The Role of Galectin-3 in Pathogen Sensing and Cell-Autonomous Defense

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Date: March 27th 2017

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular Genetics and Microbiology in the Graduate School of Duke University

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ABSTRACT

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Abstract

There is a dynamic interplay between pathogens and their hosts. This interplay has resulted in a constant struggle where the host is attempting to clear the pathogen, while the pathogen attempts to subvert the host’s immune system. The result of the back and forth has resulted in what is called the “Red Queen” hypothesis. This theory states that an adaptation by one party spurs an adaptation by the other, resulting in a never-ending conflict for supremacy.

Intracellular pathogenic bacteria have developed multiple ways in which to subvert the host’s cell-intrinsic defense systems. One common mechanism is the establishment of a pathogen containing vacuole (PV) which serves as a replicative niche for growth and shields the pathogen from many host surveillance systems. In order to effectively combat these pathogens, the host must recognize the PV as an aberrant structure and facilitate an effective defense response. One characteristic that is indispensable for pathogens that reside within PVs are bacterial secretion systems. The bacterial secretion system is critical for the pathogen to be able to interact with the host environment, modulate cellular functions, and remodel the PV to facilitate growth. There is evidence that the host can directly recognize the some of the well conserved
components of bacterial secretion systems. In this dissertation, I will show that the host has also developed a system for the detection of host ligands, which are used as a danger signal to possible damage induced by a pathogen. These damage patterns are present in the lifestyle of the pathogens, making it nearly impossible for them to avoid completely. Bacterial secretion systems cause vacuolar instability, and when a phagosome or pathogen containing vacuole is damaged, it exposes sugars from the inner leaflet of the membrane to the host cytosol. Galectins, cytosolic host glycan binding proteins, are then able to recognize the exposed sugars and bind them. Subsequently, host defense proteins including the guanylate binding proteins (GBPs) are recruited to the damaged membrane. I propose that this damage sensing ability is broadly applicable for the recruitment of host defense proteins to vacuolar pathogens.
Dedication

This dissertation is dedicated to my wife Lorin. Thank you for your unwavering love and support. Thank you for inspiring me to do better every day, and thank you for coming on this journey with me.

I also dedicate this work to my parents, Mike and Karen Feeley. Thank you for instilling in me the confidence and passion which has shaped my life.

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Bacterial infections contribute to a wide spectrum of human diseases. These diseases range from relatively minor to those that pose a significant threat with high morbidity and mortality. One class of pathogenic bacteria are intracellular pathogens. Intracellular pathogens can be either obligate intracellular, e.g. *Chlamydia* or *Rickettsia*, which only resides within host cells, or facultative intracellular e.g. *Legionella* or *Salmonella*, which can also exist outside the host as well[1]. Intracellular bacterial pathogens have evolved multiple mechanisms through which they modulate the host environment to suit their needs[2]. These tools allow the pathogen to exploit host functions to acquire nutrients, evade detection by the immune system, suppress host cell death, manipulate intracellular trafficking and form a replicative niche[3]. Pathogenic bacteria undergo four major stages of infection; Entry, Survival, Replication and Egress. This thesis will predominantly focus the relationship between bacterial pathogens and their hosts during the early stages of infection.

**1. Bacterial Invasion**

Bacteria have evolved multiple techniques for invading host cells. For example, some bacteria actively invade the host by binding to cell surface receptors which facilitate their entry into the host [4, 5]. These bacterial pathogens use specialized proteins called adhesins to bind to transmembrane receptors on the plasma membrane.
This binding with plasma membrane receptors signals to the host to internalize the pathogen[7]. Other bacteria attach to the cell surface and trigger cytoskeletal rearrangements which facilitate bacterial entry [8]. This triggered entry is initiated by the invading pathogen injecting proteins in the host cytoplasm which commences large scale actin cytoskeletal rearrangements. This cytoskeletal rearrangement results in membrane ruffling around the invading pathogen. The pocket that is formed is then closed off by actin depolarization and internalized[9]. Still other pathogens co-opt the host phagocytic pathway for entry. These bacteria co-opt the normal phagocytic activity of cells to gain entry, and subsequently block the standard cellular clearance machinery from acting on the phagosome [10-12].

Regardless of their mode of entry, invasion is only the first step. Bacteria must further modulate the host in order to successfully replicate. This is accomplished by either transforming the internalization vacuole into a replicative niche, or escaping the vacuolar environment and invading the cytosol for replication [13, 14]. In order to combat the host defense system and prevent the host from recognizing and destroying the pathogen, the pathogens produce numerous proteins called effectors. The secreted effectors are involved in multiple activities that promote virulence. These roles include, but are not limited to: scavenging for resources, developing and maintaining a replicative niche, evasion of the host defense system, and preventing premature cellular
death [15]. In order to translocate these effectors from the bacteria to the host, the bacteria utilize specialized machinery called bacterial secretion systems.

1.3 Bacterial Secretion Systems

The primary function of bacterial secretion systems is the transport of proteins across phospholipid membranes. This allows bacteria to translocate substrates from the bacterial cytoplasm to their environment. The manner in which the protein translocation occurs varies greatly between bacteria, but several conserved mechanisms have been identified [16]. Bacterial secretion systems are broadly classified based on their structure, function, and specificity. This dissertation will discuss each class of secretion, but will primarily focus on two types of secretion system that have evolved to penetrate three membranes, the type III and IV secretion systems. The type III and IV apparatuses are able to cross two bacterial membranes and one host membrane to deliver their cargo directly into the host cell. This dissertation will utilize two model pathogens, Yersinia pseudotuberculosis (type III) and Legionella pneumophila (type IV) to examine the roll of the bacterial secretion system in shaping the host response to infection. Details for each pathogen will be discussed in the appropriate sections.
1.3.1 Sec and Tat Secretion

The general secretion (Sec) and twin arginine translocation (Tat) pathways are some of the most well conserved methods for protein translocation. Proteins secreted by Sec or Tat are often destined for the periplasmic space, or in the inner bacterial membrane, but this system can also be utilized to translocate proteins out of the bacteria with the help of additional secretion systems (type II and V)[17, 18]. The Sec secretion system utilizes a membrane integrated protein channel to translocate proteins across the phospholipid membrane with the help of a motor protein, chaperones, and specific signal sequence. The central component of the Sec system is SecA [19]. SecA is an ATPase which is critical for driving the protein translocation across the membrane. SecA has also been shown to interact with the vast majority of the other components of the Sec pathway and be the receptor for the secretory proteins. [20] The chaperone protein, SecB, plays a critical role in the Sec secretion system. SecB binds to proteins destined for secretion and maintains them in their unfolded state for transport to the Sec machinery for translocation[21]. Proteins destined for secretion by the Sec system have a conserved signal peptide. This peptide is, on average 20 amino acids with a tripartite structure, consisting of a charged region, hydrophobic core and a short polar region[22].

The primary difference between the Sec and Tat pathways is that while the Sec pathway secretes unfolded proteins, the Tat pathway moves prefolded proteins across the membrane[23]. The Tat system consists of multiple membrane integrated subunits.
that together form an oligomeric pore for translocation. TatA, TatB, and TatC form the
translocon which is activated upon interaction with a Tat translocation competent
secretory protein[24]. The secretory signal is similar to the Sec system in that it is a
tripartite, charged, hydrophobic and polar sequence. However, it also has specificity for
a twin-arginine motif pattern Z-R-R-x-Φ-Φ, where Z indicates any polar residue and Φ
indicates hydrophobic residues. [25-27]

1.3.2 Type I Secretion System

The Type I secretion system (T1SS) allows for the secretion of proteins from the
bacterial cytoplasm to the extracellular space in a single step. This system allows for
secretion without the Sec or Tat pathways because it crosses both bacterial membranes
and does not require periplasmic intermediates [28, 29]. The T1SS is structurally similar
to the ATP binding cassette (ABC) transporters. ABC transporters are able to pump
toxins and small molecules, such as antibiotics, out of cells[30]. The T1SS have three
major structural components. They contain an outer membrane pore, and two inner
membrane components. The ABC cassette and the membrane fusion adaptor protein are
localized to the cytoplasmic face. The adaptor protein is responsible for recognition of
the bacterial substrates via their secretion signal[31]. The ABC cassette provides the
power for the secretion machinery. Mutations in the ATPase activity of the ABC cassette
have been shown to dramatically reduce the efficiency of type I secretion[32].
1.3.3 Type II Secretion System

The Type II Secretion System (T2SS) is utilized by a wide array of bacteria to secrete numerous substrates out of the bacterial cell. The T2SS is often considered the terminal end of the general secretory pathway. The T2SS is comprised of between 40-70 proteins depending on the bacterial species [33, 34]. The machinery of the T2SS is subdivided into 4 complexes: the pseudopilus, outer membrane complex, inner membrane complex and the secretory ATPase. The pseudopilus is a periplasmic arrangement of 5 sub-structures termed pseudopilin. These pseudopilins are structurally very similar to the bacterial Type IV pilins and also rely on prepilin protease. During secretion, the pseudopilus functions much like a piston to expel proteins across the outer membrane. [35, 36] The outer membrane complex is mainly comprised of proteins called secretins. These secretins are often, but not always arranged in a beta barrel that spans the outer membrane. Secretins are also found in type III secretion systems. These large multimeric protein assemblies are thought to be targeted to the outer membrane by proteins called pilotin.[37, 38] The inner membrane platform communicates with the secretory ATPase and the pseudopilus to coordinate the extrusion of substrates through the outer pore[39].

The T2SSs transport folded proteins from the periplasmic space out of the bacterial cell. When combined with Sec or Tat secretion, this class of secretion enables efficient transport of bacterial proteins into their environment. Some bacteria utilize this
system to secrete substrates that enhance the virulence of the bacteria. Examples include the cholera toxin of *V. cholera* \[40\], and exotoxin A of *P. aeruginosa*\[41\] Due to the fact that the T2SS secretory apparatus is housed in the outer membrane, all substrates that are exported by the T2SS must have Sec or Tat cleavage signals in the N terminal region.\[33, 42\]

### 1.3.4 Type III Secretion System

Type III bacteria secretion systems, (T3SS) often called the injectisome, are some of the more well characterized bacterial secretion systems. They have a distinctive needle and syringe like apparatus that is capable of injecting effectors across the eukaryotic cell membrane\[43\]. The T3SS is a complex of at least 20 components, depending on the bacterial species. The T3SS can be subdivided into three major components: the basal body, needle, and the tip complex. The basal body is a large protein complex that spans both the inner and outer membranes. The basal body is comprised of proteins from the secretin family. These proteins are structured into membrane embedded rings, which are attached to a central rod structure which serves as the housing for the needle complex. The needle is a hollow transmembrane structure through which the bacterial proteins are secreted. The needle itself is made up of a helical assembly of small repeating proteins attached to the core by a flexible hinge. The needle itself extends into the extracellular space \[44, 45\]. The length of the needle is strictly regulated and contains a specialized tip structure at the distal end of the needle.
The tip structure is critical for sensing the extracellular environment and host cell contact. The tip complex facilitates the insertion of the translocon into the host cell membrane to facilitate effector secretion. This specialized complex ensures that bacterial effectors are only secreted upon docking with the host cell and not aberrantly secreted into the extracellular milieu [46-48].

The T3SS has a high degree of evolutionary conservation with the type III flagellar secretion system [49]. The general architecture of both structures are very similar and the process of export of the axial flagellar components is highly analogous to export of effectors through the virulence T3SS [50, 51]. In the basal subunit, 8 proteins, including the ATPase are highly conserved between the two systems [52]. Also, the expression and hierarchical process of formation of both apparatuses are tightly regulated. They sense environmental cues and respond to external stimuli on reaching their correct physiological niche [53, 54]. However, while needle components of the T3SS share structural similarities with components of the flagellar filament, they are not the same at the amino acid level. Additionally, the substrates exported are dramatically different. The flagellar type III system exports hook and junction proteins that attach to the flagellar filament that makes up the flagella [55, 56].

The number of secreted effectors varies widely by species. Pathogens such as *Shigella* secrete dozens of effectors, where *Yersinia* secreted only a few bacterial effectors. The secretion signal for T3SS secreted effectors are usually in the N-terminus of the
substrate. These secretion signals are often bound by chaperone proteins which guide
the effectors in an unfolded state to the base of the T3SS. The bacterial effectors are then
transported through the needle and into the host cell[57]. The T3SS ATPase enhances,
but is not required for, the secretion of bacterial effectors. Substrates from Yersinia and
Salmonella have been shown to be secreted by the proton motive force in the absence of
ATPase activity[49].

1.3.4.1 Yersinia T3SS
Pathogenic Yersinia species, including pestis, pseudotuberculosis, and enterocolitica,
utilize a type three secretion system for the export of their virulence factors. These
Yersinia species share the Yop virulon. The Yop virulon is a 70kb plasmid which encodes
for the secretion system machinery, called the Ysc injectisome, as well as Yop effector
proteins which are injected into the host [58, 59]. The entire secretory apparatus is
comprised of 27 proteins. As is common for many T3SSs, the majority of the internal
components have structural similarity to basal body of the flagellum [60]. The basal
body is made up of YscC, YscD, and YscJ. YscC forms a ring in the bacterial outer
membrane, and YscD and YscJ form a ring in the inner membrane. The basal body also
includes the export apparatus, which contains the integral membrane proteins
YscRSTUV, and the ATPase complex, comprised of YscNKL. The external components
are comprised of multimeric ring structure, consisting primarily of YscF, which is tipped
with a needle like appendage of ~60nm [60, 61].
Translocon assembly is initiated by production of LcrV. LcrV polymerizes at the apical tip of the YscF needle and forms a penetrating needle tip complex [62, 63]. The needle tip complex is critical for translocation of *Yersinia* effectors[64]. At the tip, two major translocon proteins YopB and YopD are responsible for forming a pore in the host cell membrane. YopB is the larger of the two proteins with two transmembrane helices. YopD has only a single transmembrane helix [65, 66].

This Ysc injectisome serves as a hollow transportation channel for the secretion of Yops across the bacterial membranes. These Yop effectors are capable of blocking phagocytosis, altering membrane and cytoskeletal dynamics, and blocking the production of proinflammatory cytokines. For example, YopJ has been shown to acetylate critical residues in the activation loops of MAPK kinase and IKKβ to block downstream signaling [67].

The assembly and activation of the *Yersinia* T3SS is directed by several environmental cues. The initial cue is an elevated environmental temperature. Once the bacteria reach a temperature of 37 degrees centigrade, which is the ambient temperature of the host, the bacteria initiate T3SS production. The T3SS machinery is produced and assembled and a reservoir of effector Yops are produced, but the secretion channel remains closed [49, 68]. Secretion of bacterial effectors is triggered by host cell contact and low levels of environmental calcium. This sensing signal is transduced down the
translocon to the basal body, which triggers the release of YopN complex and allowing for effector transport [69-71].

The translocon itself is regulated by a secreted effector YopK. YopK has been shown to directly interact with YopD and influence the function of the secretion system. It remains unclear how YopK regulates the injectisome, but it has been suggested that YopK may form a plug which inhibits further secretion. Alternatively, YopK binding may signal across the translocon to result in structural changes or feedback inhibition of secretion[72]. This regulation is critical for pathogenesis. Over injection of cytotoxic effectors induces inflammatory response. A YopK mutant triggers caspase-1 and inflammasome activation due to hypertranslocation [73, 74].

1.3.5 Type IV Secretion System

The Type IV secretion system (T4SS) is most closely related to bacterial conjugation machinery. T4SS are often utilized by bacteria for horizontal gene transfer. Conjugative T4SS have been shown to be important for transmission of antibiotic resistance and other virulence traits. In pathogenic bacteria, the T4SS is critical for transferring bacterial effector proteins directly into the host cell. The channel in the T4SS is large enough to allow the movement of protein-protein and protein-DNA complexes [75]. The T4SS is capable of translocating large macromolecules such as protein toxins and nucleoproteins as well as smaller monomeric protein substrates. The energy for translocation is provided by cytoplasmic ATPases. They also are believed to drive the
conformational changes in the translocation complex that allows for large macromolecule transport [76]. T4SSs exist in two major classes, Type IVa and Type IVb depending on their homology to conjugal transfer system. The Type IVa system shares close homology with the plant pathogen secretion system of Agrobacterium tumefaciens, where the Type IVb is more closely related to the conjugal transfer system. Although the structural components differ, both systems rely on chaperones for effector recognition and delivery to the secretion machinery. It is also worth noting that, although the T4SS share similar machinery, the bacterial effectors produced by individual bacterial species share very little similarity. It has been proposed that the diversity among bacterial effectors due, in part, to the broad pathogen specific environments that are colonized by T4SS pathogens [77, 78]. This dissertation will focus on the Type IVb secretion system, specifically the Dot/Icm system of Legionella pneumophila, as it is the model system used in the forthcoming studies.

1.3.5.1 Legionella T4SS

*Legionella pneumophila* is a Gram-negative facultative intravacuolar bacterial pathogen[79]. The natural host for *L. pneumophila* is the freshwater amoeba, but *Legionella* is capable of adapting to a wide array of hosts. The human surrogate for the amoeba are the alveolar macrophages, which are similar enough to the native host that the bacterial effectors are effective at modulating the host. This broad specificity is in part due to its large repertoire of secreted bacterial effectors. These bacterial effectors are
delivered to the host cell by the Dot/Icm T4SS [80, 81]. The Dot/Icm secretion machinery is comprised of 27 proteins whose individual functions still remain largely unknown. There are 22 structural components and 5 chaperones involved in Dot/Icm secretion. These Dot/Icm proteins are essential for intracellular survival and bacterial virulence.

There are five Dot/Icm proteins, DotC, DotD, DotF, DotG and DotH, that appear to form a secretion apparatus that bridges the inner and outer bacterial membranes[80]. DotG, one of the largest of the system at 1048 amino acids, has been proposed to span the outer membrane. DotF has been shown to interact with DotG and may be involved in regulating substrate specificity[82]. Oligomers of DotH form the outermembrane pore, which is stabilized by interactions with DotC and DotD[83]. The majority of the inner membrane associated Dot/Icm proteins have unknown functions. One inner membrane protein, DotA is critical for bacterial effector delivery. Mutants of the DotA protein are incapable of secreting the any Dot/Icm effectors [84, 85]. DotL, DotM, and DotN are believed to be the cytoplasmic receptors for bacterial effectors[86]. Dot/Icm secretion is an energy dependent process. DotB is the cytoplasmic ATPase which drives effector secretion and the conformational changes of the secretory apparatus. The cytoplasmic proteins IcmQ, IcmR, IcmS, and IcmW are chaperone proteins important for substrate recognition and delivery [87, 88].

The Dot/Icm T4SS is capable of secreting over three hundred protein effectors [89]. The function of many of these bacterial effectors is unknown. Examining the
individual effectors is hampered by the seemingly redundant role that many effectors play. In addition, numerous effectors secreted may have host cell specific functions. Since *Legionella* has a broad host range, this has confounded the issue of identifying roles for individual effectors. There has also been no specific signal sequences identified that are responsible for Dot/Icm secretion[82]. It has been proposed that the secretion signal is embedded in the C-terminal region of bacterial effectors. Evidence to suggest this includes the fact that chaperones IcmS and IcmW binding to effector proteins triggers a conformational change in some effectors that allows presentation of a C-terminal region [90]. The T4SS plays a critical role in the pathogenesis of *Legionella pneumophila*. Mutant bacteria deficient for Dot/Icm secretion are avirulent in animal models [91, 92].

1.3.6 Type V Secretion System

The Type V secretion system (T5SS), like the T2SS, secretes bacterial proteins from the bacterial periplasm across the outer membrane into the extracellular environment. There are three main pathways for secretion of type V effectors. These pathways are classified based on the number of proteins required for effector export [93-95]. The most straightforward of these pathways is the autotransport system. These proteins destined for autotransport have specialized domains that allow for efficient self-secretion. These domains include a C-terminal domain that creates its own membrane channel for secretion. Proteins that utilize the autotransport system are initially exported to the periplasm by the Sec secretion system. Once clear of the inner
membrane, the autotransporter proteins interact with periplasmic chaperone proteins that aid in the folding and transit of proteins to the outer membrane. The C-terminal region then assembles a β-barrel pore in the outer membrane. Once the β-barrel is correctly inserted, the portion of the protein to be exported is then extruded through the pore. Once exported, the protein is cleaved and released from the pore complex by its own protease domain, or more rarely, extracellular proteases [94, 96, 97].

The second pathway is called two partner secretion. These substrates are often very large virulence proteins that rely on a cofactor that is secreted specifically to form the β-barrel. Both proteins are first transported by the sec machinery into the periplasm, where chaperone proteins stabilize the proteins in their conformational state and protect against degradation by periplasmic proteases. The helper protein subsequently forms the β-barrel in the outer membrane allowing the large protein to be exported [98, 99].

The final class of T5SSs is the trimeric autotransporter adhesin pathway. In this pathway, three individual polypeptides cooperate to form the outer β-barrel. Each individual protein only contains one third of a β-barrel. Therefore, these proteins must trimerize to form a functional β-barrel through the outer membrane. Like the other forms of Type V secretion, this trimerization and folding is facilitated by periplasmic chaperones [100, 101].
1.3.7 Type VI Secretion System

Type VI Secretion Systems (T6SSs) are multi-protein assemblies that span both bacterial membranes and inject effector proteins directly from the bacterial cytoplasm into target cells. The T6SS is composed of proteins that are evolutionarily related to components of bacteriophage tails. The T6SS is somewhat unusual in that T6SSs are capable of transporting effector proteins between bacteria as well as into eukaryotic hosts. This functionality may play a role in bacterial competition or communication between bacteria in close contact. The ability to inject effector proteins into other bacteria can provide a distinct competitive advantage. This phenomenon has been reported for T6SSs in *P. aeruginosa*, *B. thailandensis*, *V. cholerae* and *S. marcescens* [102-105].

Additionally, many pathogens that use T6SS effectors also have adjacently encoded immunity proteins. These immunity proteins are able to protect the bacteria from being harmed by effectors that are injected into the bacteria by its sibling neighbors [106, 107].

T6SSs are encoded by large, variable gene clusters that contain up to 21 genes that make up the secretion system. Thirteen of these proteins are conserved in all T6SSs, therefore considered to be the core components. These proteins assemble into two main complexes, a membrane associated body and extracellular secretion apparatus. Two core proteins, Hcp and VgrG, are believed to make up the extracellular section of the secretion machinery[108]. They form a needle like insertion device related to the bacteriophage tail spike. Upon target cell contact, this spike is believed to be thrust into
the target cell by contraction of the tail sheath-like structure that surrounds the spike [109, 110]. This T6SS sheath is long tubular structure that is found in either extended or contracted conformations. This sheath is connected to the inner membrane by a distinct basal structure.

T6SS effectors are very broad in their composition, ranging from small single domain proteins to large toxins and secreted structural components of the secretion system [102, 111]. One of the more well studied classes of Type VI effectors are the cell wall degrading effectors. These effectors are specialized for bacterial antagonism and have a specialized roll in breaking down peptidoglycan. Other T6SS effectors have also been shown to have a role in nucleic acid breakdown, phospholipases disruption, and some effectors may be pore forming toxins [112].
2.0 Pathogen Recognition

2.1 Introduction to Pathogen Sensing Mechanisms

Throughout evolution, there has been a “molecular arms race” in which host and pathogen have attempted to obtain the upper hand in the fight for survival. These dynamic host pathogen interactions have resulted in the evolution of a wide range of bacterial effectors and secretion systems, as discussed in the previous sections, as well as a multitude of pathogen sensing and host defense proteins. Direct pathogen sensing by the host is accomplished by pattern recognition receptors (PRRs). These PRRs detect pathogen-associated molecular patterns (PAMPs), which are common characteristics of pathogens that are under heavy selective pressure and therefore unable to be altered by the pathogen without suffering reduced fitness [113]. The host has also evolved methods to detect damage (or danger) associated molecular patterns (DAMPs) which allows for indirect recognition of pathogen invasion [114]. The upcoming sections will detail the methods of pathogen recognition by the host.

2.1.1 Toll-like Receptors

The Toll-like receptors (TLRs) play a critical role in the immune response by recognizing both PAMPs from bacteria and viruses and DAMPs from dying or injured cells. TLRs also perform an important immune function by activating signaling pathways that result in the downstream production of cytokines and chemokines. The TLRs are integral membrane glycoproteins. The extracellular domain contains leucine
rich repeats (LRR) which are primarily responsible for binding PAMPs. There have been eight TLR-ligand complexes crystalized to date[115]. The overall shape of the TLRs is similar, but they vary significantly in the binding pocket. Ligand binding is believed to induce dimerization of the TLRs. The intracellular region contains a Toll/IL-1 receptor homology (TIR) domain which is responsible for signaling. TLR dimerization is believed to bring the TIR domains together to initiate signaling upon receptor binding. TLR signaling is mediated by adaptor proteins including MyD88, Trif, Traf, and Tram. These adaptor proteins induce signaling cascades that result in the production of pro-inflammatory cytokines following TLR activation. [116, 117]

There are thirteen TLRs in mammals. TLRs 1 through 9 are conserved between mice and humans. Mice lack a functional TLR 10, and TLRs 11 through 13 are not found in humans. TLRs can be classified by their cellular localization. TLR1, 2,4,5,6, and 10 are expressed on the cell surface. TLRs 3, 7,8,9,11,12, and 13 are endosomal [118, 119]. The heterogeneous localization of the TLRs is due to the fact that they recognize distinct PAMPs. The cell surface expressed TLRs are predominantly responsible for detecting microbial components in the extracellular milieu. These products include lipids, lipoproteins and proteins. The endosomal TLRs are predominantly tasked with the detection of nucleic acids [120].

There are several TLRs of particular note for the detection of bacterial infection. TLRs 1 and 2 are specialized in their recognition of bacterial lipoproteins. These
substrates include porins, glycolipids, triacylated lipoproteins, and soluble lipoproteins. TLR4 recognizes lipopolysaccharide (LPS) derived from the cell wall of Gram-negative bacteria. The recognition of LPS by TLR4 is enhanced by complexing with LPS binding protein and CD14. LPS recognition by these molecules results in the internalization of TLR4 into endosomes which activates the downstream signaling pathways, both MyD88 dependent and independent. TLR5 recognizes bacterial flagellin from multiple gram negative bacteria. TLR9 recognizes unmethylated CpG motifs which are present in bacterial DNA [121, 122]. TLR13 is able to recognize single stranded RNA of bacterial origin.

2.1.2 Nod-like Receptors

Nod-like receptors (NLRs) are a family of predominantly cytosolic receptors which recognize an array of pathogenic signals. NLRs are an important line of defense for the host if the pathogen manages to evade the extracellular surveillance and invades the cytosol. NLRs are characterized by the presence of a conserved nucleotide-binding and oligomerization domain (NOD) [123]. Structurally, the NLRs are made up of three major regions. They contain an N-terminal effector domain, a NOD/NACHT domain, and a LRR domain at the C-terminus. The N-terminal domain of the NLRs consists of protein-protein interaction domains. The most common varieties are the caspase recruitment domain (CARD), pyrin domain, and baculovirus inhibitor repeat (BIR) domain. These active domains are used to classify the NLRs, with the final letter
designating the N-terminal domain [124, 125]. For example, NLRP designates a NOD-like receptor with a pyrin domain. The central NOD/NACHT region is critical for oligomerization upon activation by receptor ligand binding. The C-terminal region contains LRRs which are responsible for PAMP recognition. When no antigen is present, the LRRs in the C-terminus are thought to fold back on the NOD domain to block spontaneous oligomerization and activation[126]. NLRs are predominantly expressed in immune cells such as macrophages and dendritic cells, but can also be found in nonimmune cells. There are greater than twenty NLRs in humans and at least thirty NLRs in mice. It has been proposed that the large number of NLRs aids in the detection of a wide range of PAMPs [127, 128].

NLRs are involved in activating diverse signaling pathways. Three major downstream signaling targets of NLR signaling have been identified; they are NFκB, MAPK, and caspase -1. NOD1 (NLRC1) and NOD2 (NLRC2) have been shown to detect the presence of the peptidoglycan fragments meso-DAP and muramyl dipeptide [129, 130]. Upon activation, they stimulate NFκB and MAPK signaling through a serine threonine kinase, RIP2. This cascade leads to the production of multiple proinflammatory cytokines including TNF-α, IL-6 and IL-8. NLRs induce caspase-1 activation through the assembly of structures called inflammasomes. Inflammasomes are multi-protein complexes of NLRs and adaptor proteins, such as ASC, that come
together to mediate cleavage of the protease, caspase-1. Following cleavage of caspase-1, the cell undergoes pyroptotic cell death and secretes proinflammatory cytokines IL-1β and IL-18 [131].

NLRC4 has been shown to be a potent activator of the inflammasome upon bacterial infection. Work by Michele Swanson and Russell Vance showed that the NLRC4 inflammasome was being activated by cytosolic detection of flagellin. Bacterial mutants that lack flagellin are able to avoid the NLRC4 inflammasome [132-135]. This NLRC4 inflammasome activation is also lacking in bacteria that lack functional secretion systems. Many bacterial pathogens, including *Legionella* and *Salmonella* aberrantly secrete flagellin through their secretion system machinery. This aberrant secretion leads to the cytosolic detection of flagellin by NLRC4 and subsequent caspase-1 activation. NLRs can also be activated without direct cellular infection. Bacterial components, such as peptidoglycan can be detected in the cytoplasm after injection through bacterial secretion systems[67] or fusion of outer membrane vesicles with the plasma membrane[136].

### 2.1.3 RIG-I-like receptors

Retinoic acid-inducible gene 1 (RIG-I) like receptors (RLRs) are a type of intracellular PRRs involved in the recognition of viral RNA by the host. While the TLRs are capable of detecting viral RNAs in the extracellular environment or RNA sequestered in endosomes, RLRs are specialized to detect cytoplasmic viral RNA. There
are three RLR family members, RIG-I, MDA5 and LGP2 [137]. The N-terminal region of RIG-I and MDA5 contain tandem CARD domains, which are involved in downstream signaling through MAVs. MAVs serves as a scaffold for the downstream activation of MAPK, NFκB, and interferon regulatory factor 3[138]. LGP2 does not contain a CARD domain, and its function remains unknown. The central region of RLRs contains a DEAD box helicase domain. The DEAD box helicase enhances the affinity of RLRs to dsRNA through a conformational change[139]. The C-terminal region is well conserved between the RLR and facilitates the binding to RNA. The RLRs recognize RNA motifs that are absent on self RNAs. RIG-I has been shown to bind viral RNA containing either a 5′-triphosphated single-stranded RNA or 5′ blunt-end dsRNA with a triphosphate motif [139, 140].

2.2 Introduction to Interferon

Interferons were first described 60 years ago where Isaacs and Lindenmann identified a new factor (interferon) that had the ability to interfere with influenza virus replication [141]. Over time, it became apparent that interferon (IFN) was not one, but a family of distinct proteins that fall into three classes based on structure and receptor binding (Table 1).
The interferons comprise a group of signaling molecules that play a key role in combatting pathogens ranging from virus and bacteria to protozoa. This dissertation will discuss both Type I and Type II interferons, which play a role in antimicrobial responses. Type III IFN have similar functions to the type I IFN family, however the target cells pool is significantly smaller as the IFNλ receptor is primarily expressed on epithelial cells [142, 143].

2.2.1 Type I Interferon

Type I IFNs constitute the most numerous class of IFNs and are expressed in response to PAMP and DAMP recognition by PRRs [144]. Interferons alpha and beta are the highest expressed and are the most well studied of the family. Expression of type I interferons can be triggered following pathogen recognition. This activity is mediated by signaling cascades that result in activation of interferon response factors (IRFs) [145].

### Table 1: Interferons

<table>
<thead>
<tr>
<th>Class</th>
<th>Members</th>
<th>Major Producers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>α/β/ε/τ/κ/ω/δ/ζ</td>
<td>All cells</td>
</tr>
<tr>
<td>Type II</td>
<td>γ</td>
<td>NK Cells, T Cells</td>
</tr>
<tr>
<td>Type III</td>
<td>λ1, λ2, λ3, λ4</td>
<td>Hematopoietic cells, epithelial cells</td>
</tr>
</tbody>
</table>
IRF7 leading to the induction of IFNα/β production. These cytokines produce a heightened immune state in infected and non-infected bystander cells [146, 147].

Type I IFNs stimulate the production of hundreds of interferon stimulated genes (ISG) that influence the development of the innate and adaptive immune system. IFNα/β induce gene expression through binding with the Type I IFN receptor (IFNAR). IFNAR contains two subunits, IFNAR1 and IFNAR2. Canonically, this signal is transduced through the Janus Kinase (JAK) and signal transducer and activator of transcription (STAT) pathway [148, 149]. When no IFN is present, the cytoplasmic portions of the IFNAR1 and 2 are bound by JAKs in an inactive state. Upon IFNα/β binding to IFNAR, two JAK kinase domains are brought together by conformational changes resulting in phosphorylation and activation [150, 151]. This phosphorylation triggers STAT phosphorylation and dimerization. Once activated, the STAT proteins interact with IRF9 and translocate to the nucleus to drive ISG production [152, 153].

### 2.2.2 Type II Interferon

IFN-gamma was first identified in 1965, in which activated lymphocyte supernatants protected against the cytopathic effects of Sindbis virus [154]. Type II interferon production is most potently induced by natural killer (NK) cells and cytotoxic T lymphocytes. NK cells are primed to rapidly respond to pathogenic infections by producing IFNγ [155]. This allowed for NK cells to provide the initial wave of IFN-gamma production upon an infection. In contrast to NK cells, T cells require a sustained
activation signal to produce IFNγ [156]. This functionality allows for temporal control over the amount of IFN produced. Aberrant IFNγ expression has been associated with chronic autoimmune and autoinflammatory diseases, including inflammatory bowel disease, multiple sclerosis, and diabetes mellitus [157-159].

The signaling cascade of Type II share components of the JAK-STAT pathway with the Type I system. Synergistic effects of IFNα and IFNγ have been reported where pretreatment with IFNγ can boost the cellular response to IFNα by elevating the levels of STAT1 and IRF9 [160, 161]. The receptor complex for IFNγ, IFNGR1 and IFNGR2, is almost ubiquitously expressed in all cell types. Much like the IFNAR, the cytoplasmic subunits of IFNGR1 and IFNGR2 are bound by JAK1 and Jak2 respectively when inactivated. Following receptor binding, the JAK-STAT pathway is activated which in turn induces genes containing the gamma-interferon activation sequence in the promoter [162, 163].

IFNγ plays a critical role in antimicrobial defense. Mice deficient in IFNγ production or responsiveness are much more susceptible to intracellular bacterial infection than their wildtype peers [164, 165]. IFNγ also triggers an increase in phagocytosis and antigen presentation by major histocompatibility complex (MHC) receptors by antigen presenting cells [166].
2.3 Cell-Autonomous Immunity

Cell-autonomous immunity is the cellular self-defense program for warding off pathogenic infections. Cell-autonomous immunity is not exclusive to professional immune cells, though they are better equipped; all cells have some ability to defend themselves[143]. Cells can be primed to combat pathogens by interferon. This priming provides a baseline boost to the cells anti pathogenic capabilities by inducing production of defense proteins. These defense proteins aid in the recognition and destruction of pathogens, as well as feeding forward into interferon production. A number of these interferon stimulated genes (ISGs) control infections by directly targeting pathways and functions required during the pathogen life cycle [167, 168]. This section will detail the general pathways of control and highlight some of the key genes involved.

2.3.1 Autophagy

Macroautophagy, herein after referred to as autophagy, is a bulk degradation process for removal of unwanted materials from the cell. This process involves the coordinated actions of dozens of autophagy-related (ATG) genes, numerous adaptor proteins, and requires multiple biological signals which mediate membrane rearrangements and organelle recycling. To accomplish this task, the cell encloses the target of degradation in a dual isolation membrane structure termed and autophagosome. The autophagosome then goes through a maturation process before finally fusing with a lysosome which acidifies the compartment. The cellular
components and pathways for autophagy can also be used to clear pathogens from the cell [169-171]. When cells utilize autophagy to remove pathogens, the process is called xenophagy. Xenophagy is a form of selective autophagy that utilizes signals placed on pathogens, or PVs to target for destruction. While the precise mechanism of action and specificity determinants of xenophagy are not fully understood, several key factors have been identified [172].

The cell has several mechanisms in place for sequestering pathogens via xenophagy. The most direct utilization of autophagy for pathogen control involves fusion of bacterial containing phagosomes with lysosomes. This system relies on the recruitment of microtubule associated light chain 3 (LC3) to the phagosome. LC3 triggers the maturation of the vacuole and subsequent fusion with lysosomes. There is evidence that both *Streptococcus pyogenes*, and *Mycobacterium tuberculosis* are susceptible to this route of control. Additionally, loss of LC3 leads to increased bacterial growth in both cases [173-176].

When the host is marking cellular cargo for degradation by the autophagy machinery, it often uses molecular tags like ubiquitin. Ubiquitin is a small protein that is covalently attached to lysine residues by three enzyme classes and their scaffolding proteins, E1, E2 and E3. These ubiquitination marks, or poly ubiquitination tails act as sorting signals for protein degradation. This process is also utilized in cell-autonomous immunity where pathogens, and PVs, are marked by ubiquitin. For the intracellular
pathogens Salmonella, Shigella and Listeria, accumulation of ubiquitin requires adaptor proteins like p62 (sqstm1), NDP52 and NBR1. These adaptor proteins bind to ubiquitin via the UBA domain and mark the target for destruction by autophagy [177-179].

2.3.2 Nutrient Deprivation

In addition to direct mechanisms for bacterial control, the host has some indirect methodologies for limiting pathogen growth. During an infection, the cell attempts to make itself an inhospitable environment. The goal is to limit the availability of key nutrients such as carbohydrates, amino acids, and ions.

Intracellular pathogens have stringent metal cation requirements for growth. Limiting these resources severely hampers the pathogens ability to replicate. The host has evolved IFN-induced mechanisms to restrict the availability of Ca^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} ions[180-182]. Additionally, the host increases the Cu^{+} concentration which helps to drive the formation of reactive oxygen species (Discussed in the next section). In order to restrict iron availability, which has been shown to be critical for numerous intracellular pathogens, the host maintains iron in the ferric bound redox state (3^{+})[183]. To further inhibit the availability of Fe^{2+} and Mn^{2+}, the host uses a interferon induced efflux pump called natural resistance-associated macrophage protein 1 (NRAMP1). NRAMP1 has been shown to block the accumulation of iron by phagosomal pathogens by outcompeting the bacterial Fe^{2+} and Mn^{2+} transporters for the substrate. Finally, upon
IFN stimulation, the host downregulates transferrin, which slows the intake of iron from extracellular sources and further limits its availability [180].

Amino acid availability is also strictly regulated by the host. One critical amino acid that is controlled by the host is L-tryptophan. Indoleamine-2,3-deoxygenase (IDO) is an interferon inducible protein which degrades L-tryptophan. IDO is a haem-containing oxidoreductases that is required for the initial step of the kynurenine pathway, in which L-tryptophan is used to generate N-formylkynurenine [184, 185]. This reduction of available tryptophan has been shown to be an effective tool for the restriction of multiple pathogens including *Chlamydia, Francisella*, and *Rickettsia* [186].

2.3.3 Oxidative Damage

Oxidative damage is another non-specific method of pathogen control. The advantage of using reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) for defense is that a pathogen cannot evade this defense mechanism by changing a structure as they would for PRR evasion. ROI (1) and RNI (2) can damage DNA via guanine base oxidation, and damage lipid motifs via peroxidation. This oxidative damage is non-specific, and can damage host structures as well as pathogens. Therefore it is imperative that oxidative damage be strictly controlled[187].

One family of enzymes is the major producers of reactive oxygen species (ROS) during infection[188]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family is able to produce superoxide (O$_2^-$), and peroxynitrite (OONO$^-$).
The best studied of the group is NOX2, which is composed of heterodimer of gp91phox and p22phox which are transmembrane and three catalytic subunits, p67phox, p47phox and p40phox [190, 191]. Upon IFN stimulation NOS2 is expressed in a variety of immune and non-immune cell types. Upon phagocytosis, the transmembrane components gp91phox and p22phox attach to the phagosomal membrane. Subsequently, the catalytic subunits bind and trigger the translocation of electrons across the membrane in a phenomenon called respiratory burst [192, 193]. This targeted burst maximizes the damaging potential of the oxide radicals in a small location which increases their efficacy and reduces the potential for self-harm[11].

Another producer of oxide radicals is inducible nitric oxide synthase (iNOS) iNOS is induced by proinflammatory signals like IFN and IL-1β. It has also been shown to be upregulated by recognition of microbial components like LPS by PRRs. iNOS generates a destructive reactive nitrogen species called nitric oxide. iNOS converts L-arginine and oxygen into L-citrulline and nitric oxide in an oxidoreductase reaction. NO is capable of interacting with components of the bacterial replication machinery, nucleic acids, and metabolic enzymes[194].

The host is also adept at utilizing characteristics of the microenvironment to enhance ROS and RNI damage to pathogens. For example, by increasing the copper levels in the phagosome, it can enhance the generation of hydroxyl radicals in the phagosome to enhance bacterial killing[195]. Additionally, the microenvironment of the
32

autophagosome and autolysosome are amenable to the creation of radicals. The low pH accelerates the conversion of $\mathrm{O}_2^-$ to $\mathrm{H}_2\mathrm{O}_2$, an even more potent antibacterial agent. It also facilitates the conversion of neutralized $\mathrm{NO}_2^-$ back to the more damaging radical NO. As expected, mice deficient in NOX or iNOS [196, 197] have an impaired ability to control infection compared to wildtype mice [198].

2.3.4 Antiviral Immunity

This section will be kept brief as it goes beyond the scope of this dissertation. The cell possesses multiple cell-autonomous antiviral activities as well. Two protein families have been shown to antagonize the virus early in its lifecycle. The interferon inducible transmembrane (IFITM) proteins are capable of blocking orthomyxoviridae, alphavirus, and flaviviruses from entering the host cytosol by inhibiting the release of the virus from the endocytic pathway [199]. The tripartite motif (TRIM) proteins have been shown to restrict retroviral entry by binding to the retroviral capsid which causes premature viral uncoating and subsequent degradation. Additionally, the cytosol possesses resting nuclease activity in the form of RNases. RNase L (RNL) is an interferon induces nuclease whose activity particularly favors uracil-rich regions, preferentially cleaving after UU or UA sequences [200, 201]. RNL indiscriminately destroys host and viral RNA, but this is much more damaging to an invading virus whose ability to replicate is dependent on maintaining intact RNA. The cleaved RNA products then feed forward to produce additional interferon by stimulating PRRs [202]. Finally, there are defense proteins like


tetherin which are able to block viral budding and prevents the spread of virus to neighboring cells. All told, there are multiple avenues in which IFN-inducible restriction factors inhibit the viral lifecycle.

2.4 Interferon Inducible GTPases

The interferon inducible GTPases are a family of immunity proteins that provide protection against a wide range of pathogens. There are at least 47 family members in mammals. Each GTPase can be segregated into one of 4 classes: the myxovirus resistance (MX), very large IFN-inducible GTPases (VLIGs), immunity-related GTPases (IRG), and the guanylate-binding proteins (GBPs). These dynamin like GTPases are strongly upregulated upon stimulation with type I (MX, IRGs, and GBPs) and type II (IRGs, GBPs) IFNs. Structurally, this family contains a highly conserved G domain that is responsible for catalytic activity as well as helical domains that are required for membrane tethering. The C-terminal region appears to be critical both protein-protein and protein-lipid interactions [203, 204]. This dissertation will primarily focus on the GTPases with known antimicrobial activity, the IRGs and GBPs.

2.4.1 Myxovirus resistance

Myxovirus resistance (MX) was the first of the family to be discovered in 1963 for its ability to interfere with influenza virus replication. The MX proteins are believed to antagonize the virus at early steps in the lifecycle. MX is believed to interact directly with viral components, such as the nucleocapsid and waylay the virus before it can
reach its intended destination for replication. MX self-oligomerization is essential for its antiviral activity, and mutations in the helical or GTPase domains completely abrogate its function [205, 206].

### 2.4.2 Very Large Inducible GTPases

The very large inducible GTPases (VLIGs) have not been well studied. The VLIGs are the largest known GTPases at nearly 300 kilodaltons. VLIGs first arise in vertebrates, but the number on VLIGs varies greatly between species and their function remains unspecified to date[207].

### 2.4.3 Immunity Related GTPases

The immunity related GTPases (IRG) are a class of 47 kilodalton proteins first reported in 1992. IRG proteins have been shown to protect against protozoan and bacterial infections [208]. There are 20 IRGs in mice that can be further classified by into two subfamilies bases on the biochemical structure of their G domains. The G domains of the IRGs contain 3 GTP binding motifs, the phosphate binding P-Loop, the DxxG region and the N/T K/Q xD motif. The IRGs are classified as GKS if the possess a GX4GKS P-Loop or GMS is they have a GX4GMS P-Loop sequence. The GKS and GMS subfamilies have been shown to have different cellular functions in response to infection [209, 210]. The GKS or “effector IRGs” have been shown to target directly to pathogens vacuoles, whereas the GMS or “guard IRGs” localize to host endomembranes and prevent GKS binding [211].
In order to associate with PVs, the GKS IRGs require GTP binding. GTP binding facilitates the oligomerization and stabilization of GKS proteins on target membranes. When in the GDP bound form, the GKS IRGs are mostly cytosolic, making only transient interactions with membranes. The GMS IRGs likely play a role in ensuring that the GKS proteins cannot aberrantly target host membranes by serving as guanine dissociation inhibitors preventing GTP binding and oligomerization. The effector IRGs have been shown to target *Chlamydia trachomatis* [212-214], *Encephalitozoon cuniculi*, and the parasite *Toxoplasma gondii* [215]. Importantly, these PVs are devoid of the GMS proteins due to their atypical cell invasion mechanisms, thereby marking the vacuole as missing-self and facilitating GKS binding. However, lack of GMS proteins alone is not sufficient to drive complete GKS recruitment. In mice deficient for the GMS guard proteins, the GKS proteins aggregate in the cytosol and on select host endomembranes, such as lipid droplets, but not all host endomembranes [211]. Therefore other host membranes either contain additional, as of yet undiscovered guard proteins, or lipid droplets and PVs contain a positive selection marker. Identification of this positive signal is an active area of research.

### 2.4.4 Guanylate Binding Proteins

The guanylate binding proteins (GBPs) are a family of 65-73 kDa GTPases that were first discovered in 1979 [216]. There are 7 GBPs in humans (hGBPs) and 11 GBPs in mice (mGBPs). The hGBPs and mGBPs share significant homology, especially in the
conserved G domains. The hGBPs are all on one gene cluster, while the mGBPs are distributed in two genomic islands on chromosomes 3 and 5. The GBPs are strongly induced by IFNγ, and can also be stimulated by type I IFNs, Interleukin-1β (IL-1β), and tumor necrosis factor-α (TNFα)[217, 218].

GBPs, like all the IFN inducible GTPases, are classified as dynamin like proteins. GBPs are made up of a G domain, an assembly domain, a GTPase binding domain, and a regulatory C terminal α-helical region. Despite being dynamin like, the GBPs have poor homology with classic dynamin proteins in the G domain. The GBPs maintain a conserved G domain for GTP binding, but instead of the canonical N/T K/Q xD motif, the GBPs have a TLRD sequence which regulates its interaction with guanine [217, 219]. The GBPs are unique in their ability to bind GMP, GDP, and GTP with equal affinity. The GBPs are also unusual in their ability to hydrolyze GTP to GDP and then to GMP in sequential hydrolysis reactions. This hydrolysis is dependent on GBP oligomerization [220]. Several family members in mice and humans also contain a CaaX farnesylation motif in the C terminus which appears to be important for interactions with lipid membranes.

Despite being discovered nearly 40 years ago, the function of GBPs has only recently begun to be elucidated. The first evidence that the GBPs were involved in innate immunity was their ability to restrict vesicular stomatitis virus (VSV) and encephalomyocarditis virus (ECMV) infection in vitro [221]. While the GBPs do appear
to have some antiviral activity, they restrict a fairly shallow pool of viruses. In contrast, the GBPs have been shown to be critically important for the control bacterial and protozoan infections such as L. monocytogenes and T. gondii [222]. These result spurred additional research to try to determine the role of GBPs in innate immunity.

GBPs have been shown to have a role in several cellular processes, including immune signaling, proliferation, and innate immunity. The GBPs are part of a robust antimicrobial immune response that is effective against a wide range of pathogens. GBPs have been shown to target to PVs as well as cytosolic bacteria [223]. This targeting phenotype is depended on GTP binding and catalytic activity to properly assemble and recruit additional defense factors. Knockdown of individual GBPs has a variable effect on host defense, depending on the pathogen tested and cell line used. However, in studies where the entire murine gene cluster on chromosome 3 (mGBPChr3-/-), including GBPs 1,2,3,5, and 7, was removed, the mice had dramatically increased mortality in response to Toxoplasma gondii challenge[224]. GBPs functionality may be reduced when they are unable to heteroligomerize or some GBPs may serve a regulatory function. GBPs also have synergistic effects with other defense pathways. For example, mGBPChr3-/- mice have a partial reduction in GKS IRG targeting to T. gondii and C. trachomatis PVs [224-226]. Additionally, mutations in the guard IRGs causes GBP aggregates to form in the cytoplasm [227]. Furthermore, GBPs have been shown to interact with components of the autophagy clearance pathway including p62 and
ubiquitin. Finally, the GBPs have been shown to be important for inflammasome activation [228]. GBPs enhance the activation of the canonical NLRP3 and the noncanonical Caspase-11 inflammasomes by mechanisms that have still not been fully elucidated [229, 230].

The defense activity of the GBPs depends on their recruitment to the pathogen. However, the method by which GBPs recognize and target to pathogens has remained a mystery. It was this problem that formed the basic question for my doctoral work. In this dissertation, I will show that there is a host danger sensing pathway involved in the recruitment of GBPs to the PV.

2.5 Introduction to Galectins

The galectins family of proteins is defined by an evolutionarily conserved carbohydrate recognition domain (CRD) and strong $\beta$-galactoside binding capabilities. To date, there have been 15 galectins identified in mammals, which can be segregated into three major categories; prototype, tandem-repeat, and chimeric [231, 232]. The prototype galectins contain one CRD domain and are predominantly monomeric or dimeric. The tandem repeat galectins have two CRD domains, one N terminal and one C terminal, which are connected by a flexible linker region. Galectin 3 (Gal3) is the only member of the chimera type, which has one C terminal CRD domain and an N terminal regulatory domain [233, 234]. Gal3 is a 30kDa soluble $\beta$-galactoside binding protein. Gal3 is unusual in its ability to oligomerize through the self-assembly of the N-terminal
regulatory domain. Once oligomerized, the Gal3 complex has multiple CRD domains in close proximity which aids in carbohydrate recognition and binding. The oligomerization of Gal3 can be regulated by phosphorylation of the N-terminal regulatory domain [235].

The ability of all galectins to bind β-galactoside-containing glycans relies upon a collection of key conserved amino acids within each CRD. Interestingly, the affinity for these sugars is highly varied between family members. For example, all galectins recognize polylactosamine, but galectin 1 preferentially binds the terminal sugar modification while galectin 3 preferentially recognizes internal sugar modifications [236]. This variability in glycan binding preference for various presentations of simple sugars provides evidence for the complementary activities of each family member [237]. Galectins are primarily cytosolic, especially in immune cells, fibroblasts and endothelial cells [238]. There are however multiple galectins found on the plasma membrane. Interestingly, galectins do not have signal sequences or hydrophobic transmembrane domains that would allow for classical secretion. Therefore, the secretion of galectins is thought to be atypical and utilize the leaderless secretory pathway. This pathway is also responsible for secretion of fibroblast growth factor and macrophage migration inhibitory factor, but the system has not been well elucidated [239-242]. The galectins are expressed in the vast majority of tissues, and have roles in numerous cellular processes. These processes include: cellular proliferation, cellular adhesion, apoptosis, and
immunity. The following sections will focus on the role of galectins in innate immunity[243].

### 2.5.1 Galectins in Innate Immunity

Galectins have been shown to be important in the innate immune response. Expression of several galectins, including galectin 1, 3, and 9 are strongly upregulated during inflammation [244, 245]. Gal3 has the capability to recognize LPS from several gram negative bacteria, including *Salmonella minnesota*, *Neisseria gonorrhoeae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* [246, 247]. Galectins can also detect pathogen invasion via indirect methods. [248, 249] For many pathogens, endosomal escape is required for the pathogen to complete its lifecycle. These pathogens must enter cytosol through their PVs [250, 251]. This pathogen escape results in the breakdown of the endosomal integrity and leads to the exposure of the luminal side of the vacuole. Galectin 8 then recognizes the exposed glycans and triggers the activation of autophagy with the help of an adaptor molecule NDP52 [252]. The exposed glycans from the luminal surface of the endosomes are a DAMP which is recognized by the host [253]. This broad danger sensing mechanism has been adapted by the host for the recruitment of additional host defense proteins.
3.0 Galectin 3 recruits GBP to the PCV

Note: This section contains published work from the publication; Feeley et al, Galectin-3 directs antimicrobial guanylate binding proteins to vacuoles furnished with bacterial secretion systems. Contributions from co-authors will be explicitly noted in the text.

3.1 Introduction

Most pathogenic bacteria remodel the phagosomes that they occupy into vesicles defective for phagolysosomal fusion [254]. In addition to blocking lysosomal maturation, pathogens commonly manipulate their surrounding vacuolar compartments in a number of ways to assure nutrient acquisition. To customize phagosomes into beneficial PVs, intravacuolar bacteria use secretion systems to release effector proteins from the bacterial cytoplasm into the host cell cytosol [255]. These secretion systems are essential for bacterial virulence. Some of the structural components of bacterial secretion systems are highly conserved and shared among many distinct bacterial species. This conservation is exploited by the innate immune system, which evolved cytosolic PRRs that can detect conserved components such as the basal body rod component or the needle protein of the T3SS [67]. Distinct molecular features of these conserved T3SS structural proteins constitute PAMPs that are directly recognized by host PRRs. In addition to the direct recognition of structural components of bacterial secretion systems, the host is also able to detect the presence of secretion system indirectly by
sensing cellular perturbations caused by secreted bacterial effector proteins. These microbe-directed perturbations are often shared among different types of infections and thus constitute conserved “pattern of pathogenesis” [256]. For example, some bacterial effectors modify the lipid composition of PVs. As a potential consequence of these lipid modifications, PVs are more likely to rupture when exposed to cytoskeleton motor-dependent mechanical forces [257, 258]. Although pathogens such as *Legionella pneumophila* or *Salmonella enterica* secrete effectors to counteract destabilizing effects of membrane manipulations to maintain the PV as a replicative niche [257, 259, 260], subsets of *Legionella*-containing vacuoles (LCVs) or *Salmonella*-containing vacuoles (SCVs) nonetheless fail to maintain their membrane integrity[248, 257].

Vacuolar instability is recognized by host β-gal–binding proteins of the Galectin family[261]. Extracellular galectins can bind directly to bacteria, mediate bacteriostatic or bactericidal effects, and modify inflammatory signaling events. More recently, intracellular Galectin-3 was characterized as a sensor of vacuolar rupture that occurs when bacteria such as *Shigella flexneri* actively enter the host cell cytosol [248]. Gal3 also detects LCVs and SCVs, and Galectin-8 and -9 were also shown to associate with SCVs [228, 252, 257]. The association of Galectins with SCVs is largely dependent on host glycans restricted to the vacuolar lumen, indicating that SCVs display membrane lesions that can be recognized by the host. Galectin-8 was shown to interact with the autophagy adaptor protein NDP52 and to promote the delivery of NDP52 to SCVs, which results in
diminished intracellular bacterial survival [252]. The functional consequences of recruiting Galectin-9 or Gal3 to SCVs or other PVs have remained unexplored.

Whereas galectins are constitutively expressed in macrophages and other cell types [261], many cell-intrinsic host defense programs are activated by proinflammatory cytokines and IFNs in particular [262, 263]. IFNs robustly induce the expression of host resistance factors, including IRGs and GBPs [264, 265]. IRGs and GBPs facilitate cell-intrinsic immunity in vitro and host resistance in vivo to a broad spectrum of intracellular pathogens. In infected cells, IRGs and GBPs colocalize with intracellular bacterial pathogens residing in the host cell cytosol or within PVs [266], and several studies have suggested functional interactions between members of the IRG and GBP families [211, 224, 227, 266-268]. Although IRGs and GBPs can combat intracellular infections cooperatively, mounting evidence suggests that these two protein families also execute unique cellular functions independent of one another. For example, IRGs facilitate the delivery of ubiquitin E3 ligases to *Toxoplasma*- and *Chlamydia*-containing vacuoles independent of GBPs. GBPs, on the contrary, were reported to control the deposition of the NADPH oxidase NOX2 at *Mycobacterium*-containing phagosomes independent of IRGs [223]. These studies broadly characterize IRGs and GBPs as escorts for distinct antimicrobial factors en route to PVs. However, the mechanisms by which IRGs and GBPs detect and bind to PVs remain poorly characterized [269].
We previously demonstrated that IRGs are essential for targeting GBPs to *Toxoplasma*- and *Chlamydia*-containing vacuoles [211, 267]. In this study, we describe an IRG-independent pathway by which GBPs are delivered to PVs. We show that Gal3 and murine GBP2 (mGBP2) form protein complexes, which associate with vacuoles harboring secretion system-competent *L. pneumophila* or *Yersinia pseudotuberculosis*. We demonstrate that the secretion of translocon proteins by *Y. pseudotuberculosis* is critical for the recruitment of Gal3 and mGBP2 to *Yersinia* containing vacuoles (YCVs). We further describe a functional role for Gal3 in the delivery of mGBP2 and mGBP1 to YCVs and LCVs. Our study thus characterizes bacterial secretion apparatuses as PV-associated patterns of pathogenesis that trigger Gal3 dependent recruitment of antimicrobial GBPs.

### 3.2 Results

#### 3.2.1 GBPs Associate with LCVs Independent of ATG and IRG Proteins

Members of the GBP and IRG families of IFN-inducible GTPases have been shown to colocalize with several types of PVs [264]. To determine whether this extends to LCVs, we first monitored the subcellular localization of ectopically expressed mGBP1, mGBP2, and mGBP7 GFP-fusion proteins in murine RAW 264.7 macrophages. In these and all subsequent experiments, we used flagellin-deficient (ΔflaA) *L. pneumophila* strains to prevent the flagellin-dependent activation of the NAIP5-NLRC4 inflammasome and the subsequent pyroptotic cell death [132, 133]. We observed that ectopically expressed mGBPs decorated LCVs in RAW 264.7 cells (Figure 1), as did
endogenous mGBP2 in IFNγ primed bone marrow-derived macrophages. Recruitment of mGBP2 to LCVs was detected as early as 20 min postinfection, albeit at low frequency.
Figure 1: GBPs associate with LCVs independent of IRGs and ATG5.

Confocal images of dsRED+ L. pneumophila (L.p.)-infected RAW 264.7 macrophages expressing GFP-mGBP1, GFP-mGBP2, or GFP-mGBP7 (A) and confocal images of IFNγ primed, L. pneumophila-infected BMDMs stained with anti-mGBP2 antibody at 2 hpi (B) are shown. Percentages of mGBP2+ LCVs in IFNγ primed BMDMs were quantified in 20-min intervals for the first 2 hpi (C). Frequencies of Irgb10+ and Irga6+ LCVs in IFNγ primed WT vs. Irgm1−/−Irgm3−/− BMDMs at 2 hpi are shown (D). Frequencies of mGBP2+ LCVs in IFNγ primed WT vs. Irgm1−/−Irgm3−/− (E) or ATG5−/− BMDMs (F) are shown. Error bars represent SDs. At least three independent experiments were performed. Statistical analysis was performed by unpaired two-tailed Student’s t test (***P < 0.005; n.s., not significant). (Magnification, 63×) Credit: A, D, E, F: Pilla-Moffett, D.
The frequency of mGBP2-positive LCVs steadily increased over the course of the first 2 h of infection (Fig. 1C). LCVs also attracted the IRG proteins Irgb10 and Irgb6 (Fig. 1D), thus mimicking observations made for PVs formed by the bacterium *Chlamydia trachomatis* and the protozoan *Toxoplasma gondii* [270, 271]. We previously reported that the regulatory IRG proteins Irgm1 and Irgm3 are required for delivery of Irgb10 and Irgb6 to *Chlamydia* and *Toxoplasma* PVs [211]. Similar to these previous observations, we found that the recruitment of Irgb10 and Irgb6 to LCVs was greatly diminished in Irgm1−/−Irgm3−/− BMDMs (Fig. 1D), suggesting a shared regulatory role for Irgm1 and Irgm3 proteins in the targeting of IRG proteins to LCVs and *Chlamydia* and *Toxoplasma* PVs.

Irgm1 and Irgm3 not only control the subcellular localization of other IRGs but also are essential for the delivery of GBPs to *Chlamydia* and *Toxoplasma* PVs in mouse cells. In Irgm1+/-Irgm3+/- BMDMs, mGBP2 mislocalizes to vesicular structures, as independently confirmed in the present study (Figure 2). However, despite this altered staining pattern, mGBP2 associated with LCVs at a comparable frequency in WT and Irgm1−/−Irgm3−/− BMDMs (Fig. 1E and Fig. 2A). We then proceeded to test additional host factors known to be required for the targeting of GBPs to *Chlamydia* and *Toxoplasma* PVs for their role in the recruitment of mGBP2 to LCVs. One such host factor is the protein ATG5 [222, 225, 272, 273], which participates in autophagosome formation and the
lipidation of ubiquitin-like ATG8 proteins, including the autophagosomal marker LC3 [274].
Figure 2: Recruitment of mGBP2 to LCVs is independent of autophagy and Irgm1/Irgm3 proteins.

WT, ATG5−/−, ATG7−/−, Beclin−/− and Irgm1−/−Irgm3−/− BMMs were infected with dsRED+ L. pneumophila (L.p.) and stained with anti-mGBP2 (green). Confocal images for infected WT, ATG5−/−, and Irgm1−/−Irgm3−/− BMMs are shown in A. Percentages of mGBP2+ LCVs over the course of 2 h of infection in WT, ATG7−/−, and Beclin−/− BMMs are shown in B. At least 100 infected cells were counted per condition and experiment. Two independent experiments were performed. Error bars represent SDs. (Magnification, 63×.)
Although some mGBP2 protein mislocalized to ring-like structures in Atg5−/− BMDMs (Fig. 2A), we found that ATG5 was not required for the recruitment of mGBP2 to LCVs (Fig. 1F). Additionally, we observed that mGBP2 targeting to LCVs was independent of the autophagy proteins Beclin1 and Atg7 (Fig. 2B). Together, these data indicate that mGBP2 recruitment to LCVs is mechanistically distinct from its targeting to *Chlamydia* and *Toxoplasma* PVs.

### 3.2.2 The Presence of Bacterial Secretion Systems Dictates the Recruitment of mGBP2 to LCVs and YCVs

To characterize this newly described IRG- and ATG-independent GBP delivery system, we asked what specific properties of LCVs were recognized by the cell-autonomous immune system to assure the specific targeting of mGBP2 to LCVs. The most simplistic model posited that mGBP2 was indiscriminately recruited to the phagocytic cup or early phagosomes, and as such would target to any internalized cargo. To test this model, we fed live and dead *L. pneumophila* bacteria or latex beads to IFNγ primed BMDMs. At 2 h postinfection (hpi), mGBP2 frequently decorated LCVs formed by live bacteria but remained absent from phagosomes containing formalin-fixed or heat-inactivated bacteria or latex beads (Fig.3A). These data demonstrated that mGBP2 was exclusively recruited to vacuoles containing live bacteria. We therefore hypothesized that vacuoles containing live *L. pneumophila* featured a unique PAMP or pattern of pathogenesis that is detected by the host and prompts the deposition of
Figure 3: mGBP2 targets vacuoles containing live bacteria expressing bacterial secretion systems.

Live or dead dsRED+ L. pneumophila (L.p.) or latex beads were fed to IFN-γ–primed BMDMs for 2 h. The percentages of mGBP2-positive phagocytosed bacteria or beads are depicted (A). IFN-γ–primed BMDMs were infected with GFP+ L. pneumophila (B), GFP+ Y. pseudotuberculosis (Y.p.) (C), or mCherry+ Y. pseudotuberculosis (D) of the indicated genotypes. Representative images and percentages of mGBP2+ PVs at 2 hpi are shown. A minimum of 200 infected cells was analyzed per condition and experiment. At least three independent experiments were performed. Error bars represent SDs. Statistical analysis was performed by one-way ANOVA with Tukey’s multiple comparison test (A) and unpaired two-tailed Student’s t test (B–D; *P < 0.05 and ***P < 0.005). (Magnification, 63×)
mGBP2 at LCVs. Common to many bacterial pathogens is the use of bacterial secretion systems to deliver virulence factors across eukaryotic membranes. We therefore hypothesized that the presence of bacterial secretion systems at PVs could constitute a pattern recognizable by host GBPs. *L. pneumophila* extensively remodels its surrounding vacuole through the secretion of bacterial effector proteins by the Dot/Icm (T4SS), a process that requires the bacterial scaffolding protein dotA. We found that vacuoles containing dotA-deficient bacteria remained devoid of mGBP2 (Fig. 3B). In macrophages coinfected with ΔdotA and WT *L. pneumophila*, mGBP2 exclusively recruited to WT LCVs (Fig. 4). These data demonstrated that the host recognizes the presence of the Dot/Icm T4SS in a phagosome-intrinsic manner, resulting in the deposition of mGBP2 at WT LCVs. We reasoned that the host could detect PV-associated bacterial secretion systems by two distinct mechanisms: the host could sense the presence of the bacterial secretion apparatus or detect the activity of secreted bacterial effector proteins. To distinguish between these two mechanisms, we tested whether the apparatus of a second bacterial secretion system, the T3SS of *Y. pseudotuberculosis*, was necessary and sufficient to trigger mGBP2 delivery to vacuoles. The T3SS of *Y. pseudotuberculosis* secretes a small number of effector proteins, which includes the antiphagocytic factor YopE [275]. Therefore, WT *Y. pseudotuberculosis* can block
Figure 4: Host recognizes the presence of the Dot/Icm T4SS in a phagosome-intrinsic manner.

WT BMDMs were asynchronously coinfected with dsRED+ WT and GFP+ ΔdotA L. pneumophila at a 1:1 ratio. Colocalization of mGBP2 with LCVs was assessed by immunofluorescence at 2 hpi, and representative confocal images are shown in A. Per experiment, a minimum of 100 dually infected (WT + ΔdotA) BMDMs were assessed for mGBP2 colocalization with LCVs (B). Data are from two independent experiments. Error bars represent SDs. **P < 0.005. (Magnification, 63×)
phagocytosis by macrophages. To allow macrophages to ingest *Y. pseudotuberculosis* and to test whether secreted effectors are required for the recruitment of mGBP2 to PVs, we exposed IFNγ primed BMDMs to the “effectorless” *Y. pseudotuberculosis* ΔHOJMEK mutant strain[276], which lacks all known translocated effector proteins including YopE. At 2 hpi, approximately 15% of all YCVs formed by ΔHOJMEK stained mGBP2-positive (Fig. 3C), demonstrating that the secretion of T3SS effector proteins is not necessary to trigger mGBP2 recruitment to YCVs. We next asked whether the presence of the bacterial secretion system itself could be detected by GBPs. The T3SS system consists of structural proteins forming the base, the inner rod, and the needle. This part of the apparatus enables Gram-negative bacteria to secrete effectors across the inner and outer membranes of the bacterial cell wall. The delivery of effectors into the host cell cytosol by extracellular or vacuolar pathogens requires an additional bacterial translocon complex, which is inserted into host cell membranes [45]. We hypothesized that GBPs could sense the insertion of such a bacterial pore complex into PV membranes. In *Y. pseudotuberculosis*, the T3SS translocon complex is made of the two proteins YopB and YopD. To test our hypothesis that the host detects the YopB/YopD translocon complex, we monitored recruitment of mGBP2 to vacuoles containing the effectorless *Y. pseudotuberculosis* strain with an additional deletion in yopD (ΔDHOJMEK). Deletion of yopD largely abrogated the delivery of mGBP2 to YCV (Fig. 3C), indicating that the
presence of the YopB/YopD translocon complex is necessary for the delivery of mGBP2 to YCVs.

The secretion of Yop proteins is tightly controlled by the effector YopK. YopK-deficient strains release more translocon proteins than YopK-competent strains [68, 72, 277]. We therefore hypothesized that YopK would be able to block mGBP2 recruitment to YCVs, if mGBP2 was indeed able to detect the presence of translocon proteins in YCV membranes. To test our hypothesis, we compared mGBP2 recruitment to YCVs occupied by ΔEJ or ΔEJK, respectively. We used strains with a ΔEJ genetic background to permit phagocytosis and to avoid YopJ-mediated cell death [278, 279]. We found that vacuoles harboring the YopK deficient strain ΔEJK were decorated with mGBP2 at a higher frequency than the coisogenic control strain ΔEJ (Fig. 3D), demonstrating that YopK can protect YCVs against immune detection by mGBP2. Collectively, these data indicated that the host delivers mGBP2 to vacuolar membranes that contain components of bacterial secretion systems.

### 3.2.3 mGBP2 Colocalizes with Galectin-3 at Vacuoles Containing Bacteria Expressing Functional T3SS or T4SS

Several cytosolically localized members of the Galectin protein family were shown to associate with PVs formed by S. enterica or *L. pneumophila*. Sporadic loss of membrane integrity in PVs is thought to be the underlying reason for the association of Galectins with PVs, but the molecular cause for the inherent instability of PV membranes remains enigmatic. We asked whether the presence of bacterial secretion
Figure 5: Galectin-3 colocalizes with dotA-expressing L. pneumophila (L.p.) and YopB-/YopD-hypersecreting Y. pseudotuberculosis. (Y.p.)

YFP-Gal3–expressing iBMDMs were infected with dsRED+ L. pneumophila (A) or mCherry+ Y. pseudotuberculosis (B) of the indicated genotypes. The percentage of Gal3+ PVs at 2 hpi is depicted. A minimum of 200 infected cells were quantified per condition and experiment. At least three independent experiments were performed. Error bars represent SDs. Statistical analysis was performed by two tailed Student’s t test (**P < 0.01 and ***P < 0.005). (Magnification, 63×.)
systems could contribute to PV instability. To test this hypothesis, we monitored the subcellular localization of ectopically expressed YFP-Galectin-3 fusion protein in IFNγ primed, immortalized BMDMs (iBMDMs). As reported previously [228, 280], we observed that a sizeable percentage of WT LCVs attracted Gal3 (Fig. 5A). We found that Gale3 failed to colocalize with vacuoles containing the ΔdotA mutant, suggesting that the presence of the Dot/Icm T4SS apparatus contributes to diminished LCV integrity. The relative instability of WT LCVs compared with ΔdotA vacuoles could result from the effects of vacuolar remodeling by secreted effector proteins or the insertion of a bacterial secretion system into LCV membranes. Distinguishing between these two competing models is difficult, as L. pneumophila secretes hundreds of effector proteins and an effectorless L. pneumophila mutant strain is unavailable. We therefore pursued an alternative approach by assessing the colocalization of Gal3 with vacuoles containing ΔEJK or ΔEJ mutant Y. pseudotuberculosis strains, which differ in their secretion and assembly of the translocon complex [277]. We observed a substantial increase in Galectin-3–positive vacuoles containing the ΔEJK strain, which secretes excess amounts of the pore-forming proteins YopB and YopD. These results suggested that the insertion of translocon proteins into YCV membranes is the underlying cause for vacuolar instability (Fig. 5B). Galectin-3 and mGBP2 associated with LCVs or YCVs at comparable frequencies (Figs. 3 and 4), suggesting that these two proteins target the same subset of LCVs and YCVs. In support of this hypothesis, we observed colocalization of mGBP2
and YFP–Galectin-3 at LCVs (Fig. 6A) and at YCVs (Fig. 6B) in IFN-γ–primed BMDMs. The majority of Gal3–decorated LCVs and YCVs also stained positive for mGBP2 and vice versa (Fig. 6 C and D). As expected, ΔdotA LCVs were devoid of detectable Galectin-3/mGBP2 costaining, and only a small percentage of ΔEJ vacuoles were decorated with either host protein (Fig. 6 C and D). Together, these data demonstrated that the presence of the *Legionella* T4SS or hypersecretion of *Yersinia* T3SS translocon proteins promote the concomitant recruitment of Gal3 and mGBP2 to PVs.

3.2.4 Mouse and Human GBPs Colocalize with Galectin-3 at Sterilely Damaged Host Vesicles

Galectins bind to glycosylated proteins residing extracellularly or on the luminal face of intracellular vesicles. Ruptured vesicles expose glycosylated proteins to the host cell cytosol and thereby recruit Galectins. Because mGBP2 and Gal3 colocalize at LCVs and YCVs (Fig. 6), we asked whether mGBP2 and Gal3 could also be corecruited to sterilely damaged vesicles. To test our hypothesis, we induced damaged vesicles by hypotonic shock. As expected, cells exposed to hypotonic shock conditions accumulated Gal3 puncta, which is indicative of the formation of damaged vesicles (Fig. 7A and Fig. 8). Similarly, we observed an accumulation of mGBP2 puncta in IFNγ primed BMDMs exposed to hypotonic shock (Fig. 7A and Fig. 8) and found that approximately 20% of all Gal3 puncta formed in these cells also stained positive for
Figure 6: Galectin-3 and mGBP2 colocalize at PVs.

YFP-Gal3–expressing iBMDMs were infected with dsRED+ WT L. pneumophila (L.p.) (A) or mCherry+ WT Y. pseudotuberculosis (Y.p.) (B) and stained for endogenous mGBP2 at 2 hpi. Representative colocalization analysis and line trace are shown. The percentage of LCVs (C) or YCVs (D) staining positive for Galectin-3 only, mGBP2 only, or dual-positive for Galectin-3 and mGBP2 are shown. A minimum of 100 infected cells were quantified per condition and experiment. At least three independent experiments were performed. Statistical analysis by two-tailed student’s t test for double-positive bacteria is shown (***P < 0.005). (Magnification, 63×.)
mGBP2. Next, we exposed mouse embryonic fibroblasts (MEFs) to calcium phosphate precipitates (CPPs) known to cause endosomal damage and to recruit Gal3. We observed that more than half of all CPP-damaged, Galectin-3–positive endosomes acquired mGBP2 in IFNγ primed MEFs (Fig. 7B). As a third method to steriley induce vesicular damage, we exposed IFNγ primed BMDMs to the lysosomotropic compound L-Leucyl-L-leucine methyl ester (LLOMe), which disrupts lysosomal membranes [281]. LLOMe treatment induced the formation of Gal3 positive structures, of which approximately half also stained positive for mGBP2 (Fig. 7C). Together, these data demonstrated that mGBP2 was recruited to multiple types of damaged vesicles. GBPs constitute relatively large protein families in rodents and primates, with 11 murine and 7 human family members. We next asked whether the ability of mGBP2 to detect damaged vesicles was conserved in any of the human GBP orthologs. To address this question, we individually expressed mCherryhGBP1-7 fusion proteins in human embryonic kidney (HEK) 293T cells that also expressed YFP–Galectin-3. We then induced vesicular damage by CPP or LLOMe treatment and found that hGBP1 colocalized with ∼60% of Gal3 puncta in CPP treated HEK 293T cells and 35% of Gal3 puncta in LLOMe-treated HEK 293T cells (Fig. 7D). With the exception of a few colocalization events between hGBP2 and Galectin-3, we failed to detect recruitment of any of the other ectopically expressed human GBP paralogs to
Figure 7: Galectin-3 and GBPs colocalize at sterilely damaged vesicles.
YFP-Gal3–expressing iBMDMs were exposed to hypotonic shock, and the formation of Gal3+ puncta and the frequency of their colocalization with mGBP2 are shown (A). Endosomal damage in MEFs expressing YFP-Gal3 was induced by CPP treatment, and the quantification of Gal3+ puncta and colocalization with mGBP2 are shown by treatment (B). YFP-Gal3–expressing iBMDMs were damaged with LLOMe, and the formation of Gal3+ puncta and colocalization with mGBP2 were quantified (C). HEK 293T cells expressing YFP-Gal3 and hGBP-mCherry fusion proteins were treated with CPP or LLOMe. The percentages of YFP-Gal3 puncta colocalizing with individual hGBP orthologs are shown (D). A minimum of 100 damaged cells were quantified per condition and experiment. HS, hypotonic shock. (Magnification, 63×.) **D:** In collaboration with Piro, A.
damaged vesicles in 293T cells (Fig. 7D). Collectively, these data showed that the
targeting of GBP5s to damaged vesicles is conserved from mice to humans.

### 3.2.5 Galectin-3 and mGBP2 Form Protein Complexes

Because Gal3 and mGBP2 colocalized at damaged vesicles and at PVs, we asked
whether these proteins would also physically interact. To address this question, we used
GFP-Trap immunoprecipitations (IPs) in IFNγ primed iBMDMs transduced with an
YFP–Galectin-3 expression construct. GFP-Trap beads efficiently immunoprecipitated
YFP–Galectin-3 and coimmunoprecipitated endogenous mGBP2 from LLOMe-treated
iBMDMs, and, to a lesser degree, from untreated iBMDMs (Fig. 9A). As expected, GFP-
Trap beads failed to coimmunoprecipitate endogenous mGBP2 in untransduced control
iBMDMs (Fig. 9A). As an independent approach, we performed proximity ligation
assays (PLAs) in YFP-Galectin-3–expressing MEFs. Using antibodies directed against
YFP and mGBP2, we detected a significant increase in PLA puncta in IFNγ primed cells
treated with CPP (Fig. 9B). Collectively, these data demonstrated that Galectin-3 and
mGBP2 form protein complexes in response to vesicular damage.

### 3.2.6 Galectin-3 Promotes the Recruitment of mGBP2 and p62 to PVs.

Because Galectin-3 and mGBP2 form protein complexes that localize to PVs, we
asked whether Galectin-3 or other members of the Galectin family could control the
recruitment of mGBP2 to PVs. To test this hypothesis, we first screened a set of
Figure 8: Hypotonic shock induces mGBP2 puncta.

Cells were treated for hypotonic shock (H.S.) or left untreated. Each dot represents the mean number of Gal3 (A) or mGBP2 (B) punctae per cell and per experiment. Representative images of IFN-γ-primed iBMDMs are shown. Average of combined data from four experiments ± SEM is depicted. At least 100 cells were quantified per condition and experiment. *P < 0.05. (Magnification, 40×.) Credit: Pilla-Moffet D.
Figure 9 Galectin-3 and mGBP2 form protein complexes.

iBMDMs transduced with YFP-Gal3 retrovirus or untransduced controls were damaged with LLOMe or left untreated, and the formation of GBP2/YFP-Gal3–containing protein complexes was assessed with by GFP-Trap IPs. Whole-cell lysates and unbound and IP fractions were immunoblotted against GBP2 and YFP (A). IFN-γ–primed and unprimed MEFs expressing YFP-Gal3 were treated with CPP or left untreated, and interaction was detected by PLA and quantified. A minimum of 15 fields were quantified per treatment. Representative images (B) and quantification (C) are shown. At least three independent experiments were performed. Statistical analysis was performed by two-way ANOVA with Tukey’s multiple comparison test (**P < 0.005). NT, nontreated. (Magnification, 63×.) Credit: Figure A: Pilla-Moffett, D.
YFP-Galectin expression constructs for colocalization with LCVs. We found that Galectin-8 and Galectin-9, in addition to Gal3, associated with LCVs in iBMDMs (Fig. 10A). To determine whether LCV-resident Galectins played a role in the delivery of mGBP2 to LCVs, we interfered with the expression of individual LCV-associated Galectins by using shRNAs. We observed a reduced percentage of mGBP2-positive LCVs in IFNγ primed iBMDMs that expressed Gal3 shRNAs or, albeit to a lesser extent, Galectin-8 shRNAs (Fig. 10B). To independently interrogate the function of Gal3 in directing mGBP2 to PVs, we monitored the subcellular localization of mGBP2 in BMDM derived from Galectin-3–deficient (Gal3−/−) mice. As expected, Gal3−/− and WT BMDMs expressed comparable levels mGBP2 protein under IFN-γ priming conditions (Fig. 11). Nonetheless, recruitment of endogenous mGBP2 to LCVs (Fig. 10C) or YCVs (Fig. 10D) was significantly reduced in IFNγ primed Gal3−/− relative to WT BMDM. Ectopically expressed GFP-mGBP1 or GFP-mGBP2 also displayed reduced colocalization with YCVs in Gal3−/− iBMDM (Fig. 10E). Because Galectin-8–mediated recruitment of NDP52 to SCVs is dependent on the recognition of intravacuolar host glycans, we asked whether recruitment of mGBP2 to PVs was regulated in a similar fashion. To answer this question, we treated RAW 264.7 cells with the O-glycosylation inhibitor benzyl-GalNAc (BGN) before infection with Y. pseudotuberculosis. We found that BGN treatment
Figure 10: Galectin-3 promotes targeting of mGBP2 and p62 to LCVs and YCVs.
WT iBMDM expressing individual Galectins as YFP-fusion proteins were infected with dsRED+ L. pneumophila. The percentages of Galectin+ LCVs were quantified at 2 hpi (A). WT iBMDMs were treated with shRNA against Galectin-3, -8, and -9, and the percentages of mGBP2+ LCVs were quantified at 2 hpi (B). WT and Gal3−/− BMDMs were infected with dsRED+ L. pneumophila (C) or mCherry+ ΔEJK Y. pseudotuberculosis (D), and the percentages of mGBP2+ PVs were quantified over time. Colocalization of GFPmGBP1 and GFP-mGBP2 with ΔEJK YCVs was monitored in WT and Gal3−/− iBMDMs at 2 hpi (E). RAW 264.7 cells were treated with 2 mM BGN or DMSO control for 3 d before infection with Y. pseudotuberculosis ΔEJK. Percentages of mGBP2+ YCVs were quantified at the indicated time points (F). BMDMs of the indicated genotypes were infected with ΔEJK, and YCV colocalization with p62 (G) or ubiquitin (H) was assessed at 2 hpi. A minimum of 100 infected cells were counted per experimental condition and experiment. At least three independent experiments were performed except for the experiment shown in A, which was repeated once. Error bars represent SDs. Statistical analysis was performed by two-way ANOVA (B, G, and H), two-tailed Student’s t test (C, D, and E), and one-way ANOVA with Tukey’s multiple comparison test (F; *P < 0.05, **P< 0.01, and ***P < 0.005).
Figure 11: Expression of mGBP2 protein is comparable between WT and Gal3−/− BMDMs.

Anti-mGBP2 and corresponding anti-actin immunoblots of protein lysates taken from IFN-γ–primed and unprimed WT, Gal3−/−, and GBPchr3−/− BMDMs are shown.

Figure 12: Inhibition of host O-glycosylation reduces targeting of mGBP2 to YCVs.

RAW 264.7 cells were treated with 2 mM BGN or DMSO control for 3 d before infection with Y. pseudotuberculosis ΔHOJMEK. Percentages of mGBP2+ YCVs were quantified at indicated time points. **P < 0.01.
significantly reduced the association of mGBP2 with ΔEJK (Fig. 10F) or ΔHOJMEK vacuoles (Fig. 12), suggesting that Gal3 mediated recruitment of mGBP2 to YCVs requires recognition of host glycans. We next monitored the localization of the mGBP1-interacting protein p62 to YCVs. We observed a decrease in the percentage of p62-positive YCVs in GBP-deficient (GBPchr3−/−) and, to a lesser degree, in Gal3−/− BMDMs (Fig. 10H). This decrease in p62 recruitment correlated with a similar decrease in the number of ubiquitin-decorated YCVs in GBPchr3−/− and Gal3−/− BMDMs (Fig. 10I), indicating that GBPs recruit the ubiquitin binding protein p62 to PVs directly or, alternatively, indirectly through the activation of a PV-targeted ubiquitination pathway. Because the number of ubiquitin-positive YCVs was only partially reduced in GBPchr3−/− BMDMs, we tested whether GBP-independent YCV ubiquitination pathways existed.

We previously demonstrated an essential role for the IRG proteins Irgm1 and Irgm3 in the ubiquitination of C. trachomatis PVs in mouse cells. Here, we found that the absence of Irgm1 and Irgm3 partially reduced the number of ubiquitin-decorated YCVs (Fig. 13), suggesting that IRGs and GBPs control parallel pathways of PV recognition and ubiquitination. In agreement with a model in which IRGs and Gal3/GBPs control parallel, functionally redundant pathways of YCV and LCV recognition, we found IFNγ primed Gal3−/− BMDMs to restrict bacterial growth, similar to WT BMDMs (Fig. 14). Together, our findings demonstrate that Gal3 promotes the recruitment of mGBP2 to
LCVs and YCVs in a host glycan-dependent manner as part of a complex network of PV recognition pathways.
Figure 13: Irgm1 and Irgm3 promote ubiquitination of YCVs in BMDMs.

WT and Irgm1−/−Irgm3−/− BMMs were infected with mCherry+ Y. pseudotuberculosis ΔEJK and stained with anti-mGBP2. Percentages of mGBP2+ YCVs at 2 hpi are shown.
Figure 14: Galectin-3 is nonessential for cell-autonomous immunity toward L. pneumophila and Y. pseudotuberculosis in IFN-γ-primed BMDMs.

WT and Gal3−/− BMDMs were primed with 100 U/mL IFN-γ overnight or left unprimed and were then infected with bioluminescent ΔflaA L. pneumophila. Luminescence as relative light units (RLU) is depicted over the course of 30 h of infection (A). Similarly, IFN-γ-primed WT and Gal3−/− BMDMs were infected with the indicated strains of Y. pseudotuberculosis at an MOI of 5, and bacterial burden was assessed by immunofluorescence microscopy at 8 hpi (B). Data are representative of at least two independent experiments.
3.3 Discussion

IFN-inducible GBPs provide cell-autonomous immunity to intracellular pathogens residing within customized PVs. Although it has been shown that GBPs translocate to PVs and kill PV-resident microbes, the mechanisms by which GBPs specifically identify and bind to PVs are largely unknown. Here, we demonstrate that vacuolar disruption mediated by bacterial secretion apparatuses provides a pattern of pathogenesis, which prompts the delivery of antimicrobial GBPs to PVs.

To combat infections with PV-resident pathogens, the host must be able to discriminate between “nonself” PV membranes and endomembranes. To do so, a subset of “membrane-surveying” PRRs are predicted to detect patterns that are unique to PV membranes. One such pattern is provided by the occasional compromise of PV membrane integrity. Although pathogens such as *S. enterica* and *Mycobacterium tuberculosis* are traditionally classified as vacuolar pathogens, a proportion of these bacteria exits PVs and replicates inside the host cell cytosol [282, 283]. The host cell can detect the breakdown of these PVs and activate host resistance mechanisms [284]. The rupture of *Mycobacterium*-containing vacuoles (MCVs) by the bacterial ESX-1 virulence system, for example, triggers the recruitment of the host ubiquitin E3 ligase parkin to MCVs, leading to MCV ubiquitination and subsequent targeting and degradation of bacteria within autophagosomes [285].
Although active egress from PVs is occasionally executed by vacuolar pathogens as a strategy to promote intracellular replication, loss of membrane integrity may also occur as an unintended consequence of PV manipulations by resident bacteria. Previous studies have demonstrated that PV membrane remodeling and manipulations of PV trafficking by secreted effector proteins can stabilize and destabilize PV membranes. The present study shows that hypersecretion of *Yersinia* YopB/YopD pore proteins is sufficient to recruit the vacuolar damage sensor Gal3 to YCVs independent of the secretion of bacterial effectors. Thus, our work suggests that the insertion of bacterial translocation pores into PV membranes is in itself a pattern of pathogenesis that can be detected by the innate immune system. The tight control over the secretion of bacterial pore complexes, as illustrated by the function of YopK in limiting the translocation of YopB and YopD, is therefore likely designed as a microbial stealth strategy to avoid immune detection [73, 286]. Loss of vacuolar integrity renders the luminal side of PVs accessible to the host cell cytosol. As a consequence of increased PV membrane permeability, cytosolic galectins bind to glycans confined to the PV interior. The disintegration of PVs attracts Galectin-3, -8, and -9, but only the functional consequences of Galectin-8 recruitment to PVs was previously reported [252]. Here, we describe a function for Galectins in regulating the delivery of GBPs to PVs. We demonstrate that Gal3 and mGBP2 form a protein complex and that Gal3 facilitates the targeting of mGBP2 to PVs. Galectin-8 appears to also promote the delivery of mGBP2 to PVs, albeit
less efficiently. Galectin-8–mediated delivery of mGBP2 to YCVs or LCVs likely explains the rare occurrence of mGBP2-positive but Galectin-3–negative PVs (Fig. 6). Similar to previous observations, our data suggest that host cells detect ruptured PVs as a result of the cytosolic exposure of intravacuolar host glycans. We demonstrate that inhibition of host O-glycosylation reduces the recruitment of mGBP2 to YCVs, but not in full. Therefore, N-glycosylated host proteins or bacterial PAMPs associated with PV membranes could provide additional signals that direct Galectins and GBPs to PVs. Galectin-mediated recognition of PVs is not the only pathway by which the host can deliver GBPs to PVs. We previously reported that IFNγ priming triggers the ubiquitination of *Chlamydia* and *Toxoplasma* PVs and the ubiquitin-dependent recruitment of GBPs to these PVs. The ubiquitination of *Chlamydia* and *Toxoplasma* PVs and the subsequent recruitment of GBPs to PVs requires the IFNγ inducible GTPases Irgm1 and Irgm3. In the current study, we show that the association of GBPs with LCVs is independent of Irgm1 and Irgm3, demonstrating that GBPs can be delivered to PVs by Irgm-dependent and -independent pathways. It is currently unknown whether these two pathways mediate the translocation of all or only subsets of GBPs to PVs, whether additional GBP targeting pathways exist, and if and how pathogens interfere with Galectin-dependent attacks on PVs. Finally, our study reveals an unexpected role for GBPs in mobilizing ubiquitin to PVs. GBPs are dispensable for the ubiquitination of *Chlamydia* or *Toxoplasma* PVs in mouse cells, a process that instead requires Irgm1 and
Irgm3. In human cells, on the contrary, ubiquitination of *Chlamydia* PVs is human IRGM-independent. Here, we show that Irgm-dependent and GBP-dependent pathways target ubiquitin to YCVs inside IFNγ primed mouse macrophages. Whether these two pathways are independent from one another or ultimately converge will need to be examined in the future. The possibility of functional redundancy between IRG- and GBP-mediated cell-autonomous immunity directed at PVs may explain why GBP^{chr3−/−} and Gal3−/− BMDMs restrict intracellular growth of *L. pneumophila* or *Y. pseudotuberculosis*, similar to WT BMDMs (Fig. 13). Alternatively, Galectin-3– and GBP-dependent PV ubiquitination may play regulatory roles, for example, in modulating cellular signaling that is initiated at PVs. Although a detailed understanding of the molecular and cellular activities of PV-resident GBPs remains elusive, the present study provides a vastly improved understanding of the mechanism by which GBPs identify and dock to PVs and thereby substantially advances the emerging field of GBP biology.

3.4 Materials and Methods

3.4.1 Cell Culture, Virus Production, Transduction, and Ectopic Gene Expression

BMDMs and MEFs were derived from the indicated mouse lines. WT (C57/BL6j) and Gal3−/− [287] mice were purchased from Jackson Laboratories. GBP^{chr3−/−}, Irgm1−/−Irgm3−/−, LysM-Cre+Atg5flox/flox, LysM-Cre+Atg7flox/flox, and LysM-Cre+Beclinflox/flox mice were previously described [176, 224, 262]. BMDMs were
isolated from mouse femurs as described previously [228]. Briefly, mouse bone marrow cells were cultured in BMDM media [RPMI 1640 + 20% (vol/vol) FBS + 12% (vol/vol) macrophage colony stimulating factor-conditioned media]. Immortalized iBMDMs were generated by culturing bone marrow cells in BMDM media and the presence of J2 virus as described previously [228]. iBMDMs were grown for at least five passages after final virus treatment before experiments. MEFs were generated and cultured as described previously[270]. Raw 264.7 and HEK 293T cells were obtained from the American Tissue Culture Collection (ATCC) and cultured according to ATCC recommendations. To deplete host glycans, Raw 264.7 cells were cultured for 3 d in the presence of 2 mM BGN. At 12 h before infection, cells were plated on poly-lysine–coated glass coverslips and primed with IFN-γ. Viral particles were produced in HEK 293T cells, and transduction was performed as described previously[225]. At 48 h post transduction, cells were treated with puromycin or Blasticidin S (Invivogen), as appropriate for selection. The pLK0.1 lentiviral shRNA vectors TRCN0000301479, TRCN0000301480, and TRCN0000301477 (Gal3); TRCN0000066415, TRCN0000066416, and TRCN0000066417 (Gal8); and TRCN0000288518 and TRCN0000288440 (Gal9) were used to interfere with gene expression. More information on the constructs is available through the Broad Institute Web site (portals.broadinstitute.org/gpp/public/clone/search). M6P plasmids were used to produce recombinant murine leukemia virus (MLV) for the expression of YFP-tagged
Galectin proteins in mammalian cells, as described previously [252]. For increased transduction efficiency in iBMDMs, MLV particles were pseudotyped with vesicular stomatitis virus G protein. GFP-Gbp1, GFP-Gbp2, and GFP-Gbp7 were cloned in murine stem cell virus (MSCV) expression vectors by using standard procedures. MSCV transduction was performed as described previously [228]. All seven human GBP paralogs were cloned into the expression vector pmCherry-C1 (Clontech) to generate fusion proteins with N-terminal mCherry tags. Constructs were transfected into HEK 293 cells to monitor subcellular localization of individual hGBP fusion proteins.

3.4.2 Bacterial Strains and Infections

All L. pneumophila experiments were conducted by using a flagellin-deficient LP01 strain (ΔflaA) [133] (annotated as WT) or a coisogenic ΔflaAΔdotA strain (annotated as ΔdotA). Bacteria were grown in N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) buffered yeast extract broth at 37 °C overnight to reach postexponential phase. Infections were performed by using bacteria harvested from broth culture at an OD600 of 3.4–4. BMDMs were infected at a multiplicity of infection (MOI) of 1. WT and ΔdotA coinfection experiments were not conducted at 4 °C, but at room temperature to render infections asynchronous. Bioluminescent L. pneumophila strains and growth assays were conducted as described previously [288]. Luciferase readings were taken by using a Perkin–Elmer EnSpire plate reader. Y. pseudotuberculosis infections were carried out as follows. Bacteria were grown in 2× yeast extract tryptone broth overnight.
at 26 °C. Bacteria were then diluted into fresh media and supplemented with 20 mM sodium oxalate and 20 mM MgCl2 for 1 h at 26 °C, followed by 2 h at 37 °C, before infection. Cells were infected with an MOI of 1 (ΔEJ, ΔEJK) or 0.5 (ΔHOJMEK, ΔDHOJMEK). Where indicated, cells were primed with murine IFN-γ (Millipore) at 100 U/mL overnight (12–16 h) before infection. All Y. pseudotuberculosis strains are coisogenic to IP2666. The strains ΔEJ, ΔEJK, and ΔHOJMEK were previously described (51, 52). To generate ΔDHOJMEK, an in-frame deletion in YopD was introduced in a ΔHOJMEK background. Strains were transformed with the GFP expression plasmid pFPV25.1 (Addgene) and mCherry expression plasmid pSS128, which constitutively expresses mCherry (generated and provided by the laboratory of Sunny Shin, University of Pennsylvania, Philadelphia).

3.4.3 Sterile Vesicle Damage

Endosomal damage by CPP was performed essentially as described before (45). Briefly, cells were plated on poly-D-lysine–treated coverslips. CPPs were prepared with equal volumes of CaCl2 (256 mM) and buffer A (50 mM Hepes, 3 mM Na2HPO4, pH 7.05). CPPs were added dropwise to cells at 20% vol/vol and incubated for 4 h. Cells were fixed for 10 min in 4% (vol/vol) paraformaldehyde (PFA) and processed according to the standard immunofluorescence protocol. Hypotonic shock experiments were performed as described previously [252]. Briefly, cells were incubated for 10 min in a solution of 10% (vol/vol) PEG 1,000 and 0.5 M sucrose, followed by two washes in PBS
solution. They were then incubated in 60% (vol/vol) PBS solution in water for 3 min and returned to BMDM media for 20 min. Cells were then fixed for 10 min in 4% (wt/vol) PFA and processed for immunofluorescence. Lysosomal damage was induced by incubating cells with the lysosomotropic LLOMe at 1.5 mM for 2 h, except for the GFP-Trap experiments, in which LLOMe was used at 0.5 mM for 1 h.

3.4.4 Immunocytochemistry, PLAs, and Data Analysis

Cells were seeded onto glass coverslips in 24-well plates. Cells were treated as appropriate for the assay. Cells were fixed in 4% (wt/vol) PFA for 10 min at room temperature. Cells were then washed twice with PBS solution. For antibody staining, cells were permeabilized with 0.1% Triton X-100 for 15 min, blocked in 1% BSA supplemented with 0.3 M glycine for 30 min, and then incubated with primary antibodies for 1 h at room temperature. Subsequently, cells were incubated with secondary antibody at the appropriate dilution and treated with Hoechst 33258 to counterstain nuclei. Coverslips were then mounted to glass slides in a solution of Mowiol (Sigma) and 0.1% p-phenylenediamine (Sigma). Images were acquired on a Carl Zeiss Axio Observer.Z1 microscope or a Zeiss LSM 510 inverted confocal microscope. Colocalization and line trace analysis was performed with Fiji software (ImageJ; National Institutes of Health). To perform PLA, a Duolink In Situ Detection Kit (Sigma) was used following the manufacturer’s recommended procedure for PLA probe dilutions and incubation times, rolling circle amplification times, and polymerase
concentrations. For dual recognition of GBP2 and Gal3-YFP, the experiments were performed by using the anti-rabbit PLUS and anti-mouse MINUS secondary probes. Fluorescence image acquisition was performed on a Carl Zeiss Axio Observer.Z1 microscope or a Zeiss LSM 510 inverted confocal microscope. For all experiments, quantifications were performed from at least 10 images. High-resolution images from single scans were analyzed in Fiji to calculate the number of PLA puncta. Images were first smoothed, and a threshold was selected manually to discriminate PLA puncta from background fluorescence. When it had been selected, this threshold was applied uniformly to all images in the sample set. The built-in macro “Analyze Particles” was used to count and characterize all objects within images. The following primary antibodies and dilutions were used for immunofluorescence: rabbit polyclonal anti-Irgb10 at 1:1,000, rabbit polyclonal anti-Irgb6 at 1:1,000, rabbit polyclonal anti-GBP2 at 1:1,000, mouse anti-Ubiquitin (FK2; Enzo) at 1:500, and rabbit anti-p62/SQSTM1 (MBL International) at 1:500. For PLA assays, mouse anti-GFP at 1:1,000 (Clontech) and rabbit anti-GBP2 1:2,000 were used.

3.4.5 GFP-Trap IP and Immunoblotting

To perform GFP-Trap IP, 5 × 10^6 YFP-Gal3–expressing iBMDMs or control iBMDMs were seeded in 10-cm untreated tissue culture dishes and then primed with 100 U/mL IFN-γ overnight or left unprimed. The following day, cells were treated with 0.5 mM LLOMe for 1 h. Cells were collected mechanically and spun at 600 × g for 5 min,
resuspended, and washed twice in 1 mL PBS solution. Cells were incubated with 2 mM of the membrane-permeable cross-linker DSP (Thermo Scientific) for 30 min at room temperature. To stop the reaction, cells were incubated with 20 μM Tris, pH 7.5, for 15 min. The co-IP assay was performed with Chromotek GFP-Trap beads per manufacturer guidelines with slight modifications. To remove the cross-linking solution, cells were washed twice in cold PBS solution and lysed in 200 μL lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40) for 30 min on ice with vigorous pipetting every 10 min. The cell lysate was then spun at 20,000 × g for 10 min at 4 °C, and the supernatant was collected and diluted with 300 μL 4 °C dilution buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA); 25 μL was saved for lysate analysis. The diluted sample was mixed with washed GFP-Trap beads and incubated with end-over-end tumbling for 2 h at 4 °C. After incubation, 25 μL supernatant was saved for analysis (i.e., unbound), and the beads were washed three times in cold dilution buffer. To collect bead-bound proteins, the beads were resuspended in 50 μL 2× SDS sample buffer [120 mM Tris-Cl, pH 6.8, 20% (vol/vol) glycerol, 4% (wt/vol) SDS, 0.04% bromophenol blue, 10% (vol/vol) β-mercaptoethanol, and 20 mM DTT]. A total of 25 μL 2× SDS sample buffer was added to the 25 μL total lysate and unbound samples. SDS/PAGE was performed with BioRad 4–20% (wt/vol) precast Tris·glycine stain-free gels. Samples were run at 150 V for 45 min. Postelectrophoresis protein loading equivalency was checked by using a UV imager. Subsequently, the gel was transferred to nitrocellulose by using the
BioRad semidry transfer apparatus. The transferred blot was blocked with 1% BSA for 30 min. Primary antibodies rabbit anti-Gbp2 (1:1,000) and mouse anti-eGFP (1:1,000) diluted in 1% BSA were incubated overnight at 4 °C. Blots were washed and incubated with secondary antirabbit and anti-mouse HRP antibodies for 30 min at room temperature. The membrane was activated with ECL (Perkin-Elmer/Western Lightning). Blots were developed on the Odyssey imaging system (LI-COR Biosciences).
4. Impact and Future Directions

4.1 Overview

Intracellular pathogens have evolved numerous techniques to subvert the host response. In my dissertation research, I focused on the host factors that played a role in the recruitment of the antimicrobial IFN inducible GTPases, specifically the GBPs. In my work, I identified that the bacterial secretion system represents a pattern of pathogenesis that is inflexible from an evolutionary standpoint. The presence of bacterial secretion systems can be recognized indirectly by the host, therefore providing a broadly applicable danger signal which triggers the cell-autonomous immune response.

This work takes place in an idealized setting for the host, where the pathogen is placed at a significant disadvantage so we can probe the host response in an optimal situation. This methodology allows us to interrogate the basic cell-autonomous defenses with a more reductionist approach. Therefore, I was able to identify galectin-3 as a critical host factor for the recognition of damaged PVs by the GBPs. Interestingly, the Gal3 recognition of damaged membranes is somewhat unique, in that it doesn’t require direct recognition of PAMPs, but instead recognizes the aberrant localization of host sugars. This data is summarized in the model (Figure 15). While this study has given us a better understanding of how GBPs are recruited to PVs, there are still several questions that remain unanswered.
Figure 15: Current Model
4.1.1 Mechanism of GBP recruitment to damaged vacuoles

While my work has established a clear link between damaged vacuoles and GBP recruitment, the exact mechanism by which GBPs are recruited has not been fully elucidated. I have shown that GBP recruitment to damaged membranes is at least partially dependent on galectin-3. Loss of Gal3 has a marked reduction on the efficiency of GBP recruitment to the damaged vesicles. The residual GBP recruitment is most likely due to redundant activity of additional galectins. I have shown previously, that mGBP2 and galectin-8 also colocalise on damaged vesicles. Therefore it is possible that multiple galectins act in concert to facilitate GBP recruitment of damaged vesicles, and loss of a single galectin may only not completely abrogate targeting. Therefore, in order to fully examine the role of galectins in the recruitment of GBPs, it would require a pan-galectin knockout. It may also be feasible to specifically remove the galectins that are important for pathogen targeting. I have shown that galectins-3,-8 and-9 are specifically recruited to the PV, and therefore loss of those three galectins may be sufficient to abolish GBP targeting.

It will also be important to identify which regions of Gal3 are critical for the recruitment of GBPs to the PV. Gal3 is the only galectin in the chimera class, which is characterized by having a single CRD and a large amino-terminal domain, which has been shown to contribute to self-aggregation [231, 236, 247]. It is unknown how Gal3
interacts with GBP's and which regions of the protein are involved. Mutational analysis may provide additional information on how Gal3 facilitates GBP recruitment to damaged vesicles. Based on evidence published by other groups [252], I anticipate that mutations in the carbohydrate recognition domain would result in a loss of specificity for targeting of GBP's to damaged membranes. Thursten et al. have shown that loss of the CRD eliminates the recruitment of galectin-8 to *Salmonella* containing vacuoles. Additionally, mutations in the C-terminal domain would likely reduce galectin oligomerization as well as reduce GBP binding. Further details about the specificity may be elucidated by making chimeric galectins. This may allow for the segregation of the beta galactoside recognition activity with the GBP recruitment. These mutant galectin expressing cells could be tested in both the infection assays, as well as the sterile damage assays to determine if there is some additional specificity for host or bacterial sugars.

Another pressing question is how are galectins are able to translocate to the inner leaflet of the damaged PV membrane? The most straightforward hypothesis would be that the pores created by bacterial secretion systems are of sufficient size to allow for galectins to easily traverse the membrane. This model seems unlikely as galectins are large proteins and diffusion across the membrane seems implausible. There is the possibility that the number of translocons produced by the bacteria could result in vesicular instability, such that the membrane is destabilized when an abundance of secretion systems is produced. There is some evidence to suggest this may be partially
true, as the *Yersinia* hypersecretion mutant ΔEJK, has a significant increase in the percentage of damaged vesicles compared to the controlled translocon production in the isotype control ΔEJ [289].

There would be two assays to test for PV membrane damage. Translocon pore size can be assessed by mixing bacteria with fluorescent dextran and infecting cells. Using video microscopy, I could then assess for leakage of dextran from the PV in real time. Presumably, the bacteria lacking a secretion system would never have dextran leakage, where hypertranslocating bacteria would have increased dextran leakage. By utilizing dextrans of varying molecular weights, I would be able to determine what size the membrane disruption is. Additionally, membrane disruption can be assessed by a Beta-lactamase assay (BLAM). In a BLAM assay, a cytosolic FRET substrate can be activated when cleaved by beta-lactamase upon exposure to the cytosol. I would infect CCF2 loaded cells with bacteria expressing beta-lactamase and monitor for changes in fluorescence which would indicate a disruption of the PV and exposure of the bacteria to the cytosol.

One current hypothesis for the disruption of the PV is that it is an active, host driven process carried out by an unidentified factor. This factor could directly recognize the translocon of bacterial secretion systems and perforate the membrane. This disruption would then allow galectins access to the inner leaflet of the PV and facilitate binding to the exposed beta galactosides. Subsequently, galectins would aid in the
recruitment or stabilization of GBPs on the PV. There is also the possibility that galectins themselves may recognize bacteria through interaction with LPS [246, 249] or bacterial outermembrane vesicles on the outside of the PV. Super-resolution microscopy or electron microscopy would probably be required to determine if that is the case.

Alternatively, galectins could be secreted across the PV membrane by an unknown mechanism. Galectins have a nontraditional secretion pathway which likely uses vesicular transport machinery to transfer galectins across membranes to their destination [239, 241, 242]. It could be that some host factor recognizes the bacterial translocon and facilitates the translocation of Gal3 across an intact membrane to the luminal side. Once there, Gal3 would be able to bind the luminal sugars, but the ability to interact with GBPs or other host factors on the cytoplasmic side would still be a challenge.

We can also interrogate the order of events in which Gal3 and GBP are recruited to the PV. I have not done an extensive timecourse on Gal3 recruitment to the PV, but based on the fact that I see GBP recruitment as early as 20 minutes post infection, I expect that galectins would be recruited at similar rates. Interestingly, while loss of galectin has been shown to reduce the efficiency of GBP recruitment, loss of GBPs has no effect on the recruitment of Gal3 to the PV [228]. This suggests that there is a hierarchy of events in which galectins facilitate the recruitment of GBPs to the PV. Therefore the membrane disruption likely happens very early in the bacterial lifecycle.
Finally, it may prove useful to identify additional interacting factors involved in the recruitment of stabilization of GBPs on the PV. Using our GFP-Trap, we may be able to isolate additional components associated with the ruptured PV. We can use a candidate approach and test likely interacting partners by CO-IP or, if we can get sufficient quantities, we may be able to run mass spec analysis to identify new targets.

4.1.2 Additional Roles for Galectins

In the inhibitor studies, I was able to validate a role for Gal3 in the recognition of O-glycosylated host proteins. However, the reduction in targeting was only a partial effect. This could either mean that there is some involvement with recognition of N-glycosylated host proteins, or the GBP targeting is compensated for by some unknown mechanism. This could be further tested by infecting Cho-Lec1 cells. These cells lack GlcNAc glycosyl transferase so that N-linked carbohydrates are blocked at the Man5-GlcNAC2-Asn intermediate. This would help parse out the role of specific sugar modification on galectin binding. Gal3 is unique among the galectins, insofar as it is the only member of the chimeric group which is able to oligomerize to form a multimeric carbohydrate recognition platform. It is possible that some pathogens may have evolved techniques to interfere with Gal3 recognition of host sugars either directly or perhaps through biological mimicry.
Galectins are conserved down to sponges. It has been proposed that these galectins perform similar roles in lower species, with many orthologs having similar glycan binding capabilities. Interestingly, in the natural host of \textit{Legionella}, galectins are noticeably absent. Discoidin I and II are the most similar proteins, in so far as they are N-acetylgalactosamine-binding proteins [290-292]. This could be some evidence as to why the \textit{Legionella} have not evolved to evade any galectin mediated cell autonomous defense.

It is also unclear as to why Gal3 and mGBP2 are strongly recruited to disrupted lysosomes in the sterile damage model. Presumably, PVs that have fused with lysosomes pose limited threat to the host. It could be due to the fact that the lysosomal membrane proteins LAMP and LIMP are heavily modified. These proteins are extensively glycosylated with asparagine-linked oligosaccharides. It has been postulated that these glycans protect the underlying polypeptides from the proteolytic environment of the lysosome. These asparagine linked (N-Linked glycosylation) glycans would remain intact and would therefore serve as strong binding partners for the galectins. My hypothesis is that the recruitment of Gal3 and GBPs to lysosomes may be involved in antigen stabilization or presentation by cells. This could lead to changes in cytokine signaling. MHC presentation rate/ability have not yet been tested in either Gal3 or GBP knockout cells.
4.1.3: Impact

This study provides new evidence for the role of a host glycan recognition event that triggers the recruitment of immune surveillance proteins to the site of infection. I have demonstrated that vacuolar disruption mediated by bacterial secretion apparatuses provides a pattern of pathogenesis, which prompts the delivery of antimicrobial GBPs to PVs. The loss of membrane integrity may occur as an unintended consequence of PV manipulations by resident bacteria or there may be an unidentified host factor that recognizes the bacterial secretion systems and triggers PV disruption. This study provides further evidence for the necessity of tight regulation of secretion systems by pathogens. Hypersecretion of bacterial translocons increases vacuolar instability and leads to an increase in GBP recruitment to the PV. Well adapted pathogens must therefore limit the amount of bacterial translocon produced in order to avoid immune detection while still allowing for secretion of bacterial effectors to modulate the host environment. Finally, this study has shown that loss Gal3 negatively impacts the host’s ability to target PVs for destruction through ubiquitination. In some infections, loss of ubiquitination may lead to impaired pathogen control. Therefore, the role of galectins in innate immunity is an area of research that may still yield important discoveries in the field.
References


Biography

Eric Michael Feeley was born July 28th 1987 in Newton, MA. Eric grew up in Northborough, MA and it was there that Eric met his high school sweetheart and future wife, Lorin. Eric attended Providence College, in Providence, RI where he was first introduced to scientific research by Fr. Nicanor Austriaco. Eric began his research career studying programmed cell death in the budding yeast saccharomyces cerevisiae.

Following his graduation, Eric took a research technician position at the Ragon Institute with Dr. Abraham Brass. Eric’s research primarily focused on identifying viral restriction factors for influenza virus. Under Dr. Brass tutelage, Eric contributed to the publication of six scientific manuscripts, including his first author publication: “IFITM3 inhibits influenza A virus infection by preventing cytosolic entry”. Eric left the Ragon Institute in 2011 to pursue a PhD at Duke University. There he joined the laboratory of Dr. Jörn Coers, where he studied the cell-autonomous response to intracellular pathogens. In the Coers lab, Eric contributed to two publications, including his first author paper “Galectin-3 directs antimicrobial guanylate binding proteins to vacuoles furnished with bacterial secretion systems” which was published in PNAS in 2017.