapter 4: Future Perspectives and Unanswered Questions

Lysozyme is a multifaceted antimicrobial

We conclude that *L. monocytogenes* resistance to lysozyme is an essential determinant of pathogenesis. Indeed, many bacterial pathogens are lysozyme-resistant (Davis and Weiser ; Vollmer 2008). The role of lysozyme appears to be multifaceted. We and others have shown lysozyme activity can kill bacteria and stimulate inflammation, but in other contexts it has been shown to inhibit inflammation. Ganz et al showed *Micrococcus luteus* induced increased inflammation in LysM- mice, suggesting peptidoglycan degradation by lysozyme is required to down-regulate inflammation (Ganz, Gabayan et al. 2003). Thus one role of lysozyme may be to degrade potentially pro-inflammatory PGN fragments associated with commensal or non-pathogenic bacteria, which may be a strategy to avoid mounting an inappropriate inflammatory response. This may be similar to a mechanism whereby host enzymes inactivate bacterial LPS to avoid prolonged tolerance and increased inflammation (Munford and Varley 2006). However, lysozyme also serves to generate peptidoglycan fragments that are important immunostimulatory ligands during infection. Davis suggests that modified peptidoglycan, that is more resistant to hydrolysis by lysozyme, results in generation of larger fragments, which might be differentially detected by a host (Davis and Weiser). More recently, Davis showed that lysozyme activity generates NOD2 ligands, which promote clearance of *S. pneumoniae* (Davis, Nakamura et al. 2011). Thus, lysozyme functions to kill lysozyme-sensitive bacteria and to generate immunostimulatory PGN fragments during infection in some cases, but also to destroy immunostimulatory fragments in others. The ability of lysozyme to act from within cells (both in phagosomes and cytosol) and extracellularly highlights its role as a versatile antimicrobial factor that contributes to multiple aspects of host defense during bacterial infection.

Lysozyme activity coupled with cytosolic receptor activation

After discovering lysozyme, Alexander Fleming famously declared “We shall hear more about lysozyme one day” (Fleming 1922; Fleming 1932) and, indeed, his prediction has held true and will likely continue to. Lysozyme has been a remarkably well-studied antimicrobial, however, many aspects of its function remain unknown, especially in vivo. Lysozyme sensitivity, bacteriolysis, and generation of ligands that activate innate immune pathways are clearly linked (Sauer, Witte et al. ; Ginsburg 2002; Davis, Nakamura et al. 2011; Rae, Geissler et al. 2011). Surprisingly, this connection does not seem compartmentalized to the extracellular space and the vacuole, where it was presumed that lysozyme activity was restricted. Rather, lysozyme activity can exert itself on cytosolic bacteria and generate ligands that activate cytosolic sensors (Shimada, Park et al. ; Davis, Nakamura et al. 2011). It is likely that lysozyme-induced bacteriolysis leads to activation of additional cytosolic sensors besides NRLC3, NOD2, and AIM2. Cytosolic nucleotide sensors such as STING, MAVS, and RIGI potentially detect PAMPs generated by lysed bacteria and may very well contribute to the outcome of infection.

Lysozyme interactions with commensal and non-pathogenic bacteria

Lysozyme activity kills bacteria and generates various PGN fragments that may increase or decrease inflammation. But for a pathogen, so what, they simply become resistant to lysozyme ("Pathogens do whatever they want."- D. Portnoy). So what is the selective pressure for a host to produce lysozyme if it is useless against pathogens? One possibility is that lysozyme serves to regulate commensal populations, to keep them compartmentalized, and to prevent them from generating immunostimulatory ligands. The role of lysozyme in gut microbiology and gut immunology will be an interesting avenue of inquiry to follow. Perhaps lysozyme's role is more nuanced than previously thought. Is its primary function to preserve and regulate commensal homeostasis and to inhibit immune responses to non-threatening microbes rather than to simply lyse any bacteria in its path?

***L. monocytogenes* peptidoglycan fragments**

PGN is an excellent candidate PAMP because it is unique to bacteria, ubiquitous, abundant and essential. Gram-positives release PGN as they modify and rearrange their walls during replication. It is unsurprising that hosts have developed PGN sensors, especially in the cytosol. Why don't NOD1, NOD2, or TLR2 seem to play a significant role in inhibiting *L. monocytogenes* infection? *L. monocytogenes* PGN contains both NOD1 and NOD2 ligands and it is predicted that lysozyme plus the activity of *L. monocytogenes* own autolysins would generate them. For instance, lysozyme (or NamA) plus the activity of p60 would generate MDP (Lenz, Mohammadi et al. 2003). However, Ami, an amidase would presumably destroy it. There are additional carboxypeptidases and amidases that could also act to ablate this PAMP. *L. monocytogenes*' ability to avoid NOD receptor activation may be a clue that it tightly controls the PGN fragments that it generates and releases. *L. monocytogenes* express many seemingly redundant and overlapping autolysins and understanding their precise roles and coordinate activities will be an important undertaking. There is evidence that PGN fragments are not only detected by the host, but also by bacteria and may act as second messenger molecules. Resuscitation promoting factors in *M. luteus* and *M. tuberculosis* have autolysin activity similar to lysozyme and lytic transglycosylases and generate PGN fragments that can induce sporulation (Keep, Ward et al. 2006). Do *L. monocytogenes* autolysins act to destroy NOD1, NOD2 or other potentially immunostimulatory ligands? Do *L. monocytogenes* act to generate PGN fragments that can act as second messengers? *L. monocytogenes* express two putative resuscitation promoting factors. Does the acetylation state of *L. monocytogenes* PGN affect generation of fragments that may be detected by hosts or other bacteria?

Bacteriolysis as a pattern of pathogenesis

Perhaps bacteriolysis is a signature event detected by a host. The subsequent rapid release of multiple ligands that signify death (PAMP post mortem) may result in simultaneous activation of multiple immune receptors and subsequent strong, but perhaps unsustained, signaling (Vance, Isberg et al. 2009). Perhaps the response to bacteriolysis influences downstream events. Indeed, LLO/Pgd/Oat-minus *L. monocytogenes*, which undergo increased bacteriolysis, suppressed immunity more than LLO-minus. Presumably, there were equivalent amounts of potential PAMPs delivered during

immunization, but their temporal release and subsequent detection were the difference between logs of immunity (Fig 3.7B, C).

Pgd regulation

It has been reported that *L. monocytogenes* Pgd expression is upregulated in vivo (Camejo, Buchrieser et al. 2009), however, there is little evidence in the literature to suggest how Pgd might be regulated. Oat activity is regulated in *B. subtilis* by the σV operon, which is strongly induced by lysozyme (Guariglia-Oropeza and Helmann 2011). Indeed, stationary and mid-log phase Pgd-minus *L. monocytogenes* seem to have differential susceptibility to lysozyme (personal observation). What regulates Pgd? Thomas Burke has identified a pathway involving a small RNA that is required for lysozyme resistance. What activates this pathway? Does de-acetylation also act as a marker for directing autolysins? If so, are there multiple pathways that regulate Pgd expression and its activity?

Pgd and Oat inhibitors as alternatives to antibiotics

Antibiotic resistance is an enormous threat to public health and development of alternative antibiotic classes and antimicrobial treatments is critical. A good antimicrobial target is specific and unique to pathogens, thus cell wall associated enzymes such as Pgd and Oat are excellent candidates. Indeed, efforts to identify inhibitors of Pgd and Oat are underway. Ideally, such compounds would render pathogens susceptible to lysozyme, making them avirulent and allowing the host to clear infection. Inhibitors must be specific only to bacterial Pgd or Oat, yet with activity against a broad range of species-specific enzymes. *B. cereus* N-deacetylase acts on *B. subtilis* and *H. pylori* PGN, suggesting a potentially conserved mechanism of action (Psylinakis, Boneca et al. 2005). Recently, the crystal structure of *S. pneumoniae* PgdA was solved and the catalytic mechanism of deacetylation was determined (Blair, Schuttelkopf et al. 2005). This information has allowed virtual screening of libraries of small molecule inhibitors and two potential inhibitors have been identified (Bui, Turk et al. 2011). Similarly, an inhibitor of *N. gonorrhoeae* O-acetylase, Ape1, has been identified in a high throughput screen and prevented growth in vitro (presumably by inhibiting lytic transglycosylase activity) (Moynihan and Clarke 2011). Will inhibitors of Pgd and Oat be a viable strategy to treat antibiotic resistant infections?

Conclusion

This work reinforces the notion that lysozyme resistance is a critical virulence determinant. It also supports the idea that lysozyme is a highly dynamic antimicrobial defense whose primary role may not be what Fleming and the field had assumed for decades. Indeed, “We shall hear more about lysozyme.” -A. Fleming.

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