Mechanisms of Host-Defense Against Intracellular Bacterial Pathogens Through The PI3K/Akt Host Signaling Pathway

Dissertation
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Abstract

Intracellular bacterial pathogens have developed mechanisms to exploit host cells as a part of their lifecycle and these pathogens manipulate or suppress signaling events by the host to accomplish this. Elucidating host signaling and defense mechanisms is essential for advancing our understanding of the pathogenesis caused by many different microbes and for discovering novel therapeutic targets. Two different Gram-negative intracellular bacterial pathogens will be examined in the following chapters, *Francisella tularensis* and *Burkholderia cenocepacia*. Both organisms are capable of replicating within mononuclear phagocytes and thus subvert the immune clearance mechanisms of these cells. These bacterial pathogens also activate the PI3K/Akt signaling pathway, which regulates numerous cellular functions including immune responses. The focus of the following studies is to understand how the PI3K/Akt pathway is regulated during microbial infection and to investigate the role of this pathway in host-defense.

The first part of this dissertation investigates the role of microRNAs as regulators of the immune response against *Francisella*. Previously it has been shown that activation of the PI3K/Akt pathway is beneficial to the host by enhancing NF-κB activation, the production of pro-inflammatory cytokines, promoting macrophage killing of bacteria, and overall host resistance to *Francisella*. These processes are all inhibited by the phosphatase SHIP, which limits Akt activation. Here it is shown that a microRNA, miR-
155, negatively regulates SHIP to promote pro-inflammatory cytokine production following infection. The most interesting finding was that this microRNA is differentially induced between low virulence and high virulence *Francisella* subspecies. This study uncovers a key mechanism by which PI3K/Akt signaling is altered during the microbe-host interaction.

In the second part of this dissertation the regulation of miR-155 during *Francisella* infection is explored in detail. This microRNA is essential for many immune functions and is also oncogenic. Therefore it is important to understand the regulation of this miR in a variety of contexts. Here it is shown that miR-155 is induced through an indirect response to infection and can be transferred through soluble bacterial factors. The induction requires NF-κB activation to up-regulate Fos and Jun transcription factors, which mediate transcription of the precursor to this microRNA.

The third and final data section to this dissertation explores the role of PI3K/Akt signaling in the host-response to *Burkholderia*. This is a highly antibiotic resistant organism with limited therapeutic options. It causes an opportunistic respiratory infection in susceptible individuals who have a dramatic inflammatory response leading to progressive morbidity and mortality. Here it is shown that Akt promotes NF-κB activation and the production of pro-inflammatory cytokines from *Burkholderia* infected phagocytes. This occurs through a unique IKK-independent, GSK3β-dependent mechanism. Thus this provides a potential novel therapeutic target for the treatment of *Burkholderia* infections.
The overall goal of these studies was to determine novel mechanisms of PI3K/Akt regulation during bacterial infection and to broaden the overall understanding of this pathway as a key regulator of host-defense.
This work is dedicated to my dearest family
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Publications

1. Kishore V. L. Parsa, Jonathan P. Butchar, Murugesan V. S. Rajaram, Thomas J. Cremer and Susheela Tridandapani (2008), The tyrosine kinase Syk promotes phagocytosis of Francisella through the activation of Erk, Molecular Immunology, 10: 3012-3021


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Major Field: Molecular, Cellular, and Developmental Biology Program
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>B.c.</td>
<td><em>Burkholderia cenocepacia</em></td>
</tr>
<tr>
<td>Bec</td>
<td><em>Burkholderia cepacia</em> complex</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin-A</td>
</tr>
<tr>
<td>BIC</td>
<td>B-cell integration cluster</td>
</tr>
<tr>
<td>BMM</td>
<td>Bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BTK</td>
<td>Bruton tyrosine kinase</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>F.n.</td>
<td><em>Francisella tularensis</em> subspecies <em>novicida</em></td>
</tr>
<tr>
<td>F.t.</td>
<td><em>Francisella tularensis</em> subspecies <em>tularensis</em></td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>IFNβ</td>
<td>Interferon beta</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1-beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>JNK</td>
<td>C-jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani broth</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MiR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MyrAkt</td>
<td>Myristoylated Akt</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>PBM</td>
<td>Peripheral blood monocyte</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDK1</td>
<td>3'-phosphoinositide-dependent kinase-1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PtdIns3,4P₂</td>
<td>Phosphatidylinositol 3,4 bisphosphate</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PtdIns3,4,5P₃</td>
<td>Phosphatidylinositol 3,4,5 trisphosphate</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time RT-PCR</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2 domain-containing inositol 5’-phosphatase 1</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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The following chapter will introduce key concepts in host-defense against intracellular bacterial pathogens that are examined in-depth in this dissertation. The broad focus of work done in this thesis is to understanding the signaling pathways that control the immune response to intracellular Gram-negative bacterial pathogens and the numerous means of regulation. This work is undertaken to enhance our understanding of the host-pathogen interaction and to work towards the goal of identifying novel therapeutic targets for the treatment of the diseases caused by infection with these pathogenic organisms.

1.1 Role of monocytes and macrophages in host-response to bacterial pathogens

Mononuclear phagocytes such as monocytes and macrophages are key components of the innate immune system and are essential for controlling bacterial pathogens\textsuperscript{1-3}. Monocytes circulate through the blood and are the immature precursor to the fully differentiated macrophage, which takes residence in various tissues. Major functions of these cells are phagocytosis\textsuperscript{4-6}, antigen presentation\textsuperscript{7}, and the production of inflammatory mediators that signal to other cells of the immune system\textsuperscript{8}. Some pathogenic microorganisms have the ability to subvert these functions of monocytes/macrophages\textsuperscript{9} and hijack these cells\textsuperscript{10}. Many intracellular bacterial pathogens such as \textit{Listeria, Shigella, Francisella}, and \textit{Burkholderia} have developed ways to evade the
killing mechanisms of these immune cells, thus transforming these cells into niches for replication

Both monocytes and macrophages express a variety of pattern recognition receptors to sense microbes, which include toll-like receptors. Activation of the TLRs leads to signaling through the MyD88 and/or TRIF adaptor molecules. Down-stream signaling activates a number of signaling transducing kinases, which include the MAPK, PI3K, and IKK. These signaling events lead to the activation of a variety of transcription factors to initiate the transcription of immune response genes.

1.2 PI3K signaling pathway functions in host-defense to pathogens

PI3Ks are signal transducing enzymes that phosphorylate PtdIns at the free 3-hydroxyl position. There are three general classes of PI3Ks; class IA/IB, class II, and class III. These classes differ in substrates, products, and functional consequences. It is the class I PI3Ks which are of greatest importance in the current study as it is the class I PI3Ks that promote the conversion of PIP\(_2\) to PIP\(_3\). It is PIP\(_3\) that activates Akt through PDK1 at the plasma membrane (Figure 1.1). The catalytic and regulatory subunits of the PI3Ks are directly recruited to a variety of membrane bound receptors, which include the TLRs. Thereby TLR ligands activate PI3K; however, the function of PI3K in the response to TLR ligands has been controversial.

Akt/PKB is one of the downstream targets of PI3K activation. Other targets of PI3K signaling include Ras, BTK, PDK-1, and GEFs. In a broad context PI3K/Akt signaling is important for embryonic development, cancer, insulin response, and immune cell function. While the focus of this thesis is on the role of PI3K/Akt signaling in
innate immune response, there is also an important role for this pathway in adaptive immune cells\textsuperscript{23}. Interestingly, multiple bacterial pathogens suppress Akt activation\textsuperscript{24,25}.

Akt itself has a number of down-stream targets, which include IKK\textalpha and GSK3\beta among others\textsuperscript{17}. Both proteins are of particular interest to studies in host response because they regulate NF-\kappaB activation and thus many key immune responses. It has been shown that Akt can directly phosphorylate IKK\textalpha\textsuperscript{26} and NF-\kappaBp65 phosphorylation at serine-536\textsuperscript{27} to promote NF-\kappaB nuclear translocation and gene transcription. GSK3\beta is an interesting molecule in terms of immune function because GSK3\beta\textsuperscript{-/-} mice are embryonic lethal and have impaired NF-\kappaB activation\textsuperscript{28}. However, it also has been shown that under a variety of conditions GSK3\beta promotes NF-\kappaB activity\textsuperscript{29-31}. Thus there is great

![Figure 1.1: Model of the PI3K signaling and down-stream targets.](image)

PI3K can be activated by TLR receptor activation. PI3K converts PIP\textsubscript{2} into PIP\textsubscript{3} second messenger. PDK1 and Akt localize at the plasma membrane whereby Akt becomes active. Akt influences NF-\kappaB and the production of inflammatory mediators by directly promoting IKK\alpha activation or inhibiting GSK3\beta activation.
complexity in this signaling pathway and it remains to be fully understood.

1.3 Inositol phosphatases regulating PI3K signaling

PI3K signaling is tightly regulated by a multiple mechanisms. The inositol phosphatases PTEN and SHIP are important regulators of PI3K signaling by converting PIP<sub>3</sub> to PIP<sub>2</sub>. PTEN catalyzes the conversion of PtdIns(3,4,5)P<sub>3</sub> to PtdIns(4,5)P<sub>2</sub>, while SHIP catalyzes PtdIns(3,4,5)P<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>. Both phosphatases have similar functions, yet there are also differences between the two<sup>32</sup>. The studies in this thesis have focused on the role of SHIP as a regulator of PI3K/Akt signaling during bacterial infection; however, that is not to imply that PTEN is not of importance.

There exist two forms of SHIP that differ in expression patterns, substrate specificity, and in the structure of their non-catalytic region. SHIP1 is expression is expressed in hemopoietic cells<sup>33</sup>, while SHIP2 is more broadly expressed<sup>34</sup>. Viable knockout mice for both SHIP1<sup>33</sup> and SHIP2<sup>34,35</sup> have been created and used experimentally. Major functions of SHIP1 in phagocytes are to regulate FcγR-mediated phagocytosis<sup>36</sup>, superoxide production<sup>37</sup>, and inflammatory cytokine production<sup>38,39</sup>. The studies undertaken in this thesis focus exclusively on SHIP1 and thus SHIP1 will be referred to as simply “SHIP” for the rest of this manuscript.

It is interesting to note that bacteria can secrete phosphatases which regulate PI3K signaling within host cells. *Salmonella dublin* secretes a protein known as SopB that has phosphatase activity *in vitro* and alters inositol phosphate signaling *in vivo*<sup>40</sup>. The phosphatase activity of SopB has also been shown to manipulate the *Salmonella*-containing vacuole to promote the formation of a favorable intracellular environment<sup>41,42</sup>. 
This highlights the importance of phosphatidylinositol signaling during bacterial infections and finding additional examples of pathogens that directly manipulate host signaling in a similar fashion would be of considerable interest.

1.4 MicroRNA regulation

MicroRNAs function to modulate protein expression by targeting specific mRNAs. This is presumed to occur by partial complementarity at the 3’UTR, which results in inhibition of protein translation or mRNA degradation. Currently the exact means by which miRs inhibit mRNA translation are not well understood. A single miR can have numerous mRNA targets. Also mRNAs can be targeted by multiple miRs; however, it is important to note that the number of miR binding sites on an mRNA does not always correlate with degree inhibition. In fact when multiple miR binding sites are in close proximity on an mRNA, the ability to down-regulate the target is impaired when the miRs that target those sites are co-expressed.

It’s becoming accepted that miRs are key regulators of host immune response as they can fine tune the expression of signaling and effector molecules that control the host’s defenses. Early screening experiments identified a small select number of miRs, miR-132, miR-146a/b, and miR-155, that are responsive to TLR activation. Additional miRs have since been found to be responsive to TLR activation, miR-21, miR-29b and miR-147. Furthermore the expression of TLRs themselves, components of signal transduction, and the immune regulatory cytokines IL-10 and TGFβ are directly regulated by multiple miRs. Thus not only is miR expression regulated by the presence of...
microbes, but miRs can also influence the hosts ability to respond to microbes by targeting signaling pathways and direct mediators of host response.

1.5 Francisella tularensis

*Francisella tularensis* is a Gram-negative intracellular bacterial pathogen which causes the febrile and potentially fatal disease of Tularemia. What makes this pathogen particularly troublesome is that the most virulent subspecies require a very low dose to cause disease in humans and there is yet to be a fully effective vaccine. Due to these features this pathogen could be used as a biological weapon and thus it has been classified as a category A select agent by the United States Centers for Disease Control. There are different subspecies of *Francisella* which differ in virulence\(^{57,58}\). *Francisella tularensis* subspecies *tularensis* (type A strain) has the highest virulence in humans, subspecies *holartica* (type B strain) is less virulent, and subspecies *novicida* is virtually avirulent in humans yet retains virulence in mice, allowing its use as a model system\(^ {59}\). However, immunocompromised humans can become susceptible to *F.n.* infection\(^ {60}\).

Mononuclear phagocytes have been well studied for their response to *Francisella* because it was discovered that this bacterium can subvert the killing mechanisms of these immune cells and replicate within them\(^ {61}\). These cells express TLR2 and it is this surface expressed TLR which is critical for NF-κB activation and pro-inflammatory cytokine production\(^ {62}\). The intracellular life cycle involves the host cell taking up the bacterium through looping phagocytosis where it becomes enclosed within a phagosome\(^ {63}\) (Figure 1.2). The uptake can be mediated through the complement, mannose, Fcγ\(^ {64}\), and scavenger receptors\(^ {65}\). Within an hour of entering the host cell the bacterium escapes the
phagosome to gain access to the cytosol. Entry to the host cytosol triggers the production of IFNβ, IL-1β, and caspase-1 activation. The cytosolic pattern recognition receptors of Francisella are Pryin and Aim-2. Phagosomal escape mutants of Francisella have been generated; MglA−, IglC−, and ABCH− are examples. Once in the cytosol the bacterium can replicate. Replication in the cytosol requires the IglD gene on the Francisella pathogenicity island. At the late stages of infection the cytosolic bacteria become enclosed within vacuoles resembling autophagosomes. This induction of autophagy appears to be beneficial for the host as it has been shown that an autophagy inducing agent inhibits Francisella survival within macrophages. However, Francisella also suppresses many of the mediators of autophagy, presumably to counter this favorable host response.

Figure 1.2: Depiction of the intra-macrophage lifecycle of Francisella. 1Francisella enters the host cell through looping phagocytosis and is enclosed within a phagosome. 2The vacuole acidifies and the bacterium escapes into the cytosol within 1 hour. Entry into the cytoplasm results in inflammasome/caspase-1 activation. 4In the cytoplasm the bacteria replicate for a period of time. 5At the late stages of infection the bacteria become enclosed within autophagosomes, which acidify. 7The end result of infection is host-cell death and bacterial spread.
It is worth noting that infection is not limited to mononuclear phagocytes. It has been reported that a *Francisella* mutant which cannot replicate within macrophages can still be pathogenic *in vivo* and infect other cell types\(^7^9\). Regardless, it has still been shown that macrophages alone can promote *in vivo* resistance to a host infected with *Francisella*\(^8^0\), thus it is important to understand the macrophage response to this pathogen.

One of the intriguing aspects of *Francisella* infection is that there is a remarkable lack of inflammatory response during the early stages of infection *in vivo*; furthermore, this infection can suppress the response against a secondary challenge with a potent inflammatory stimulus\(^8^1\). However, it is not the case that an inflammatory response is completely absent. While human dendritic cells do not response to *F.t.* by producing pro-inflammatory cytokines, monocytes do produce these molecules and the difference is due to CD14 expression between these cells\(^8^2\). Furthermore, *F.n.* clearly induces the production of various pro-inflammatory cytokines\(^8^0;8^3;8^4\) and direct comparisons show that it is greater than what is induced by *F.t.*\(^9;5^3\). In the early stage of infection there is a delay in response, but at the later stages of infection there is actually a strong inflammatory response which is counter productive\(^8^5\). It is tempting to speculate that it is the lack of cytokine production during the early stages of *Francisella* infection is what prevents the host from mounting a proper immune response and this is why the host succumbs, while if the immune system was properly alerted during the initial stages of infection the host would be better able to resolve the infection. In fact treatment with a synthetic TLR ligand to ‘prime’ the immune systems can be host protective against *Francisella* infection\(^8^6\).
1.6 *Burkholderia cenocepacia*

*Burkholderia cenocepacia* is also a Gram-negative intracellular bacterium and is a member of the Bcc. This is an opportunistic respiratory pathogen, thus causes disease in a select population of individuals. Susceptible individuals include those with cystic fibrosis and chronic granulomatous disease where *B. c.* infection causes a progressive life threatening condition termed ‘cepacia syndrome’ and there are limited therapeutic options as these bacteria are highly antibiotic resistant\(^87\);\(^88\). Adding to this problem, this infection can be acquired by human to human transmission\(^89\).

The mononuclear phagocyte response to *B. c.* and the intracellular lifecycle of this pathogen in general is rather poorly understood. Much of the *in vitro* research done on *B. c.* has utilized lung epithelial cells since they are relevant to the site of infection\(^90\);\(^91\), but more recently a role for the macrophage has emerged\(^92\). Previously it was believed that this pathogen simply survived within phagocytes by evading phagosome-lysosome fusion, yet did not replicate\(^93\). More recently it seems to become accepted that this bacterium does replicate within these cells and this has been aided by the development of methods and mutants that get around the issue of antibiotic resistance\(^91\);\(^94\).

It is known that susceptible individuals with cystic fibrosis have a detrimental inflammatory response\(^95\) and dysregulated NF-κB activity\(^96\);\(^97\). Clinically it has been shown that administration of corticosteroids is associated with more favorable patient outcome when infected with *Burkholderia*\(^98\). While this does not enhance bacterial clearance, it limits harmful inflammation to benefit the patient. Understanding how the host pro-inflammatory response is regulated during *Burkholderia* infection will provide new therapeutic targets for the treatment of infected individuals.
This thesis will explore the regulation of the host inflammatory response to two different bacterial pathogens. The host response to *B. c.* is an interesting contrast to that of *Francisella*. Whereas *B. c.* triggers an overly robust inflammatory response by the fact that it expresses a highly stimulatory form of LPS\(^{99,100}\) and the bacterium itself can directly activate the TNF receptor\(^ {101}\) and TLR5\(^ {102,103}\), which has been shown to contribute to virulence. *Francisella* expresses a modified form of LPS that does not activate TLR4\(^ {104}\) and at least in the early stages of infection there is minimal to no inflammatory cytokine response\(^ {81,105}\). MyD88\(^{-/-}\) mice are hyper-susceptible to *Francisella* infection\(^ {106,107}\), yet they are resistant to *Burkholderia* infection\(^ {108}\). It may be fair to generalize the inflammatory response to *Francisella* as overly subtle, while in *Burkholderia* it is overt. Therefore, it may be more favorable to enhance this response against *Francisella*, yet tone it down against *Burkholderia* infection.

The prevailing theme of this thesis is that the PI3K/Akt signaling pathway is an important means of regulating host immune response against intracellular Gram-negative bacterial pathogens. Here it is demonstrated that microRNAs are key regulators of this pathway, thus this has uncovered a previously unknown means by which the activation of Akt can be controlled. The role of a down-stream target of Akt, GSK3\(\beta\), has been investigated in the context of microbial infection to broaden our understanding of the connection between Akt and the inflammatory response. These studies detail how host signaling pathways control immune function and determining how they are regulated are essential for the advancement of new therapeutic strategies that target the host, as opposed to the microbe\(^ {109}\). Such strategies will move to greater importance in the future as new multi-drug resistant microbes emerge.
Chapter 2 describes the studies that were undertaken to uncover how SHIP expression is modulated during infection with *Francisella*. The key finding is that low virulence *F. novicida* induces the expression of miR-155 to down-regulate SHIP and enhance the host’s pro-inflammatory response; however, high virulence *F. tularensis* elicits strikingly less miR-155 induction and inflammatory response.

Chapter 3 provides an in-depth examination of the molecular mechanisms by which miR-155 expression is regulated. Not only is this microRNA important for the host response against *Francisella*, it is also important for other disease states including cancer and autoimmunity. Therefore, clearly defining the mechanisms of miR-155 regulation is of great interest to a broad audience.

Chapter 4 examines a role for PI3K/Akt signaling in host-response to the opportunistic pathogen *Burkholderia cenocepacia*. There is a particularly strong inflammatory response against this pathogen and in susceptible individuals the infection progresses to a severe life-threatening condition. This chapter describes the studies showing that Akt promotes the inflammatory response to *B. cenocepacia*, yet does not influence the bacterium’s ability to infect or replicate within mononuclear phagocytes. Therefore, targeting Akt may be useful therapeutic target in the treatment of cepacia syndrome.
Chapter 2: MiR-155 Induction by *Francisella novicida*, Yet Not the More Virulent *Francisella tularensis* Down-regulates SHIP Expression and Enhances Host-Response

2.1 Abstract

*Francisella tularensis* is an intracellular Gram-negative bacterium that causes the disease tularemia and is known for its ability to subvert host immune responses. Previously the Tridandapani laboratory has identified the PI3K/Akt pathway and SHIP as critical modulators of host resistance to *Francisella*. This chapter furthers the understanding of the role of SHIP during *Francisella* infection because not only does this phosphatase regulate host-response during infection, but its own expression is regulated during the course of infection. Here it is shown that SHIP expression is down-regulated in both monocytes and macrophages following infection with *F. tularensis novicida* (*F.n.*). To determine the mechanism of this negative regulation we explored the possibility that microRNAs (miRs) target SHIP. Currently there is only one miR that is predicted to target SHIP and that is miR-155. We examined miR-155 expression *in vitro* and *in vivo* during *Francisella* infection, and find that this miR is highly responsive to infection. Luciferase reporter assays confirmed that miR-155 down-regulates SHIP specifically through the 3’UTR. Further experiments showed that miR-155 and *BIC*, the gene that encodes miR-155, were up regulated within four hours of infection in primary human monocytes. This induction was dependent on MyD88 and did not require
inflammasome activation. Functional studies show that miR-155 positively regulated pro-inflammatory cytokine release in human monocytes infected with Francisella, as consistent with negatively regulating SHIP. We find a contrast in this response between the highly virulent type A SCHU S4 strain of Francisella tularensis (F.t.) and the less virulent F.n. Monocytes infected with F.n. induce miR-155 expression and down-regulate SHIP to enhance pro-inflammatory responses. However, miR-155 is minimally induced by F.t., which helps explain the lack of both SHIP down-regulation and pro-inflammatory response and may account for the virulence of F.t.

2.2 Introduction

Francisella tularensis is a highly infectious Gram-negative bacterium that can infect and replicate within immune cells63;110;111. Exposure to only ten colony forming units can be lethal to humans, therefore the CDC has classified this pathogen as a Category A select agent57;58;112. Additionally there is no FDA approved vaccine available, thus discovering novel therapeutics is a pressing need. Debatably there are five known subspecies of Francisella, it is F.n. that is the least harmful to humans, while F.t. has the highest virulence113. However, studies using F.n. are relevant for two reasons. First it is of use in a model system because it shares a similar intracellular lifecycle to F.t. and leads to tularemia-like pathologies in mouse models57. The second is that a comparative study can be conducted to determine differences in response among two organisms with high degrees of genetic similarity, yet differences in pathogenesis.

It is clear that effective host cell defense against Francisella is subverted by numerous mechanisms. Indeed, interferon response114, toll-like receptor (TLR)
signaling, autophagy, and antigen presentation are all found to be compromised by Francisella. Even though Francisella is Gram-negative, it presents a modified form of lipopolysaccharide (LPS) that does not strongly activates TLR4. Of the TLRs it is TLR2 which is of major importance and it works in conjunction with TLR1 and TLR6. Anecdotally it is interesting that Francisella is recognized by TLR2 because pairing with TLR6 can block protective inflammatory responses against Yersinia pestis by inducing high levels of IL-10. The tactic of preventing the host from responding to infection makes it important to find ways to enhance host immune function. By understanding these host responses and how Francisella undermines them will allow for novel therapeutic strategies.

One important response downstream of TLR stimulation is signaling through the PI3K/Akt pathway. The Tridandapani laboratory has shown that PI3K/Akt activation is host-protective against F.n. infected mice. Activation of this pathway is subject to negative regulation and one of the key regulators is SHIP. Expectedly, this phosphatase has been demonstrated to be important during Francisella infection. BMM lacking SHIP display increased NF-κB activity and enhanced cytokine production, resembling the responses seen with constitutively active Akt. These earlier findings show that the PI3K/Akt pathway and SHIP as critical regulators of the host-response to Francisella (Figure 2.1).
Figure 2.1: Model of the PI3K/Akt pathway function during Francisella infection. Francisella is recognized by TLR2 and triggers down-stream activation of PI3K, in turn activating Akt through the generation of PIP3. Akt promotes NF-κB activity to promote the production of pro-inflammatory cytokines. Akt activation inhibits bacterial growth within macrophages, shows signs of increased phagosomal maturation, and leads to increased host-resistance to a lethal challenge in vivo. The phosphatase SHIP inhibits these processes by negatively regulating the activation of Akt.

Further examination of the role of SHIP in host-response to Francisella showed that infection with the less virulent F.n. leads to SHIP down-regulation. To determine how this was mediated we entertained the possibility that miRs may play a role. MiRs can regulate both transcript and protein levels of specific genes through targeted interactions with the 3’UTR of mRNA transcripts. Hypothesizing that miRs regulate SHIP expression was reasonable since it was shown that treatment with TLR ligands can induce miR-155 expression and bioinformatics predicted SHIP to be regulated by miR-155 (www.targetscan.org). Oddly enough, at the same time we were investigated this
hypothesis, multiple reports on the subject of miR-155 targeting SHIP were also underway\textsuperscript{120;121}. In addition to this report of miR-155 regulating immune response to \textit{Francisella}, there has been studies examining the role of this miR in \textit{Helicobacter pylori} infection\textsuperscript{122} and viral infections\textsuperscript{123;124}.

In this chapter, it is shown that \textit{Francisella} infection reduces SHIP expression and this at least in part is mediated through the induction of miR-155. It is of special interest that the less virulent \textit{F.n.} subspecies strongly induces miR-155 while the virulent \textit{F.t.} subspecies does not. Expression of miR-155 is a component of host defense against \textit{Francisella} and here there is a definitive difference in miR-155 response to these two subspecies, which may help explain the success of \textit{F.t.} as an infectious agent in humans.

2.3 Materials and Methods

\textit{Cell and Reagents.} Human monocytic THP-1 cells and RAW264.7 murine macrophages were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in RPMI-1640 (Gibco-BRL, Rockville, MD) supplemented with 5\% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), L-glutamine, penicillin (10,000 U/ml) and streptomycin (10,000 \(\mu\)g/ml) (Invitrogen, Carlsbad, CA). Chinese hamster ovary (CHO) cells were obtained from the ATCC and cultured as previously described\textsuperscript{125}. The BAY 11-7085 IKK inhibitor and the Sn50 NF-\(\kappa\)B peptide inhibitor were a generous gift from Dr. Denis Guttridge (The Ohio State University). LY294002 (20\(\mu\)M), U0126 (2.5\(\mu\)M) and SP00125 (5\(\mu\)M) were purchased from Calbiochem (San Diego, CA). SB216763 (5\(\mu\)M) and DMSO vehicle control (0.2\%) was obtained from Sigma-Aldrich (St. Louis, MO). Cytochalasin-D was obtained from Biosource.
(Camarillo, CA) and used at a concentration of 5μg/ml to inhibit phagocytosis as previously described\textsuperscript{126}.

\textit{Transgenic and knockout mice.} C57BL/6J WT and TLR2 signaling defective transgenic mice (TLR2\textsuperscript{tm1kir\textsuperscript{J}}) were purchased from The Jackson Laboratory (Bar Harbor, MA). Femurs from WT and MyD88\textsuperscript{-/-} were kindly provided by Dr. Thirumala-Devi Kanneganti (St. Jude Children’s Research Hospital). WT and caspase-1\textsuperscript{-/-} mice were provided by Dr. Amal Amer (The Ohio State University) WT and miR-155\textsuperscript{-/-} (B6.129S7-Mirn15\textsuperscript{5tm1Brd}) mice were purchased from the Mutant Mouse Regional Resource Centers (MMRRC) (Columbia, MO)

\textit{Bone marrow-derived macrophages isolation.} Mice were sacrificed according to Institutional Animal Care and Use Committee approved protocol and the femurs were removed. Bone marrow was flushed from the femurs and cultured in DMEM (Invitrogen, Carlsbad, CA) with 10\% heat-inactivated FBS, 30\% sterile filtered L-cell conditioned media, 0.1\% β-mercaptoethanol (BioRad, Hercules, CA), and penicillin/streptomycin for six to seven days. Dr. Stéphanie Seveau (The Ohio State University) provided the murine fibroblast cell line L929. These cells were grown to confluence, approximately 6 days, in minimum essential media (Invitrogen, Carlsbad, CA) supplemented with 10\% heat-inactivated FBS (HyClone, Logan, UT), non-essential amino acids, sodium pyruvate and penicillin/streptomycin (Invitrogen, Carlsbad, CA). The conditioned media from the L929 cells was collected, passed through a 0.2μm filter and used as the source of growth factors to drive differentiation of the bone marrow cells into macrophages. When the
BMMs were fully differentiated they were collected by washing 3 times in sterile PBS, scraped, and then plated overnight in 12-well tissue culture plates. To confirm the purity of harvested macrophages, CD11b\(^+\) staining was assessed by flow cytometry.

*Peripheral blood monocyte isolation.* Human peripheral blood monocytes (PBM) were isolated from leukopacks by centrifugation through a Ficoll gradient followed by CD14-positive Magnet-Assisted Cell Sorting (MACS, Miltenyi Biotec, Auburn, CA) according to manufacturer instructions as previously described\(^9\). Flow cytometry analysis for CD14 expression showed a minimum of 98% purity for each sample. PBM were used immediately after isolation and cultured in RPMI-1640 containing 10% heat-inactivated FBS and L-glutamine.

*Bacterial infections.* Infections were conducted in 5% or 10% FBS-containing RPMI-1640 without antibiotic. *F. novicida* U112 (JSG1819) and *F. tularensis* subspecies *tularensis* (SCHU S4) were provided by Dr. John Gunn (OSU). Bacteria were grown on Chocolate II agar plates (Becton, Dickinson and Company, Sparks, MD) at 37 °C. All *F.t.* infections were conducted by CDC approved select agent users at The Ohio State University BSL3 Select Agent facility in accordance with the BSL3 bio-safety plan. All *F.t.* infected samples and matched reference samples were decontaminated in accordance with approved protocols by the BSL3 advisory committee to ensure effective killing of microbes before removal from the facility. Optical density measurements taken at 600nm (1.0 OD = 5x10\(^9\) bacteria) were used to determine bacterial density for MOI calculations and were verified by plating the inoculum overnight and counting CFUs. Heat-killing
(HK) of *Francisella* was accomplished by heating at 98 °C for 10 minutes. Paraformaldehyde (PFA)-killed *F.n.* was prepared by treating bacteria with 4% PFA for 30 minutes and then washing with PBS three times to remove residual PFA. Effective killing of microbes was done by plating on chocolate II agar and looking for growth. Pulse-chase infections were done by infecting for 2 hours, removing the media, washing the cells with sterile PBS to remove non-internalized bacteria, and then incubating in media containing 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA) for 30 minutes to kill extracellular bacteria. Phagocytes were then washed again and incubated in media containing a lower dose gentamicin (10μg/ml) for the duration of the time course.

*In vivo infections with Francisella.* FVB/n background wild-type mice were injected intraperitoneally with either 200 CFU of *F.n.* suspended in PBS or PBS alone in accordance with Institutional Animal Care and Use Committee protocols as done previously. The infectious dose was verified by plating the inoculum on chocolate agar and counting CFUs. Mice were euthanized at 24, 48 or 72 hours post-infection and none of the mice died of infection during this time course. The liver, lungs, and spleen were harvested, passed through a 70μm cell strainer (BD, Bedford, MA) to get cell suspensions, centrifuged briefly, and then resuspended in TRIZOL® reagent (Invitrogen, Carlsbad, CA). RNA extraction was then performed according to manufacturer instructions (Invitrogen) as done before.
CFU assays. At the time of collection cells were lysed in 0.1% SDS (Sigma-Aldrich, St. Louis, MO) diluted in PBS for 5 minutes, this was followed immediately by serial dilutions in PBS and plating on Chocolate II agar overnight to obtain countable single colonies as previously described\textsuperscript{126}.

ELISA cytokine measurements. Sandwich ELISAs for human TNF\(\alpha\) were purchased from R&D Systems (Minneapolis, MN). Human IL-1\(\beta\) and IL-6 ELISA kits were obtained from eBioscience (San Diego, CA) as done previously\textsuperscript{84}.

Western blot analysis. T\(\text{N}1\) lysis buffer (50mM Tris [pH 8.0], 10nm EDTA, 10M Na\(_4\)P\(_2\)O\(_7\), 10 nM NaF, 1% Triton-X 100, 125nM NaCl, 10nM Na\(_3\)VO\(_4\), 10\(\mu\)g/ml of both aprotinin and leupeptin) was used to collect whole cell lysates. Protein matched samples were separated on acrylamide gels and transferred to nitrocellulose membranes. Anti-SHIP antibodies were provided by Dr. K. Mark Coggeshall (University of Oklahoma) and Upstate Cell Signaling Solutions (Lake Placid, NY). Anti-TLR2, Akt, and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pSerAkt, pJNK, pERK, pP38 antibodies were purchased from Cell Signaling (Beverly, Massachusetts) HRP-conjugated secondary antibodies followed by enhanced chemiluminescence was used to develop westerns (GE, Buckinghamshire, UK) as previously described\textsuperscript{38}. 
Quantitative Real-Time PCR. Samples were lysed in TRIZol® reagent (Invitrogen, Carlsbad, CA) and RNA isolation was completed according to the manufacturer’s instructions. 10 to 100 ng of total RNA were used for reverse transcription which was measured by a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). cDNA was generated with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) utilizing specific reverse transcription primers for hsa-miR-155 RNU44, RNU48, mmu-miR-155, sno412 or sno202 (Applied Biosystems, Foster City, CA). Corresponding PCR primers were used with TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Branchburg, NJ). cDNA synthesis for BIC mRNA expression was primed for reverse transcription with 0.8nM of random hexamer (Applied Biosystems, Foster City, CA) and assayed by qRT-PCR using custom Taqman probes spanning exons 2-3 (Hs01374570) (Applied Biosystems, Foster City, CA). Reverse transcriptase minus and no cDNA template minus controls were conducted, which showed no signs of contamination. Samples were run in triplicate for each experiment, and each experiment was performed at least 3 times. Relative expression is calculated as $2^{\Delta \Delta CT}$ (Target – CT Housekeeping Gene). This was then converted to fold-change for simplicity.

Transfection. 5 μg of pcDNA3.1 vector control or pcDNA3.1 expressing exon 3 of the human BIC gene was used for each transfecting 10x10^6 PBM. Amaxa solution T was used for electroporation with program Y-01 as previously described. Optimal miR-155 plasmid-driven expression was found between 12 and 14 hours post-transfection by qRT-
PCR analysis, with approximately 70% of PBM recovered and viable as measured by trypan blue staining. Therefore, infections were performed 14 hours post-transfection.

Construction of psiCHECK/INPP5D. A fragment of the INPP5D 3’UTR was PCR-amplified using the forward primer (5’->AGC CCT CAG TGA GCT GCC ACT GAG TCG ->3’) and reverse primer (5’->GAG TGA GAA AGG CAC AAT TTA ATT GG- >3’). This was subcloned into the PCR2.1 vector following the manufacturer’s protocol (Invitrogen). Plasmid DNA was isolated from the transformed colonies and verified by dideoxy chain termination sequencing. Then the fragment was removed from the PCR2.1 plasmid by digestion with EcoRI and blunt-end ligated into the psiCHECK vector downstream of the f-luc reporter gene. The final construct was verified with dideoxy chain termination sequencing.

Luciferase reporter assay. Dual-luciferase reporter assays were conducted with the psiCHECK vector with or without the 3’ UTR of SHIP, co-transfected with 50 ng of the pRL-CMV Renilla luciferase vector (Promega, Madison, WI) in CHO cells. These cells were also transfected with 0 to 50 nM synthetic miR precursor of miR-155 or scrambled miR control (Ambion) and luciferase reporter vectors using lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 48 hours post transfection the cells were lysed with passive lysis buffer (Promega) and dual-luciferase activity was assayed using a luminometer as previously described125.
2.4 Results

**SHIP expression is down-regulated during F.n. infection**

It was previously shown that the pro-inflammatory cytokine response to *Francisella* infection requires the activation of the PI3K/Akt pathway and that this response is inhibited by SHIP\(^{38,80}\), a basally expressed negative regulator of the PI3K/Akt pathway\(^{128}\). Further examination of these findings had directed us to examine the expression of SHIP during the course of *F.n.* infection. Here we examined SHIP protein expression in human monocytic THP-1 cells that were infected at an MOI of 100 for 5, 10, or 24 hours. The results demonstrate a dramatic down-regulation of SHIP in infected cells versus uninfected controls (Figure 2.2A). In the lower panel there is a re-probe of the same membrane with actin antibody to verify equal loading of protein in all lanes. Down-regulation of SHIP in response to *F.n.* was also observed in primary cells. BMM (Figure 2.2B) or PBM (Figure 2.2C) were infected with *F.n.* for 24 hours at an MOI of 50. Having established that SHIP protein is down-regulated with infection, we next examined mRNA expression. PBM infected with *F.n.* for 24 hours at an MOI of 50 also show substantial down-regulation of mRNA expression (Figure 2.2D). Even though we observe mRNA degradation, it is still reasonable hypothesize that miRs are down-regulating SHIP expression because they influence mRNA degradation and not strictly protein expression\(^{129}\). It is important to note that the cells were still viable even after 24 hours of infection, with as high as 90% viability observed.
Figure 2.2: SHIP expression is down-regulated by *F. n.* infection. A THP-1 cells were infected with *F. n.* at an MOI of 100 for the time points indicated. B BMMs and C PBM were infected with *F. n.* at an MOI of 50 for 24 hours. Cell lysates were resolved by western blotting with antibodies against SHIP and the membranes were re-probed with Actin as a loading control. D PBM were infected with *F. n.* at an MOI of 50 for 24 hours were at that time RNA was collected as SHIP expression was assayed by qRT-PCR. The graphs represent mean and standard deviation of samples in triplicate. All data were analyzed by paired student’s t-test (* indicates p value < 0.05). R, resting/uninfected cells.

MiR-155 targets the 3’UTR of SHIP

When searching for a mechanism to account for this down-regulation of SHIP, we explored the possibility that microRNAs (miRs) may be involved. The online bioinformatics program TargetScan (http://www.targetscan.org) identified miR-155 as the only predicted miR to bind SHIP mRNA on its 3’ UTR (Figure 2.3A). However, the predictions are not always accurate and must be verified experimentally. To directly determine the ability of miR-155 to specifically target the 3’UTR of SHIP we performed
**Figure 2.3:** SHIP expression is repressed by miR-155. A Predicted interaction between miR-155 and the 3'UTR of SHIP (INPP5D) mRNA. B Normalized luciferase activity in cells transfected with the 3'UTR of SHIP (psiCHECK-INPP5D) or with vector alone (psiCHECK), and cotransfected with a control Renilla luciferase vector. Synthetic miR-155 or non-specific (scrambled) miRs were subsequently transfected at concentrations of 0, 10, 25 and 50 nM. Luminometer readings were taken 48 hours post-transfection. The graph represents f-luc expression normalized to r-luc expression, then normalized to percent maximal response.

the standard dual-luciferase reporter assays. This was done with an f-luc luciferase reporter alone (psiCHECK) or the same reporter with the 3’UTR of SHIP (psiCHECK-INPP5D). These reporters were separately transfected into Chinese hamster ovary (CHO) cells using Lipofectamine 2000. To account for differences in transfection efficiency a pRL-CMV Renilla luciferase construct was co-transfected with the psiCHECK vectors. Synthetic miR-155 or scrambled miRs were used to manipulate miR-155 expression and were co-transfected into the cells at concentrations ranging from 10 nM to 50 nM. After 48 hours the cells were washed, lysed, and the dual-luciferase activity in the transfectants was measured. As predicted miR-155 suppressed the luciferase activity of psiCHECK-INPP5D but not of psiCHECK alone, indicating that miR-155 targeted the 3’ UTR of SHIP (Figure 2.3B). As an additional control, cells were transfected with psiCHECK-
INPP5D followed by a scrambled miR, and these showed no decrease in luciferase production. This establishes that miR-155 does directly inhibit SHIP expression. This is supported by the reports from other groups examining the ability of miR-155 to target SHIP\textsuperscript{120,121}.

*MiR-155 is induced in vitro and in vivo by F.n. infection*

Since SHIP is basally expressed\textsuperscript{130}, the expression of the negative regulator must be induced for SHIP to be down-regulated during *F.n.* infection. Having found that miR-155 is a negative regulator of SHIP, we next examined whether mature miR-155 and *BIC*, the non-protein coding gene that encodes miR-155, mRNA were indeed induced by infection. Before conducting any direct experiments on miR-155 expression we first examined data sets from an mRNA and a separate miR array analysis of monocytes that had been infected with *F.n.* at an MOI of 100 for 24 hours. Functional miR-155 originates from the 3 exon of a ‘gene’ known as BIC (Figure 2.4A). As a side note, even though this ‘gene’ does not produce a functional protein it was know that it was important in oncogenesis even before the discovery of miRs though a non-protein function\textsuperscript{131,132}, which we now know is miR-155\textsuperscript{133,134}. The mRNA analysis of infected monocytes shows that the BIC gene is highly induced during *F.n.* infection (Figure 2.4B). Correspondingly we find that miR-155 is also highly up-regulated in the miR array (Figure 2.4C).
Figure 2.4: BIC and miR-155 are induced during *F.n.* infection. A

Representation of the non-protein coding human BIC gene, not to scale. B

Microarray mRNA analysis of BIC expression and C global miRNA analysis in
human monocytes infected with *F.n.* at an MOI of 100 for 24 hours.

To validate these findings, human PBM were infected with *F.n.* and the
expression of miR-155 and *BIC* were measured by qRT-PCR. The results show a dose-
dependent induction of mature miR-155 (Figure 2.5A) as well as *BIC* (Figure 2.5B) in
PBM infected at an MOI 1, 10, or 50 for 24 hours. Next the time course of miR-155 and
*BIC* induction was examined. There is a gradual increase in both mature miR-155 (Figure
2.5C) and *BIC* mRNA (Figure 2.5D) starting as early as 4 hours and continuously
increasing up through 24 hours when infected with *F.n.* at an MOI of 50. The kinetics of
miR-155 induction is roughly consistent with previous findings in murine macrophages
stimulated with toll-like receptor ligands\textsuperscript{119}. 

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Figure 2.5: Validation that BIC and miR-155 are induced during *F.n.* infection. PBM were infected with *F.n.* for 24 hours at the indicated MOI then assayed for A miR-155 expression or B BIC expression by qRT-PCR. PBM were infected at an MOI of 50 with *F.n.* for the indicated time points then qRT-PCR was used to measure C miR-155 and D BIC expression. Data is represented as fold change. All graphs in this chapter represent the mean and standard deviation of triplicate samples unless specifically stated otherwise.

Having shown that miR-155 is induced *in vitro*; we tested whether *F.n.* infection led to miR-155 induction *in vivo*. FVB/n mice were challenged with 200 CFU of *F.n.* or with PBS, delivered intraperitoneally. The animals were sacrificed at 24, 48, and 72 hours post-infection (6 per time point). Immediately after sacrificing, the organs were harvested. RNA was extracted from cell suspensions and was assayed for mature miR-155 by qRT-PCR (Figure 2.6A). Tissue-specific patterns of miR-155 matched those previously reported\textsuperscript{125}, where basal expression was highest in the spleen and lowest in the liver. Results show that *F.n.* infection increases miR-155 expression in all organs tested by 72 hours post-infection. The delayed kinetics *in vivo* should be expected since it was observed that miR-155 induction was also delayed in pure cultures of monocytes. Bacterial burden was also examined in a portion of these mice and there is a rough
correlation between the amount of bacteria present and the expression of miR-155 (Figure 2.6B).

**Figure 2.6: MiR-155 is induced during *F.n.* infection in vivo.** FVB/N mice were injected with 200 CFU of *F.n.* via IP injection. Mice were sacrificed at the indicated time points where the lung, liver, and spleen were removed. RNA was extracted from the organs and assayed by qRT-PCR for miR-155 expression. Data is represented as fold change (n=6). Bacterial burden was measured in parallel by CFU assay (n=2).

**Bacterial viability contributes to miR-155 induction**

Given that *F.n.* induces miR-155, a simple follow-up experiment is to determine if bacterial viability was required for this response. PBM were infected / treated with live, paraformaldehyde-killed or heat-killed *F.n.* at an MOI of 50 or equivalent for 24 hours. Killed bacteria were able to induce miR-155 expression, but the levels were lower than what is induced by live bacteria (Figure 2.7A). Hence, bacterial viability and possibly bacterial protein synthesis contribute to miR-155 induction. This is fitting since it is reported that maximal NF-κB induction elicited by *Francisella* requires bacterial protein synthesis.\(^{135}\)

Next we sought to determine if the time of exposure to bacteria influences miR-155 induction in monocytes. So we are asking if a single exposure sufficient for miR-155 induction or if there is an additive increase in miR-155 expression based upon the contact
time of bacteria with host cells. Here PBM either exposed to a 50 MOI of *F.n.* for the full 24 hour period (Infected) or an infection where cells were exposed to bacteria for 2 hours followed by the removal of extracellular bacteria (Pulsed). The PBM exposed to *F.n.* for the entire 24 hour period showed a stronger induction in miR-155 than those exposed for only 2 hours (Figure 2.7B). Conversely, levels of miR-155 induced by killed bacteria were similar between the continuous exposure and the 2-hour exposure. These results show that a single brief exposure to live or dead *F.n.* is sufficient for inducing miR-155, but prolonged exposure to viable bacteria elicits continuously higher levels of expression.

![Graph](image)

**Figure 2.7:** Bacteria viability and prolonged contact is required for maximum miR-155 induction. 
A. PBM were infected/treated with live, paraformaldehyde-killed, or heat killed *F.n.* at an MOI of 50 for 24 hours. MiR-155 expression was assayed by qRT-PCR. B. PBM were continuously infected (Gray) or given a transient pulsed infection (Black) of live or killed *F.n.*

**MiR-155 induction by *F.n.* requires TLR signaling**

Previous studies have showed that TLR2 is critical for the recognition of *Francisella* and the production of pro-inflammatory cytokines. MyD88 is the adaptor protein required for down-stream signaling by all TLRs except TLR3 and TLR4. To test for the involvement of TLR signaling in miR-155 induction we infected BMM from WT or MyD88−/− mice with *F.n.* at an MOI of 50 for 24 hours. WT macrophages showed strong induction of miR-155, whereas the miR-155 response in MyD88−/− macrophages
was absent (Figure 2.8A). Confirming the functional absence of MyD88, we found that TNFα was not secreted by the MyD88−/− macrophages following infection (Figure 2.8B). This should be expected given that TNFα induction is MyD88-dependent107. Hence, the induction of miR-155 by F.n. is MyD88-dependent. To more specifically determine the receptor mediating miR-155 induction we tested the role of TLR2 by infecting macrophages from WT or signaling defection TLR2tm1kr/J mice. These macrophages were infected for 8 hours with F.n. at an MOI of 50 and miR-155 expression was assayed by qRT-PCR. Macrophages from WT mice induce miR-155 expression upon infection, but TLR2 signaling mutant macrophages do not (Figure 2.8C). To confirm the expression of non-functional TLR2 we conducted western blots for TLR2 itself and for down-stream signaling pathways. TLR2 is expressed in both BMM sets as expected (Figure 2.8D); however, the signaling mutant TLR2tm1kr/J macrophages do not activate PI3K/Akt, MAPK, or NF-κB pathways as evidence of the signaling defect62 (Figure 2.8E).
Figure 2.8: MiR-155 induction is dependent upon MyD88 and TLR2. A. BMMs from WT and MyD88-/- were infected with F.n. at an MOI of 50 for 24 hours. Then miR-155 expression was assayed by qRT-PCR. B. TNFα was assayed by ELISA from the media of matching samples from part A. C. BMMs from WT and TLR2 signaling mutant cells were infected with F.n. at an MOI of 50 for 8 hours, then assayed for miR-155 expression by qRT-PCR. D. WT or TLR2 mutant BMMs were checked for TLR2 expression by western blot. E. These BMMs were infected for 5, 20, 40, or 60 minutes. Cells were lysed and subject to western examining the indicated proteins.

We next tested the involvement of PI3K and the MAPKs, downstream mediators known to be activated during Francisella infection. PBM were pretreated with the following small molecule inhibitors LY294002 (PI3K inhibitor), U0126 (ERK inhibitor), SP00125 (JNK inhibitor), SB203580 (p38 inhibitor) or DMSO vehicle control for 30 minutes. After pretreatment, cells were infected with F.n. for 6 hours at an MOI of 50. PI3K inhibition completely blocked miR-155 induction (Figure 2.9A). Of the MAPKs it was ERK and JNK that were required for miR-155 induction, but p38 was dispensable. This is consistent with a report in B cells examining the mechanism of miR-155 induction by B-cell receptor activation. Given the results we can conclude that miR-155
induction in response to F.n. requires the downstream signaling through PI3K, ERK, and JNK.

It is well established that NF-κB is a critical mediator of TLR signaling as well as a myriad of other cellular responses\textsuperscript{139}. However, its role in miR-155 induction has been unclear since one report showed that it was essential\textsuperscript{123}, while another concluding that it was not\textsuperscript{138}. We then surmised that its involvement might be dependent upon the nature of the stimulus, so we examined the role of NF-κB within the context of Francisella infection. Canonical NF-κB signaling occurs when the IKK complex is phosphorylated and targets IκB for degradation. Removal of IκB allows for the transcription factor to translocate to the nucleus and initiate gene transcription\textsuperscript{140}. To test NF-κB dependency PBM were pretreated with either the IKK inhibitor BAY 11-7085 or with DMSO vehicle control for 90 minutes. Cells were then infected with F.n. at 50 MOI for 6 hours. Inhibition of NF-κB by BAY 11-7085 completely blocked the F.n.-induced miR-155 response (Figure 2.9B). As a functional readout of NF-κB inhibition, TNFα secretion was measured after F.n. infection with or without BAY 11-7085. As expected, there was no detectable TNFα release in the presence of BAY 11-7085 (Figure 2.9C). As a side note it was shown by lactate dehydrogenase (LDH) assay that the cytotoxicity was less than 10% after incubation with BAY 11-7085 for 8 hours, confirming that the cells were viable. For an alternative way to confirm the requirement for NF-κB, the peptide inhibitor Sn50 was used as in the previous experiment and found to block miR-155 induction (Figure 2.9D). Collectively, these results show that F.n.-induced miR-155 induction requires NF-κB activation.
Figure 2.9: MiR-155 induction is dependent upon PI3K, MAPK, and NF-κB. A PBM were pretreated with DMSO or inhibitor for 30 minutes, and then infected with *F.n.* at an MOI of 50 for 6 hours. MiR-155 expression was assayed by qRT-PCR. B PBM were pretreated with DMSO or IKK inhibitor for 30 minutes, and then infected with *F.n.* at an MOI of 50 for 6 hours. The expression of miR-155 was assayed by qRT-PCR. C An ELSIA was used to measure TNFα from matched media in part B. D RAW 264.7 cells were pretreated with the NF-κB inhibitor Sn50 for 30 minutes, then infected with *F.n.* at an MOI of 50 for 6 hours followed by qRT-PCR analysis for miR-155 expression.

*F.n.*-mediated miR-155 induction is independent of caspase-1 inflammasome activation

One highly studied aspect of *Francisella* infection is the ability to escape from the phagosome, because that allows the bacterium to gain access to its replicative niche. However, in the cytosol the bacterium is sensed by the inflammasome, which leads to caspase-1 processing and IL-1β release. To test for inflammasome involvement in the induction of miR-155, we infected WT or caspase-1−/− BMMs with *F.n.* at an MOI of 50 for 8 hours. Then miR-155 expression was measured by qRT-PCR. It was found that both WT and caspase-1−/− macrophages responded in a similar way (Figure 2.10A). Therefore, *F.n.*-induced miR-155 expression is caspase-1-independent. To verify lack of caspase-1 function in these BMMs, IL-1β release was measured from the media of matched samples.
from part A of this figure. Results showed that, as expected\textsuperscript{66}, IL-1β secretion by caspase-1\textasciitilde macrophages was impaired (Figure 2.10B).

**Figure 2.10: MiR-155 induction is caspase-1 independent.** A BMM from WT or caspase-1\textasciitilde mice were infected at an MOI of 50 for 8 hours. MiR-155 expression was assayed by qRT-PCR. B Supernatant from matching samples in part A was assayed by IL-1β ELISA.

*Phagosomal escape and intracellular replication is not required for miR-155 induction*

Having shown that miR-155 induction is caspase-1 independent, this highlights two issues. One being that caspase-1 is not directly required for miR-155 induction and the second that IL-1β is not an important mediator in miR-155 induction during *F.n.* infection even though that cytokine has been shown can induce miR-155 expression under certain conditions\textsuperscript{141}. However, IFNβ is produced when *F.n.* is sensed in the cytosol\textsuperscript{66,142} and it has been reported that IFNβ can induce miR-155 expression\textsuperscript{119}. To directly test the role of phagosomal escape we made use of two different phagosomal escape-defective mutants of *F.n.* The MglA transcription factor is an important regulator of the *Francisella* pathogenicity island and is required for phagosomal escape\textsuperscript{71}. Primary PBM were infected with *F.n.* or MglA\textasciitilde *F.n.* for 24 hours at an MOI of 50. MiR-155 induction as measured by qRT-PCR shows no significant difference (Figure 2.11A). Alternatively PBM were infected with *F.n.* or the quadruple acid phosphatase mutant of
*F. n.* (ABCH’), which is also defective in phagosomal escape. Again there is no difference in miR-155 induction when phagosomal escape is impaired (Figure 2.11B). Therefore, miR-155 induction is not dependent upon phagosomal escape. Despite reports that IFNβ is produced by murine macrophages once *F. n.* is sensed in the cytosol, we were unable to detect IFNβ protein production from infected monocytes and levels of this cytokine may be too low to be relevant for mediating miR-155 induction. In light of these findings it is more likely that miR-155 induction is mediated through cell surface receptor activation of TLR2 and that intracellular sensing is entirely irrelevant to the up-regulation of this miR.

![Graph](image)

**Figure 2.11:** Phagosomal escape is not required for miR-155 induction. A PBM were infected with *F. novicida* or MglA- mutant of *F. novicida* at an MOI of 50 for 24 hours. B PBM were infected with *F. novicida* or ABCH- mutant *F. novicida* at an MOI of 50 for 24 hours. MiR-155 expression was assayed by qRT-PCR.

*MiR-155 induction by Francisella does not require bacterial internalization*

Given that TLR2 activation through MyD88 was absolutely critical for miR-155 induction, yet phagosomal escape was not required, we hypothesized that cell surface contact with the bacteria is sufficient to induce miR-155 expression. To test this possibility we pretreated PBM with an actin polymerization inhibitor, cytochalasin-D (CytoD), or DMSO vehicle control for 30 minutes to inhibit phagocytosis. PBM were
then infected with *F.n.* at an MOI of 50 for 6 hours and miR-155 expression was measured by qRT-PCR (Figure 2.12A). In parallel the effectiveness of cytochalasin-D was tested by CFU assay (Figure 2.12B). Finding that miR-155 induction was comparable between vehicle control and cytochalasin-D treatments, indicates that internalization of *Francisella* was not required during the early stages of infection for a miR-155 response.

**Figure 2.12: MiR-155 induction is independent of host cell entry.** A PBM were treated with DMSO or cytochalasin-D for 30 minutes and then infected with *F.n.* at an MOI of 50 for 6 hours. MiR-155 expression was assayed by qRT-PCR. B PBM were infected in parallel to the samples from part A of this figure with or without cytochalasin-D. At 6 hours post-infection CFU assays were conducted to enumerate the number of internalize bacteria.

MiR-155 promotes pro-inflammatory cytokine production during *F.n.* infection

It was previously shown that activation of the PI3K/Akt pathway promotes the pro-inflammatory response to *F.n.*\(^{80}\), which is repressed by SHIP\(^{38}\). Consistent with this SHIP\(^{-/-}\) macrophages display enhanced pro-inflammatory cytokine production in comparison to WT macrophages infected with *F.n.* Given that miR-155 is induced in response *F.n.* infection and that miR-155 negatively regulates SHIP expression, we hypothesized that miR-155 exerts its pro-inflammatory function against *Francisella* by
repressing SHIP. Thereby more miR-155 leads to less SHIP and more enhanced PI3K/Akt signaling accompanied with increased pro-inflammatory cytokine production. To test the function of miR-155, PBM were transfected with either exon 3 of \textit{BIC} (encoding the mature miR-155) or an empty vector. 22 hours after transfection qRT-PCR analysis for miR-155 expression was conducted to determine if over-expression was effective. Indeed over-expression resulted in an approximately 3-fold increase in miR-155 compared to vector-only transfection (Figure 2.13A). The same samples were then measured for SHIP mRNA qRT-PCR, and results showed that miR-155 over-expression alone was sufficient to significantly decrease SHIP mRNA (Figure 2.13B). To determine the functional consequence of miR-155 during \textit{F.n.} infection, PBM were infected 14 hours after transfection with \textit{F.n.} at an MOI of 50 for 8 hours and the secretion of TNF\(\alpha\) (Figure 2.13C) and IL-6 (Figure 2.13D) quantified by ELISA. Both cytokines were significantly enhanced in PBM over-expressing miR-155 as compared to vector control. MiR-155 over-expression yields results similar to what was seen with SHIP\(^{-/-}\) macrophages infected with \textit{F.n.} These results are consistent with the hypothesis that miR-155 promotes pro-inflammatory cytokine production largely by down-regulating SHIP.
Figure 2.13: MiR-155 promotes pro-inflammatory cytokine production. A PBM were transfected with vector or miR-155 over-expression plasmid, then assayed for miR-155 expression 22 hours post-transfection. B RNA from part A was assayed for SHIP expression by qRT-PCR. C PBM with vector of miR-155 over-expression plasmid and 14 hours post-transfection were infected with F.n. at an MOI of 50 for 8 hours. Media from the cells was assayed by ELSIA for TNFα. D Media from part C was measured for IL-6 by ELISA.

Loss of miR-155 impairs pro-inflammatory cytokine production during F.n. infection

Since miR-155⁻/⁻ mice have been generated and were available we conducted the reverse experiment to the one done above. If miR-155 over-expression promotes pro-inflammatory cytokines, then miR-155⁻/⁻ cells should have reduced production. Here WT and miR-155⁻/⁻ BMM were assayed by qRT-PCR to confirm the loss of miR-155 expression. Indeed WT cells express basal levels of the miR, but the KO fails to show any amplification (Figure 2.14A). Next those same RNA samples were assayed by qRT-PCR for SHIP expression and when miR-155 is absent there is increased SHIP expression (Figure 2.14B). Then functional experiments were conducted with these BMMs where they were infected with F.n. at an MOI of 50 for 8 hours. The media of the
infected cells was screened for IL-6 production by ELSIA. Consistent with the role of miR-155 inhibiting SHIP expression to promote pro-inflammatory cytokine production, the miR-155\(^{-/-}\) BMM had reduced IL-6 production as compared to WT control (Figure 2.14C).

**Figure 2.14: Loss of miR-155 results in lower pro-inflammatory cytokine production.** A WT or miR-155\(^{-/-}\) BMM were assayed for miR-155 expression by qRT-PCR. B RNA samples from part A were assayed for SHIP expression by qRT-PCR. C WT and miR-155\(^{-/-}\) BMM were infected with *F. novicida* at an MOI of 50 for 8 hours. The media was assayed for IL-6 expression by ELISA.

MiR-155 inhibits intra-macrophage growth of *F. n.*

One function of miR-155 identified in this study was to promote pro-inflammatory cytokine production in response to *F. n.* However, there are potentially many other functions of this miR that influence the microbe-host interaction. Another study on the role of the PI3K/Akt pathway in *F. n.* infection by the Tridandapani laboratory showed that SHIP promotes intra-macrophage bacterial growth by inhibiting phagosomal maturation. Loss of SHIP results in reduced CFUs *in vitro* and *in vivo*\(^{127}\).
Therefore miR-155 over-expression, which reduces SHIP expression, should result in reduced intra-macrophage growth. Here we transfect RAW 264.7 murine macrophages with vector or miR-155 over-expression vector, which resulted in greater than 10-fold over-expression (Figure 2.15A). The over-expression was greater in RAW 264.7 macrophages than in PBM due to the higher transfection efficiency in RAW 264.7 cells. At 14 hours post-transfection the macrophages were infected with *F.n.* at an MOI of 50 and CFU assays were conducted. During the first hour of infection CFUs were comparable between vector and miR-155 over-expressing cells. This indicates that phagocytosis of *F.n.* was not influenced. However, at 8 and 24 hours post-transfection there are significantly few CFUs in miR-155 over-expressing cells (Figure 2.15B), thus intra-macrophage replication was repressed by miR-155. This function of miR-155 is consistent with expected results based of the earlier findings showing that Akt activation promotes improved intra-macrophage control of *F.n.*

**Figure 2.15: MiR-155 inhibits intra-macrophage bacterial growth.** A RAW 264.7 macrophages were transfected with vector or miR-155 plasmids. B 14 hours post-transfection cells were infected with *F.n.* at an MOI of 50. CFU assays were conducted at 1, 8, or 24 hours post-infection to quantify intra-macrophage growth of *F.n.*
**Virulent F.t. induces marginal levels of miR-155 and pro-inflammatory cytokines**

Here we showed that infection the low virulence F.n. induces robust miR-155 induction in monocytes/macrophages. MiR-155 promotes TNFα and IL-6 production, while also having an inhibitory effect on the intra-macrophage growth of F.n. Next we examined whether this miR-155 response was similar with the highly virulent F.t. PBM were infected with F.n. or F.t. for 24 hours at an MOI of 50. The expression of miR-155 (Figure 2.16A) and BIC (Figure 2.16B) was assayed by qRT-PCR. F.t infection led to modest miR-155 and BIC expression, yet it was significantly lower than that elicited by F.n. It is worth noting that BIC and mature miR-155 expression parallel each other, therefore, it is unlikely that BIC would interfere with miR processing as the means of preventing miR-155 expression. We have previously shown that the rates of attachment of F.n. and F.t. are comparable with human monocyctic cells. While there may be minor differences in the phagocytosis of F.n. versus F.t., earlier it was shown that the induction of miR-155 is not dependent upon internalization into host cells, so that would not account for the differential induction of miR-155 between the two subspecies. The more likely explanation is that F.t. specifically compromises the signaling pathways that mediate the induction of miR-155. Indeed the TLR\(^9,105\) and PI3K/Akt pathways\(^9,25\), which we found to be critical for miR-155 induction, are specifically impaired during F.t. infection.

MiR-155 negatively regulates SHIP and its induction is minimal following F.t. infection, so it would be predicted that F.t. infection would be less able down-regulate SHIP than during F.n. infection. We infected PBM with F.n. or F.t. for 24 hours. SHIP down-regulation was apparent with infection by F.n. but not by F.t. (Figure 2.16C). This
was repeated in THP-1 monocytes infected with either \textit{F.n.} or \textit{F.t.}, which yielded similar results (Figure 2.16D).

To determine if miR-155 suppression or lack of induction was specific to the virulent \textit{F.t.} subspecies (\textit{tularensis}, type A) used, we tested an alternative virulent \textit{F.t.} subspecies (\textit{holarctica}, type B). PBM were infected with \textit{F.n.}, virulent type A \textit{F.t.} (F.t. A) or virulent type B \textit{F.t.} (F.t. B) at an MOI of 50 for 24 hours. As expected, \textit{F.n.} induces miR-155 expression, whereas both virulent \textit{F.t.} subspecies lack clear induction of miR-155 (Figure 2.16E). This indicates that it is a general feature of the virulent subspecies of \textit{Francisella} to suppress or lack the factor to induce miR-155 expression.

\textbf{Figure 2.16: MiR-155 is minimally induced by high virulence \textit{F.t.} and lacks SHIP down-regulation.} \textsuperscript{A} PBM were infected with \textit{F.n.} or \textit{F.t.} at an MOI of 50 for 24 hours. MiR-155 expression was assayed by qRT-PCR. \textsuperscript{B} mRNA from part A was assayed by qRT-PCR for SHIP expression. \textsuperscript{C} PBM or \textsuperscript{D} THP-1 were infected with \textit{F.n.} or \textit{F.t.} at an MOI of 50 for 24 hours, then lysed and subject to western blotting for SHIP. \textsuperscript{E} PBM were infected with \textit{F.n.}, \textit{F.t.} (type A isolate), or \textit{F.t.} (type B isolate) at an MOI of 50 for 24 hours. MiR-155 expression was assayed by qRT-PCR.
To further follow-up on these findings, we examined the production of TNFα and IL-6 in response to *F.n.* and *F.t.* Both of these cytokines have been now shown to be regulated by miR-155 in this study and therefore should be produced at lower levels in response to *F.t.* than to *F.n.* Here PBM were infected at an MOI of 50 of *F.n.* or *F.t.* for 24 hours and the levels of TNFα (Figure 2.17A) and IL-6 (Figure 2.17B) were determined by ELISA. Concordantly, the production of both cytokines was significantly lower in *F.t.*-infected samples than *F.n.*-infected samples. The lack of miR-155 induction after infection with the virulent subspecies may be a key contributing factor in the lack of inflammatory response.

**Figure 2.17:** *F.t.* infected monocytes have reduced production of miR-155-dependent pro-inflammatory cytokines. PBM were infected with *F.n.* or *F.t.* at an MOI of 50 for 24 hours. The media was then screened for A TNFα or B IL-6 production by ELISA.

To summarize, we find that miR-155 is strongly induced *in vitro* and *in vivo* by *F.n.* through the TLR signaling pathway and NF-κB activation; however, this does not require intracellular sensing. Of greatest interest it was shown that infection with the highly virulent *F.t.* leads to minimal induction of miR-155 as well as reduced ability to down-regulate SHIP expression. This is one factor that contributes to the lower pro-inflammatory cytokine production elicited by *F.t.*, which may in part account for its highly virulent nature.
2.5 Discussion

This study had two major achievements. First it demonstrates that miRs are important regulators of host-response to bacterial pathogens. The role of miRs in viral pathogenesis has caught on relatively quickly\textsuperscript{143;144}, though interest in miR involvement for bacterial pathogenesis has been slow to develop. Second is that it advances the understanding of the role PI3K/Akt pathway and SHIP during \textit{Francisella} infection by showing a dynamic interaction between the host and microbe in regulating this pathway.

Here we show that miR-155 is a positive regulator of pro-inflammatory cytokine response to \textit{Francisella}. Though this miR has also emerged as an important player in many other processes that include cancer\textsuperscript{134}, innate immune response\textsuperscript{119}, adaptive immune response\textsuperscript{145}, physiology\textsuperscript{125}, and developmental disabilities\textsuperscript{146}. With the wide range of cellular processes and diseases regulated by this miR, it is of paramount interest to understand its function.

With regard to host-response, the first study on pathogen-induced miR-155 expression was done with the avian leukosis virus (ALV). This showed that this virus induced high levels of \textit{BIC} expression in infected chickens and that this was highly correlated with B-cell lymphoma\textsuperscript{131;132}. More recent studies on miR-155 and viruses have found that multiple viruses encode functional orthologs of miR-155\textsuperscript{124;147}. This implies that miR-155 expression may actually benefit certain viruses. The oncogenic potential of this miR could be exploited by viruses to promote host-cell survival. Another possibility, which has been supported, is that miR-155 helps maintain latent viral genomes through the negative regulation of TRIF signaling by miR-155 inhibition of IKK\textgreek{e}\textsuperscript{148}. 
On the host side, it has been shown that deletion of miR-155 leads to system-wide changes in immune function\textsuperscript{149-151}. Lymphocyte responses are compromised in mice for which miR-155 has been genetically deleted. When miR-155\textsuperscript{-/-} mice are vaccinated with tetanus toxin fragment C there is overall reduced IgM and switched antigen-specific antibody production due to defects in B-cell function. MiR-155\textsuperscript{-/-} T cells show impaired IL-2 and IFNγ production after immunization\textsuperscript{149} and they exhibit a bias toward a Th2 response\textsuperscript{150}. Antigen presentation is affected, as miR-155\textsuperscript{-/-} dendritic cells were less able to activate T cells expressing a transgenic receptor for ovalbumin in the presence of that protein\textsuperscript{149}. It is quite astonishing that a single miR serves such an essential role in multiple immune cell types.

This chapter examined the role of miR-155 in monocytes and macrophages within the context of host response to bacterial pathogens. Previous studies by the Tridandapani laboratory have shown that PI3K/Akt pathway is host-protective against \textit{Francisella} infection\textsuperscript{80} and SHIP negatively regulates this response\textsuperscript{38}. Due to the importance of this pathway we were interested in the intricacies of SHIP regulation during \textit{Francisella} infection. Because there was such a dramatic down-regulation of SHIP during \textit{F.n.} infection and there were no clear mechanisms to account for this at the start of the study, we examined the possibility that miRs may were playing a role. It was encouraging that we found that monocytes and macrophages induce miR-155 in response to \textit{F.n.} infection and that this miR down-regulates SHIP. During the original preparation of this manuscript it was also reported by O’Connell \textit{et al.} that miR-155 can directly target SHIP\textsuperscript{120}. The data in this chapter is consistent with their findings, so the importance of miR-155 induction in response to \textit{Francisella} is well supported. Also mice over-
expressing miR-155 develop a similar myeloproliferative phenotype\textsuperscript{152} to that of SHIP\textsuperscript{−/−} mice\textsuperscript{33}, which gives further confidence as to the connection of SHIP and miR-155.

Multiple TLR ligands are capable of inducing miR-155\textsuperscript{119}, and given that the host-response to \textit{Francisella} is almost exclusively dependent upon TLR2 it is logical that we find miR-155 induction is TLR2/MyD88-dependent. This finding is interesting because while there are many cell surface receptors that are engaged and that are of importance in the host response to \textit{Francisella}\textsuperscript{64,65}, it appears that TLR2/MyD88 is of absolute importance for miR-155 induction. Signaling transduction through ERK and JNK were required for miR-155 induction, which is in agreement with the finding by Yin \textit{et al.}, which showed of ERK- and JNK-dependent BCR induction of miR-155\textsuperscript{138}. While in contrast to Yin \textit{et al.}\textsuperscript{138} but in agreement with Gatto \textit{et al.}\textsuperscript{123}, who used a viral protein to induce miR-155 in B-cells, we found that activation of NF-κB was absolutely required for miR-155 induction by \textit{Francisella}. This point will be further addressed in chapter three. However, it is fitting that inhibition of PI3K blocked miR-155 induction, as PI3K controls NF-κB activation in response to \textit{Francisella}\textsuperscript{80}. It appears as though cell type, cellular context and/or the nature of stimulus may affect the intracellular machinery that controls miR-155 expression.

TNF\textsubscript{α} and IFN\textsubscript{β} are two cytokines produced during \textit{Francisella} infection that can potentially contribute to miR-155 induction. Though it has already been shown that these cytokines work through the TNF\textsubscript{α} receptor, but direct TLR stimulation can induce miR-155 independently of TNFR. Therefore it seems likely that autocrine / paracrine signaling not required. This is likely the case with \textit{Francisella}, as it has been shown to activate TLR2\textsuperscript{62}. While cytosolic sensing of \textit{Francisella} by the inflammasome triggers multiple
host response events\textsuperscript{67}. Finding that bacterial internalization, phagosomal escape, and caspase-1 are not required for miR-155 induction, we conclude that this host response is predominately driven by cell surface TLR2 signaling. This is another issue that will be addressed in chapter three.

The most interesting finding of this chapter was that the low virulence \textit{F.n.} strongly induces miR-155 and down-regulate SHIP, whereas the highly virulent \textit{F.t.} elicits minimal miR-155 response and an inability to down-regulate SHIP. This is the first report to demonstrate differentially regulation of a single miR between two bacteria of different subspecies while also inversely correlating with virulence. However, there is not a general trend of reduced miR expression in \textit{F.t.} infected cells. We have unpublished data showing that there are at least four miRs with expression that is inversely related to miR-155 during \textit{F.n.} or \textit{F.t.} infection. There is also one miR that is equally induced by \textit{F.n.} and \textit{F.t.} (data not shown), thus there is specificity in the miR response between these different subspecies of \textit{Francisella}. While we are still exploring the mechanism by which \textit{F.t.} prevents miR-155 induction, we do know that \textit{F.t.} preferentially down-regulates both TLR2 and components of the PI3K/Akt pathway, whereas \textit{F.n.} does not. Therefore, the mechanism to prevent miR-155 induction may be as simple as disruption of these signaling components, which we show are required for miR-155 induction.

The current study shows that miR-155 positively regulates TNF\textgreek{a} and IL-6 production in response to \textit{Francisella}. Given that miR-155 has been shown to target the negative regulator SHIP and that this phosphatase negatively regulates NF-\textgreek{xB} activation and pro-inflammatory cytokine production, we conclude that miR-155 is pro-inflammatory within the context of \textit{Francisella} infection. While PI3K/Akt and SHIP have
been shown to have varying effects on the pro-inflammatory response\textsuperscript{20}, it has already been shown that SHIP negatively regulate neutrophil responses to TLR2 activation\textsuperscript{39}, which is what is used by \textit{Francisella}. While this study highlights the connection between miR-155 inhibition of SHIP and the pro-inflammatory cytokine response, there are other relevant targets of miR-155 that many be important. MiR-155 has been shown to target SOCS\textsuperscript{153}, which also inhibits the inflammatory response\textsuperscript{154}. It has also been suggested that miR-155 may regulate TNF\textalpha mRNA stability to promote its production, yet that was never experimentally tested\textsuperscript{155}. Therefore there may be many mechanisms by which miR-155 promotes the inflammatory response. One final point is that there may actually be conditions where miR-155 serves to antagonize the pro-inflammatory response\textsuperscript{156}, which may be due to the nature of the stimulus or cell type. Chapter five will revisit this point.

In conclusion, this chapter identifies a new aspect in the regulation of the PI3K/Akt pathway for host-defense against \textit{Francisella}. We see that miR-155 is highly induced by \textit{F.n.} to enhance the host’s ability to respond to infection by inhibiting SHIP. The induction of miR-155 occurs by TLR2/MyD88 signaling to NF-\kappa B. MiR-155 promotes pro-inflammatory cytokine production and inhibits intra-macrophage bacterial growth. Lastly, we find that this miR is differential induced by infection with different \textit{Francisella} subspecies. The expression of miR-155 inversely correlates with virulence and this implies that differences in miR responses may at least partially account for the highly pathogenic nature of \textit{F.t.} versus \textit{F.n.}
Chapter 3: MiR-155 Induction by *Francisella* Occurs via Soluble Bacterial Factors and Requires de novo Host Protein Synthesis

3.1 Abstract

The previous chapter showed that miR-155 negatively regulates SHIP to promote favorable host-response against *Francisella*. Many essential mediators of miR-155 induction during *F.n.* infection were identified. However, additional aspects of miR-155 regulation remain to be fully understood. This miR regulates numerous aspects of immune response, physiology, and cancer, thus it is important for many cellular processes. It is known that this miR is regulated by toll-like receptor ligands, cytokines, microbial infection, and even nutrient deficiency. Here is a detailed examination of how miR-155 is regulated during *F.n.* infection. There are two key findings in this study; first miR-155 requires indirect NF-κB signaling to up-regulate fos/jun transcription factors to drive AP-1 transcription of the BIC gene. Second miR-155 can be induced through soluble factors that originate from *Francisella*, but not the host. This study advances the understanding of the complex regulation of miR-155 during microbial infection.
3.2 Introduction

MicroRNAs (miRs) are modulators of gene expression and generally function to fine tune protein expression, as opposed to mediating dramatic shifts in expression\textsuperscript{157}. However, their dysregulation has been shown to play a critical role in numerous pathogenic conditions\textsuperscript{158-161}. A great deal of the work regarding miR regulation and function has been done within the context of cancer or developmental biology\textsuperscript{43}; however, miRs are poised to make a major contributions to the field of microbial pathogenesis. Indeed groups studying viral pathogenesis have embraced miRs as key regulators of host processes that influence immune response and thus microbe persistence. Despite the intriguing nature of miRs there have been few studies examining the role of miRs in bacterial pathogenesis\textsuperscript{53,122}.

There are several miRs that have been identified to be responsive to inflammatory stimuli and that also regulate different aspects of the immune system\textsuperscript{46}. Of those miRs, MiR-155 has received a great deal of attention because of its connection to cancer\textsuperscript{134} and role as a regulator of inflammation\textsuperscript{162}. This miR originates from a non-protein coding gene known as BIC\textsuperscript{133}. BIC mRNA and processed mature miR-155 have been shown to be induced by TLR ligands, interferon, and pro-inflammatory cytokines\textsuperscript{119}. But most importantly this miR has been shown to be required for normal innate and adaptive immune responses\textsuperscript{149,155}. Thus there is the need to understand the regulation and function of miR-155, particularly within the context of microbial infections.

The previous chapter showed that miR-155 is induced by and regulates the host-response to the intracellular Gram-negative bacterium \textit{Francisella tularensis}, which is the causative agent of the zoonotic disease known as Tularemia\textsuperscript{113}. Most importantly there
were differences in miR-155 induction depending upon the subspecies used to infect monocytes. It was *F.t.*, which is regarded as having the highest virulence in humans that gave minimal to no induction of miR-155, whereas *F.n.*, which rarely causes disease in humans, gave strong induction of this miR. This is quite interesting because miR-155 is regulated by a diverse number of stimuli\(^{119;123;125;141;163;164}\), yet here we have found a pathogen that potentially suppresses the expression of this miR. By studying the mechanisms of miR-155 induction during *Francisella* we can gain insight into how this miR may be negatively regulated.

To reiterate the main points on from the previous chapter, we found that miR-155 negatively regulated the phosphatase SHIP, which limits Akt activation. Within the context of *Francisella* infection Akt promotes NF-κB activity, thus enhancing the production of pro-inflammatory cytokines. Akt also promotes phagosome maturation, which may be due to the effect on NF-κB, because activation of this transcription factor has been reported to promote phagosome-lysosome fusion\(^ {165}\). Increase phagosome maturation will prevent the bacterium from escaping and inducing caspase-3 dependent macrophage cell death\(^ {127}\). It is important to mention that caspase-3 cell death is a major factor in the pathogenesis of *F.t.* infection as opposed to capase-1 cell death\(^ {166}\). The ability of Akt to increase resistance against an *in vivo Francisella* challenge is likely due to the production of pro-inflammatory mediators and increased macrophage killing of bacteria. We conclude that, miR-155 induction benefits the host by promoting the activation of Akt through the inhibition of SHIP.

What we previously learned about the mechanism of miR-155 induction was that cell surface signaling through TLR2 and MyD88 was required, as well as down-stream
signaling through ERK, JNK, and PI3K. Finally there was a strong dependence on NF-κB. In this chapter we uncover new details about the mechanism through which miR-155 is induced. Here we find that miR-155 induction requires new host-protein synthesis and thus is not directly induced by infection. Coupled with this we find the conditioned media from infected cells can induce miR-155 expression; thus there are soluble factors can mediate miR-155 induction. This presented the likely explanation that the host produces an autocrine/paracrine acting factor to induce miR-155. Yet surprisingly we find that the factor which induces miR-155 originates from the bacterium and not the host cell. To reconcile the requirement for new host protein synthesis we looked at the possibility of an NF-κB dependent transcription factor being required to directly promote BIC/miR-155 transcription. Indeed Fos and Jun up-regulation occurred through an NF-κB-dependent mechanism to promote miR-155 induction through AP-1 driven transcription. This chapter provides a more comprehensive picture of how miR-155 is regulated during Francisella infection.

3.3 Materials and Methods

Cells and reagents. RAW 264.7 cells were obtained from ATCC and cultured in RPMI-1640 (Gibco-BRL, Rockville, MD) which was supplemented with 5% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) and L-glutamine (Invitrogen, Carlsbad, CA) as done before. The BAY-7085-11 IKK inhibitor (5μM) was provided by Dr. Denis Guttridge (The Ohio State University) and was dissolved in DMSO. The ERK inhibitor UO-126 was obtained from Calbiochem. DMSO vehicle control (0.2%) was obtained from Sigma-Aldrich (St. Louis, MO). Cycloheximide was purchased from
Sigma-Aldrich (St. Louis, MO) and dissolved in sterile endotoxin-free water. Brefeldin A in DMSO was obtained from BioLegend (San Diego, CA) and used as recommended by the manufacturer. Brefeldin A is reported to have some antimicrobial activity, so we used CFU assays to determine if the concentration of Brefeldin A was microbicidal to F.n. We found no effect on Colony Forming Units at the concentration used in the experiments with up to 20 hours of exposure. Recombinant human IFNβ was obtained from PBL Interferon Source (Piscataway, NJ). The passive lysis buffer for lucifearse assays and the luciferase assay reagent were obtained from Promega (Madison, WI). Sterile filtering units of 0.22μm were obtained from Millipore (Bedford, MA). SiRNA against c-Fos (sc-44200) was purchased from Santa Cruz Biotechnology (Santa Cruz, SD).

**Antibodies.** Phosphorylated-Stat1, c-Fos and c-Jun antibodies were purchased from Cell Signaling (Beverly, Massachusetts). The actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Neutralizing antibodies to human TNFα were obtained from R&D systems (Minneapolis, MN). Neutralizing antibodies to human IFNβ were obtained from PBL Interferon Source (Piscataway, NJ) and were pre-incubated with samples before adding to cell culture as recommended by the manufacture.

**Peripheral blood monocyte isolation.** Human peripheral blood monocytes (PBM) were isolated from leuckopacks using centrifugation through a Ficoll gradient followed by CD14-positive selection by Magnet-Assisted Cell Sorting (MACS, Miltenyi Biotec, Auburn, CA) as done earlier⁹. Purity was greater than 98% CD14⁺.
Bacterial infections. *F. novicida* U112 (JSG1819) was provided by Dr. John Gunn (The Ohio State University), and grown on Chocolate II agar plates (Becton, Dickinson and Company, Sparks, MD). For infection bacteria was re-suspended in RPMI1640 culture media without antibiotic. The culture was quantified by a spectrometer at 600nm wave length.

Quantitative real-time RT-PCR. At the time of collection all media was removed and cells were lysed in TRIzol® reagent (Invitrogen, Carlsbad, CA) for a minimum of 15 minutes. Phenol/cholorform RNA isolation was completed according to the manufacturer’s instructions. Reverse transcription to generate cDNA used 10 to 100 ng of total RNA. Mature human or mouse miR-155 expression was assayed with TaqMan® Universal PCR Master Mix, No AmpEraseH UNG (Applied Biosystems, Branchburg, NJ) by qRT-PCR and normalized to an internal control; RNU44 for human and sno412 for mouse (Applied Biosystems, Foster City, CA) as previously described. In this chapter miR expression data is presented as relative expression=$2^{\Delta(CT_{miR-155}-CT_{House Keeping Gene})}$.

Relative copy number (RCN) for human TNFα mRNA was calculated as previously described and primed for cDNA synthesis with 0.8 nM random hexamer (Applied Biosystems, Foster City, CA).

Western blot analysis. At the time of collection all media was removed and cells were lysed in TN1 buffer (50mM Tris [pH 8.0], 10 nm EDTA, 10M Na₄P₂O₇, 10 nM NaF, 1% Triton-X 100, 125nM NaCl, 10nM Na₃VO₄, 10 μg/ml of both aprotinin and leupeptin. Protein matched whole cell lysates were electrophoretically separated on 10% acrylamide
gels. After separation the proteins were transferred to nitrocellulose membranes, and then probed with antibody of interest. Detections were performed with HRP-conjugated secondary antibodies followed by development with enhanced chemiluminescence western blotting substrate (Pierce, Rockford, IL) as previously described.

Transfections. Due to low transfection efficiency in PBMs, roughly 20%, RAW 264.7 macrophages were used for all transfection studies. 10-12x10^6 RAW 264.7 cells were transfected by electroporation in Amaxa solution V with program U-14 as previously described. 2μg of AP-1 luciferase reporter was used for each transfection. 4.0μM of siRNA against c-Fos was used for knock-down experiments.

3.4 Results
MiR-155 induction by Francisella requires new host-protein synthesis

A relatively straight forward, but informative experiment to conduct was to determine if miR-155 induction was a direct effect or an indirect effect of infection with F.n. Here we made use of the eukaryotic protein synthesis inhibitor cycloheximide. PBM were infected with F.n. at an MOI of 50 for six hours in the presence or absence of cycloheximide. We found that the presence of cycloheximide significantly impaired miR-155 induction (Figure 3.1A), which is somewhat surprising since the BIC gene has an NF-κB site. To verify the effectiveness of cycloheximide at specifically blocking host protein synthesis we examined the mRNA and protein production of a direct response gene, TNFα. As expected TNFα mRNA was induced equally well in the presence or absence of cycloheximide (Figure 3.1B). This is also important because it shows that NF-
κB function was not fundamentally disrupted in the presence of cycloheximide.

Importantly, only the protein production was significantly reduced in the presence of cycloheximide (Figure 3.1C). These controls verify that cycloheximide only blocked host-protein synthesis and not the mRNA induction of a direct response gene to *F.n.* infection.

![Graph A](image1)

**Figure 3.1**: MiR-155 induction requires new host protein synthesis. A PBM were pre-treated with cycloheximide or vehicle control, and then infected with *F. novicida* at an MOI of 50 for 6 hours. MiR-155 expression was assayed by qRT-PCR and represented as relative expression. B TNFα mRNA was measured by qRT-PCR from Figure 3.1 samples and represented as relative copy number. C TNFα protein was measured by ELISA from the samples in Figure 3.1. Graphs represent average and standard deviation of triplicate samples.

*MiR-155 induction can be mediated through soluble secreted factors*

The previous experiment found that miR-155 induction required host protein synthesis and there are at least two possible reasons that host-protein synthesis might be required; the first is that miR-155 is induced through a host autocrine/paracrine acting...
factor and the second is that a transcription factor that is not basally expressed must be produced to mediate BIC/miR-155 transcription. So next we tested to see if soluble factors present during the course of infection can mediate the induction of miR-155. Here PBM were infected at an MOI of 50 for 24 hours. From that first set of cells RNA was collected to assay miR-155 expression and the media from those cells was also collected. That conditioned media was put through a sterile filtering device to remove bacteria, and then mixed at a proportion of 1:1 with fresh culture media. This was then used to culture new PBM for a 24 hour period for which RNA was then collect from. Removal of bacteria from the conditioned media was validated by plating a sample of the filtered media on chocolate agar plates. No growth was observed by 48 hours post plating. MiR-155 expression from the two sets of PBM, directly infected cells or cell treated with conditioned media (CM), was assayed by qRT-PCR (Figure 3.2A). MiR-155 expression was induced by factors present in the conditioned media of infected cells even with the absence of bacteria present. Therefore miR-155 induction by host cells does not require direct contact with the bacterium and can be mediated through soluble factors. It is important to note that this method does not discriminate host factors from bacterial factors.

Earlier we reported that miR-155 induction is NF-κB-dependent and under different conditions it has been reported that there is a requirement for NF-κB activity in the regulation of miR-155\textsuperscript{123}. Having shown that miR-155 can be induced through conditioned media of infected cells, we next tested to see if the induction of miR-155 through conditioned media was NF-κB-dependent or if this stage of response is NF-κB-independent. This experiment started by infecting PBM with \textit{F.n.} at an MOI of 50 of 24
hours, and then the media from those cells was collected and sterile filtered to generate conditioned media. Fresh PBM were then infected directly with *F.n.* at an MOI of 50 for 6 hours or cultured in the conditioned media for 6 hours. Prior to infection/stimulation the PBM were pretreated with DMSO or IKK/NF-κB inhibitor BAY 11-7085. RNA from these PBM isolated and assayed for miR-155 expression by qRT-PCR (Figure 3.2B). While miR-155 is again induced by soluble factors from conditioned media we find that regardless of direct infection or conditioned media stimulation there is a requirement for NF-κB to induce miR-155 expression.

![Figure 3.2: Soluble factors can induce miR-155 expression in a NF-κB-dependent mechanism.](image)

*Figure 3.2: Soluble factors can induce miR-155 expression in a NF-κB-dependent mechanism.* A PBM were infected with *F. novicida* at an MOI of 50 for 24 hours (R & Fn), then the conditioned media (CM) was sterile filtered mixed 1:1 with fresh media and used to culture fresh PBM for 24 hours (CM R & CM Fn). MiR-155 expression was assayed by qRT-PCR. B Conditioned media from infected cells was generated as in Figure 3.2A. PBM were pre-treated with DMSO or BAY 11-7085, and then infected with *F. novicida* at an MOI of 50 or sterile filtered conditioned media for 6 hours. MiR-155 expression was assayed by qRT-PCR.

**TNFa and IFNb are not major mediators of Francisella induced miR-155 expression**

The first logical explanation for miR-155 requiring new synthesis by the host and the ability for the induction to be transmitted through conditioned media was that miR-155 induction was mediated by a cytokine. This makes sense because it has been shown
that TNFα, IFNβ, and IFNγ can induce miR-155119. Its already been shown that macrophages produce TNFα105 and IFNβ66 during *Francisella* infection. Therefore, we used neutralizing antibodies against both cytokines to determine if either cytokine was required for miR-155 induction. To address the role of TNFα, PBM were infected at an MOI of 50 for 6 hours with *F.n.* alone, *F.n.* with TNFα neutralizing antibody, or *F.n.* with isotype control antibody. RNA was collected to assay miR-155 expression by qRT-PCR and the data shows that neutralizing TNFα does not block or even slightly impair miR-155 induction (Figure 3.3A). To roughly gauge if the neutralizing antibody was binding and blocking TNFα, an ELISA for TNFα was run on the media from figure 3.3A. There is an effect of the neutralizing antibody on blocking the ability of TNFα to be recognized by ELISA, yet the isotype antibody has effect on TNFα levels (Figure 3B).

**Figure 3.3: TNFα is not required for *F.n.*-induced miR-155 expression.** A PBM were untreated or pre-treated with TNFα neutralizing antibody or isotype control antibody, then infected with *F.n.* at an MOI of 50 for 6 hours. RNA was collected and assayed for miR-155 expression. B The matching media samples from part A were assayed for human TNFα ELISA.
Having excluded TNFα, we next examined a role for IFNβ in *F. n.*-induced miR-155 expression. Due to technical issues, for the neutralizing antibody against IFNβ to be effective, media samples containing IFNβ had to be pre-incubated with the neutralizing antibody for one hour as recommended by the manufacturer. Thus we had to make use of conditioned media, which was obtained as in the same way it was done in the earlier experiments. After culturing uninfected or infected (MOI of 50) PBM for 24 hours, the media was sterile filtered to remove bacteria. New PBM then received the conditioned media (CM:R or CM:Fn) pre-incubated with IFNβ neutralizing antibody or isotype control. Direct infected PBM (R or Fn) were included as a reference. These samples were infected/stimulated for 6 hours. RNA was isolated and the samples were assayed for miR-155 expression. The results show that neutralization of IFNβ did not impair miR-155 induction as compared to infection only samples or isotype control antibody infected samples (Figure 3.4A). To verify that the IFNβ neutralizing antibody was effective, PBM were treated with recombinant IFNβ alone, IFNβ with neutralizing antibody, or IFNβ with isotype control antibody. PBM were exposed to those conditions for 15 minutes and then lysed. Cell lysates were probed for phosphorylated STAT1, which is activated IFNβ. Under these conditions the neutralizing antibody blocked STAT1 phosphorylation (Figure 3.4B) indicating effective neutralization of IFNβ.

While TNFα and IFNβ have been shown to induce miR-155, we find that during *F. n.*-induced miR-155 expression neither cytokine plays a critical role in mediating the effect. Additional experiments conducted below will support this finding that host-cytokines are of marginal importance in mediating miR-155 induction. Furthermore the
levels at which these cytokines are produced during infection are likely not sufficient to contribute to the induction of miR-155.

**Figure 3.4: IFNβ is not required for *F. n.*-induced miR-155 expression.**

A Conditioned media from PBM that were uninfected (CM:R) or infected (CM:Fn) with *F. n.* at an MOI of 50 for 24 hours was sterile filtered and incubated with neutralizing antibody or isotype control antibody at 37°C for one hour to allow binding. Fresh PBM were then cultured in the four CM conditions shown above or direct infection (R and Fn) as a reference point. RNA was collected and miR-155 expression was assayed by qRT-PCR. B Neutralization of IFNβ was verified by pre-treating media with recombinant IFNβ (500 U/ml) with or without neutralizing antibody or isotype control antibody for one hour. PBM were exposed to media alone, IFNβ, IFNβ plus neutraling antibody, or IFNβ plus isotype control for 15 minutes. Cells were lysed and subject to western blot for phosphorylated STAT1, followed by actin re-probe.

**Cytokine release is not required for miR-155 induction**

With the two prime candidates, TNFα and IFNβ, of the soluble secreted factors that induce miR-155 expression excluded, we explored the possibility that autocrine/paracrine acting cytokines are of marginal importance in miR-155 induction by *F. n.* While we know that miR-155 induction can be mediated through the conditioned media of infected cells, it is possible that the primary factor that induces the expression originates from the bacterium, perhaps a soluble TLR ligand, as opposed to a host produced factor. The first experiment tested to see if cytokine release was required for
miR-155 induction. This was done by using the exocytosis inhibitor, brefeldin A, which is commonly used for intracellular cytokine staining assays and prevents cytokine release\textsuperscript{168}. PBM were pretreated with the vehicle control DMSO or brefeldin A for 30 minutes. These cells were then infected with \textit{F.n.} at an MOI of 50 for 16 hours. RNA was extracted from these samples and miR-155 expression was assayed by qRT-PCR. The data indicates that inhibition of cytokine release does not impede miR-155 induction (Figure 3.5A). To provide confidence in these findings we included controls showing that demonstrating that brefeldin A treated PBM had not only impaired cytokine release, but also promoted accumulation of these cytokines within the cell. The extracellular levels of TNF\textalpha are significantly reduced in the presence of brefeldin A (Figure 3.5B), though the intracellular levels of TNF\textalpha are significantly increased in the presence of brefeldin A (Figure 3.5C).
Figure 3.5: Cytokine release is not required for miR-155 induction by F.n. ^PBM were treated with DMSO or brefeldin A, then infected with F.n. at an MOI of 50 for 16 hours. RNA was then isolated and assayed for miR-155 expression by qRT-PCR. B-C The media from additional PBM samples run in parallel was collected as well as the cell lysate from the matched samples. Both the Bmedia and Ccell lysate were assayed for TNFα production by ELISA.

An alternative way to examine the role of host cytokines in F.n.-induced miR-155 expression was to test the ability conditioned media from responsive versus non-responsive cells to induce miR-155. In the absence of TLR2 or MyD88 the production of pro-inflammatory cytokines in response to F.n. infection is essentially eliminated.62,107 We already know that MyD88−/− macrophages do not produce pro-inflammatory cytokines or induce miR-155 expression from chapter two. Therefore the conditioned media from MyD88−/− BMMs will lack host pro-inflammatory cytokines, yet contain bacterial factors at comparable levels to the conditioned media of WT BMMs. So if WT cells respond equally well to the conditioned media from WT or MyD88−/− infected BMM, then the soluble factor most likely originates from the bacterium since the miR is induced even though host factors are lacking in the MyD88−/− conditioned media. For this
experiment BMMs from WT and MyD88\textsuperscript{-/-} mice were uninfected or infected with \textit{F.n.} at an MOI of 50 for 24 hours. This generated conditioned media that was then sterile filtered and used to stimulate new WT macrophages. RNA was obtained from the original cells that were used to generate the conditioned media as well as the WT macrophages that were treated with the two types of conditioned media. The results (Figure 3.6) show that WT macrophages infected with \textit{F.n.} (Bars 1, 2) or the conditioned media of WT macrophages (Bars 3, 4) have a strong induction of miR-155 as expected and seen in figure 3.2A. However, WT macrophages treated with the conditioned media of infected MyD88\textsuperscript{-/-} macrophages also have a strong induction of miR-155 (Bars 5, 6), despite the fact that the conditioned media from the MyD88\textsuperscript{-/-} macrophages is absent of pro-inflammatory cytokines. Finally as seen before in figure 2.8A, the MyD88\textsuperscript{-/-} macrophages do not respond to \textit{F.n.} infection (Bars 7, 8). This experiment dissociates host inflammatory cytokines from the ability to induce miR-155 expression.
**Figure 3.6: MyD88-dependent cytokine production is not required for miR-155 induction.** WT (bars 1&2) or MyD88−/− (bars 7&8) BMM were infected at an MOI of 50 for 24 hours and assayed for miR-155 expression. The media from those two cell types was sterile filtered to generate conditioned media (CM). New WT BMMs were cultured in the conditioned media from WT (bars 3&4) or MyD88−/− (bars 5&6) cells for 24 hours, and then RNA was isolated to assay miR-155 expression.

The conditioned media experiments above indicate that host cytokine production is not critical for miR-155 induction. An important point to remember is that soluble bacterial factor will be transferred through conditioned media in addition to the host-produced cytokines. In light of this, we next examined if soluble factors from bacteria alone can induce miR-155 expression. Here *F. n.* was cultured in RPMI-1640 for 24 hours at a density of 3.5x10^8 bacteria / ml to generate a new type of conditioned media. That density of bacteria is comparable to an MOI of 50 for 5x10^6 cells in 1ml, which is used in the standard infection. This media was sterile filtered to remove bacteria, but retains factors shed by the bacteria. So this media only contains soluble bacterial factors and no soluble host factors. PBM were directly infected with *F. n.* at an MOI of 50 or cultured with is bacterial conditioned media for 24 hours. MiR-155 expression was highly induced with direct infection and with the sterile conditioned media containing soluble bacterial
products (Figure 3.7). Collectively based on this series of experiments we conclude that miR-155 expression can be induced through filterable factors of bacterial origin and not host origin.

**Figure 3.7: Soluble bacterial factors are sufficient to induce miR-155 expression.** Bacterial conditioned media (CM) was generated by culturing *F. n.* in cell culture media at a concentration of $3.5 \times 10^8$ bacteria / ml for 24 hours, which was then sterile filtered. PBM were infected with *F. n.* at an MOI of 50 for 24 hours (R & Fn) or stimulated with the conditioned media for 24 hours. RNA was extracted from these samples and assayed for miR-155 expression by qRT-PCR.

**MiR-155 induction is mediated through NF-κB-dependent up-regulation of fos and jun**

Thus far we find that miR-155 induction is mediated through soluble bacterial factors and not host-produced factors, which answers one question. However, there still remained the question as to what was the newly synthesized protein which is required for miR-155 induction. Since the newly produced protein is not a secreted factor, it is quite possible that it is an inducible transcription factor. It is known that Fos and Jun transcription factors act at AP-1 sites and their expression is transiently induced in response to inflammatory stimuli. A detailed study of the BIC/miR-155 promoter showed that there are AP-1, NF-κB, and Ets sites. Though only the AP-1 site was found to be critical for BIC/miR-155 transcription. It was shown that MAPK
phosphorylation/activation was required for mediating miR-155 induction, yet NF-κB activity was never examined\textsuperscript{138}. This is in agreement with our data because while we find that there is a requirement for NF-κB activation, its activation alone is not sufficient for miR-155 induction. Therefore, it is conceivable that the activation of NF-κB may be up-regulating AP-1 transcription factors that directly regulate BIC/miR-155 transcription. In fact there is evidence that NF-κB regulates c-Fos expression\textsuperscript{172}.

To test this hypothesis we examined c-Fos and c-Jun expression in PBM that were infected with \textit{F.n.} at an MOI of 50 for 4 hours in the presence or absence of the NF-κB inhibitor (BAY 11-7085). Notably the expression of both c-Fos (Figure 3.8A) and c-Jun (Figure 3.8B) were almost undetectable by western blot under resting conditions, yet both are strongly up-regulated by infection. Additionally the up-regulation of these transcription factors is NF-κB-dependent, and would also be blocked by cycloheximide as they are newly synthesized proteins. This accounts for the dependence of miR-155 induction on new host-protein synthesis and indirect NF-κB activity.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.8.png}
\caption{Fos and Jun transcription factors are induced during \textit{F.n.} infection through an NF-κB dependent mechanism. A-B PBM were pretreated with DMSO vehicle control of BAY 11-7085 IKK/NF-κB inhibitor and were then infected with \textit{F.n.} at an MOI of 50 for four hours. Cells were lysed and subject to western blotting for A c-Fos or B c-Jun expression.}
\end{figure}
Having shown that the expression of AP-1 transcription factors is NF-κB-dependent, we next examined if AP-1 activity as measured by a luciferase reporter was also NF-κB-dependent. Macrophages were transiently transfected with the AP-1 luciferase reporter and 14 hours post-transfection these cells were infected with *F.n.* at an MOI of 50 for 1, 4, 8, or 24 hours. Samples were collected and assayed for luciferase activity (Figure 3.9A). On a purely observational note, the pattern of AP-1 activity induced by *F.n.* infection matches the induction of miR-155 as shown in Figure 2.5A. To test if AP-1 activity has a dependence on NF-κB, macrophages expressing the AP-1 reporter were pretreated with vehicle control, IKK/NF-κB inhibitor (BAY), and ERK inhibitor (UO) then infected with *F.n.* at an MOI of 50 for eight hours. Cell lysates were then assayed for luciferase activity and reported as % increase over resting control for the three separate conditions. ERK was previously shown to be required for Fos/Jun up-regulation and AP-1 activity\(^{138}\), so we used the ERK inhibitor UO126 as a positive control and it significantly inhibited AP-1-driven luciferase activity (Figure 3.9B). But additionally we see that inhibition of NF-κB by BAY 11-7085 also inhibits AP-1 activity, which would be expected since the induction of AP-1 transcription factors was also NF-κB-dependent.
**Figure 3.9: F.n. infection induces AP-1 transcription through NF-κB.** A RAW 264.7 cells were transfected with AP-1 luciferase construct. 14 hours post-transfection cells were infected with F.n. at an MOI of 50 for the indicated time points. Cell lysates were collected and tested by luciferase assay. B As in part A, macrophages were transfected with AP-1 luciferase construct. 14 hours post-transfection cells were treated with DMSO, BAY 11-7085, or UO126. Then cells were infected with F.n. at an MOI of 50 for eight hours.

Lastly, to directly show the connection between AP-1 transcription factors and miR-155 induction we used a siRNA approach to knock-down c-Fos expression. Here macrophages were transfected with siRNA control or c-Fos siRNA. One hour post-transfection the cells were infected with F.n. at an MOI of 50. At four hours post-infection samples were collected for lysates to demonstrate knock-down of inducible c-Fos expression (Figure 3.10A). Then at 16 hours post-transfection the remaining cells were collected and assayed for miR-155 expression. Control macrophages induce miR-155 expression as seen before, yet when c-Fos is knocked-down the induction of miR-155 is greatly impaired (Figure 3.10B). Therefore we conclude that NF-κB indirectly regulates miR-155 induction through the up-regulation of AP-1 transcription factors and activity.
3.5 Discussion

It has been known that miR-155 is induced by TLR ligands, interferon, and pro-inflammatory cytokines\(^{119}\). The previous chapter showed clearly that miR-155 induction required TLR2 and MyD88 signaling during \textit{Francisella} infection, which is logical and somewhat expected. However, there were intricacies in the regulation of miR-155 that at first glance were quite puzzling. Two seemingly related questions were addressed in this chapter; while miR-155 can be induced through soluble factors and requires new protein synthesis, the mechanism accounting for this was unanticipated.

The first major finding in this chapter was that miR-155 induction can be transferred through sterile filtered media. Therefore, it seems likely that, a cytokine such as TNF\(\alpha\), IFN\(\beta\), IFN\(\gamma\),\(^{119}\) or even IL-1\(\beta\)\(^{141}\) would promote miR-155 through autocrine/paracrine signaling. We specifically neutralized TNF\(\alpha\) and IFN\(\beta\), yet find that blocking either cytokine alone does not impair miR-155 induction. Monocytes and macrophages are not considered major sources for IFN\(\gamma\), thus this cytokine was unlikely

**Figure 3.10: F.n.-induced MiR-155 expression is dependent upon c-Fos up-regulation.** A RAW 264.7 cells were transfected with siRNA control of c-Fos siRNA. One hour post-transfection cells were infected with \textit{F.n.} at an MOI of 50 for 4 hours. Cells were lysed and subjected to western blot for c-Fos expression, then re-probed with actin. B Additional samples run in parallel with part B were infected for 16 hours, followed by RNA isolation and assay for miR-155 expression by qRT-PCR.
to influence miR-155 induction in this study. In chapter two it was shown that miR-155 is induced independently of caspase-1, thus IL-1β would not be implicated either. In this chapter we further ruled out the possibility of host cytokines being the major mediators of miR-155 induction during *F.n.* infection by two experiments. The brefeldin A experiment showed that host cytokine release was not required for miR-155 induction. The experiment utilizing conditioned media from responsive (WT) versus non-responsive (MyD88-/-) BMMs showed that host produced cytokines did not influence miR-155 induction. This lead us to the possibility is that there are soluble factors originating from the bacterium which are the critical factors for miR-155 induction. This was supported by the finding that media that had been cultured with *F.n.* for 24 hours and filtered could efficiently induce miR-155 expression. While *Francisella* is Gram-negative, it is unlikely that LPS is the soluble factor mediating the induction of miR-155 since *Francisella* LPS is a poor TLR4 agonist. Plus we already found that the induction of miR-155 was TLR2-dependent. However, *Francisella* expresses multiple lipoproteins that activate TLR1/2 or TLR2/6 and thus are potential soluble bacterial factors that induce miR-155 expression.

The question still remained as to if miR-155 was induced as a direct effect of infection. Chapter two presented data showing that NF-κB activity was essential for the induction of this miR. A similar requirement for NF-κB has also been demonstrated in Epstein–Barr virus induced miR-155 expression. However, NF-κB transcription alone is not sufficient for *F.n.* induced miR-155 expression because in this chapter we find that there is a requirement for new protein synthesis. Even though there is an NF-κB site at the promoter of the BIC gene, a detailed study in B-cells which found that out of the Ets,
NF-κB, and AP-1 sites at the promoter of the BIC gene, it is only the AP-1 site which is critical for BIC/miR-155 up-regulation. Upon further examined of the role of AP-1 transcription, we find the expression of c-Fos and c-Jun transcription factors are inducible and it occurs through an NF-κB-dependent mechanism. Thus NF-κB is indirectly required for miR-155 induced due to its ability to mediate the expression of c-Fos and c-Jun, which transcriptionally drive BIC/miR-155 expression. With regard to the signaling kinases involved we previously found that inhibition PI3K blocked miR-155 induction, which fits this model because PI3K promotes NF-κB activity. The MAPKs, JNK and ERK, were also shown to be required for miR-155 induction, which is logical since both signaling kinases promote the expression and dimerization of c-fos and c-jun, thus influencing AP-1 transcription which is critical for miR-155 induction.

To summarize, here we have uncovered new details on the molecular mechanisms of miR-155 induction by bacterial infection. Soluble factors originating from F. n. and not the host drive miR-155 induction. These factors activate TLR2 and MyD88, which transfer the signal through PI3K, ERK, and JNK. While NF-κB activity is required for miR-155 induction, it is not sufficient. But NF-κB mediates c-Fos and c-Jun expression to promote AP-1 transcription and BIC/miR-155 expression. This chapter shows the complexity in the regulation of this immunoregulatory microRNA (Figure 3.11).
**Figure 3.11: Mechanism of *F.n.*-induced miR-155 expression.** *Francisella* activates TLR2 and MyD88 through direct contact or through soluble secreted factors. MyD88 allows for activation of PI3K, ERK, and JNK signaling kinases. The signaling kinases allow for NF-κB to initiate gene transcription, which produces Fos and Jun transcription factors. Fos and Jun dimerize to promote gene transcription at AP-1 sites. BIC/miR-155 is transcriptionally up-regulated by AP-1.
Chapter 4: AKT Inactivates GSK3β to Promote NF-κB Activity and the Inflammatory response against *Burkholderia cenocepacia*

4.1 Abstract

*Burkholderia cenocepacia (B.c.)* is an environmental bacterium that can cause opportunistic lung infections in immunocompromised individuals, particularly those with cystic fibrosis. Infections in susceptible patients are associated with exacerbated inflammation leading to rapid decay of lung function and in some cases resulting in cepacia syndrome, which is characterized by a fatal acute necrotizing pneumonia and sepsis. It has been known that *B.c.* can survive within macrophages by preventing phagosome-lysosome fusion, but surprisingly little is known on macrophage responses to the intracellular infection. This chapter examined the role of the PI3K/Akt signaling pathway in *B.c.*-infected monocytes and macrophages. In light of the previous finds about the role of this pathway in regulating the pro-inflammatory response and intramacrophage killing of Gram-negative intracellular bacteria, we examined how targeting this pathway may be of interest in therapeutic strategies to combat *B.c.* infections. We show that PI3K/Akt activity was required for NF-κB activity and the secretion of pro-inflammatory cytokines during infection with *B.c.* Though phagocytosis and intracellular survival of the bacteria were not directly affected by activation of this pathway.
Mechanistically we find an unexpected means by which Akt exerts influence over NF-κB and pro-inflammatory cytokine production. In contrast to previous observations in epithelial cells infected with other Gram-negative bacteria, Akt did not enhance the phosphorylation of IKKα/β or NF-κB p65 (serine-536), but rather inactivated GSK3β, a negative regulator of NF-κB transcriptional activity. This chapter describes the mechanism by which PI3K/Akt/GSK3β regulate of NF-κB activity and indicate that this pathway is a potentially unique therapeutic target for controlling excessive inflammation upon B.c. infection, without given a growth advantage to the bacterium.

4.2 Introduction

*Burkholderia cenocepacia* is a member of the *Burkholderia cepacia* complex (Bcc). This opportunistic respiratory pathogen represents a threat to immunocompromised individuals, particularly those with cystic fibrosis\(^87;88;90\). What is particularly troublesome is that these bacteria have naturally acquired resistant to many antibiotics\(^173;174\), therefore there are limited therapeutic options. Because of the lack of therapeutics, it is critical to gain an understanding of host immune responses to this organism, since that may provide new means to overcoming the current limitations for treatment.

A major factor in the morbidity and mortality caused by *B. cenocepacia* infection is a counterproductive inflammatory response, which causes collateral tissue damage\(^99;175\). Fittingly, administration of anti-inflammatory corticosteroids has been associated with favorable patient outcome during *B. cepacia* infection\(^98\). Further evidence of a detrimental inflammatory component in disease progression is show by *in vivo*
studies demonstrating that TLR/MyD88 driven inflammation promotes host susceptibility to the host, as MyD88−/− mice show a survival advantage over WT mice challenge with B.c. TLR5 in particular seems to plays a key role in promoting exacerbated inflammation in susceptible individuals.

Activation of TLRs results in the production of pro-inflammatory cytokines, such as TNFα. This cytokine activates numerous signaling pathways through the TNF receptor, resulting in NF-κB activation yet also trigger signals to initiate cell death. This cytokine has been highlighted as a major mediator of mortality in an in vivo mouse model of B.c. infection because TNFα−/− mice were resistant to a challenge lethal of B.c., while WT mice succumb. Therefore by understanding how TNFα is regulated during infection that will lead to more effective treatments by countering the deleterious effects of this cytokine.

Recent studies have shown the importance of macrophages during B. cenocecpacia infection, as they are a site of bacterial replication much like lung epithelial cells. Also there is a connection with macrophage trafficking of bacteria and the CFTR-defect in individuals with cystic fibrosis. BMMs with the CFTR mutation or WT BMM treated with a CFTR-inhibitor show delayed phagolysosomal fusion compared to control. This is one mechanism that helps to explain the persistence of B.c. in individuals with cystic fibrosis, since their macrophages are be less able to control the intracellular bacteria. The other important role of monocytes and macrophages in B.c. infection is due to the fact that they are major sources of inflammatory cytokines such as TNFα and IL-8, which contribute to the hyper-inflammatory state following B. cenocecpacia infection.
PI3K/Akt signaling regulates numerous biological functions, including the pro-inflammatory response to TLR signaling as was shown in the earlier chapters. However, the effect of this pathway on inflammatory response differs depending upon several factors that remain to be fully understood\(^{20}\). So in this chapter we investigated the role of PI3K/Akt signaling on IKK/NF-\(\kappa\)B activation and the ensuing pro-inflammatory response from mononuclear phagocytes infected with \(B.\) \(c.\) We show that PI3K and Akt promote NF-\(\kappa\)B activity as well as pro-inflammatory cytokine production. However, this is not mediated through enhanced IKK nor NF-\(\kappa\)B p65 phosphorylation. Rather, PI3K/Akt to inactivate GSK3\(\beta\), a downstream repressor of NF-\(\kappa\)B in this study. Together these findings indicate that targeting PI3K/Akt/GSK3\(\beta\) signaling may be of therapeutic value within the context of \(B.\) \(cenocepacia\) infection.

4.3 Materials and Methods

**Cells and reagents.** As in the previous chapters, RAW 264.7 cells obtained from ATCC were cultured in RPMI-1640 (Gibco-BRL, Rockville, MD) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), L-glutamine, penicillin (10,000 U/ml) and streptomycin (10,000 \(\mu\)g/ml) (Invitrogen, Carlsbad, CA). The BAY 11-7085 (5\(\mu\)M) IKK/NF-\(\kappa\)B inhibitor was provided as a generous gift from Dr. Denis Guttridge (The Ohio State University). LY294002 (20\(\mu\)M) PI3K inhibitor was purchased from Calbiochem (San Diego, CA). SB-216763 (2\(\mu\)M) GSK3\(\beta\) inhibitor was purchased from Sigma (St. Louis, MO). DMSO vehicle (0.2%) came from Sigma-Aldrich (St. Louis, MO). The Akt inhibitor, Akt(X) (10\(\mu\)M) was obtained from Calbiochem (San Diego, CA) and dissolved in water. Antibodies directed against phosphorylated Akt-
serine-473, phosphorylated IKKα-serine-180/β-serine-181, phosphorylated NF-κBp65-serine-536, phosphorylated GSK3α-serine-21/β-serine-9, phosphorylated GSK3β-serine-9, and GSK3β were purchased from Cell Signaling (Beverly, Massachusetts). Loading control antibodies against Akt and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

*Bone marrow-derived macrophages.* WT and MyrAkt expressing mice were sacrificed according to institution-approved animal care and use protocols. Bone marrow cells were collected and differentiated as previously described with MCSF (R&D systems, Minneapolis, MN) and polymyxin-B (CalBiochem, San Diego, CA)\(^{38}\). This method provided greater consistency than the L-cell conditioned media method and thus was used for the present study.

*Peripheral blood monocyte isolation.* Human PBM were isolated from leucopacks by centrifugation through a Ficoll gradient followed by CD14-positive selection by Magnet-Assisted Cell Sorting (MACS, Miltenyi Biotec, Auburn, CA) according to manufacturer instructions as previously described\(^9\).

*Bacterial infections.* All infections were conducted in 5% or 10% heat-inactivated FBS-containing RPMI-1640 without antibiotic. *Burkholderia cenocepacia* K56-2 isolate was grown in L.B. broth (Sigma, St. Louis, MO) for approximately 14 hours to post-logarithmic phase. Optical density at 600 nm was taken to assess the density of cultures and calculate the multiplicities of infection (1.0 OD = 1.2x10\(^9\) bacteria). Serial dilutions
of cultures and plating on L.B. agar to count colony forming units was done to verify accuracy of the MOI calculations. Before infection, bacterial cultures were centrifuged, washed, and resuspended in macrophage culture media. An MOI of 5 was used for all infections. Heat killed bacteria were prepared by heating at 56\(^0\)C for 1 hour. Paraformaldehyde-killed bacteria were prepared by treating in 1% PFA for 1 hour followed by 3 washes in PBS to remove residual PFA. The prepared cultures were plated on LB and monitored for growth to ensure effective killing of microbes.

**ELISA cytokine measurements.** Cell-free supertennants were screened by sandwich ELISA. Human TNF\(\alpha\), mouse TNF\(\alpha\), mouse IL-6, and mouse RANTES ELISA kits were obtained from R&D Systems (Minneapolis, MN) and human IL-6 and human IL-8 ELISA kits were obtained from eBioscience (San Diego, CA). The manufacture’s instructions were followed as done before\(^38\).

**Western blot analysis.** As done in the previous chapters monocytes and macrophages were lysed in T\(_N\)1 buffer (50mM Tris [pH 8.0], 10 nm EDTA, 10M Na\(_4\)P\(_2\)O\(_7\), 10 nM NaF, 1% Triton-X 100, 125nM NaCl, 10nM Na\(_3\)VO\(_4\), 10 \(\mu\)g/ml of both aprotinin and leupeptin. Whole cell lysates were separated on 10% acrylamide gels, followed by transferred to nitrocellulose membranes, and finally probed with antibody of interest. After incubation with the primary antibody, a HRP-conjugated secondary antibody was used to detect the protein with enhanced chemiluminescence western blotting substrate (Pierce, Rockford, IL) as previously described\(^38\).
**CFU assays.** These assays took advantage of the aminoglycoside-sensitive strain of K56-2 and were conducted as done by Hamad *et al.*\(^9^4\). BMM were infected with *B.c.* then placed on a rocker at room temperature for 5 minutes. Next the cells were centrifuged at room temperature at a speed of 400 x g for 4 min. At 1 hour post-infection the media was removed and cells washed with PBS. Media containing 50 μg/ml of gentamicin was added for 30 minutes. We found that under these conditions >98% of the aminoglycoside sensitive K56-2 strain was killed, though less than 12% of the parental K56-2 strain was killed under the same conditions. At the end of the incubation time with antibiotics, the cells were again washed in PBS to remove antibiotic, then either lysed in 0.1% Triton-X for the one hour infection time. For the 8-and 24-hour time point the cells were incubated in fresh culture media, until the time of lysis. Cell lysates were serially diluted ten-fold in PBS immediately after macrophage lysis, less than 5 minutes, and plated on L.B. agar plates. Between 24 and 36 hour post-plating, CFUs were counted. The alternative method for the CFU assays was to use the dual-antibiotic combination method. These assays were conducted exactly as done above, with two exceptions. First that parental strain K56-2 was used as opposed to the MH1K deletion mutant of K56-2. Second extracellular bacteria were killed with gentamicin (500μg/ml) plus ceftazidime (250μg/ml), instead of gentamicin only.

**LDH cell death assay.** BMM were exposed to the treatment conditions for the indicated time points. Then media and cell lysates was collected. These samples were assayed with the Lactate Dehydrogenase-based In-Vitro Toxicology Assay kit from Sigma (Saint...
Louis, MO) according to the manufacturers instructions as we have previously described\textsuperscript{127}. Data is expressed as LDH release = (Media\textsubscript{abs}/(Media\textsubscript{abs}+Lysate\textsubscript{abs})) \cdot 100.

**Luciferase assays.** Transfected RAW 264.7 macrophages were uninfected or infected for 5 hours with B.c. At the end of the infection period cells were washed with PBS and then lysed in 200 μl of Luciferase Cell Culture Lysis Reagent (Promega, Madison, Wisconsin). Samples were assayed for luciferase activity using the Luciferase Assay Reagent (Promega), as previously described\textsuperscript{80}. Graphs represent percent increase over matched uninfected control samples.

**Transfection.** Over expression of GSK3β was accomplished by transfecting RAW 264.7 cells with 5 μg of pcDNA vector with or without wild-type human GSK3β, Addgene plasmid 14753, (Addgene, Cambridge, MA). For transfection 10\times10^6 cells were resuspended in Amaxa solution V and used program U-14 for electroporation as previously described\textsuperscript{80}. Infections were conducted 14 hours post-transfection to allow time for sufficient over-expression, which was confirmed by western blotting of GSK3β. NF-κB reporter assays used 2 μg of NF-κB luciferase reporter plasmid per transfection.

4.4 Results

**PI3K signaling is activated in human monocytes by B.c. infection**

PI3K activation leads to generation of phosphatidylinositol-(3,4)-bisphosphate and phosphatidylinositol-(3,4,5)-trisphosphate, which result in phosphorylation and activation Akt at the plasma membrane\textsuperscript{22}. To first determine if PI3K/Akt signaling might
be involved in mediating the host pro-inflammatory response to *B.c.* we looked for phosphorylation of Akt in PBM infected with *B.c.* Cells were lysed after 20, 40, or 60 minutes of infection and subject to western blotting. Indeed there is rapid phosphorylation of Akt within 20 minutes of infection (Figure 4.1A). Next we examined if this response also happens in BMMs and there is also clear phosphorylation/activation of Akt, which is sustained for at least the first 5 hours of infection (Figure 4.1B). We next sought to determine if the activation of Akt require bacterial viability. Thus PBM were infected with *B.c.* at an MOI of 5 of live bacteria or treated with comparable quantities of heat-killed or paraformaldehyde-killed *B.c.* Akt is still be activated in PBM treated with killed *B.c.*, even though to a slightly lesser degree (Figure 4.1C). Thus bacterial viability may influence Akt activation, but it is not absolutely required.

**Figure 4.1:** The PI3K/Akt pathway is activated by live or dead *B.c.* A PBM were infected with *B.c.* at an MOI of 5 for 20, 40, or 60 minutes. 'R' represents resting or uninfected samples. Western blots on cell lysates were done to measure phosphorylated-Akt-Serine-473, followed by reprobes for total Akt as a loading control. B BMM were infected with *B.c.* at an MOI of 5 for the indicated time points. Lysates were subject to western blot for phosphorylated-Akt-Serine-473 and re-probed with actin. C PBM were infected or treated with live *B.c.*, heat-killed *B.c.*, or paraformaldehyde-killed *B.c.* equivalent to an MOI of 5. Samples were subject to western blotting as in part A.
**PI3K activation is required for the pro-inflammatory response to B.c.**

PI3K signaling drives numerous cellular processes, including the production and release of pro-inflammatory mediators\(^\text{20}\). So next we asked if PI3K/Akt regulates the production of inflammatory cytokines in human monocytes. To determine the function of PI3K on host-inflammatory response PBM were pretreated with DMSO vehicle control or the PI3K inhibitor LY294002, and then infected with *B.c.* at an MOI of 5 for eight hours. First it was tested to see if the inhibitor was effective under these conditions by western-blotting of phosphorylated Akt-serine-473. Infection induces high levels of phosphorylated Akt, while this was blocked in PBM given the PI3K inhibitor and infected (Figure 4.2A).

Then the functional consequences of inhibiting PI3K activity on production of pro-inflammatory cytokines were examined. Matched supernatant from the samples in part A of the pervious figure was collected and pro-inflammatory cytokine production assayed by ELISA. Infected PBM showed strong induction of TNF\(\alpha\), IL-6, and IL-8 as compared to uninfected cells as would be expected. Though PBM pretreated with the PI3K inhibitor produced significantly reduced levels of the inflammatory mediators (Figures 4.2B-D). Thus the inflammatory response to *B.c.* by monocytes and macrophages requires PI3K signaling.
Figure 4.2: PI3K is required for pro-inflammatory cytokine production in response to B.c. A PBM treated with DMSO vehicle control or LY294002 (20μM) were infected with B.c. at an MOI of 5 for eight hours. Cells were lysed then subject to western blotting for phospho-Serine-473-Akt followed by actin reprobe. B-D Pro-inflammatory cytokine production of PBM from Figure 1B was measured by ELISAs from cell-free supernatants for human C TNFα, D IL-6, or E IL-8. All graphs in this chapter represent mean and standard deviation of triplicate samples. * denotes a p value < 0.05 from Student’s t-test.

Akt promotes the pro-inflammatory response to B.c.

Akt is one of the major targets of the class I PI3K. Since we find that PI3K is required for the monocyte / macrophage pro-inflammatory response to B.c., we next examined if Akt activation could also be linked to the pro-inflammatory response in infected PBMs. PBM were pre-treated with the Akt inhibitor Akt(X) or vehicle control and infected with B.c. for 8 hours. Again we first verified the effectiveness of the inhibitor by western blot of phosphorylated Akt-serine-473. There was a clear inhibition of phosphorylated Akt in the presence of Akt(X) as compared to the control infected sample (Figure 4.3A), yet this inhibitor was less effective at blocking Akt phosphorylation than LY294002. Functionally we saw that Akt was also required for pro-
inflammatory cytokine production because inhibition of Akt led to reduced production of TNFα, IL-6 and IL-8 compared to cells pretreated with vehicle control (Figures 4.3C-D). The reduction in IL-6 never reached statistical significance, which may be due to limited effectiveness of this inhibitor; however, a reduction was consistently observed in numerous experiments.

**Figure 4.3**: Akt is required for pro-inflammatory cytokine production in response to *B. c.* PBM were pre-treated with H₂O vehicle control or Akt(X) (10μM) for 30 minutes, and then infected with *B. c.* at an MOI of 5 for 8 hours. A Cell lysate was subject to western blot for phosphorylated Akt-serine-473 and re-probed with Actin. B-D Media from the matched samples was assayed by ELISA for B TNFα, C IL-6, and D IL-8.

The findings above provide evidence that Akt activation is required for cytokine production following *B. c.* infection. To test whether Akt could drive production of these cytokines, we made use of BMMs from WT mice or from mice expressing MyrAkt. We verified the expression of MyrAkt in the BMMs by western blotting for phosphorylated-Akt-473. WT BMMs have barely detectable levels of this protein basally, but the MyrAkt expressing BMMs show strong levels of the transgene with phosphorylated Akt (Figure
4.4A). These cells were infected with B. c. for 8 hours and then ELISAs were used to assay the production of TNFα, IL-6 and RANTES. RANTES, a member of the IL-8 superfamily of cytokines, was screened as opposed to IL-8, because mice do not have IL-8. MyrAkt expressing BMM had significantly enhanced production of all three cytokines upon B. c. infection (Figure 4.4B-D).

![Figure 4.4](image)

**Figure 4.4:** Akt can promote pro-inflammatory cytokine production in response to B. c. A WT and MyrAkt BMM were lysed and subject to western blot of phosphorylated Akt-serine-473, then re-probed with Actin. B-D WT and MyrAkt BMM were infected with B. c. at an MOI of 5 for 8 hours. The media was screened for the production of B TNFα, C IL-6, and D Rantes.

**Optimization of CFU assays for assaying B. c. phagocytosis and intracellular replication**

CFU assays with *Burkholderia* are particularly complex because the standard gentamicin protection assays are ineffective at killing B. c., due to antibiotic resistance. To get past this issue we used two different methods. First we used the gentamicin sensitive mutant of K56-2, termed MH1K. Here the aminoglycoside efflux pump, which confers resistance to gentamicin, has been deleted. Importantly this mutant only differs from the
parental strain by the sensitivity to gentamicin and not intracellular trafficking or replication. We verified the sensitivity to gentamicin by culturing parental strain K56-2 and the mutant MH1K strain in macrophage culture media for 15 minutes in low dose gentamicin, 50 μg/ml. Then serial dilutions were made and plated on LB agar to quantify CFUs, with data expressed as total recovered CFUs (Figure 4.5). Greater than 98% of the mutant MH1K strain was killed with gentamicin, while less than 12% of the parental K56-2 strain was killed at the same dose. This functionally confirms the mutant strain’s sensitivity to gentamicin.

Figure 4.5: K56-2 versus MH1K susceptibility to gentamicin. Parental K56-2 or MH1K aminoglycoside efflux pump deletion mutant of K56-2 were incubated in cell culture media for 15 minutes. The two conditions were media alone (Ctrl) or media containing 50μg/ml gentamicin (Gentamicin). After the treatment period samples were serially diluted and plated on LB agar.

The second method was with the dual antibiotic treatment of gentamicin (500μg/ml) and ceftazidime (250μg/ml) as done by Sajjan et al. This combination is effective at killing greater than 98% of B.c. First we tested the ability of these antibiotics to kill or prevent growth of B.c. in broth culture. B.c. was cultured in LB broth spiked with gentamicin (500μg/ml) and/or ceftazidime (250μg/ml) for the indicated time course. Either antibiotic alone was rather ineffective at inhibiting growth, though the combination was effective (Figure 4.6A). To determine the potential toxicity to mononuclear
phagocytes, BMM were cultured with the same dose of gentamicin and ceftazidime for one hour and the LDH assay was used to assess cell death. No observable cell death was detected as treated samples were equal to untreated and showed virtually 0% LDH release (Figure 4.6B). Thus this antibiotic combination is well tolerated by the host cells. To explore this further we tested varying concentrations of antibiotics to optimize bacterial killing while preserving host-cell survival. Here B.c. was cultured in cell culture media for one hour with the indicated concentrations of gentamicin and ceftazidime (Figure 4.6C). Concentrations above 500 μg/ml of gentamicin and ceftazidime were more effective than the initial tested concentration of 250 μg/ml gentamicin and 500 μg/ml ceftazidime. Cell viability was tested with these varying concentrations of antibiotics in PBM. The number of cells was counted prior to treatment and one hour post-treatment to assay potential toxic effects. There was no clear effect on cell viability except for when cultured at the highest tested dose, 1000 μg/ml of both gentamicin and ceftazidime (Figure 4.6D). In light of this data we used a concentration of 500 μg/ml of gentamicin and ceftazidime for the CFU assays, since that dose was highly effective at killing B.c. without overt toxicity to the host cells.
**Figure 4.6:** Dual antibiotic combination of gentamicin and ceftazidime effectively kills *B. c.* without killing host cells. A. *B. c.* was cultured in bacterial growth media in the presence or absence of 500 μg/ml ceftazidime and/or 250 μg/ml gentamicin for the indicated time points. Bacterial growth was measured by optical density (OD) at 600 nm. B. BMM were cultured with 500 μg/ml ceftazidime and 250 μg/ml gentamicin for 1 hour and assayed for cell death with LDH assay. C. *B. c.* were incubated in cell culture media for 1 hour in the indicated concentrations of ceftazidime and gentamicin, then serially diluted and plated on LB agar to enumerate CFUs. Data is shown as % of bacteria to no antibiotic control. D. PBM were treated with the indicated concentrations of ceftazidime and gentamicin. Viable cells were counted with typan blue dye at the starting time point (black line) and 1 hour post-treatment (gray line). Data is expressed as % viable cells to untreated control, which is equal to 100%.

**PI3K/Akt do not regulate uptake nor intra-macrophage replication of *B. cenocepacia***

PI3K signaling has been reported to influence both phagocytosis and intra-macrophage survival of Gram-negative bacteria.\(^{178,181}\) However phagocytosis of Gram-negative bacteria is not always PI3K dependent.\(^{126,182}\) To evaluate the role of this signaling pathway in macrophage phagocytosis of *B. c.*, we investigated bacterial uptake and replication in BMM treated with the PI3K inhibitor or MyrAkt expressing BMMs. Both methods described above are used to assess the involvement of PI3K/Akt on phagocytosis and intracellular survival of *B. c.* in BMMs. First WT BMMs were pre-
treated primary with DMSO or LY294002, and infected with *B. cenocepacia* MH1K at an MOI of 5 for one hour. After one hour of infection non-internalized bacteria were washed away and killed with the low dose gentamicin (50 μg/ml) for thirty minutes. CFUs were measured at one hour of infection to estimate bacterial uptake. Cells were washed again, lysed, serially diluted, and plated on LB agar to measure CFUs. Even though we saw earlier that PI3K was critical of the production of pro-inflammatory cytokines, we find no effect on the phagocytosis of *B.c.* (Figure 4.7A). Next for examining the role of Akt on phagocytosis we used WT and MyrAkt expressing BMMs. These cells were infected with MH1K at an MOI of 5 for 1 hour and CFUs were assayed as in part A of this experiment. Again we find that there is no difference in phagocytosis of *B.c.* even when the PI3K/Akt pathway is manipulated (Figure 4.7B).

As an alternative method we used the dual combination of gentamicin (500 μg/ml) and ceftazidime (500 μg/ml) with the parental K56-2 strain of *B.c.* Again we see that inhibition of PI3K does not influence phagocytosis (Figure 4.7C). The MyrAkt BMM had marginally reduced, yet significant, uptake of *B.c.*, < 0.2 log difference, as compared to WT (Figure 4.7D). It is unclear as to why recovered CFUs were less in MyrAkt expressing BMMs than WT when using the dual antibiotic method of CFUs; however, this appears spurious as PI3K inhibition had no effect on phagocytosis using either CFU method. Also WT and MyrAkt expressing BMMs showed comparable phagocytosis when using the MH1K mutant to assess phagocytosis. Collectively we interpret these results to indicate that PI3K and Akt do not influence macrophage phagocytosis of *B.c.*
Figure 4.7: Phagocytosis of *B. c.* is PI3K/Akt-independent. A-B The MH1K mutant of *B. c.* was used for CFU assays studying the role of PI3K/Akt on phagocytosis. A BMM were pre-treated with DMSO vehicle control or LY294002 then infected with *B. c.* for 1 hour at an MOI of 5 and CFUs were quantified. B WT and MyrAkt expressing BMM were infected as done in part A, and then CFUs were quantified. C-D Parental K56-2 *B. c.* was used in combination with dual antibiotic treatment of 500 μg/ml gentamicin and ceftazidime. C Again BMM were pre-treated with DMSO vehicle control or LY294002 then infected with *B. c.* for 1 hour at an MOI of 5 followed by CFU quantification. D WT and MyrAkt expressing BMM were infected as done in part C, and then CFUs were quantified.

To gauge the intra-macrophage survival and replication of *B. c.* CFU assays were conducted at time points of 1, 8, and 24 hours post-infection. The first hour is reflective of phagocytosis, while eight and twenty four time points represent intracellular replication. As done in figure 4.7 BMM were pre-treated with DMSO vehicle control of LY294002 PI3K inhibitor, and then infected with MH1K *B. c.* at an MOI of 5 for one hour. At that time extracellular bacteria were washed away and killed with 50 μg/ml gentamicin. Cells were washed again and the one hour time point was lysed and plated on LB agar to count CFUs, while the eight and twenty four hour time points were cultured in fresh culture media until the indicated time points at which they were lysed and plated on LB agar too. PI3K inhibition with LY294002 shows no effect on intracellular replication.
of B.c. at the time points tested (Figure 4.8A). An identical experiment was carried out in WT versus MyrAkt expressing BMMs as opposed to DMSO versus LY294002 treatment. Consistent with the finding that PI3K does not influence intra-macrophage replication, MyrAkt expressing BMM show similar intracellular replication as WT BMMs (Figure 4.8B).

As a complementary method, the dual antibiotic combination of gentamicin and ceftazidime was used to assay intra-macrophage growth with the parental K56-2 B.c. As done in figure 4.8A BMM were pre-treated with DMSO vehicle control of LY294002 PI3K inhibitor, and then infected with K56-2 parental B.c. at an MOI of 5 for one hour. At that time extracellular bacteria were washed away and killed with 500 μg/ml of gentamicin and ceftazidime. Cells were then washed again where the one hour time point was lysed and plated on LB agar, while the eight and twenty four hour time points were cultured in fresh culture media until those time points at which they were also lysed and plated on LB agar, followed by CFU enumeration. The data agrees with figure 4.8A that PI3K does not influence intra-macrophage replication of B.c. (Figure 4.8C). As done in figure 4.8B, an identical experiment was run with WT versus MyrAkt expressing BMMs using the K56-2 B.c. used with the dual antibiotic combination method for CFU assays. While there were subtle, yet significant, differences in CFUs between WT and MyrAkt BMMs in this experiment, the general trend at twenty four hours post-infection is that Akt activation does not influence intracellular replication of B.c. (Figure 4.8D). However, there was greater variability with the high dose dual antibiotic combination of gentamicin and ceftazidime method, than the MH1K gentamicin sensitive mutant with low dose gentamicin treatment. In principle the gentamicin sensitive mutant of B.c. is preferable to
using parental K56-2 B.c. with high levels of antibiotics, since that exposure may result in unintended off target effects\textsuperscript{94}.

Collectively we interpret the data to mean that PI3K/Akt activity is dispensable for bacterial internalization and intra-macrophage replication, yet the PI3K/Akt pathway is required for the pro-inflammatory response. These findings indicate that PI3K/Akt can be targeted to limit inflammation without altering intra-macrophage dynamics of B.c.

**Figure 4.8: Intra-macrophage replication of B.c. is PI3K/Akt-independent.**

A The MH1K mutant of B.c. was used for CFU assays studying the role of PI3K/Akt on intra-macrophage replication. BMM were pre-treated with DMSO vehicle control or LY294002 then infected with B.c. for 1 hour at an MOI of 5 and CFUs were quantified. Non-internalized bacteria were washed away and killed with gentamicin (50 μg/ml), then macrophages were incubated in fresh culture media for 8 and 24 hours. B WT and MyrAkt expressing BMM were infected as done in part A, and then CFUs were quantified. C-D Parental K56-2 B.c. was used for CFU assays measuring intra-macrophage replication. C Again BMM were pre-treated with DMSO vehicle control or LY294002 then infected with B.c. for 1 hour at an MOI of 5. Non-internalized bacteria were washed away and killed with gentamicin and ceftazidime (500 μg/ml). Cells were then cultured in fresh media and CFUs were determined at the indicated time points. D WT and MyrAkt expressing BMM were infected as done in part C, and then CFUs were quantified.
IKK/NF-κB are activated by B.c. and required for inflammatory responses

Thus far it was shown that PI3K/Akt promotes inflammatory responses to B.c. without influencing uptake or replication in macrophages. To determine how PI3K and Akt promote this inflammatory response we examined activation of the IKK/NF-κB pathway. There have been reports showing that Akt directly activates the IKK complex as well as NF-κBp65 phosphorylation at serine-536, which enhances its transcriptional activity\textsuperscript{183-185}. Thus this may account for the influence of Akt over the production of inflammatory cytokines. PBM were infected with B.c. for 20, 40 and 60 minutes, and IKK phosphorylation was measured by western blotting. Results show that there was phosphorylation of IKKa and β within the first 20 minutes of infection (Figure 4.9A). Phosphorylation of the IKK complex triggers a number of events that free NF-κB for nuclear translocation and gene transcription\textsuperscript{186}. The NF-κBp65 is regulated by phosphorylation, which enhances its transcriptional activity\textsuperscript{183}. We examined the status of serine-536 phosphorylation on NF-κBp65, as it has been shown to be regulated by Akt\textsuperscript{187}, and found that it was also rapidly phosphorylated in response to B. cenocepacia infection (Figure 4.9B).

To test the requirement of IKK signaling for mediating the pro-inflammatory responses to B.c., PBM were pre-treated with an IKK inhibitor, BAY 11-7085, before infection. In the presence of this inhibitor, B.c.-driven TNFα, IL-6, and IL-8 production was greatly diminished in compared to control following infection (Figures 4.8C-E). These results show that IKK signaling to NF-κB is a critical mediator of pro-inflammatory responses to B.c. In light of these finding that PI3K/Akt promoted cytokine
production after infection and IKK signaling was also required, the possibility arose that PI3K/Akt drove IKK/NF-κB activation.

**Figure 4.9**: IKK is activated and is required for *B. c.*-induced production of inflammatory cytokines. A-B PBM were infected with *B. c.* at an MOI of 5 for the indicated time points. Cells were lysed and probed for A phosphorylated IKKα-serine-180/β-serine-181 and B phosphorylated NF-κBp65-serine-536. C-E PBM were pre-treated with DMSO or the IKK inhibitor BAY 11-7085. Cells were then infected with *B. c.* at an MOI of 5 for eight hours. Media from the cells was assayed by ELISA for C TNFα, D IL-6, and E IL-8.

**Activation of IKK/NF-κB and PI3K/Akt are independent events**

The above data indicate that both PI3K/Akt and IKK/NF-κB are required for the monocyte/macrophage inflammatory response to *B. c.* To test if the two pathways were linked, the activation of both was examined in the presence or absence of specific inhibitors. PBM were pre-treated with DMSO vehicle control, PI3K inhibitor (LY294002) or IKK inhibitor (BAY 11-7085), and then infected with *B. c.* for the indicated time points. To our surprise, the PI3K inhibitor did not impair IKKα/β phosphorylation (Figure 4.10A) nor NF-κBp65-Ser536 phosphorylation (Figure 4.10B).
despite blocking Akt phosphorylation. The IKK inhibitor blocked IKKα/β and NF-κBp65-Ser536 phosphorylation but not Akt phosphorylation (Figure 4.10C). These results demonstrate the specificity of the inhibitors and show that the two pathways being studied here are activated independently of the other following B.c. infection.

**Figure 4.10**: IKK and NF-κBp65 phosphorylation is independent of PI3K. A-C PBM were pre-treated with DMSO, PI3K inhibitor (LY294002), or IKK inhibitor (BAY 11-7085), and then infected with B.c. at an MOI of 50 for the indicated time points. Cells were lysed and subject to western blot. Membranes were probed with antibodies against phosphorylated A IKKα-serine-180/β-serine-181, B phosphorylated NF-κBp65-serine-536, or C phosphorylated Akt-serine-473. The blots were re-probed with antibodies against actin or Akt.

Recalling the earlier results (Figure 4.4) showing that MyrAkt-expressing macrophages produced higher levels pro-inflammatory cytokines than WT, we then examined IKK/NF-κB phosphorylation in the MyrAkt-expressing macrophages. WT and MyrAkt macrophages were infected with B.c. for the indicated time points. In agreement with the previous figure, Akt activation status did not influence the phosphorylation of IKK (Figure 4.11A) nor NF-κB (Figure 4.11B). While there is a requirement for PI3K/Akt activation for cytokine production (Figures 4.2-4.4), these results indicate that
either PI3K/Akt signaling is required in parallel to NF-κB for inflammatory cytokine production, or alternatively that the effect of PI3K/Akt on NF-κB is downstream of NF-κB activation itself.

![](image1)

**Figure 4.11: Akt activation does not influence IKK or NF-κBp65 phosphorylation.** A-B WT and MyrAkt BMMs were infected with B.c. at an MOI of 5 for the indicated time points. Cell were lysed and subject to western blot. Blots were probed with A phosphorylated IKKα-serine-180/β-serine-181 or B phosphorylated NF-κBp65-serine-536. Then blots were re-probed for actin.

PI3K activation is required for NF-κB transcriptional activity.

To test if PI3K/Akt influenced NF-κB-driven gene transcription, we transiently transfected RAW 264.7 cells with a NF-κB luciferase reporter to assay NF-κB transcription. 14 hours post-transfection cells were pretreated with PI3K inhibitor (LY294002) or IKK inhibitor (BAY 11-7085). Then they were then infected with B.c. for 5 hours. Luciferase activity measured by luciferase assay NF-κB. There is a marked increase in NF-κB activity upon infection, and as expected the IKK inhibitor (BAY 11-7085) blocked this response. Though, the PI3K inhibitor (LY294002) also strongly impaired NF-κB activity (Figure 4.12). This confirms that PI3K signaling is required for NF-κB-driven gene transcription following B.c. infection. Therefore, we concluded from all of the results that in infected mononuclear phagocytes, PI3K influences NF-κB activity, but does so downstream of initial IKK activation.
GSK3β is phosphorylated and inactivated by PI3K during B.c. infection

One prominent downstream target of Akt is GSK3β. There have been reports that this molecule can both promote28 and repress29-31 NF-κB activity. This molecule is normally active until phosphorylated at serine-21 of GSK3α188 or serine-9 of GSK3β189. Here PBM were infected with B.c. for 20, 40, or 60 minutes, and we examined both GSK3α and GSK3β phosphorylation by western blotting. B.c. infection induces strong phosphorylation of GSK3α/β at Serine 21 and 9 (Figure 4.13A). More specific examination of Serine 9 on GSK3β, by an antibody specific to only that phosphorylated form of GSK3β, confirmed its phosphorylation following infection (Figure 4.13B).

It has been reported that Akt controls this inhibitory phosphorylation of GSK3β190, so we tested whether PI3K activity during B.c. infection was required for GSK3α/β phosphorylation. PBM pretreated with either DMSO vehicle control or the PI3K inhibitor LY294002, were infected with B.c. Western blotting shows rapid phosphorylation of GSK3α/β in vehicle control treated samples. However, presence of the PI3K inhibitor strongly attenuated this infection-mediated phosphorylation (Figure

**Figure 4.12: PI3K is required for B.c.-induced NF-κB activity.** RAW 264.7 macrophages were transfected with an NF-κB luciferase reporter. 14 hours post-transfection the cells were infected with B.c. at an MOI of 5 for five hours. Cell lysates were collected and assayed for luciferase activity. Data is expressed as % increase over uninfected control.
This demonstrates that inhibitory phosphorylation of GSK3β following *B. c.* infection is PI3K dependent.

**Figure 4.13: GSK3β is phosphorylated and inactivated in a PI3K-dependent mechanism during *B. c.* infection.** A-B PBM were infected with *B. c.* at an MOI of 5 for the indicated time points, then lysed for analysis by western blot. Membranes were probed for A phosphorylated GSK3 α-serine-21 / β-serine-9 and re-probed with actin. B An identical membrane was probed for phosphorylated GSK3 β-serine-9 alone and re-probed with GSK3β. C PBM were pre-treated with DMSO vehicle control or PI3K inhibitor (LY294002), and then infected with *B. c.* at an MOI of 5 for the indicated time points. Cell lysates were resolved by western blotting for phosphorylated GSK3 α-serine-21 / β-serine-9 and re-probed with actin.

**Pharmacologic inhibition of GSK3β enhances NF-κB activity and cytokine production**

Next we tested the function of GSK3β on NF-κB and inflammatory response during *B. c.* infection, using the NF-κB luciferase reporter as done in Figure 4.12. Reporter-transfected macrophages were treated with DMSO vehicle control or with the GSK3β-specific inhibitor SB216763. Following treatment, the cells were infected with *B. c.* for 5 hours. Inhibition of GSK3β led to increased NF-κB reporter activity compared to vehicle control (Figure 4.14A). Thus, within the context of *B. c.* infection of mononuclear phagocytes, GSK3β functions as a repressor of NF-κB activity. To test the functional outcome on cytokine production with GSK3β inhibition, we pre-treated macrophages with DMSO vehicle control or SB216763, then infected with *B. c.* and
collected supernatants from the infected macrophages to measure TNFα production by ELISA. Results showed a significant increase in TNFα with the GSK3β inhibitor (Figure 4.14B). Importantly these results are in agreement with the earlier findings that both PI3K and Akt promote NF-κB activity, because these signaling molecules inactivate GSK3β. Thus we have connected signaling data on pathway activation to functional data on cytokine production, which is all in agreement with one another.

![Figure 4.14: Inhibition of GSK3β promotes NF-κB activity and pro-inflammatory cytokine production.](image)

**Over-expression of GSK3β represses NF-κB and the pro-inflammatory response**

To obtain further evidence of the function of GSK3β as a repressor of NF-κB and inflammatory response to *B.c.* infection, we co-transfected macrophages with an NF-κB luciferase reporter plus vector control or wild-type GSK3β plasmid. In support of our findings in the previous figure, macrophages over-expressing GSK3β showed reduced NF-κB activity compared to vector control in response to *B.c.* infection (Figure 4.15A). Aside from regulating inducible NF-κB activity, GSK3β over-expression decreased basal...
NF-κB activity by up to 50%. Functionally we examined TNFα production in GSK3β over-expressing macrophages. Macrophages were transfected with vector or GSK3β plasmid, and then 14 hours after transfection the cells were infected with B.c. for 24 hours. TNFα production was assayed by ELISA, which showed that cells over-expressing GSK3β had reduced production of this inflammatory cytokine (Figure 4.15B). GSK3β over-expression was confirmed by western blotting (Figure 4.15C).

**Figure 4.15: GSK3β over-expression inhibits B.c.-induced NF-κB activity and the production of pro-inflammatory cytokines.**

A) RAW 264.7 macrophages were transfected with NF-κB luciferase reporter construct plus vector or GSK3β over-expression plasmid. 14 hours post-transfection cells were infected with B.c. at an MOI of 5 for five hours. Cell lysate was assayed for luciferase activity and expressed as % increase over uninfected control samples. B) RAW 264.7 macrophages were transfected with vector or GSK3β over-expression plasmid. 14 hours post-transfection cells were infected with B.c. at an MOI of 5 for 24 hours. C) Vector or GSK3β transfected RAW 264.7 cells were lysed 14 hours post-transfection and subject to western blot analysis. Membranes were probed with GSK3β and re-probed with actin. Numerical values below the top blot represent densitometry quantification.

To briefly summarize, our results show that PI3K/Akt and IKK/NF-κB activation are required for pro-inflammatory cytokine production in response to B.c. However, instead of Akt driving NF-κB activation through enhancing IKK phosphorylation, PI3K/Akt signaling goes through a parallel pathway to inactivate the inhibitory function of GSK3β on NF-κB (Figure 4.16).
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Activity through IKK. Akt can directly phosphorylate IKK\(\alpha\) to mediate NF-\(\kappa\)B nuclear translocation as well as gene transcription\(^{26}\), though one factor that this depends on is the ratio of IKK\(\alpha\) to IKK\(\beta\) within the cell\(^{191}\). Through a different mechanism PI3K can promote the phosphorylation and activation of NF-\(\kappa\)Bp65 through Akt independently of \(\text{I}\kappa\B\) degradation\(^{27}\). Thus there is precedence for involvement of PI3K/Akt in NF-\(\kappa\)B activation.

**Figure 4.16**: Model of signaling pathways mediating *B. cenocepacia*-induced NF-\(\kappa\)B activity and pro-inflammatory cytokine production. Infected monocytes and macrophages induce rapid phosphorylation / activation of Akt and the IKK complex, both of which are required for the activation of NF-\(\kappa\)B and the production of pro-inflammatory cytokines. These signaling molecules are phosphorylated / activated independent of on another. While IKK triggers canonical NF-\(\kappa\)B activation, Akt functions to repress its down-stream target GSK3\(\beta\), which inhibits *B. c.*-induced NF-\(\kappa\)B activation and cytokine production.

4.5 Discussion

This chapter demonstrates that signaling through the PI3K/Akt/GSK3\(\beta\) pathway is an important regulator of NF-\(\kappa\)B activity and the production of pro-inflammatory cytokines during *B. c.* infection. Akt has previously been shown to regulate NF-\(\kappa\)B activity through IKK\(^{184}\). Akt can directly phosphorylate IKK\(\alpha\) to mediate NF-\(\kappa\)B nuclear translocation as well as gene transcription\(^{26}\), though one factor that this depends on is the ratio of IKK\(\alpha\) to IKK\(\beta\) within the cell\(^{191}\). Through a different mechanism PI3K can promote the phosphorylation and activation of NF-\(\kappa\)Bp65 through Akt independently of \(\text{I}\kappa\B\) degradation\(^{27}\). Thus there is precedence for involvement of PI3K/Akt in NF-\(\kappa\)B activation.
While there have been studies of inflammatory responses to Gram-negative bacteria in intestinal epithelial cells showing that PI3K/Akt inhibition resulted in decreased NF-κBp65 phosphorylation\textsuperscript{187,192}, thus providing one potential mechanism by which PI3K/Akt regulate the inflammatory response, we did not observe this. Within the context of \textit{B}\.\textit{c}\.\textit{-infected mononuclear phagocytes, it was only the inactivation of GSK3β which connected PI3K/Akt to regulatory control over NF-κB activity and cytokine production. Here we do not find evidence for PI3K or Akt promoting IKK or NF-κBp65 phosphorylation. Yet both the species of bacteria and the type of host cell, epithelial cells versus monocytes / macrophages, may be responsible for the disparities between those earlier studies\textsuperscript{187,192} and the work presented here. Thus this chapter describes a unique IKK-independent mechanism by which Akt regulates NF-κB activity and the production of pro-inflammatory cytokines during bacterial infection.

The results in this chapter are also in agreement with Hii \textit{et al.}\textsuperscript{193}, where PI3K was critical for pro-inflammatory responses of HEK293T cells infected with \textit{Burkholderia pseudomallei}. However, it remains to be determined if the mechanism of GSK3β inhibition by PI3K/Akt is also similar, as the nature of \textit{B}\.\textit{cenocepacia} versus \textit{B}\.\textit{pseudomallei} infection is quite different\textsuperscript{194}. The PI3K/Akt pathway is tightly regulated at many levels, such as by phosphatases\textsuperscript{38} and microRNAs\textsuperscript{53}. Thus it is possible that these two unique, yet related pathogens, differentially affect these regulators to influence the role of this signaling pathway in host-response.

The function of GSK3β in the inflammatory response is variable and depends upon a number of factors that are not entirely understood\textsuperscript{195}. Early on there was strong support for the role of GSK3β in promoting NF-κB activation\textsuperscript{28}. However, numerous
elegant studies in more recent years have shown a role of GSK3β for inhibiting NF-κB activation\textsuperscript{29-31,196}. Thus there is great complexity in this signaling pathway and GSK3β is serving dramatically different functions under unique conditions. It is possible that factors specific to \textit{B.c.} influence not only the mode of PI3K/Akt-mediated NF-κB activation but also the role of GSK3β. The results in this study are well supported since the functional results of manipulating three different signaling molecules (PI3K, Akt, and GSK3β) are all in alignment with the direct signaling connections between them.

The function of the PI3K/Akt pathway on the production of pro-inflammatory cytokines was a major focus of this chapter, though we also examined the role of this pathway on the uptake and intracellular replication of \textit{B.c.} There was a report showing that PI3K inhibition by wortmannin inhibits intracellular replication of \textit{B.c.} within epithelial cells, presumably through an effect on autophagy, though it was not specifically tested\textsuperscript{178}. This would have been of great interest in this current study because not only would inhibiting PI3K/Akt reduce the detrimental inflammatory response, though it also reduces intracellular survival. However, we find that in macrophages PI3K/Akt activation does not influence the uptake or intracellular replication of \textit{B.c.} The difference between these two studies may be reflective of macrophage versus epithelial cell or even LY294002 versus wortmannin pan-PI3K inhibition. However, we also find through genetic manipulation of Akt that this pathway does not influence macrophage phagocytosis or the intracellular replication of \textit{B.c.} So in this study we find that PI3K and Akt both regulate the production of inflammatory cytokines without affecting \textit{B.c.} uptake or survival, which is interest in its own regard because in principle this pathway can be
therapeutically targeted to limit inflammation without inadvertently given the bacteria a growth advantage in host cells.

The next step in these studies will require testing the role of the PI3K/Akt pathway of morbidity and mortality *in vivo*. This can be accomplished by using the complex animal models of *B. c.* infection in susceptible animals, gp91<sup>phox</sup>−/− mice<sup>197</sup> or vinblastine-immunosuppressed mice<sup>108</sup>. This will be required to determine the *in vivo* consequences of manipulating PI3K/Akt/GSK3β on bacterial growth, cytokine production, and overall host-resistance to infection. Based on the findings in this chapter it would be predicted inhibition of Akt will promote host-resistance, since bacterial burden is unaffected, though there will be reduced production of detrimental inflammation.

One critical aspect related to this finding is that the *B. c.* isolate used in this study represents the virulent ET12 lineage that is associated with cepacia syndrome-related deaths. This lineage directly binds to the TNF receptor (TNFR1) to induce MAPK activation and IL-8 production<sup>101</sup>. Importantly, one of the major effects of TNFR1 activation is activation of the pro-inflammatory transcription factor NF-κB<sup>177</sup>. This strongly implies that inhibition of PI3K/Akt may be an ideal therapeutic strategy in cepacia syndrome, since it regulates NF-κB at a point downstream of initial IKK activation. Hence, it would be effective at limiting the inflammatory response even against this direct activation of the TNFR by the bacterium. It is imperative for these results to be examined *in vivo* in order to translate these findings to the clinic.
Chapter 5: Summary and Future Perspectives

Innate immunity is an ancient and highly conserved system used by multi-cellular organisms to defend themselves against invaders\textsuperscript{198,199}. A fundamental concept in this system is the ability to sense a conserved set of danger molecules from microbes through pattern recognition receptors\textsuperscript{200}. In the most basic sense the immune system is given the responsibility of discerning self from non-self. However, it is fair to simplify that and say that the immune system is given the duty of alerting the host of any danger, regardless of the origin\textsuperscript{201}. Not only is innate immunity important for combating microbial infection, but it is also of great importance in cancer\textsuperscript{202}, autoimmunity\textsuperscript{203}, traumatic injuries\textsuperscript{204}, and neurodegenerative diseases\textsuperscript{205}. Innate immunity is a truly exciting area of study due to recent advance in our understanding of how innate immunity is triggered, though now the challenge is to understand the complex regulation within this system in order for us to manipulate the response in the hopes of resolving many disease states. With regard to microbial pathogenesis these advances in our understanding of innate immunity may clear the way for novel classes of therapeutics that combat microbial infections by targeting the host, as opposed to strictly the pathogen\textsuperscript{109}.

The major focus of chapters two and three was to examine the role of miRs in the host-response to Francisella infection. MiRs are a fascinating topic in molecular biology right now as they provide a means of regulation that was unknown until recently\textsuperscript{206}. 

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Indeed in chapter two we hypothesized that the down-regulation of SHIP during *F.n.* infection involved miRs. The Tridandapani laboratory knew for some time that *F.n.* infection down-regulated SHIP, though *F.t.* did not. The mechanism was lacking until we looked at the role of miR-155 as a negative regulator of SHIP. Thus there is immense potential in miRs regulating key aspects of the immune response and currently we are only at the tip of the iceberg given that a large proportion of the genome is subject to miR regulation\textsuperscript{207}.

The fourth chapter gets back to fundamental questions about the role of the PI3K/Akt pathway in regulating the host immune response to a diverse number of pathogens. There are similarities and differences in the host-responses regulated by this pathway during infection with *Francisella* or *Burkholderia*. While there has been substantial work done on the PI3K/Akt pathway and inflammation\textsuperscript{20}, there is much that still remains unknown. Collectively this work establishes that PI3K/Akt signaling regulates key aspects of the innate immune response to intracellular bacterial pathogens and there is a dynamic interaction between the host and microbe that alters the activation of this critical pathway during the course of infection.

MiR-155 is a regulator of host-response functions during bacterial infection

The aforementioned studies on miR-155 demonstrate that SHIP is a relevant target during *Francisella* infection. Indeed the results of miR-155 over-expression\textsuperscript{53} recapitulate the results of deficiency of the studied target, SHIP\textsuperscript{38}. Though, this should not be interpreted that SHIP is the only relevant target of miR-155. To determine the contribution of SHIP as the key target of miR-155 versus alternative targets a number of
experiments can be undertaken. One experiment is to over-express miR-155 in SHIP deficient macrophages to see if there is any enhancement of host response, cytokine production or intracellular replication, from increased miR-155 expression even though the target of interest has been removed. Thus if miR-155 expression enhances host-response in SHIP knockout macrophages, then there are other relevant targets that contribute the responses seen in chapter two. Based on the number of predicted targets it seems likely that there will be other relevant targets of miR-155 that influence the host’s ability to respond to infection.

SOCS1 is a reported target of miR-155\(^\text{153}\) that is particularly interesting because it alos negatively regulates the inflammatory response\(^\text{154}\). As it so happens, an earlier report by the Tridandapani laboratory showed that SOCS1 mRNA was highly induced in \textit{F.t.} infected cells (low miR-155 expression), though not induced with \textit{F.n.} infection (high miR-155 expression)\(^9\). As expected SOCS1 mRNA inversely correlates with miR-155 expression and this is differently expressed depending on the subspecies of \textit{Francisella} used to infect host cells. Alternatively it has been speculated that miR-155 directly influences TNF\(\alpha\) expression through some direct interaction affecting mRNA translation or stability\(^\text{155}\). It is also demonstrated that miR-155 promotes inflammation in the context of autoimmune disease states\(^\text{208}\). So, collectively these reports support the role of miR-155 as an enhancer of immune activation.

There also exist reports that indicate miR-155 serves to repress, rather than enhance immune activation\(^\text{156}\). One report indicates that miR-155 inhibits MyD88 expression\(^\text{209}\), yet this is odd since the most recent version of targetscan (www.targetscan.org) does not list MyD88 as a potential target of miR-155. Another
report indicates that miR-155 attenuates NF-κB activation through negative regulation of IKKe\textsuperscript{148}, which is important for TLR3 and TLR 4 signaling down-stream of TRIF to activate NF-κB. However, miR-155 inhibition of IKKe should not be expected to be of any consequence in TLR2 signaling, which is used by \textit{Francisella} in chapter 2. In fact SHIP itself has been reported to have different effects on the inflammatory response, where this molecule suppresses TLR2 mediated responses\textsuperscript{38,39}, though enhances TLR4 responses\textsuperscript{210}. This indicates that the nature of the stimulus many dictate the role of miR-155 and SHIP in promoting or inhibiting the inflammatory response.

One issue with miRs is that they modulate or fine tune gene expression, hence their effects are somewhat minor on protein expression\textsuperscript{157}. It is well supported that miR-155 negatively regulates SHIP\textsuperscript{53;120;121;211}, though this is only one mechanism and there must be other important mechanisms to regulate this phosphatase. There are some interesting observations about the connection between miR-155 and SHIP from the literature and our own unpublished work. Chapter two shows that miR-155 is induced during \textit{F.n.} infection and SHIP expression is also dramatically reduced\textsuperscript{53}. However, work by O’Connell \textit{et al.} show that LPS stimulated macrophages also induces miR-155 expression, yet there is an overall increase in SHIP expression after LPS treatment and this is despite the fact that this miR still targets SHIP in that study\textsuperscript{120}. Unpublished work by the Tridandapani laboratory indicates that R848, a TLR7 ligand\textsuperscript{212}, induces miR-155 expression though has no observable effect on SHIP protein expression. So SHIP expression does not always inversely correlate with miR-155 expression, thus there must be other overriding mechanisms that control SHIP expression depending on the nature of the stimulus. This is not to discount the importance of the connection between miR-155
and SHIP, because in vivo studies done with miR-155 over-expressing mice develop a similar phenotype as SHIP deficient mice\textsuperscript{33,152}, though this is just to point out some interesting anomalies on this subject that require additional work.

Mechanisms of miR-155 regulation and suppression

Initially the mechanism of miR-155 induction by\textit{Francisella} was quite perplexing, though upon further examination in chapter three the mechanism was clarified. Early on we observed no difference in the induction of this miR in the phagosomal escape mutants. This was concerning because phagosomal escape promotes IFN\textbeta\textsuperscript{66} and IL-1\beta expression\textsuperscript{67}, and both of these cytokines can induce miR-155 expression\textsuperscript{119,141}. Also there was a requirement of new host protein synthesis and it was found that soluble factors from the conditioned media of infected cells could transfer miR-155 responsiveness. We initially interpreted this to mean that an autocrine/paracrine acting cytokine mediated the induction. Yet neutralization of TNF\alpha and IFN\beta had no effect on\textit{Francisella}-induced miR-155 expression.

After challenging our initial hypothesis, we tested the possibility that it was a soluble bacterial factor and not a host factor that induced miR-155 expression. Indeed bacterial conditioned media alone is sufficient to induce miR-155 expression in phagocytes. Coupled with this, we find that caspase-1 and MyD88-dependent cytokines are not critical mediators of miR-155 induction. It is cell surface TLR2 mediated signaling which is the essential factor for regulating miR-155 expression during\textit{Francisella} infection. Finally we reconcile the issue with the requirement for new host protein synthesis. While NF-\kappa B is necessary for miR-155 induction, it is not sufficient.
What this transcription factor does is up-regulate the expression of AP-1 transcription factors that are responsible for directly mediating transcription of the BIC/miR-155.

It is worth mentioning that there have been recent reports showing that IL-10\textsuperscript{164} and TGF\(\beta\)\textsuperscript{125} inhibit miR-155 induction. The focus in the work of chapter three was to determine how \textit{F.n.} mediates miR-155 induction. Suppressors of miR-155 induction were not a focus of this study, so it remains to be determined what the role of IL-10 or TGF\(\beta\) is during \textit{F.n.}-mediated miR-155 induction. These findings are pertinent to the issue of \textit{F.n.} versus \textit{F.t.} miR-155 induction because there was clearly a lower induction of miR-155 during infection with highly virulent \textit{F.t.} subspecies as compared to low virulence \textit{F.n.} subspecies, which was shown in figure 2.16. To briefly explore the possibility that miR-155 induction was being suppressed by the virulent subspecies of \textit{Francisella}, \textit{F.t.} A and \textit{F.t.} B, through higher induction of IL-10 or TGF\(\beta\), the microarray data base that was generated previously\textsuperscript{9} was examined. \textit{F.n.} infected monocytes yield high miR-155 induction, while \textit{F.t.} A and \textit{F.t.} B infected monocytes have low miR-155 induction (Figure 5.1). But the expression of IL-10 or TGF\(\beta\) mRNA does not inversely correlate with miR-155 induction by infection with various subspecies of \textit{Francisella}. Thus it is unlikely that either of these anti-inflammatory cytokines is responsible for the reduced miR-155 induction in monocytes infected with the high virulence subspecies of \textit{Francisella}.
Figure 5.1: Microarray analysis of IL-10 and TGFB1 expression in Francisella infected monocytes. A-B PBM were infected with an MOI of 50 of F.n., F.t. type A, or F.t. type B for 24 hours. RNA was processed and subjected to microarray analysis. Graphs represent the log fold change in mRNA expression of A IL-10 and B TGFβ. * indicates p value <0.05 as compared to uninfected samples.

Future studies are required to determine how F.t. suppresses or evades miR-155 induction. This could be address by conduction co-infections of F.n. and F.t. If the response of the mixed infection is low like with F.t., then there is likely a dominant suppressive factor present in F.t. Though if the response in a mixed infection is more like that of F.n., then it seems more likely that F.t. simply lacks the ability to induce this miR as opposed to actively suppressing its expression. Based on some of our earlier studies and unpublished data it seems that F.t. actually has a mechanism of active suppression. First of all we see that F.t. down-regulates the expression of TLR2, PI3K p85, and Akt, though F.n. does not\(^9\). Additionally F.t. can make PTEN hyperactive to inhibit PI3K and NF-κB signaling\(^25\).
Akt as a regulator of host-response to *Burkholderia*

Chapter five took several key steps in showing that PI3K signaling is a key component of the host-response to *Burkholderia* infections. Functionally we see that this pathway is essential for the production of pro-inflammatory cytokines from mononuclear phagocytes. This is important because during *B.c* infections there is a counter productive inflammatory response that leads to pathology\(^\text{108}\).

There was an interesting contrast with the studies on intra-macrophage replication of *Burkholderia* and the earlier studies with *Francisella*. While with both pathogens PI3K signaling is dispensable for phagocytosis, this pathway promotes the intracellular control of *Francisella*, yet not *Burkholderia*. This is likely due to the fact that these organisms have differences in their intracellular lifecycle. Both organisms prevent phagosome maturation, though *Francisella* replicates in the cytosol\(^\text{63}\) while *Burkholderia* replicates in a vacuole associated with the ER much like *Legionella*\(^\text{91}\).

Future studies should investigate the role of PI3K/Akt/GSK3β inhibitors *in vivo*. The animal models for *B.c* infection are somewhat complicated because it requires the use of a genetically susceptible mouse\(^\text{197}\) or immunosuppression\(^\text{108}\). The immunosuppression method can be used in a comparison of host resistance to *B.c* in WT versus MyrAkt mice. It is reasonable to speculate that the MyrAkt mice will have increased susceptibility as they will produce higher levels of the pro-inflammatory cytokines. Alternatively WT mice can be administered the PI3K inhibitor wortmannin *in vivo* to test the function of PI3K signaling\(^\text{24}\) and this may provide increased resistance against *B.c* infection.
In conclusion these findings have advanced our knowledge of the role of the PI3K/Akt signaling pathway as a key component of the innate immune response and show that this pathway is dynamically regulated during microbial infection. It seems likely that the role of PI3K/Akt signaling will be pathogen specific. By this it is meant that in the case of one pathogen signaling through this pathway is beneficial, though in the case of another pathogen it could be detrimental. Thus we must fully understand the consequences of targeting this signaling pathway in a variety of contexts. While there are obstacles to translating these findings to novel therapeutics, such as the lack of therapeutically viable inhibitors of many of these molecules and the potential for unintended consequences from manipulating the immune response, these studies have at provided insight on the host-response to intracellular bacterial pathogens. Many intriguing scientific questions remain in this field and this provides fertile ground for future studies to advance this important subject of research.
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Appendix: Differences in miR-155 Induction in Primary Cells Versus Cell Lines

Infected with *Francisella*

Cell lines are often used for *in vitro* experiments due to their ease of use and cost-effectiveness. However, in studies involving miR analysis primary cells are highly preferable because they are more efficient at miR processing. Cancerous cells have a general pattern of reduced miR expression as compared to normal cells\(^{213}\). This issue arose in early stages of our miR-155 studies when THP-1 monocytic cells were being used to assay miR-155 expression. While miR-155 was induced in response to *F.n.* infection, the response was less than a 2- to 3-fold increase. This minimal response was rather concerning during the preliminary studies on the role of miR-155 during *Francisella* infection. However, when we examined the miR-155 response to *F.n.* infection in primary human monocytes, there was up to a 60-fold increase.

Therefore, it became necessary to directly compare the miR-155 response in THP-1 cells versus PBM. Here both types of monocytes were infected with *F.n.* at an MOI of 50 for 24 hours in parallel. Basal expression of miR-155 was actually similar between the two cell types. While qRT-PCR analysis shows that miR-155 is significantly induced over uninfected samples in both THP-1 and PBM, the inducible expression of miR-155 was significantly higher in PBM than in THP-1 cells (Appendix Figure 1). So the
response still occurs in both cell types, but it is more pronounced in primary cells and thus is a better cell type to study the phenomena of miR response to infection.

Appendix Figure 1: Primary human monocytes have greater miR-155 responsiveness than monocytic cell lines. In parallel THP-1 monocytes and PBM were infected with F.n. at an MOI of 50 for 24 hours. RNA was extracted and assayed for mature miR-155 expression. * designates p value <0.05 for R versus F.n. of the same cell type, while † indicates p value <0.05 for F.n. versus F.n. of the different cell types.

Our initial array analysis was done with THP-1 monocytes and while there were several miRs up-regulated or down-regulated with infection, the differences were quite minimal (Figure 2.4). It seems likely that had primary monocytes been used there would have been additional miRs identified as responsive to infection.

One final comment about technical issues surrounding the studying miR expression is that there is a general effect of cell density on miR expression. Cells cultured at high density have increased processing of a broad range of miRs as compared with cells cultured at low density\textsuperscript{214}. Therefore, experiments should be kept at a consistent cell density. These are important issues to keep in mind for future miR studies.
since this is an emerging field and fundamental technical issues with regard to miR studies are still being worked out.