BURKHOLDERIA SP. MODULATION OF HOST IMMUNE RESPONSE GENES AND PATHWAYS: POTENTIAL INSIGHTS INTO HOST-PATHOGEN INTERACTIONS

by

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(Under the Direction of Robert J. Hogan and Kaori Sakamoto)

ABSTRACT

Among the *Burkholderia* genus, *Burkholderia mallei (Bm)* and *Burkholderia pseudomallei (Bp)* represent pathogens capable of causing fatal infections in animals and humans. Disease manifestations range from acute septicemia to chronic infection, wherein the facultative intracellular behavior of these agents promotes long-term persistence within host tissues. This ability to thrive intracellularly is thought to be related to exploitation of host immune response genes and pathways. As compared to *Bp*, less is known regarding the molecular strategies employed by *Bm* to modulate host immune responses and evade intracellular killing. To fill the gaps in our understanding of the interface between *Bm* and host immune cells as part of the effort to develop effective countermeasures, we designed an intracellular survival assay with human Mono Mac-6 (Mm6) monocytes and profiled the transcriptome of *Bm*-infected monocytes on a genome-wide scale. Gene expression changes in *Bm*-infected monocytes were compared to those of monocytes infected with the relatively non-pathogenic, genetically-related, *Burkholderia thailandensis (Bt)* and to uninfected monocytes. Negative regulators of inflammation, IL-17 signaling, and Pattern Recognition Receptor (PRR) pathways were represented among significantly modulated genes and pathways in infected monocytes. To investigate the role of negative regulators of inflammation in the pathogenesis of *Bm*, we developed a nucleofection system for gene silencing in Mm6 monocytes and explored functional inhibition studies. Our efforts to define interactions between

Bm and IL-17 signaling during intracellular infection also led us to evaluate the potential priming effects of IL-17A stimulation on Mm6 monocytes. While the role of negative regulators of inflammation and IL-17 signaling in *Bm* infection could not be conclusively elucidated, this research provides a novel understanding of the relationship between components of innate immunity induced during infection, such as soluble PRRs, and *Burkholderia* sp. Following the upregulation of the soluble PRR, pentraxin-3 (PTX3), by *Bm*- and *Bt*-infected monocytes, we report on PTX3 as a novel opsonin of *Bt* and *Bp*, together with the evasion of PTX3 recognition by *Bm*. While studies with *Bt* did not suggest a role for PTX3 in potentiating complement-mediated lysis, opsonophagocytosis, or replication within monocytes, the role of PTX3 in *Bm* pathogenesis remains to be clarified.

INDEX WORDS: Human Mm6 monocytes, Negative regulators of inflammation, IL-17 signaling, PTX3, Opsonophagocytosis, Soluble pattern recognition receptor, *Burkholderia thailandensis*, *Burkholderia mallei*, *Burkholderia pseudomallei*

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DEDICATION

I dedicate this dissertation to my 93-year old, French, spitfire grandmother, Marie Louise Augier, whose heart's desire has always been to become a veterinarian and a scientist and whose strong will and perseverance have always been exemplary and inspirational.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to research interests and approach.

Burkholderia mallei (Bm) is a facultative, intracellular bacterial pathogen responsible for causing highly-fatal infections in solipeds and humans.[1-3] While studies have shown that *Bm* can replicate in host phagocytes,[2, 4-10] in part, by dampening the host immune response to intracellular infection,[4, 11] little is known of specific immunomodulatory mechanisms utilized by this pathogen to evade host defense mechanisms, promote intracellular replication, and persist within host tissues.[2] The inability of natural immunity to effectively control infection with *Bm*, together with the poor antibiotic efficacy and lack of effective vaccines against this agent, underscore the need for further investigations of the *Bm*-host interface as part of the global effort to identify novel medical countermeasures.[1, 2]

This project seeks to help fill this knowledge gap by identifying immune response genes and pathways modulated during *Bm* infection of immune cells, with the aim to shed light onto molecular mechanisms contributing to intracellular replication. Towards this end, we developed an intracellular survival assay system with human Mono Mac-6 (Mm6) monocytes, through which we could conduct genome-wide transcriptome profiling of *Bm*-infected monocytes, using GeneChip Human Transcriptome Array 2.0. The transcriptome profile of *Bm*-infected human monocytes was compared to that of monocytes infected with the closely genetically-related, relatively avirulent, *Burkholderia thailandensis (Bt)* and to uninfected monocytes. Our laboratory is the first to describe human monocyte gene modulation by *Bm* or *Bt* on a global, genome-wide scale, with ~9500 host genes exhibiting differential expression ($p \leq 0.001$) **(Chapter 2)**. Negative regulators of inflammation **(Chapter 4)**, as well as IL-17 signaling **(Chapter 5)**, and pattern recognition receptor (PRR) pathways **(Chapter 6)** were significantly

represented among modulated genes and pathways in infected monocytes. To investigate the role of these genes and pathways in the pathogenesis of *Bm*, we established gene silencing techniques **(Chapter 3)**, explored functional inhibition of target genes **(Chapter 4)**, and investigated the potential for modulated genes to prime the immune response when expressed at high enough levels prior to infection (**Chapters 5** and **6**). Specifically, we examined the potential role of IL-17 signaling in priming human monocyte antimicrobial defenses against intracellular bacterial replication **(Chapter 5)** and explored the potential for soluble PRRs, such as pentraxin-3 (PTX3), to serve as opsonins for *Bt*, *Bm*, or *Bp*, promote complement-mediated lysis and/or opsonophagocytosis, as well as influence intracellular replication within human monocytes (*Bt* only) **(Chapter 6)**. While the role that bacterial modulation of host genes representing negative regulators of inflammation and the IL-17 signaling family member, IL-17A, may play on successful intracellular persistence could not be conclusively determined (discussed in **Chapters 4** and **5**, respectively), our investigations of the relationship between PTX3 and *Bt*, *Bm*, and *Bp* culminated in the novel identification of this PRR as an opsonin capable of binding to *Bt* and *Bp*, but not *Bm* **(Chapter 6)**. Finally, conclusions from our studies and future directions are discussed in **Chapter 7**.

This literature review first introduces the studied bacterial agents, *Bm* and *Bt* **(Chapter 1.2)** and the human monocytic cell line employed to study their intracellular behavior **(Chapter 1.3)**. The microarray platform utilized to characterize the transcriptome profile of *Bm*- or *Bt*-infected human monocytes is then described in **Chapter 1.4**. **Chapter 1.5** reviews negative regulation of the host immune response by the closely-related, pathogenic bacterium, *Burkholderia pseudomallei (Bp)*, and finally, **Chapter 1.6** provides background on the studied PRR, PTX3, and its interactions with infectious agents.

1.2 *Burkholderia mallei* and *Burkholderia thailandensis*.

Burkholderia genus

The *Burkholderia* genus represents a large bacterial family comprising over 30 species occupying a wide range of ecological niches from contaminated soils to the respiratory tract of humans and

animals.[12] While many *Burkholderia* species represent non-pathogenic, soil-dwelling bacteria (eg., *Burkholderia thailandensis*), some can cause fatal infections in animals and humans, such as the facultative intracellular pathogens, *Burkholderia mallei* and *Burkholderia pseudomallei*.[12] As compared to *Bp*, less is known of the specific molecular basis for dysregulation of host immune responses by *Bm* during intracellular infection. Thus, this project seeks to provide a better understanding of the molecular *Bm*-host interface via initial studies with the genetically-similar, *Bt*, which has been historically used as a model system to study the intracellular behavior of *Bm* in the Biosafety Level 2 (BSL-2) laboratory.[13, 14]

Burkholderia mallei

Burkholderia mallei is a gram-negative, encapsulated, non-motile, facultative, intracellular bacillus and host-adapted deletion clone of the highly genetically similar pathogen, *Bp*.[1, 13, 15] *Bm* is responsible for causing glanders, a highly contagious and fatal zoonotic disease primarily affecting solipeds (horses, mules, and donkeys).[1] Glanders can also affect humans, and it is almost always fatal without antibiotic treatment.[1] Given its prior (and potential) use as a bioterrorism weapon and highly fatal nature, *Bm* is classified as a Tier 1 Select Agent by the Centers for Disease Control and Prevention.[1-3] The disease is still endemic in parts of the Middle East, Asia, North Africa, and South America [1, 3] and has recently gained attention due to outbreaks in Brazil [16, 17] and a single case of a confirmed, subclinically-infected, serologically-positive horse with PCR-positive skin scabs in Germany in 2014 (as reported by the OIE).[111] Transmission of glanders to susceptible hosts occurs through ingestion, aerosol, or percutaneous routes, and disease manifestations are characterized by respiratory, cutaneous, and lymphatic, ulcerative lesions, abscesses, or granulomas, and/or septicemia.[1, 3] Glanders can manifest as an acute (mules and donkeys) or chronic (horses) disease, with nearly 90% of horses developing chronic or latent infections prior to death and, thus, serving as reservoirs for the maintenance and spread of the disease.[1, 3] Similar to *Bp*, *Bm* can chronically persist within host tissues following apparent clinical resolution.[1] The ability of *Bm* (and *Bp*) to thrive within host cells and tissues is thought to be related to

exploitation of immune response signaling pathways. As compared to *Bp*, relatively little is known regarding the immunomodulation of host genes and pathways that allows for the successful intracellular survival of *Bm* within host cells.[4, 11] Altogether, the lack of an effective vaccine and lengthy courses of multiple antibiotics needed to combat glanders underscore the need to gain a more thorough understanding of the pathogenesis of *Bm* as part of the effort to develop novel medical countermeasures against this agent.[18]

To date, the molecular interface between *Bm* and host immune cells has been predominantly examined from a bacterial genetics standpoint and largely in murine systems.[5-10, 19-21] More specifically, studies have largely focused on characterizing the role of *Bm* virulence factors in the context of adherence, invasion, and intracellular survival within host cells, using murine macrophage cell lines (MH-S, RAW 264.7, or J774. 2 cells).[5-10, 19-21] Additional studies have also examined the transcriptional profile of select *Bm* genes during intracellular infection of macrophages.[5]

Given the prevailing focus on bacterial genetics over the years, the potential mechanisms by which *Bm* modulates host genes and pathways to promote intracellular survival still remain poorly defined. Topological analyses of *Bm*-host protein interactions performed to fill this knowledge gap have provided evidence for *Bm* proteins to globally target intracellular host signaling processes, particularly those related to the immune response.[22] A more focused investigation by Memisevic and colleagues identified a possible interaction between host signaling components central to Toll-like receptor (TLR) signaling, such as TRAF-6 and IκBα, and the *Bm* protein, BMAA0728 (TssN).[23] Together with these *in silico* analyses, limited studies have assessed the molecular impact of *Bm* intracellular infection on host immune responses *in vitro*, with a primary focus on cellular activation and/or select cytokine profiles. The relatively weak activation of murine macrophages by *Bm*, as compared to other gram-negative bacteria, has suggested the potential for this bacterium to directly or indirectly alter host cytokine signaling and TLR pathways.[11] For instance, in contrast to *Escherichia coli*-infected macrophages, *Bm*-

infected murine macrophages produced reduced levels of both IFN-dependent genes and mediators (IFN-β and NO) and cytokines (TNF-α, IL-6, IL-10, GM-CSF, and RANTES).[11] In addition, *Bm*-induced protein expression of iNOS was only observed at higher multiplicities of infection and later time points after intracellular infection, as compared to *E. coli*-stimulated macrophages.[11] In fact, 10 to 100-fold more *Bm* than *E. coli* were required to stimulate murine macrophages to produce similar levels of IFN-β and nitric oxide (NO).[11] Furthermore, *Bm*-infected, primary human monocyte-derived macrophages and alveolar type II pneumocytes released high levels of IL-10 in the early stages of infection, with a delayed induction of pro-inflammatory cytokines (IL-6, TNF-α).[4] This altered cytokine signaling is thought to aid *Bm* in successful intracellular replication prior to host detection and the induction of an effective immune response.[4] Overall, this differential macrophage activation may be related to the *Bm* lipopolysaccharide (LPS), which, similarly to the *Bp* LPS, bears a penta-acylated lipid A with Ara4N in almost half of its molecules and appears to be less efficient at activating macrophages when compared to enterobacterial LPS.[24] Consistent with this, murine bone marrow-derived macrophages stimulated with purified *Bm* LPS exhibited a significant reduction in mRNA expression or secretion of IL-6, TNF-α, and IL-1β, as compared to *E. coli* LPS-treated macrophages, suggesting a significantly lower biological activity.[24]

These *in vitro* studies in both murine and human systems altogether provide evidence for *Bm*-induced modulation (and specifically, downregulation) of host immune responses. However, the limited knowledge of specific molecular strategies employed by *Bm* to manipulate host defense mechanisms during intracellular replication poses a challenge towards the design of effective therapeutic interventions.[4, 11] As part of advancing the understanding of the *Bm*-host interface, this dissertation project seeks to extend on the studies by Brett *et al*. 2008 and Lu *et al*. 2012 by assessing global host transcriptional profile changes during intracellular infection of monocytes on a genome-wide scale in the biologically-relevant human host.

Burkholderia thailandensis

Burkholderia thailandensis is a gram-negative, acapsular, motile bacillus and environmental saprophyte endemic to the moist soils and stagnant waters of Southeast Asia and northern Australia.[14, 25] Although considered relatively non-pathogenic compared to *Bm* or *Bp*, this bacterium does display a high degree of DNA sequence homology to these pathogenic species and, in particular, possesses several homologs of known virulence factors expressed by these related pathogenic bacteria.[14, 25] Like *Bm* and *Bp*, *Bt* is also capable of replicating within several eukaryotic cells, polymerizing host cell actin to facilitate intra- and intercellular spread, as well as stimulating cell fusion and multinucleated giant cell formation.[14] Despite similarities to the intracellular lifestyle of these pathogenic sp. *in vitro*, *Bt* is very rarely pathogenic to humans[26-28] and only exhibits virulence in animal models of infection when high inoculum doses are used.[29-32] For these collective reasons, *Bt* is considered a tractable model system to study aspects of *Bm* (and *Bp*) pathogenesis.[14]

As a well-established model system for the study of pathogenic *Burkholderia* species, the interactions of *Bt* with host cells have been extensively investigated in both human and murine systems.[21, 33-46] Most of the studies have focused on the invasion, adherence, or intracellular survival characteristics of *Bt*, as a comparison to those of *Bp*, across human epithelial cell lines (HeLa, A549, HEK 293) or professional phagocytes (primary monocyte-derived dendritic cells or macrophages, monocytes, mononuclear cells, THP-1) and murine macrophage cell lines (J774.1/2, RAW 264.7) or primary bone marrow-derived macrophages or neutrophils.[21, 33-37, 39, 40, 42-46] However, out of this body of research, fewer studies have investigated the responses of host cells to *Bt* infection, and these have focused primarily on select cytokine profiles, TLR engagement, or surface receptor changes following infection.[34, 36, 42, 46] As compared to *Bm*, some large-scale host response analyses have been conducted for *Bt* and include a human genome-wide assessment of transcriptional responses of A549 human lung epithelial cells to *Bt* infection[35] and an RNA interference screen of the human kinome to identify kinases that may

facilitate *Bt* intracellular survival in human THP-1 monocytes.[41] Given the limited understanding of host responses to *Bt* infection and how those may compare to the *Bm*-host interaction, this dissertation project builds upon prior work with the novel characterization of global human monocyte transcriptional responses to *Bm* or *Bt* infection.

1.3 Human monocytic cell line: Mono Mac-6.

The Mono Mac-6 cell line (Mm6) was established from the peripheral blood of a 64-year-old, male patient with acute monocytic leukemia.[47] This cell line is characterized by round, individual or small clusters of cells in suspension (with a small percentage of multinucleated giant cells) that can sometimes be loosely adherent.[47] These cells were assigned to the monocyte lineage based on morphological, cytochemical, and immunological criteria[47] and possess several features of mature blood monocytes, such as constitutive phagocytosis, cytokine production, and adherence to endothelial cells.[48-50] This cell line also produces reactive oxygen species and expresses non-specific esterase, as well as a broad range of surface markers **(Table 1.1)**. Among these markers, CD14 (together with TLR4) has relevance for the response to bacterial LPS, and though a specific receptor has not yet been characterized for PTX3, CD11b and CD32 (FcγRII) have been suggested to contribute to the opsonophagocytic activity of this PRR.[51] Most of the markers displayed by this cell line are shared with mature peripheral blood monocytes,[47, 50, 52-54] and, for this reason, Mm6 cells have often been used as an *in vitro* model for monocytes.[48] For the study of macrophage responses, Mm6 monocytes can be induced to terminally differentiate by various stimuli, including prostaglandin E2 (PGE_2), LPS, and tetradecanoyl-phorbol-13-acetate (phorbol 12-myristate 13-acetate, PMA).[50]

As a model system for the study of host monocytes, Mm6 cells have been extensively studied in the context of the host-pathogen interaction for several microorganisms, including *Burkholderia cepacia*,[55-57] *Pseudomonas aeruginosa*,[55, 56, 58] *Stenotrophomonas maltophilia*,[55, 56] *Escherichia coli*,[55, 56, 59] *Neisseria gonorrhoeae*,[60] *Bartonella henselae*,[61] *Mycobacterium tuberculosis*,[62] *Legionella* sp.,[52, 53, 63]

Table 1.1. Select cellular markers expressed by Mm6 monocytes.

Surface markers	Characteristics	Reference
CD14	Mediates innate immune response to bacterial lipopolysaccharide	[47, 50, 52, 53]
CD11a (LFA-1)	Integrin, mediates cell adhesion	[53]
CD11b	Integrin, mediates cell adhesion, phagocytosis of complement-coated particles	[52]
CD13	Involved in the metabolism of regulatory peptides	[47]
CD15	Adhesion molecule, mediates phagocytosis, chemotaxis	[47]
CD33	Adhesion molecule, mediates sialic acid-dependent binding to cells	[50]
CD18	Integrin, mediates cell adhesion, cell surface-mediated signaling	[52]
CD29	Integrin, monocyte/macrophage trafficking, activation, signal transduction and/or adhesion	[52]
CD54 (ICAM-1)	Intercellular adhesion molecule	[52, 53]
CD58	Adhesion molecule	[53]
CD71	Transferrin receptor protein 1, necessary for cellular iron uptake by the process of receptor-mediated endocytosis	[52, 53]
CD68	Scavenger receptor family member, promotes phagocytosis and macrophage recruitment/activation	[47]
HLA-DR	MHC Class II cell surface receptor involved in antigen presentation	[53]
CD64 (FcγRI)	High-affinity receptor for the recognition of the Fc portion of non-complexed monomeric IgG	[54]
CD32 (FcγRII)	Low-affinity receptor for the recognition of the Fc portion of aggregated IgG or antibodies complexed with multivalent antigens	[54]
CD23 (FcεRII)	Low-affinity receptor for IgE, C-type lectin	[50]

CD, cluster of differentiation; LFA-1, leukocyte function-associated antigen-1; ICAM-1, intercellular adhesion molecule-1; HLA-DR, human leukocyte antigen D related; FcR, fragment, crystallizable region receptor.

Toxoplasma gondii,[64] and *Chlamydia pneumoniae*.[65] The vast majority of these studies has focused on characterizing the adherence, phagocytic, and intracellular survival properties of the studied organisms in Mm6 monocytes. Among these, many have included a succinct analysis of select host responses, such as a specific cytokine response repertoire (and associated signaling components), respiratory burst generation, and select surface marker expression changes to microorganisms or to associated moieties.[52, 53, 55-63, 65] To our knowledge, however, only two studies have assessed gene expression profile changes of Mm6 monocytes on a large scale in the context of microbial infection or associated moieties.[64, 66] McPhillie *et al*. 2016 utilized RNA and miRNA sequencing to characterize the transcriptome profile of Mm6 monocytes infected with the EGS strain of *Toxoplasma gondii*, [64] while Heller and colleagues [66] conducted a small-scale, 86-target-based microarray study of Mm6 transcriptomic changes

following exposure to LPS- and PMA-treatment. The transcriptome profile of human Mm6 monocytes has, to date, not been evaluated in the context of a response to any bacterial infection. Not only does the work presented herein showcase the first, comprehensive, genome-wide analysis of transcriptional changes in human Mm6 monocytes exposed to bacteria, but it also represents the novel reporting of the global host response to *Bm* or *Bt* infection.

1.4 GeneChip Human Transcriptome Array 2.0: microarray technology.

DNA microarray technology utilizes microchips containing anchored arrays of short DNA elements (ie, probes) for the large-scale interrogation of gene expression.[67] Target nucleic acid samples are labelled and applied to the arrays, and relative gene expression is determined following hybridization of nucleic acid sequences to specific probes on the array and subsequent detection of hybridization events.[67] In this research project, our laboratory utilized GeneChip Human Transcriptome Array 2.0 (HTA 2.0) (Affymetrix) as the DNA microarray platform to assess the transcriptome profile of human Mm6 monocytes infected with *Burkholderia mallei* or *Burkholderia thailandensis*. GeneChip HTA 2.0 represents a high-resolution, *in-situ* synthesized, DNA oligonucleotide probe microarray that interrogates all transcript isoforms of the entire human genome.[68] To achieve this, GeneChip HTA 2.0 features >6 million distinct probes targeting coding transcripts, exon-exon splice junctions, as well as non-coding transcripts.[68] More specifically, this high probe density translates into a median of 150 probes per transcript, 10 probes per exon, and 4 probes per exon-exon splice junction. This platform is also equipped with several built-in controls for total target RNA preparation, cDNA hybridization, washing, and staining procedures.

Affymetrix platforms, such as the GeneChip HTA 2.0, are configured with an effort to address known limitations of DNA microarrays and have been found to correlate highly with RNA sequencing and real-time quantitative PCR in terms of their capacity to measure relative gene expression profiles.[69] In particular, GeneChip HTA 2.0 was built using an algorithm-based probe design to prevent cross-

hybridization, has a high dynamic range (~3 logs) allowing for the identification of both low and high abundance transcripts, and possesses built-in probe sets dedicated to correcting background signal. Furthermore, the array is annotated using a knowledge base curated from multiple sources including RefSeq, Ensembl, UCSC (known genes and lincRNA transcripts), Vertebrate Genome Annotation Database, Mammalian Gene Collection (v10), www.noncode.org, lncRNA db, and Broad Institute, Human Body Map lincRNAs and TUCP catalog. However, in contrast to RNA sequencing technology, GeneChip HTA 2.0 and other DNA microarrays depend on these genome annotations for probe selection and are, therefore, unable to detect novel transcripts and isoforms.[69, 70] Given that the project presented herein seeks to characterize the modulation of known human genes during infection with *Burkholderia mallei* and *Burkholderia thailandensis*, rather than identifying links between *Burkholderia* sp. and novel host genes, this particular feature was considered an advantage in helping focus our research efforts.

1.5 Negative regulators of inflammation and the *Burkholderia* genus.

Inflammatory responses must be tightly regulated to maintain immune homeostasis and prevent autoimmune and inflammatory diseases.[71] Towards this end, an important regulatory mechanism involves the enhanced expression of negative regulators of inflammation, which target TLRs or other inflammatory cascades and are necessary to maintain a homeostatic balance between immune activation and inhibition within the host.[71-73] The capacity for negative regulators to fine-tune and dampen inflammatory responses makes them vulnerable for exploitation by pathogens. In fact, several organisms, including *Bp*, have been reported to augment expression of these regulators to dampen host responses to intracellular infection and promote persistence within host cells. While this relationship is well documented for *Bp*, there is currently limited knowledge of immunomodulatory mechanisms employed by *Bm* to direct successful intracellular replication in host cells.[4, 11, 22, 23] This dissertation project seeks to bridge this gap in knowledge by globally investigating potential immunomodulatory mechanisms utilized by *Bm* to persist within human monocytes and specifically determining whether

this pathogen also induces negative regulators of inflammation to support its intracellular lifestyle. Given the close genetic relatedness of *Bm* to *Bp*, **Chapter 1.5** represents a comprehensive and pertinent review of host responses to *Bp* infection with an emphasis on immunomodulatory and immunoevasive strategies employed by this organism.

Immunomodulation during melioidosis

Downregulation of innate immune response components has been primarily demonstrated for *Bp*, particularly during the course of adaptation to the intracellular lifestyle in host phagocytes *in vitro*, as well as in murine models and human cases of melioidosis. Immunomodulation during melioidosis is characterized by increased expression of host negative feedback genes,[74-86] increased expression of anti-inflammatory cytokines and mediators,[79, 82] increased levels of immunosuppressive enzymes,[75, 87] and decreased pro-inflammatory cytokine production,[79, 84] resulting in defects in phagocyte function.[75, 84-87]

Melioidosis-induced suppression of host innate immunity

In vitro data suggest that *Bp* can efficiently replicate within host phagocytes, such as macrophages, through reduced cellular activation and suppression of antimicrobial responses.[75, 85-87] *Burkholderia pseudomallei*-induced modulation of host cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE_2), and Arginase 2 (Arg2) pathways represents a proposed mechanism for evasion of macrophage killing.[87] The bacterium rapidly upregulates host COX-2, PGE_2, and Arg2 following invasion of murine macrophages.[87] Prostaglandin E2 acts to suppress the intracellular level of NO, a key antimicrobial molecule that aids in killing of intracellular pathogens, such as *Bp*.[87, 88] Prostaglandin E2 exerts this function through the upregulation of Arg2, whose activity results in impairment of macrophage NO synthesis and bactericidal function **(Table 1.2)**.[75, 87] The link between COX-2, PGE_2, and Arg2 and impairment of macrophage microbicidal activity was confirmed by chemical inhibition of the COX-2 enzyme, which resulted in enhanced nitrite production and intracellular killing of *Bp*.[87]

Murine melioidosis models and human septic melioidosis cases also provide evidence for downregulation of innate immunity.[75] Upregulation of Arg2 was noted in murine liver and spleen early in the course of intravenous infection and was accompanied by increased iNOS expression, suggesting a competitive interaction between these two enzymes and the possibility of decreased NO production and depressed macrophage antibacterial activity **(Table 1.2)**.[75] Later in the course of this infection, the anti-inflammatory cytokine, IL-10, was upregulated within these infected murine tissues **(Table 1.2)**.[75] Upregulation of this regulatory cytokine was also observed 3 days post-infection in both the serum and lung of mice aerogenically infected with *Bp*, with continued significant increases in serum IL-10 by day 7 post-infection.[89] In an intranasal melioidosis model, mice infected with 3×10^3 colony-forming units (CFU) of wild-type (WT) *Bp* 1026b also showed upregulation of Arg2 in lung tissue, wherein a significant increase in both pulmonary Arg2 and PGE_2 expression correlated with disease progression.[87] The implication of Arg2 and PGE_2 in the pathogenesis of murine pneumonic melioidosis was confirmed by selective COX-2 inhibition, which resulted in improved host survival and clearance of pulmonary bacteria.[87] In a different murine intranasal melioidosis model, low levels of pulmonary TNF-α and IL-6, and splenic IFN-β and IL-6, were observed in mice infected with 100 CFU of WT *Bp* KHW.[84] This cytokine profile contrasted with the heightened inflammatory responses noted in the tissues of mice challenged with *Bp* KHW tssM mutant through the same route, suggesting an additive role for direct modulation of innate immunity by secreted bacterial virulence factors, such as TssM.[84] In humans, increased mRNA expression of anti-inflammatory cytokines and mediators (IL-10, IL-4, TNFR1, and IL-1RA) **(Table 1.2)** was observed in monocytes of Thai patients suffering from septic melioidosis, where a significant increase in IL-1RA and TNFR1 expression predominated in nonsurvivors.[82] Other studies demonstrated decreased levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-8) in the whole blood of Thai patients with septic melioidosis, with a trend towards enhanced IL-10 levels, following *ex vivo* stimulation with *E. coli* LPS, *Bp*, or *Bt*.[79] Hence, *Bp*-mediated suppression of host innate immunity in both murine and human

Table 1.2 Negative regulation of host immune responses by *Burkholderia pseudomallei* and *Burkholderia mallei*.

Regulators	Modulated by	Gene expression	Affected pathway	Possible mechanism	Significance of negative regulation	References
Arginase 2	*Bp*	Upregulated	TLR (TRIF-dependent)	Competes with iNOS for L-arginine substrate	Increased bacterial replication in murine macrophages, increased pulmonary bacteria, decreased murine host survival	1, 2
CIS	*Bp*	Upregulated	JAK/STAT	Inhibition of STAT1, 5	–	3-5
DUSP8, DUSP16	*Bp*	Upregulated	MAPK	MAPK phosphatases	–	17
IL-1RA	*Bp*	Upregulated	JAK/STAT	Inhibition of IL-1β signaling; Induced by IL-4	Predominates in human septic melioidosis nonsurvivors	18-20
IL-4	*Bp*	Upregulated	JAK/STAT-6, TLR	Inhibition of IL-1β and TNF-α signaling and classical activation of macrophages	–	18
IL-10	*Bp, Bm*	Upregulated	TLR, JAK/STAT	Blocks induction of pro-inflammatory cytokines Inhibition of STAT-1 signaling	–	1, 20-23
IRAK-M (IRAK-3)	*Bp*	Upregulated	TLR4, 9	Inhibits IRAK-1/4 phosphorylation	Predominates in human septic melioidosis nonsurvivors	1, 20
NF-κBIA, NF-κBIE	*Bp*	Upregulated	TLR	Sequestration of NF-κB	Predominates in human septic melioidosis nonsurvivors (NFκBIA)	3-5, 24-
SARM-1	*Bp*	Upregulated	TLR3, 4	Inhibition of TRIF activation	Increased bacterial replication in murine macrophages	26
SIRPα	*Bp*	Failure to downregulate	TLR3, 4	Recruitment of SHIP-1, SHIP-2	Increased bacterial replication in murine macrophages	
SOCS3	*Bp*	Upregulated	JAK/STAT, TLR3, 4	Inhibition of JAK/STAT-1, 3, 4, and TRAF3, 6	–	3-5, 25
TNFAIP3 (A20)	*Bp*	Upregulated	TLR2, 3, 4, 9	De-ubiquitylation of TRAF6; NF-κB suppressor	–	1, 22
TNFR1	*Bp*	Upregulated	TNF-α signaling	Cooperative IL-12p40 inhibition with TNF-α	Predominates in human septic melioidosis nonsurvivors	20, 27

Arginase 2; CIS, cytokine-inducible Src homology 2-containing protein; DUSP8, DUSP16, dual specificity phosphatase (DUSP); IL-1RA, interleukin-1 receptor antagonist; IL-4, interleukin-4; IL-10, interleukin-10; IRAK-M (IRAK-3), interleukin-1 receptor-associated kinase-M; NF-κBIA, NF-κBIE, nuclear factor κ-light-chain-enhancer of B cells (NF-κB) inhibitor α, NF-κB inhibitor ε; SARM-1, sterile-α and armadillo motif containing protein-1; SIRPα, signal regulatory protein-α; SOCS3, suppressor of cytokine signaling 3; TNFAIP3 (A20), tumor necrosis factor (TNF)-α induced protein 3; TNFR1, TNF-α receptor 1; TLR, Toll-like receptor; JAK/STAT, Janus kinases-signal transducers and activators of transcription; TRAF, TNF receptor-associated factor; iNOS, inducible nitrous oxide synthase; MAPK, Mitogen activated protein kinase; *Bp, Burkholderia pseudomallei; Bm, Burkholderia mallei.*

Modified from Aschenbroich, Sophie A., Eric R. Lafontaine, and Robert J. Hogan. "Melioidosis and glanders modulation of the innate immune system: barriers to current and future vaccine approaches." *Expert review of vaccines* 15.9 (2016): 1163-1181.

systems through the described molecular mechanisms altogether contributes to reduced phagocyte activation and impaired microbicidal mechanisms, thereby promoting bacterial replication and a poor disease outcome in the host.

Negative regulators of toll-like receptor and cytokine signaling

Active modulation or evasion of TLR signaling by *Bp* may contribute to the reduced macrophage activation characteristic of melioidosis.[74, 77, 78, 84, 88] Unlike other gram-negative bacteria, *Bp* fails to activate genes downstream of the TRIF-dependent (MyD88-independent) TLR pathway, such as IFN-β and iNOS, in mouse macrophages.[84-86, 90, 91] The failure of intracellular *Bp* to induce IFN-β may be linked to a lack of activation of IFN-regulatory factor 1 (IRF-1)[86, 91] and STAT-1,[91] both of which represent transcription factors necessary for increased iNOS expression.[91] This immunomodulatory strategy employed by *Bp* is believed to contribute to evasion of macrophage killing through decreased macrophage activation, abrogation of NO production, and subsequent impairment of antimicrobial activity and persistence of intracellular bacteria.[84-86, 90, 91]

More recent *in vitro* and *in vivo* evidence in both murine and human systems suggests that failure to activate signaling components downstream of TLR and cytokine pathways may, in part, stem from a *Bp*-mediated induction of negative regulators of the host immune system that act to dampen or abrogate these signaling pathways. Increased expression or lack of down-regulation of host intracellular immune system inhibitors, such as SOCS3,[80, 81] CIS,[80, 81] SARM-1,[77, 78] or SIRPα[74, 77] has been described *in vitro* during infection of mouse macrophages with *Bp* **(Table 1.2)**. Each of these negative regulators may contribute to failed TRIF-dependent TLR pathway activation during melioidosis. SOCS3 and CIS also interfere with macrophage responses to interferons through inhibition of JAK/STAT signaling during *Bp* infection.[81] By suppressing genes (iNOS) or transcription factors (e.g. STAT-1, IRF-1) downstream of cytokine or TRIF-dependent TLR pathways, increased expression of host SOCS3 and CIS during intracellular *Bp* infection would ultimately result in loss of phagocytic killing capacity and increased

bacterial replication.[80, 81] Following endocytic uptake into phagocytes, *Bp* further promotes both resistance to phagocytic killing and attenuation of pro-inflammatory cytokine responses through the upregulation of host SARM-1 and failure to downregulate SIRPα.[74, 77, 78] These negative regulators exert their effects by preventing the induction of IFN-β and iNOS and by reducing the rate of degradation of the NF-κB inhibitor, IκBα.[74, 77, 78] The critical role of SARM-1 and SIRPα in the pathogenesis of *Bp* was confirmed following siRNA-mediated knockdown of these negative regulators, which restored IFN-β and iNOS expression and increased IκBα degradation, thereby contributing to reduced intracellular bacterial replication.[74, 77, 78]

There is also evidence for *in vivo* induction of host negative regulators whose targets represent downstream signaling components of the TRIF-dependent or – independent, TLR or cytokine pathways. Murine hepatic and splenic tissues displayed upregulation of NFκBIA, NFκBIE, SOCS and DUSP family members, IRAK-M (IRAK-3), and TNFAIP3 (A20) following intravenous challenge with *Bp* **(Table 1.2)**.[75] The authors suggest that the early excessive inflammatory cytokine response noted in their acute infection model may be due to delayed upregulation of these negative feedback genes, which occurred 42 h post-infection.[75] Upregulation of IRAK-M was also noted, particularly within lung tissue, of mice intranasally challenged with *Bp*.[83] In a cohort of human septic melioidosis patients, mononuclear cell expression of IRAK-M and NFκBIA has been shown to be increased and highest in nonsurvivors.[79, 82] In a second cohort of patients from Thailand, whole blood exhibited increased expression of not only IRAK-M and NFκBIA, but also SOCS3 and TNFAIP3, although correlations with patient survival were not investigated.[76]

Altogether, modulation of host negative regulators of TLR and cytokine signaling likely play a role in immune evasion and perhaps translate clinically into rapid disease progression during melioidosis.[79, 82, 87] This notion is supported by the established correlations between increased expression of these immunoregulators with mortality or increased tissue bacterial burden in human

and/or murine systems.[79, 82, 87] During infection, WT *Bp* may use these immunosuppressive mechanisms to modulate the host immune system in favor of bacterial replication through: (1) suppression of NF-κB, as noted *in vitro* following *Bp* ligation of TLR-transfected HEK 293T cells or murine macrophages, and (2) indirect inhibition of LPS-mediated phosphorylation (and therefore proteosomal degradation) of $IKK_{\alpha/\beta}$ and IκBα, resulting in a dampened inflammatory response.[84] While bacterial-induced expression of host negative regulators of the immune system **(Table 1.2)** likely contributes to suppression of NF-κB activation and muted activation in infected cells, a significant additive or synergistic role for bacterial virulence determinants is highly probable. For example, the secreted bacterial deubiquitinase, TssM, is likely to attenuate TLR signaling through specific inhibition of NF-κB and ISRE activation following deubiquitination of IκBα (NF-κB inhibitor), TRAF-6, and TRAF-3 (TLR and TNF-αR pathway signal transducers).[84] These insights into the intracellular pathogenesis of *Bp* suggest that this organism has evolved strategies to not only indirectly modulate host signaling pathways through negative regulators and immunosuppressive enzymes, but also to directly modulate these pathways through virulence factors,[84] thus promoting a more suitable environment for colonization, dissemination, and disease progression.

The immunomodulatory role of LPS in murine and human systems

Mass spectroscopy of the *Bp* LPS lipid A moiety reveals modifications unique to *Bp*, such as 4-amino-4-deoxy-arabinose (Ara4N) capping both phosphate groups and penta-acylation with long-chain fatty acids $C_{14:O}$ (2-OH).[92] Among these modifications, the length, number, and position of fatty acyl chains can affect LPS bioactivity.[92 24] Furthermore, the positively-charged Ara4N residue can contribute to weak immunological properties through partial neutralization of the negatively-charged phosphate backbone necessary to interact with positively-charged amino acids of the TLR4 molecule, thus potentially dysregulating or dampening TLR signaling.[24, 92] These unique structural modifications may be

partially responsible for the less stimulatory nature and low-level immunological activities associated with the *Bp* LPS molecule and the reduced activation of purified *Bp* LPS-treated murine macrophages (RAW 264.7, peritoneal-derived) and human monocytes (THP-1).[92, 93] In particular, the *Bp* LPS structure may be partially responsible for weak induction and/or responses to type I and type II interferons resulting in decreased iNOS expression and NO production in *Bp*-infected murine macrophages.[81, 86] As compared to macrophages exposed to *E. coli* or *Salmonella typhi* LPS, purified *Bp* LPS has been described to elicit reduced activation of murine macrophages by one order of magnitude,[93, 94] with decreased and delayed NO and TNF-α mRNA expression and release,[93, 94] as well as decreased and delayed iNOS protein expression.[94] Consistent with these results, purified *Bp* LPS-treated, differentiated, THP-1 human monocytes also produced significantly lower levels of TNF-α, IL-6, and IL-10, as compared to monocytes exposed to purified *E. coli* LPS.[92] Overall, the interaction between the unique *Bp* LPS structure (particularly Ara4N) and macrophage surface receptors may contribute to a reduced rate of signal transduction, which would help explain the reduced macrophage activation kinetics noted in purified *Bp* LPS-stimulated macrophages, as compared to those exposed to enterobacterial LPS.[94]

Although experiments performed using murine macrophages and human monocytes have provided evidence for *Bp* LPS as a poorly stimulatory molecule, this belief has been challenged by recent work undertaking *ex vivo* stimulation of whole human or murine blood with purified *Bp* LPS.[95, 96] Whole-blood cytokine responses in Thai patients evaluated *ex vivo* following stimulation with purified *Bp* LPS were significantly higher or comparable in magnitude to those induced by *E. coli* or *Salmonella minnesota* LPS, respectively.[95] The authors surmised that differential cytokine responses in their study may be related to: 1) potential pre-existing exposure of Thai subjects living in a melioidosis endemic area to *Bp* with priming of the innate immune system, resulting in a heightened, *Bp* LPS-specific, inflammatory response, and/or 2) LPS preparations and isolation techniques, which may have modified the O-antigen moiety (OPS) structure in the LPS preparation.[95] In a subsequent study that also evaluated

ex vivo stimulation of both whole human and murine blood with purified *Bp* LPS also demonstrated strong inflammatory responses (as evidenced by high TNF-α secretion), which were comparable to those elicited by *E. coli* LPS.[96] However, purified *Bp* LPS-treated murine peritoneal macrophages from this same cohort of mice produced markedly reduced TNF-α as compared to *E. coli* LPS-treated macrophages.[96] While these data suggest a role for the *Bp* LPS as a potent driver of cytokine responses in whole blood, work with human and murine macrophages does indicate an immunomodulatory effect by the *Bp* LPS molecule on macrophage activation and defense mechanisms. In the context of melioidosis, LPS-induced immunomodulation likely represents an additional mechanism by which the bacterium dampens host immune system responses, with reduced macrophage activation promoting intracellular replication, rapid colonization of host tissues, and dissemination within the infected host. Collectively, these findings indicate that the *Bp* LPS-TLR axis deserves further investigation in both human and murine cell lines and whole blood.[95]

Research focused on the role of the *Bp* LPS in intracellular survival within phagocytic cells also indicated a possible contribution for OPS in modulation of phagocytic activation and defense mechanisms. In contrast to WT *Bp*, an OPS mutant displayed increased susceptibility to macrophage antimicrobial activity in both murine macrophages (RAW 264.7)[91] and primary human macrophages freshly isolated from the whole blood of healthy Thai volunteers,[97] which corresponded to increased activation of transcription factors (STAT-1 and IRF-1) and genes (IFN-β and iNOS) downstream of the TRIF-dependent TLR pathway in murine macrophages.[90, 91] The OPS mutant additionally stimulated the transcription of pro-inflammatory cytokines associated with increased IκBα degradation.[78, 91] The ability of the OPS mutant to activate the TRIF-dependent TLR pathway may be related to its ability to suppress SARM-1 and SIRPα, a feature absent in WT *Bp*.[74, 77, 78] Altogether, these findings indicate that the WT *Bp* OPS may contribute to reduced macrophage activation, evasion of intracellular killing, and decreased pro-inflammatory cytokine secretion, potentially through OPS-mediated upregulation of SARM-1 and

failure to downregulate SIRPα.[74, 77, 78, 91] In addition to the inherent immunomodulatory properties of the *Bp* OPS, antigenic variability has been identified across OPS molecules in several human clinical *Bp* isolates.[98] The presence of OPS variants suggests the ability of *Bp* to modulate OPS expression in order to evade immune clearance.[98] More importantly, these findings suggest the possibility that current vaccine candidates may not recognize these novel antigenic variants, resulting in evasion of immune responses and the establishment of a persistent infection in the host.

1.6 Interactions between pentraxin-3 and infectious agents.

Pentraxins are a superfamily of evolutionarily conserved, multifunctional, soluble PRRs that represent a critical component of host innate immunity against microorganisms.[99] The pentraxin superfamily is divided into two subfamilies, the short and long pentraxins.[99] The classical short pentraxins, C-reactive protein and serum amyloid P, represent acute phase proteins produced by the liver, while the prototype long PTX3 is rapidly produced and released by a wide range of other cell types **(Figure 1.1)**.[99, 100] Pentraxin-3 production by these cells is enhanced by pro-inflammatory stimuli (e.g.,

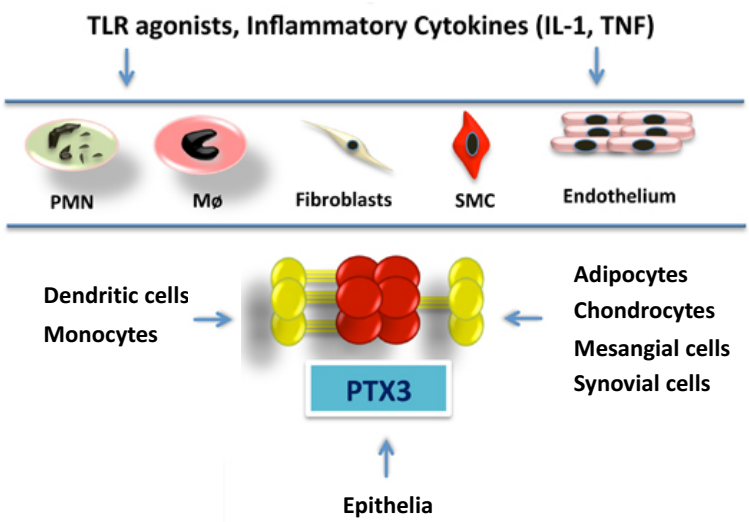

Figure 1.1 Cellular production of pentraxin-3.

Adapted from: Magrini, Elena, Alberto Mantovani, and Cecilia Garlanda. "The Dual Complexity of PTX3 in Health and Disease: A Balancing Act?." *Trends in molecular medicine* 22.6 (2016): 497-510.

TLR engagement, IL-1β, TNF-α) or by direct recognition of microbes or microbial components, leading to significant increases in plasma from physiological concentrations (~2 ng/mL) to 200-800 ng/mL.[99, 100] Following its production, PTX3 fulfills its myriad functions, some of which include: 1) microbial recognition, 2) complement activation and modulation, 3) opsonophagocytic activity, and 4) conferring host resistance to select pathogens *in vivo*.[51, 101-105]

Pentraxin-3 and the recognition of microorganisms

Pentraxin-3 can bind to several bacterial, fungal, and viral agents or microbial moieties **(Table 1.3)**, leading to activation of various antimicrobial effector mechanisms (e.g., complement, phagocytosis and NO production by host phagocytes, etc.) and, thus, promoting host resistance to infection.[99, 100]

Table 1.3 **PTX3-pathogen interface**

Microbial Ligands	Implications of PTX3-ligand interactions	References
Microorganisms		
Bacteria		
Neisseria meningitidis (acapsular form)	Amplification of antibody response, protective role (*in vivo*)	104
Klebsiella pneumoniae (select clinical isolates)	Unknown	
Salmonella typhimurium	Unknown	102
Pseudomonas aeruginosa RP73	Deficiency associated with accelerated death	103
Pseudomonas aeruginosa PA01	Unknown	103
Escherichia coli CFT073	Deficiency associated with enhanced susceptibility and exacerbated inflammation Enhanced opsonophagocytosis (*in vitro*)	105
Fungi and yeasts		
Paracoccidiodes brasiliensis	Enhanced phagocytosis and killing (*in vitro*)	106
Aspergillus fumigatus	Enhanced phagocytosis (*in vitro, in vivo*) Deficiency associated with accelerated death	102 51
Aspergillus flavus	Unknown	102
Aspergillus niger	Unknown	102
Viruses		
Cytomegalovirus	Protective role (*in vivo*)	99

Influenza virus	Protective role (*in vivo*)	[109]
Coronavirus (MHV-1)	Protective role (*in vitro, in vivo*)	[99]
Microbial moieties		
Neisseria-derived OMVs	Enhanced antibody responses	[104]
GNA0667, GNA1030, GNA2091	Unknown	[104]
Kp OMVs	Unknown	[107]
*Kp*OmpA	Enhanced inflammation and leukocyte counts (*in vivo*)	[107]
Zymosan	Enhanced phagocytosis (*in vitro*)	[106]

Pseudomonas aeruginosa RP73 (clinical isolate); *Pseudomonas aeruginosa* PA01 (laboratory strain); *Escherichia coli* CFT073 (uropathogenic strain); *Paracoccidiodes brasiliensis* (yeast form); MHV-1, murine hepatitis virus strain-1; OMVs, outer-membrane vesicles; GNA0667, GNA1030, GNA2091, meningococcal antigens; *Kp*, *Klebsiella pneumoniae*; OmpA, outer membrane protein A of gram-negative enterobacteriaceae; Zymosan, *Saccharomyces cerevisiae* (yeast)-derived particle.

Although PTX3 is able to recognize and target a diverse range of microbial ligands, this PRR does not bind to certain microorganisms, such as some strains of *Klebsiella pneumoniae* (ATCC-27736),[101] certain *Escherichia coli* strains,[102, 106] *Burkholderia cepacia*,[102, 107] or encapsulated *Neisseria meningitides*,[104] nor does it recognize LPS.[108] Also of note is that PTX3 has, to date, not been studied in the context of pathogenic *Burkholderia* sp., such as *Bm* or *Bp*, or in relation to the non-pathogenic, related organism, *Bt*. Therefore, this dissertation project presents novel findings related to the interface between PTX3 and *Bt*, *Bm*, or *Bp* and the potential relevance of these interactions to host defense mechanisms **(Chapters 2** and **6)**.

Integrated innate immunity: Pentraxin-3 and the complement cascade

Pentraxin-3 is involved in activation and/or modulation of complement through the classical, lectin, and alternative pathways and can fine-tune activation of these pathways to prevent excessive host tissue injury[99] **(Table 1.4)**. Towards promoting complement activation, PTX3 binds to initiators of both the classical and lectin complement pathways **(Table 1.4).**[110, 113-115] Initiators of the classical complement pathway (CCP), such as the C1 complex, can interact with PTX3.[110] In particular, the binding

Table 1.4 **PTX3-complement interface**

Ligands	Role of PTX3-ligand interaction	Reference
C1 complex (including C1q)	Classical pathway activation, inhibition	110
Factor H	Alternative pathway inhibition	111
C4BP	Classical and lectin pathway inhibition	112
MBL	Lectin and classical pathway activation	113
M-Ficolin (Ficolin-1)	Lectin pathway activation	114, 115
CFHR1	Unknown	111
CFHR5	Classical pathway activation	111
L-Ficolin (Ficolin-2)	Lectin pathway activation	114
H-Ficolin (Ficolin-3)	Unknown	114

C4BP, complement regulator C4b-binding protein; MBL, mannose-binding lectin; CFHR1, Factor H-related protein 1; CFHR5, Factor H-related protein 5.

interaction between PTX3 and C1q, the C1 complex recognition subunit of the CCP, is as efficient as that seen for C1q and IgG, and can result in either activation or inhibition of the CCP, depending on the spatial arrangement of the C1q-PTX3 interaction.[110] Activation of the CCP via interaction with C1q occurs only when PTX3 is immobilized (solid-phase), which mimics the binding of PTX3 to a microbial surface.[110] Alternatively, the interaction of PTX3 and C1q in the fluid-phase results in complement inhibition.[110] This inhibitory effect likely stems from PTX3-mediated competitive blocking of interaction sites involved in C1q-immunoglobulin binding or in C1q binding to complement activators.[110] Pentraxin-3 also interacts with recognition molecules and initiators of the lectin pathway, such as Ficolin 1 (M-Ficolin),[114, 115] Ficolin-2 (L-Ficolin),[114] mannose-binding lectin (MBL),[113] and Ficolin-3 (H-Ficolin).[114] Specifically, the recruitment of Ficolin-1 to PTX3-coated surfaces resulted in enhanced deposition of C4 (a downstream complement component), suggesting activation of the lectin pathway.[115] Similarly, PTX3 coating of microbial surfaces was shown to enhance deposition of Ficolin-2, also resulting in increased C4 deposition and, therefore, the potential for complement activation, as compared to microbial surfaces not pre-opsonized with PTX3.[114] Lastly, heterocomplex formation between PTX3 and MBL provides a

unique cross-talk between lectin and classical pathway components via the recruitment of C1q and enhanced deposition of C3 (central complement pathway component) and C4, presumably culminating in complement activation.[113] Altogether, while PTX3 is capable of binding to several initiators of the complement cascade and indirectly recruit central or downstream complement components (C3 or C4) to microbial surfaces,[100] this PRR has not been shown to directly activate the complement cascade (eg., enhance deposition of active complement protein cleavage products).

In addition to binding to initiators of the complement cascade, PTX3 also interacts with regulatory components to prevent exaggerated complement-driven inflammatory responses.[112, 116] The binding of PTX3 to the main soluble alternative pathway regulatory protein, Factor H (FH), was found to result in increased deposition of iC3b (the breakdown product of the opsonin, C3b) onto apoptotic cell surfaces, thereby preventing excessive complement activation.[116] PTX3 can also bind to C4b-binding protein (C4BP), the main regulator of the classical and lectin pathways.[112] This PTX3-C4BP interaction is primarily described to occur in association with the extracellular matrix and apoptotic cells and helps prevent escalation of complement activation.[112] Additionally, PTX3 can interact with complement-related proteins, such as Factor H-related protein 1 (CFHR1) and Factor H-related protein 5 (CFHR5).[111] Unlike the interactions with FH, the PTX3-CFHR5 binding interaction prevents binding of FH to PTX3, with enhanced C1q binding to CFHR5-PTX3 heterocomplexes and formation of the alternative pathway C3 convertase, thus potentiating the complement cascade.[111]

The role of pentraxin-3-complement interactions at the microbial surface

Pentraxin-3 has been shown to recruit and augment deposition of initiators of the classical and lectin pathways (C1q, ficolins, MBL), as well as central and downstream effectors of the complement cascade (C3, C4) onto a range of microbial surfaces.[113, 114] These interactions have been predominantly described for PTX3 and fungal organisms. Specifically, PTX3 cooperates with the lectin pathway initiator, MBL, on the surface of *Candida albicans*, to recruit C1q and mediate complement activation, as

suggested by enhanced deposition of C3 and C4 onto yeast surfaces.[113] Similarly, PTX3 has been shown to synergize with another lectin pathway initiator, Ficolin-2, on the surface of *Aspergillus fumigatus*, wherein PTX3-pre-opsonized *A. fumigatus* conidia exhibit enhanced binding of Ficolin-2, resulting in increased C4 deposition as a corollary of complement activation.[114]

Given the suggested role of PTX3 as a mediator of complement activation, its capacity to further coordinate the terminal lytic pathway of complement has been investigated, although to a very limited extent.[103] Aside from the findings presented in this dissertation with *Bt* **(Chapter 6)**, only one other study has investigated the potential involvement of PTX3 in complement-mediated bacterial lysis.[103] This study conducted cytolytic assays with *Pseudomonas aeruginosa* and PTX3 in the presence of 1%, 5%, or 10% serum (as a source of active complement), but results did not demonstrate PTX3-mediated amplification of the terminal lytic phase of complement against this bacterium.[103] Through this study, the authors indicated that these results effectively excluded a role for this PRR in amplifying complement-dependent lysis as a possible explanation for the beneficial effects of PTX3 treatment noted during *P. aeruginosa* infection *in vivo*.[103] In summary, while these studies have demonstrated the capacity for PTX3 to recruit and augment deposition of complement initiators and central or downstream effectors onto microbial surfaces, the ability of this PRR to direct the activation of the terminal phase of complement (ie., the membrane attack complex) to achieve lysis of targeted pathogens has not been described to date.

Opsonophagocytic properties of pentraxin-3

Pentraxin-3 can play a role in the opsonophagocytic removal of selected pathogens directly or by generating further opsonins via complement activation.[51, 102, 103, 105, 106, 112, 113] Although the exact mechanisms associated with PTX3-mediated opsonophagocytosis are still under investigation, this activity appears to require an active complement system and an intact FcγR-pathway.[100, 117] Several studies provide evidence for the serum-dependence of this PTX3-inherent property, across both macrophages and neutrophils, in human and murine systems, and in a wide range of organisms.

Studies assessing the opsonophagocytic potential of PTX3 towards bacteria are few and have focused on the interaction of PTX3-opsonized bacteria with neutrophils. In one study with *Pseudomonas aeruginosa* RP73, PTX3-pre-opsonization of bacteria was found to amplify phagocytosis by murine neutrophils, in the presence of murine whole blood.[103] This pro-phagocytic effect was found to be independent of C1q but dependent on C3 and FcγR.[103] In agreement with these findings, PTX3 also promoted enhanced phagocytosis of *P. aeruginosa* by murine alveolar neutrophils in the presence of normal human serum (NHS) or in C1q-depleted serum, but not in C3-depleted serum or in neutrophils derived from FcγR-deficient mice.[103] Another study similarly demonstrated that PTX3-pre-opsonization of uropathogenic *Escherichia coli* (UPEC) CFT073 enhanced phagocytosis by human and murine neutrophils and induced accelerated phagosomal maturation in the presence of whole blood, suggesting a role for exogenous PTX3 in promoting earlier bacterial disposal.[105] Together with these findings, neutrophils from PTX3$^{-/-}$ mice were shown to display defective phagocytic activity towards UPEC (which could be dramatically improved by PTX3-pre-opsonization of this organism), further highlighting the importance of PTX3 in effective bacterial phagocytosis.[105]

While a paucity of bacterial studies exists assessing the opsonophagocytic role of PTX3, there is, in contrast, a vast body of work detailing this PTX3-inherent property towards the fungal agent, *Aspergillus fumigatus*. In fact, PTX3-mediated enhanced phagocytosis has been demonstrated *in vitro* and *in vivo*, in both murine and human systems, and in a range of phagocytic cells (macrophages, dendritic cells, and neutrophils) in the context of this fungal organism.[51, 102] Pentraxin-3 deficiency has been described to negatively impact phagocytic activity, as evidenced by defective internalization of *A. fumigatus* conidia by PTX3-null murine alveolar macrophages.[102] This finding both indicated an essential role for PTX3 in mediating phagocytosis of this agent and suggested implications for a PTX3 macrophage receptor.[102] Pentraxin-3 deficiency additionally had detrimental effects on phagocyte defense mechanisms, as demonstrated by impaired killing of *A. fumigatus* conidia by PTX3-null murine alveolar

macrophages.[102] However, this collective impairment of phagocytic and conidiocidal activities could be restored by the addition of PTX3 to $PTX3^{-/-}$ murine macrophage cultures.[102] Furthermore, exogenous PTX3 was also able to potentiate these activities in macrophages from $PTX3^{+/+}$ mice.[102] Not only did Garlanda *et al*. (2002) demonstrate a critical role for PTX3 in phagocytosis and microbicidal activity against phagocytosed organisms, these authors also uncovered a role for this PRR in modulating phagocyte activation, as evidenced by defective responses of dendritic cells from $PTX3^{-/-}$ mice to *A. fumigatus* conidia.[102] These impaired responses were characterized by reduced IFN-γ and IL-12 production and lack of MHCII or CD86 upregulation that could be reversed by the addition of PTX3.[102]

Further studies with *Aspergillus fumigatus* also demonstrated a pro-phagocytic effect of PTX3 on human and murine neutrophils both *in vitro* and *in vivo*.[51] Pre-opsonization of *A. fumigatus* conidia with recombinant human PTX3 (rhPTX3) significantly amplified phagocytosis of this agent by human neutrophils in the presence of NHS, as compared to non-opsonized conidia.[51] Of note, when pre-opsonized conidia were washed of unbound rhPTX3, the phagocytic index was comparable to that noted in the presence of unbound rhPTX3, suggesting that conidial opsonization (rather than unbound PTX3-mediated phagocyte activation) was responsible for enhanced phagocytosis.[51] Similar studies performed with murine bone marrow-derived neutrophils in the presence of NHS showed that pre-opsonization of conidia with PTX3 augmented phagocytosis by WT neutrophils and restored the phagocytic ability of PTX3-deficient neutrophils.[51] PTX3-inherent opsonic activity was abolished in the presence of heat-inactivated NHS, Factor B (alternative pathway component)- or C3-depleted NHS, or in the presence of an anti-CD11b or FcγRIIA antibody.[51] However, the phagocytic index was unaffected by depletion of C1q, C4, or terminal pathway (membrane attack complex) component, C5.[51] In addition to these *in vitro* findings, the opsonophagocytic activity of PTX3 has also been shown *in vivo* in mice intratracheally infected with $8x10^7$, heat-inactivated, FITC-labeled, *Aspergillus fumigatus* conidia pre-opsonized with rhPTX3.[51] Following infection with the pre-opsonized agent, there was a significant increase in the

intracellular mean fluorescent intensity and in the percentage of FITC-positive $Ly6G^{+}CD11b^{+}$ cells, with significant increases in the phagocytic index of neutrophils as compared to mice infected with non-opsonized conidia.[51]

The PTX3-fungal interaction has been briefly studied for another fungal organism, *Paracoccidiodes brasiliensis*. In a single study, macrophages from PTX3 transgenic (Tg) mice both showed enhanced phagocytosis and fungicidal activity toward *P. brasiliensis*, leading to a significant reduction in CFUs recovered after 24 h, as compared to the CFUs recovered from WT macrophages.[106] The authors surmised that this reduction in CFUs may be related to enhanced NO production by Tg macrophages.[106]

Finally, the interaction of PTX3 with yeast or yeast-like particles has also been briefly examined for zymosan and *Candida albicans*, respectively. In the case of zymosan (Zy), macrophages from PTX3 Tg mice showed improved phagocytosis of this yeast particle.[106] Similarly, WT macrophages exposed to exogenous PTX3 displayed enhanced Zy uptake that paralleled that seen for Tg macrophages.[106] In addition to enhancing the phagocytic index of WT macrophages, there was also an overall increased number of macrophages internalizing Zy particles.[106] Similarly, PTX3 was shown to enhance opsonophagocytosis of the yeast organism, *Candida albicans*, by human neutrophils in the presence of serum containing MBL.[113]

Collectively, this research suggests that the opsonophagocytic activity of PTX3 may be dependent on both serum (and specifically, an active complement system) and accessible phagocytic receptors, such as FcγRs and CD11b. In addition to serving as an opsonin, some studies propose that PTX3 may also impact phagocyte activation and antimicrobial defenses to promote enhanced intracellular killing of some phagocytosed microorganisms. Furthermore, while there has been a predominant focus on studying the interface between PTX3 and *A. fumigatus*, relatively fewer studies have examined the role of this soluble PRR with bacterial agents. There is likewise a lack of studies assessing the impact of PTX3-pre-opsonized bacteria on the phagocytic or intracellular killing ability of

monocytes or macrophages. The research detailed in this dissertation represents the first body of work to delineate the potential opsonophagocytic activity of PTX3 against a member of the *Burkholderia* genus and in the context of human monocytes.

Pentraxin-3-mediated microbial resistance in vivo

The therapeutic potential of PTX3 in mediating resistance to host infection has been extensively documented in rodent models of infection for several bacterial and fungal pathogens, but has not, to date, been examined in the context of the *Burkholderia* genus.[51, 101-105] Throughout the literature, this therapeutic activity has been noted indirectly or directly in studies with PTX3-transgenic, PTX3-null, or WT animals, as well as with animals receiving rPTX3 protein. Across these studies, beneficial effects associated with PTX3 expression range from improved host survival, balanced inflammatory and cellular recruitment responses, as well as decreased tissue lesions and colonization.

Pentraxin-3-mediated host resistance to pulmonary infection was noted for both *Klebsiella pneumoniae (Kp)* and *Pseudomonas aeruginosa*.[101-103] In a PTX3-Tg murine model of *Kp* infection, mice infected with $3x10^5$ CFU of *Kp* ATCC 27736 exhibited 33% recovery from infection, and lethality was delayed in the remaining 67%; for mice infected with $3x10^4$ CFU *Kp*, 80% of mice survived the challenge.[101] In this model, PTX3-Tg mice showed enhanced pulmonary TNF-α production, as well as greater neutrophil influx and phagocytosis of bacteria by pulmonary neutrophils.[101] Of note, these PTX3-associated beneficial effects were observed even in the absence of detectable PTX3 binding to this *Kp* strain, suggesting that this PRR can direct the clearance of bacterial pathogens *in vivo* through binding-independent mechanisms, perhaps by modulating host cytokine profiles and promoting recruitment of phagocytes to sites of infection.[101] Similarly, the capacity for PTX3 to promote host resistance to pulmonary bacterial infection has been demonstrated in $PTX3^{-/-}$ and WT mice infected with *Pseudomonas aeruginosa*. In one study, PTX3-null mice infected with *Pseudomonas aeruginosa* ATCC 10145 exhibited accelerated death and increased pulmonary CFUs.[102] During chronic pulmonary

infection with *Pseudomonas aeruginosa* RP73, WT mice infected intratracheally with $2x10^6$ of bacteria and concurrently receiving daily intraperitoneal (IP) injections of rhPTX3 starting with the day of infection, were shown to exhibit a significant reduction in pulmonary colonization, as compared to mice that did not receive rhPTX3.[103] Similar decreases in pulmonary CFUs were noted when rhPTX3 treatment was instituted 7 days post-challenge and continued for the duration of the challenge period, indicating that the immunostimulating effects of PTX3 remain effective even in the face of an established infection.[103] However, treatment with rhPTX3 for 3 to 7 days duration post-infection did not confer demonstrable therapeutic efficacy, possibly because continuous, long-term treatment is necessary to combat chronic infection.[103] In addition to reducing pulmonary bacterial load, rhPTX3 also promoted a balanced inflammatory response, with decreased pulmonary pro-inflammatory cytokine levels and airway neutrophil recruitment, as evidenced by reductions in severity of histological lesions of pneumonia.[103] Follow-up studies of acute *P. aeruginosa* infection ($5x10^7$ of RP73 strain) further demonstrated that PTX3-mediated therapeutic activity was retained in C1q-deficient mice, but abrogated in C3-deficient and Fc common γ chain-deficient mice, further highlighting the potential dependence of PTX3-mediated antimicrobial activity on a complex interplay between active complement and FcγRs.[103]

In addition to promoting host resistance against pulmonary bacterial pathogens, the therapeutic effects of PTX3 have likewise been noted for other bacterial agents, such as *Neisseria meningitidis (Nm)* and UPEC CFT073, in rat and mouse models of infection, respectively. In the case of *Nm*, rhPTX3 administered to infant rats at the time of IP infection with $4x10^4$ CFU of MenB strain 2996 was found to significantly reduce blood CFUs 18 h post-IP infection, as compared to vehicle-treated animals.[104] While this work highlighted the therapeutic activity of rPTX3, the study with UPEC focused on infection dynamics of UPEC-infected $PTX3^{-/-}$ mice in a transurethral urinary tract infection model.[105] In this model, $PTX3^{-/-}$ mice exhibited early and persistent exacerbated inflammation, higher levels of bladder and

kidney bacterial burdens, as well as enhanced neutrophil infiltration, chemokine levels, and tissue damage, as compared to WT mice.[105] Overall, the authors suggested that PTX3-deficient mice exhibited defective recognition and clearance of this pathogen.[105]

The therapeutic potential of PTX3 has furthermore been consistently demonstrated in $PTX3^{-/-}$ and WT murine models of pulmonary aspergillosis. Pentraxin-3-null mice subjected to pulmonary aspergillosis all died 3 days post-infection, in contrast to WT mice.[102] This high susceptibility to infection could be reversed following intratracheal (at time of challenge), intravenous, or intraperitoneal (on day 1 and 2 post-challenge) administration of purified PTX3, with markedly improved survival and reduced pulmonary CFUs.[102] Given the decreased pulmonary IFN-γ and IL-12 levels, and increased IL-4 levels in $PTX3^{-/-}$ mice, enhanced susceptibility in these animals was thought to be related to impaired Th1 immunity, as well as defective intracellular uptake of conidia by alveolar macrophages and dendritic cells.[99, 102] In a different model of pulmonary aspergillosis, WT mice intranasally infected with $2x10^7$ of unopsonized *A. fumigatus* conidia and treated with intranasal PTX3 displayed limited inflammation and restrained fungal tissue colonization, while PTX3-treatment was completely ineffective in providing resistance to disease in FcγR-deficient mice, thus providing further support for the importance of Fc receptors in PTX3 activity.[51]

Taken together, as was noted for *in vitro* experiments, PTX3-mediated *in vivo* immunomodulatory effects may also be dependent on active complement and accessible FcγR. Furthermore, these data suggested that the capacity for PTX3 to confer host resistance to infection may be related to its ability to promote a balanced inflammatory response (with a Th1 bias noted in one study) and modulate the recruitment as well as the phagocytic and/or antimicrobial activity of inflammatory cells. The reduction in microbial burdens noted following PTX3 treatment likely stems from PTX3-mediated enhancement of phagocytosis and/or intracellular killing by host phagocytes. Of note, these studies further suggested that PTX3-mediated therapeutic activity can extend beyond a

simple “priming” effect on innate immune responses, as protective efficacy can also be achieved in the face of an establishment of infection.

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CHAPTER 2

THE TRANSCRIPTOME PROFILE OF *BM*- AND *BT*-INFECTED HUMAN MONOCYTES

2.1 Introduction.

Over the years, the intracellular pathogenesis of *Bm* has largely been examined from a bacterial genetics standpoint, with a primary focus on elucidating virulence determinants responsible for driving infection and replication within host cells (as reviewed in **Chapter 1**). However, less attention has been given to studying the potential for *Bm*-directed modulation of the host immune response to allow for intracellular persistence. Towards filling this knowledge gap, limited studies have investigated the molecular impact of *Bm* intracellular infection on host immune responses, through the study of cellular activation and/or select cytokine profiles. These studies have suggested that *Bm* may represent a weaker activator of macrophages (as compared to other gram-negative bacteria), given reduced or delayed levels of IFN-dependent genes and mediators or pro-inflammatory cytokines and the early induction of anti-inflammatory cytokines in murine or human systems.[1, 2] This dissertation project seeks to build upon these studies by assessing for *Bm*-induced modulation of host immune response genes and signaling pathways during intracellular infection on a genome-wide scale. Through a global transcriptome study, we sought to gain insights into molecular mechanisms contributing to evasion of innate immunity and survival of *Bm* within immune cells.

To accomplish this, we first established a cell culture system in which to study the intracellular pathogenesis of *Bm* **(Chapter 2.2)**. We selected the Mm6 human monocytic cell line based on relevance to the susceptible human host and to the blood-bacteria-monocyte interface, as well as the ability of monocyte-macrophages to support *Bm* replication and dissemination to distant organs. Using human Mm6 monocytes, we designed a highly-reproducible, intracellular survival assay in the BSL-2, with *Bt*,

prior to adapting this assay to study the *Bm*-monocyte interface in the BSL-3. Following the development of these systems, we utilized a large-scale, modified intracellular survival assay to identify potential immunomodulatory mechanisms utilized by *Bm* to promote effective replication within human monocytes, in the context of the GeneChip Human Transcriptome Array 2.0 **(Chapter 2.3)**. In this transcriptome analysis, global changes in human monocyte gene expression induced by *Bm* infection were compared to those of *Bt*-infected monocytes. Evidence for modulation of host immune response genes and pathways was then subsequently assessed both at the gene-level and at the pathway-level (the latter using Ingenuity Pathway Analysis) **(Chapters 2.4 and 2.5**, respectively**)**, prior to conducting mechanistic studies described in subsequent Chapters.

2.2 The intracellular lifestyle of *Burkholderia mallei* or *Burkholderia thailandensis* in Mm6 monocytes.

Cell Culture

The human monocytic cell line (Mm6) was used as a tool to study the interaction of *Burkholderia mallei* and *Burkholderia thailandensis* with human monocytes. Mono Mac-6 cells were obtained from the German Culture Collection (DSMZ no.: ACC 124), Braunschweig, Germany and were maintained in 24-well plates in 1 mL of complete medium consisting of RPMI 1640 with 25 mM HEPES, 10% fetal calf serum, 1% L-Glutamine, 1% non-essential amino acids, 1% sodium pyruvate, and 10 µg/mL of recombinant human insulin (Santa Cruz Biotechnology, CAS 11061-68-0) in a humidified 5% CO_2 atmosphere at 37°C. Cell densities were maintained between $2.5x10^5$-$1.0x10^6$ cells/mL, passaged every 3-4 days, and cultured until approximately passage 35. *Burkholderia mallei* ATCC23344 was cultured at 37°C using Brucella medium (BD) supplemented with 5% (vol/vol) glycerol under BSL-3 conditions. *Burkholderia thailandensis* DW503, an *amrAB-oprA* efflux pump mutant of the parental strain *Bt* E264 that is highly sensitive to kanamycin, was cultured at 37°C using Luria-broth (LB) medium under BSL-2 conditions.

Intracellular survival assays to study human monocytic responses to infection with Burkholderia sp.

Our laboratory represents the first to develop an intracellular survival assay system to study the pathogenesis of *Burkholderia mallei* in the context of a human monocytic cell line. Our overarching aim in designing this assay was to develop a highly reproducible system in which to study immune response gene or pathway regulation by *Bm* in comparison to *Bt*, to gain potential insights into pathogenic mechanisms specific to *Bm* or common to both species, which contribute to successful intracellular replication. To accomplish this, human Mm6 monocytes were seeded in a 24-well plate at $1x10^6$ cells/mL prior to infection with *Bt* DW503 or *Bm* ATCC23344. Bacterial suspensions were prepared from plate-grown bacteria resuspended in PBS to an optical density at 580 nm (OD_{580}) of ~1.4. Twenty-five µL of this suspension (for *Bt* DW503; corresponding to a MOI of 1:40) or of a ten-fold dilution of this suspension (for *Bm* ATCC23344; corresponding to a MOI of 1:4) were used to infect human monocytes. Bacteria-monocyte contact was promoted via centrifugation at 400 x g for 4 min. For assays with *Bt* DW503, Mm6 monocytes were infected for 2 h, washed, and subsequently treated with 50 µg/mL of kanamycin (MP Biomedicals) for an additional 2 h to reduce extracellular bacteria. Monocytes were again washed to remove residual antibiotic. Subsequently, a subset of monocytes was lysed with 0.5% saponin for 5 min at 37°C. These monocyte lysates were then serially diluted, plated onto LB-agar plates, and incubated at 37°C for 48 h. The remaining subset of monocytes was re-plated with complete medium for an additional 6 h of incubation at 37°C in a 5% CO_2 atmosphere. This subset of monocytes was then washed, lysed with saponin, and resulting lysates were then serially diluted, plated, and incubated for 48 h at 37°C. For assays with *Bm* ATCC23344, a similar protocol was followed with minor modifications (1 h infection, 1.5 h kanamycin treatment, 7.5 h of additional incubation for a subset of monocytes, plating on Brucella-agar medium). For both assays, following incubation of plated lysates for 48 h at 37°C, CFUs representative of bacteria internalized by monocytes following an initial infection phase and kanamycin treatment (phagocytosis) or internalized by monocytes that underwent an additional 6 h (*Bt*) or 7.5 h

(*Bm*) of incubation (replication) were enumerated. Intracellular growth indices were then determined by dividing the CFUs representative of bacteria internalized at the replication time point by the CFUs representing internalization during phagocytosis.

Data from a series of intracellular survival assays performed with *Bm* ATCC23344 or *Bt* DW503 and human Mm6 monocytes are presented in **Figure 2.1**. These data demonstrate that *Bm* or *Bt* replicate significantly within human monocytes (***p=0.0005 or ****p<0.0001, respectively). Intracellular growth indices of *Bm* or *Bt* in human monocytes are comparable and average ~3.2 and ~3.5, respectively. The fact that a ten-fold dilution of a *Bm* suspension at an OD_{580} of ~1.4 replicates in human monocytes to the same rate as an undiluted *Bt* suspension may be related to pathogenic mechanisms specific to *Bm*.

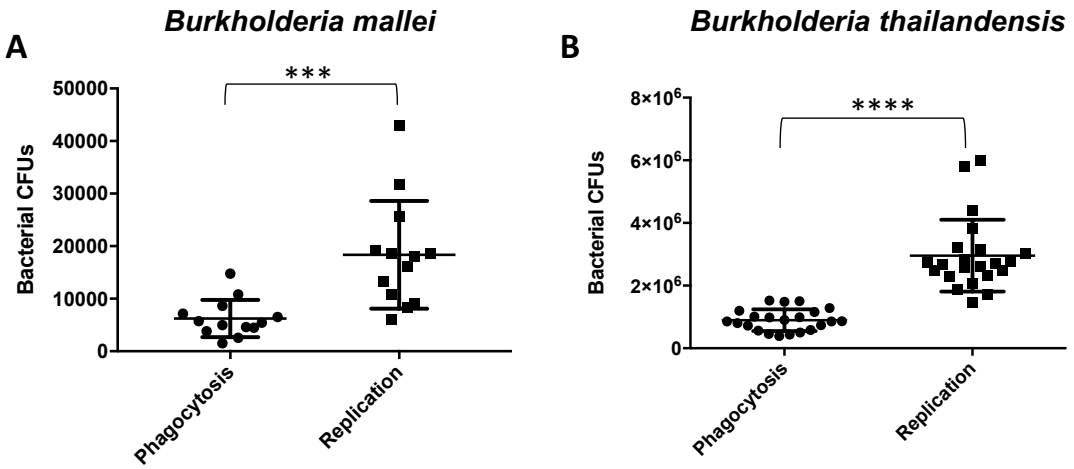

Figure 2.1. *Burkholderia mallei* and *Burkholderia thailandensis* replication in human Mm6 monocytes. **(A)** *Bm* exhibits significant intracellular growth within human monocytes between phagocytosis (2.5 h postinfection) and replication (10 h postinfection) time points (unpaired t-test***p=0.0005). **(B)** *Bt* exhibits significant intracellular growth within human monocytes between phagocytosis (4 h postinfection) and replication (10 h postinfection) time points (unpaired t-test ****p<0.0001).

2.3 Genome-wide transcriptome profiling of human monocytes infected with *Bm* or *Bt.*

Gene expression

Transcriptome profiling of human Mm6 monocytes infected with *Bm* ATCC23344 or *Bt* DW503

was conducted in the context of a large-scale, modified, intracellular survival assay. To capture differential expression of host monocyte genes during infection, the transcriptome of *Bm*- or *Bt*-infected Mm6 monocytes was assessed at 1 h, 3 h, or 6 h postinfection and compared to that of uninfected Mm6 monocytes incubated for 1 h or 6 h. At the indicated time points postinfection (1, 3, or 6 h) or post-incubation (1 h or 6 h), quintuplicate sets of *Bm*- or *Bt*-infected Mm6 monocytes or uninfected monocytes were washed and centrifuged at 2400 x g for 3 min prior to RNA extraction. Human Mm6 monocyte pellets were then resuspended in lysis buffer RLY (Isolate II RNA Mini Kit, Bioline, Inc.) supplemented with β-mercaptoethanol, and following vigorous vortexing, 10% of the resulting lysate was plated onto Brucella-agar plates (*Bm*) or LB-agar plates (*Bt*) prior to transferring lysates to -80°C until further processing. Plates were held at 37°C for 4 days to demonstrate absence of bacterial growth prior to RNA extraction under BSL-2 conditions. Total RNA was extracted from monocyte lysates using Isolate II RNA Mini Kit (Bioline) according to manufacturer's instructions. Quantification of RNA yields and $260/280_{nm}$ and $260/230_{nm}$ absorbance ratios were assessed using NANODROP 2000 Spectrophotometer (Thermo Scientific), and RNA integrity was evaluated via agarose gel electrophoresis. Out of the quintuplicate monocyte-derived total RNA samples representative of each condition, quadruplicate total RNA subsets with $260/280_{nm}$ and $260/230_{nm}$ absorbance ratios ≥ 2.0 were included in the microarray analysis. Subsequently, cDNA was synthesized from these total RNA subsets and labeled using the GeneChip Whole Transcriptome PLUS Reagent kit according to Affymetrix protocols. Ten µg of labeled and fragmented cDNA were then hybridized onto GeneChip Human Transcriptome 2.0 arrays for 16 h at 45°C. Following washing and staining with Affymetrix Fluidics Station 450, arrays were scanned using the Affymetrix GeneChip Scanner 3000 7G Plus. The quality of hybridization was determined using internal controls present within the GeneChip Human Transcriptome Array 2.0.

Microarray Analysis

The data obtained across ~70,000 probe sets present within the GeneChip Human Transcriptome Array 2.0 were reduced to 26,831 probe sets with a corresponding gene entrez ID. Following normalization of the dataset using Robust Multichip Average, as implemented in Partek® software (Partek Inc., St. Louis, MO, USA), unsupervised and supervised analyses were performed. For the unsupervised analysis, the probe sets whose signals varied the most across the dataset were first determined by calculating the coefficient of variance (CoV). Probe sets that exceeded a CoV of 0.5 were then subjected to principal component analysis and hierarchical clustering to visualize the distribution of the dataset and determine the extent of a characteristic host cell response within each of the treatment groups **(Table 2.1)**. A hierarchical clustergram generated in dCHIP[3] showed that quadruplicate monocyte

Table 2.1. Gene expression profile conditions for infected or uninfected human Mm6 monocytes.

Treatment Groups	**Legend**
*Bm*1	Monocytes infected with *Bm* for 1h
*Bt*1	Monocytes infected with *Bt* for 1h
*Bm*3	Monocytes infected with *Bm* for 3h
*Bt*3	Monocytes infected with *Bt* for 3h
*Bm*6	Monocytes infected with *Bm* for 6h
*Bt*6	Monocytes infected with *Bt* for 6h
Uninfected1	Uninfected monocytes incubated for 1h
Uninfected6	Uninfected monocytes incubated for 6h with 50 µg/mL of kanamycin

subsets representative of each treatment group clustered together (data not shown), indicating that each treatment elicited a specific and distinct transcriptome profile in human Mm6 monocytes. To avoid introducing uncontrolled variables into our experimental design, we used Mm6 monocytes of the same passage and subjected to identical experimental conditions, reagent preparations, and RNA isolation protocols.

Following the initial assessment of the human Mm6 monocyte response to each treatment condition, a supervised analysis was conducted in BRB Array Tools (developed by Dr. Richard Simon and

the BRB-ArrayTools Development Team) to investigate gene expression differences between *Bm*- or *Bt*-infected monocytes as compared to uninfected Mm6 monocytes. Supervised class prediction utilizing a random variance model for univariate F tests revealed that out of 26,831 probe sets, 9491 probe sets were differentially expressed among the treatment groups at a significance of $p<0.001$. Subsequently, leave-one-out cross-validation (LOOCV) studies were performed to assess the reproducibility of the datasets and test the abilities of the significant probe sets to truly distinguish between the classifiers (treatment groups). In these LOOCV studies, each array was left out in turn and a classifier was derived for the eight treatment groups by selecting probe sets significant at $p<0.001$. The significant probe sets were then used with the 1st and 3rd nearest neighbor prediction model to predict the class identity of the array that was left out and not included when the classification model was constructed. With eight treatment groups, by chance alone one would expect an error rate of 87.5%. However, in our study, the classifiers performed flawlessly and correctly predicted the treatment groups with 100% accuracy. This finding indicates that gene expression differences can be used to distinguish between the treatment groups with a high level of confidence. Subsequently, fold changes were calculated between *Bm*- or *Bt*-infected or uninfected monocyte groups using only genes significant at $p<0.001$ and exhibiting 100% correct classification by LOOCV.

2.4 Modulation of negative regulators of inflammation in *Bm*- or *Bt*-infected human monocytes.

A subset of genes representing negative regulators of inflammation was found to be upregulated by *Bm*, *Bt*, or both *Burkholderia* species, during infection of human Mm6 monocytes **(Figure 2.2)**. Among these, SOCS3, PTGS2 (COX-2), TNFAIP3 (A20), NFKBIA, NFKBIE, DUSP8, and DUSP16 have also been reported to be upregulated during infection with *Bp* in human and murine systems, and in the case of PTGS2 (COX-2), found to correlate with enhanced intracellular replication of *Bp* in macrophages (as reviewed in **Chapter 1**). To determine the role that these negative regulators of inflammation play in

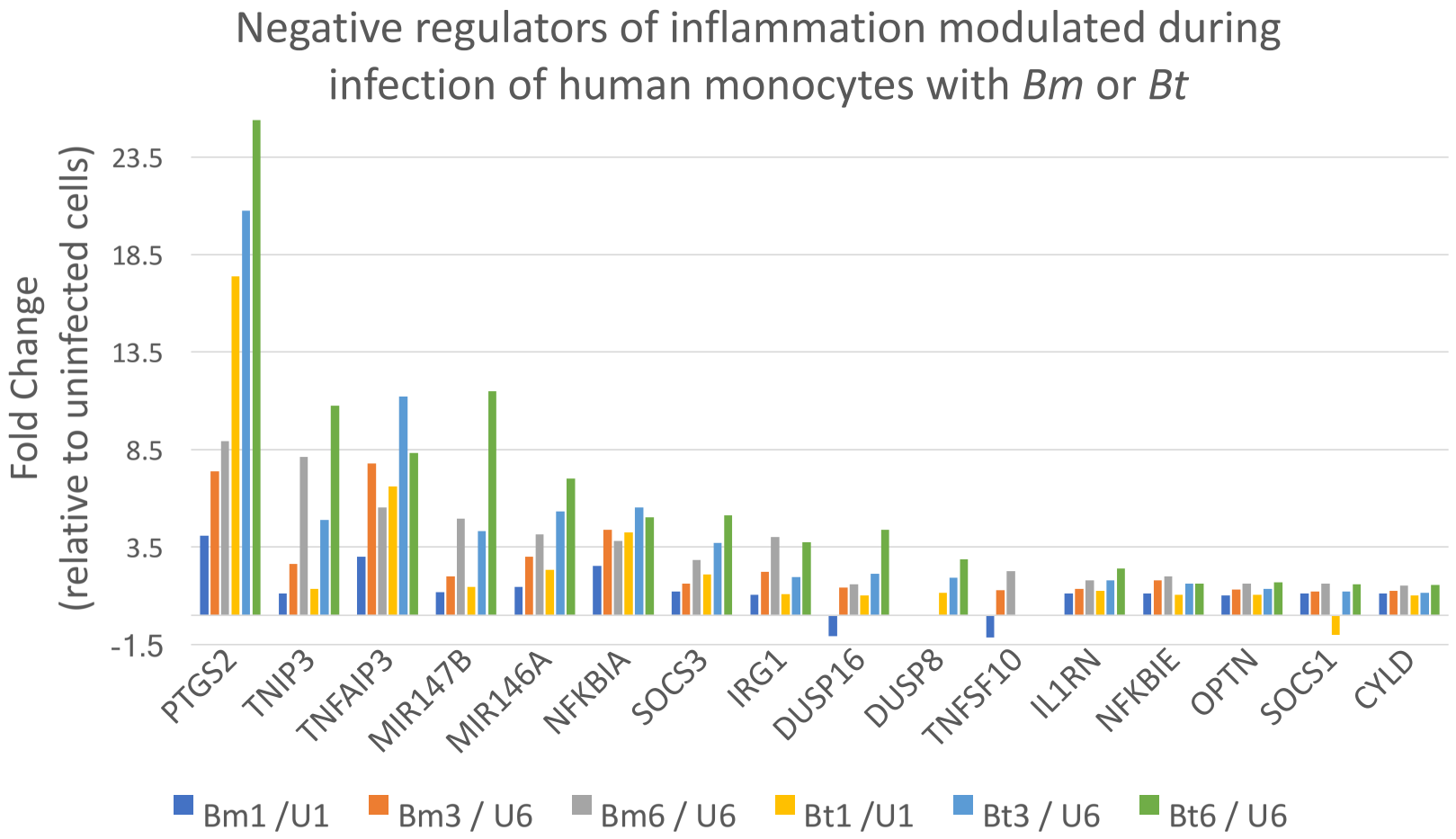

Figure 2.2. Negative regulators of inflammation modulated during *Bm* or *Bt* infection of human monocytes.
The fold change represents the mRNA expression of host genes in infected monocytes relative to the uninfected state ($p<0.001$). A fold change >1 indicates increased gene expression (upregulation), while a fold change >-1 indicates decreased gene expression (downregulation). Bm1/U1, *Bm*-infected human monocytes relative to uninfected monocytes at 1 h; Bm3/U6, *Bm*-infected human monocytes for 3 h relative to uninfected monocytes at 6 h; Bm6/U6, *Bm*-infected human monocytes relative to uninfected monocytes at 6 h; Bt1/U1, *Bt*-infected human monocytes relative to uninfected monocytes at 1 h; Bt3/U6, *Bt*-infected human monocytes for 3 h relative to uninfected monocytes at 6 h; Bt6/U6, *Bt*-infected human monocytes relative to uninfected monocytes at 6 h.

the intracellular pathogenesis of *Bm* within human monocytes, we pursued gene silencing and functional inhibition approaches detailed in **Chapters 3** and **4**.

2.5 Modulation of immune response pathways during *Bm* or *Bt* infection of human monocytes.

Gene Ontology

In addition to investigating the role of negative regulators of inflammation at the gene-level, we wished to more broadly mine the array data for biologically relevant information regarding potential infection-driven immune response pathway regulation. To accomplish this, the 9491 probe sets that were differentially impacted at a significance level of $p<0.001$ were used to populate experimentally observed, known, human canonical pathways, using Ingenuity Pathway Analysis (IPA Qiagen). IPA identifies those pathways that are overrepresented, and significance was determined using a *p*-value statistic of less than 0.05 or an activation score (z-score), with values of $2<Z<-2$ considered significant. The *p*-value assigned to each canonical pathway is determined based on the overlap of genes in our transcriptome dataset with those of known, published pathways, while the z-score is determined by comparing the gene expression directionality within our dataset to known activation or inhibition gene expression patterns in documented, published pathways. The most highly significant human canonical pathways enriched in *Bm*- or *Bt*-infected human monocytes, as ranked by *p*-value and z-score, are represented in the subsequent hierarchical clustergrams (**Figures 2.3** and **2.4**, respectively).

Altogether, analyses performed in IPA demonstrated significant enrichment for several immune response-related canonical pathways in the transcriptome of *Bm*- or *Bt*-infected human monocytes. Among these enriched pathways, we focused our studies on understanding the role of IL-17 signaling and Pattern Recognition Receptor (PRR) pathways during *Bm* infection. The role of IL-17 cytokine family members in the intracellular pathogenesis of *Bm* has not been previously explored, and the interactions between *Bm* and soluble, membrane-anchored, or intracytoplasmic PRRs, are likewise poorly understood. As part of the IL-17 signaling pathway, both the well-described role of IL-17A in host

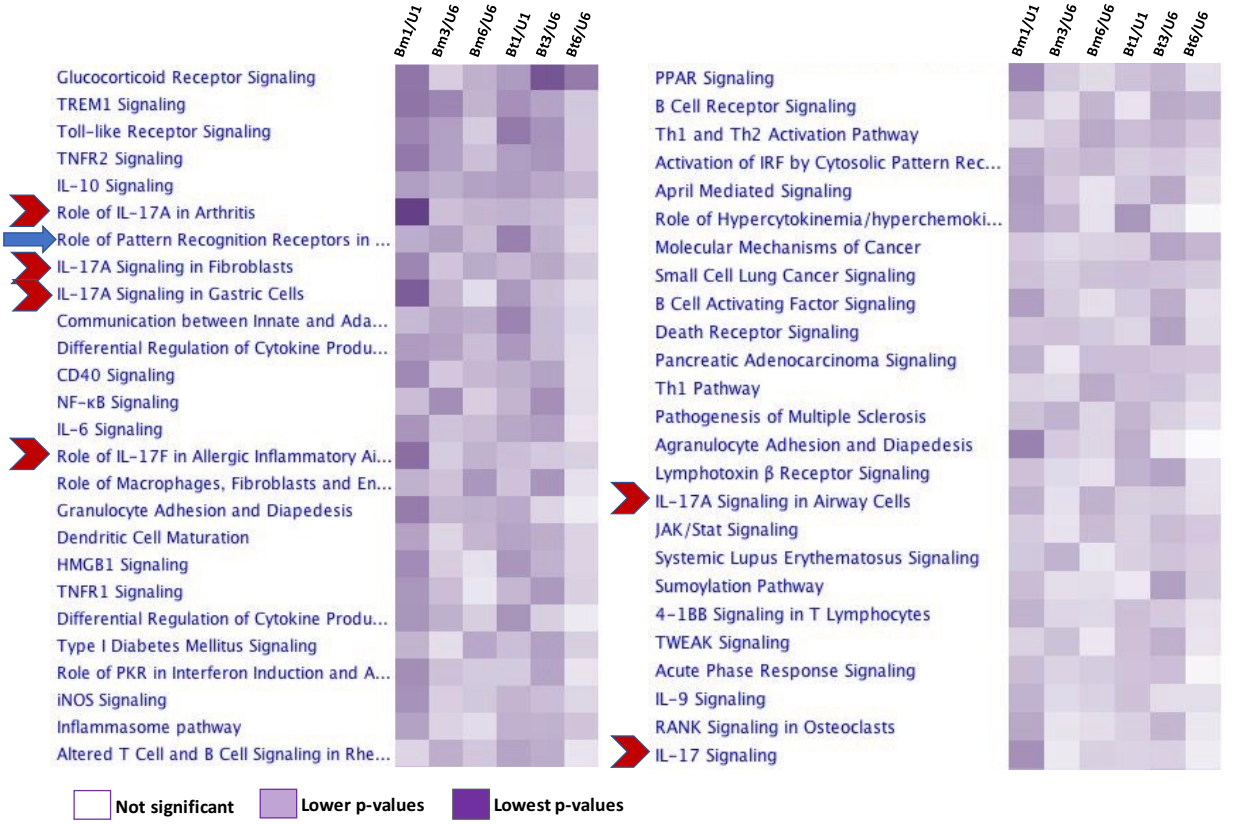

Figure 2.3. Modulation of human canonical pathways in *Bm*- or *Bt*-infected Mm6 monocytes. Significantly enriched pathways are identified in *Bm*- or *Bt*-infected monocytes as compared to uninfected monocytes. Among modulated canonical pathways, several IL-17 signaling pathways (red arrowheads) are overrepresented ($p<0.0231$). The Role of Pattern Recognition Receptors pathway (blue arrow) is also represented as a significantly modulated and enriched pathway ($p<3.96\times10^{-6}$ (*Bm*) and $p<8.32\times10^{-4}$ (*Bt*)). Bm1/U1, *Bm*-infected human monocytes relative to uninfected monocytes at 1 h; Bm3/U6, *Bm*-infected human monocytes for 3 h relative to uninfected monocytes at 6 h; Bm6/U6, *Bm*-infected human monocytes relative to uninfected monocytes at 6 h; Bt1/U1, *Bt*-infected human monocytes relative to uninfected monocytes at 1 h; Bt3/U6, *Bt*-infected human monocytes for 3 h relative to uninfected monocytes at 6 h; Bt6/U6, *Bt*-infected human monocytes relative to uninfected monocytes at 6 h.

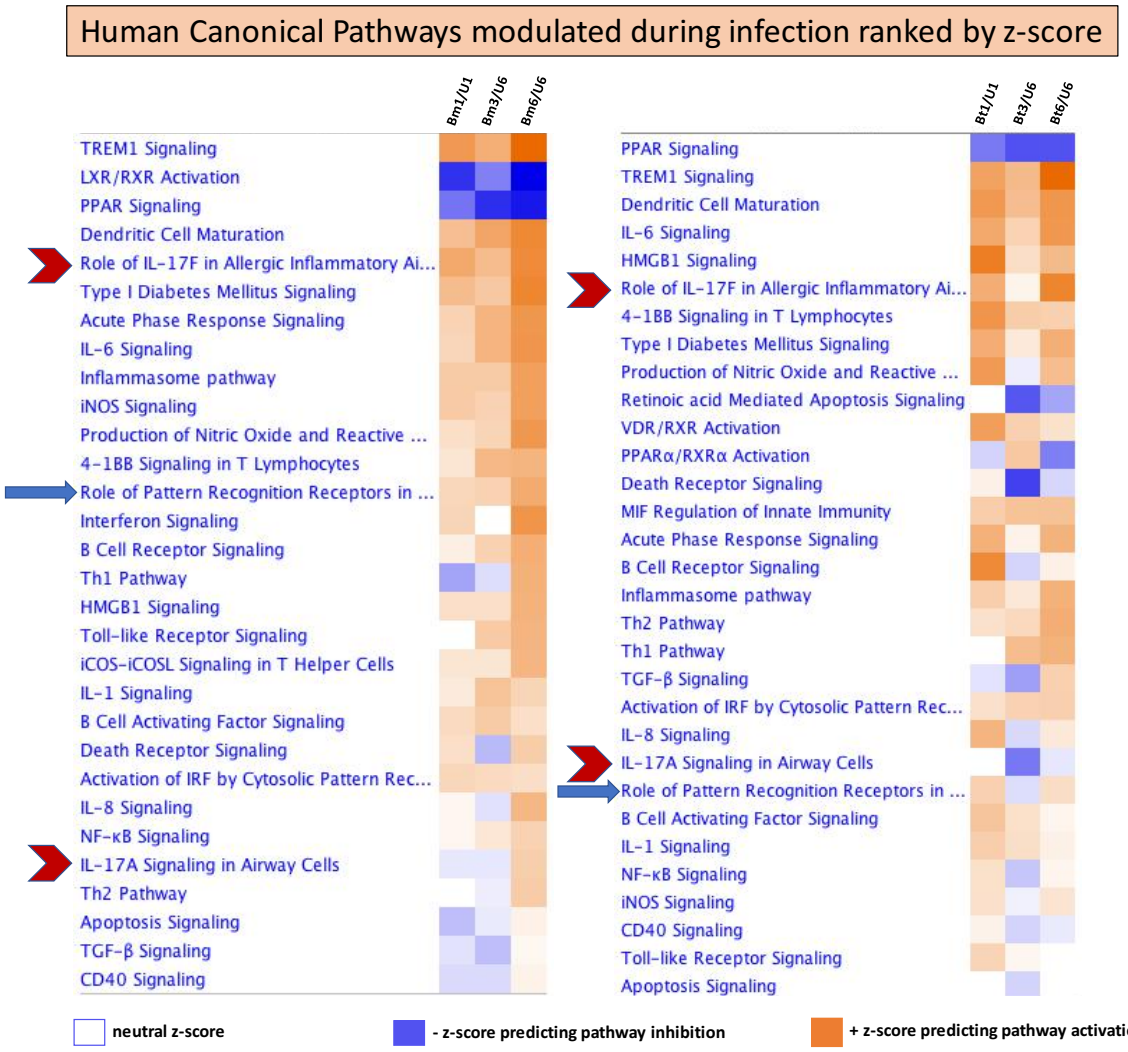

Figure 2.4. Upregulation or downregulation of canonical pathways in *Bm*- or *Bt*-infected monocytes. Significantly activated (upregulated) or inhibited (downregulated) pathways are identified in *Bm*- or *Bt*-infected human monocytes as compared to uninfected monocytes. Among significantly enriched IL-17 signaling pathways, the IL-17F pathway (red arrowheads) is significantly upregulated (z score ≥ 2.8) during *Bm* or *Bt* infection, while the IL-17A pathway (red arrowheads) shows a trend toward upregulation (*Bm*-infected monocytes) or downregulation (*Bt*-infected monocytes), over time. The Role of Pattern Recognition Receptors pathway (blue arrows) is upregulated in *Bm*- and *Bt*-infected monocytes, with a z-score statistic reaching significance only for *Bm*-infected monocytes. Bm1/U1, *Bm*-infected human monocytes relative to uninfected monocytes at 1 h; Bm3/U6, *Bm*-infected human monocytes for 3 h relative to uninfected monocytes at 6 h; Bm6/U6, *Bm*-infected human monocytes relative to uninfected monocytes at 6 h; Bt1/U1, *Bt*-infected human monocytes relative to uninfected monocytes at 1 h; Bt3/U6, *Bt*-infected human monocytes for 3 h relative to uninfected monocytes at 6 h; Bt6/U6, *Bt*-infected human monocytes relative to uninfected monocytes at 6 h.

defense and its immunostimulatory effects on phagocytic cells[4-15] sparked our interest in exploring the immunotherapeutic applications of this cytokine in potentially priming host monocytes towards clearance of *Bm* (through initial studies in *Bt*) **(Chapter 5)**. Towards gaining a better understanding of the relationship between PRRs and *Bm*, we further mined the transcriptome data, at the gene-level, for evidence of modulation of specific monocyte-expressed PRRs. Among these, PTX3, NALP3 (NLRP3), RIG-1, MDA-5, and C3 were found to be upregulated in *Bm*- or *Bt*-infected human monocytes, with PTX3 exhibiting the highest gene expression fold change **(Figure 2.5)**. We selected PTX3 for further study

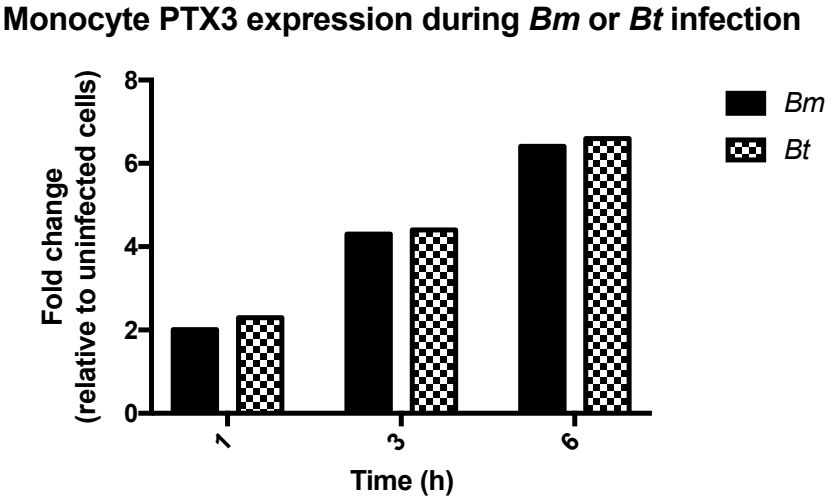

Figure 2.5. Human monocytes upregulate expression of PRR, PTX3, during infection with *Bm* or *Bt*. PTX3 gene expression significantly increases in a time-dependent manner, with highest expression at 6 h postinfection ($p<0.001$).

based on high gene expression fold change in *Bm*- and *Bt*- infected monocytes and given the known involvement of this PRR in microbial recognition, complement activation and modulation, opsonophagocytosis, and host resistance to several pathogens.[16-26] Given these properties, we were interested in defining the role of PTX3 in the pathogenesis of *Burkholderia* sp. by assessing the capacity for this PRR to recognize and opsonize *Bt*, *Bm*, or *Bp* and conducted initial studies with *Bt* to determine whether following pre-opsonization, PTX3 could bolster the bactericidal activity of complement against

inherently complement-resistant bacteria,[27, 28] promote opsonophagocytosis and intracellular killing, or, alternatively, mediate enhanced bacterial replication within human monocytes **(Chapter 6).**

2.6 Summary.

We report on the first, human, monocytic cell line system developed to study the intracellular pathogenesis of *Bm*. This cell line system has relevance to both the susceptible human host and the blood-monocyte-bacteria interface. To determine whether modulation of host immune responses may, in part, be driving successful intracellular survival of *Bm* in host immune cells, we conducted human genome-wide transcriptome profiling of *Bm*-infected human monocytes and included the relatively non-pathogenic *Burkholderia* sp., *Bt*, as a comparison. Transcriptome-based analyses revealed modulation of several immune response-related genes and pathways, including negative regulators of inflammation as well as IL-17 signaling and PRR pathways. Several negative regulators of inflammation were found to be upregulated by *Bm*- or *Bt*-infected monocytes, indicating possible bacterial modulation of host monocyte gene expression to dampen antimicrobial responses and promote intracellular replication. **Chapter 4** represents a thorough exploration of the role of these negative regulators in the intracellular pathogenesis of *Bt* (as a model for their potential role in *Bm* infection) or in their effects on pro-inflammatory mechanisms in monocytes. With regards to IL-17 signaling, *Bm*- or *Bt*- infected monocytes significantly upregulated the IL-17F signaling pathway over time but differed in their expression of the IL-17A signaling pathway, with a trend towards pathway upregulation (*Bm*-infected monocytes) or downregulation (*Bt*-infected monocytes) across time. Studies aimed at elucidating the role of the IL-17A pathway in the intracellular survival of these *Burkholderia* sp. are pursued in **Chapter 5**, with a focus on investigating the capacity for this cytokine to prime monocyte antimicrobial defenses towards reducing intracellular bacterial replication. Finally, the PRR pathway is activated over time for both *Bm*- or *Bt*-infected monocytes, with the soluble PRR, PTX3, exhibiting the highest gene expression fold change, among monocyte-expressed PRR pathway members. The role of PTX3 as a potential opsonin of *Bm*, *Bt*,

and *Bp*, as well as its cooperative involvement in integrated innate immunity with complement and host monocytes are discussed in **Chapter 6** in the context of *Bt* infection.

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CHAPTER 3

ESTABLISHING GENE SILENCING TECHNIQUES IN HUMAN MONOCYTES

3.1 Introduction.

Supervised statistical analysis of the transcriptome dataset revealed ~9500 differentially-modulated host genes during the infection of human monocytes with *Bm* or *Bt* **(Chapter 2)**. Negative regulators of inflammation and members of the IL-17 signaling and pattern recognition receptor pathways were represented among several modulated host immune response genes and pathways. To investigate the role of genes of interest in the intracellular replication of *Bm* or *Bt* within human monocytes, we employed RNA interference (RNAi)-mediated gene silencing approaches, such as small-interfering RNAs (siRNAs) or lentiviral vectors expressing short hairpin RNAs (shRNAs). Small-interfering RNAs or short hairpin RNAs act by catalytically silencing protein expression via binding to complementary mRNA sequences of target transcripts and promoting mRNA degradation or translational repression of targeted genes.[1] The ability of siRNAs and shRNAs to silence gene expression stems from their capacity to mimic endogenous, cytoplasmic microRNAs (miRNAs), allowing them to harness the pre-existing, miRNA-driven, endogenous RNAi pathway responsible for the critical post-transcriptional regulation of the host genome.[2, 3] Following cellular uptake of siRNAs or shRNAs, these RNA duplexes are processed by the Dicer enzyme and integrated into the RNA-induced silencing complex (RISC), whereby the guide strand of the RNA duplex allows RISC to target mRNA sequences of interest for silencing.[2, 3] The introduction of siRNAs or shRNAs into target cells is accomplished through two predominant techniques: transfection or electroporation.[3] Transfection is typically based on the use of cationic lipid-based reagents, which interact with negatively-charged nucleic acids to form lipoplexes taken up by cells via endocytosis.[3] In contrast, electroporation involves the application of an external

electric field, which is thought to transiently increase the plasma membrane permeability by introducing pores through which nucleic acids directly enter.[3]

While siRNA and shRNA-based gene silencing approaches can be very effective in adherent and epithelial cell lines, the successful delivery of nucleic acids into monocytes or macrophages is known to be challenging.[4] Cell lines belonging to the monocyte-macrophage lineage, such as human Mm6 monocytes, tend to be refractory to transfection, potentially due to their active nuclease machinery against foreign cytoplasmic nucleic acids.[3-8] Only a few transfection approaches have been shown to reliably and efficiently deliver nucleic acids into monocyte-macrophages, and these are frequently accompanied by reduced cellular viability and/or alterations in cellular behavior.[6] Given this, we selected Accell siRNAs **(Chapter 3.2)** as our initial gene silencing platform due to their reported ability to ensure siRNA uptake into difficult-to-transfect cells without the necessity for additional transfection reagents, viral particles, or instruments (eg., electroporator). In addition to Accell siRNA delivery systems, Nucleofector technology (modified electroporation) **(Chapter 3.3)**, transfection reagent-based nucleic acid delivery methods **(Chapter 3.4)**, and lentiviral-vector based shRNA approaches **(Chapter 3.5)** were also undertaken and are hereafter discussed.

3.2 Accell siRNA delivery systems: gene silencing techniques in human Mm6 monocytes.

Assessing the viability of human monocytes exposed to Accell siRNAs under serum-free conditions

These assays were aimed at developing an optimized transfection system with Accell siRNAs and human Mm6 monocytes. As a first step in the optimization process, both the introduced siRNAs and the Accell siRNA Delivery Media were evaluated for their effects on Mm6 monocyte viability. To accomplish this, Mm6 monocytes seeded at $1x10^6$ cells/500 µL were incubated with 0.25 µM of Accell Green (FAM-labeled) Non-targeting (NT) siRNA (D-00-1950-01 GE Dharmacon) or Accell Human GADPH siRNA SMARTpool (D-001930-01 GE Dharmacon) in serum-free Accell siRNA Delivery Media (B-005000 GE Dharmacon) reportedly formulated to ensure Accell siRNA uptake while maintaining cell health. Another

subset of Mm6 monocytes was incubated in Accell siRNA Delivery Media, in the absence of siRNAs, to independently assess the effect of this serum-free medium on cellular viability. At 24, 48, and 72 h post-transfection, cellular viability was evaluated via Trypan blue staining. For all tested conditions (with or without siRNAs), cell death was negligible at 24 h but ranged from 30-50% at 48 h to ≥ 70% at 72 h post-transfection. Altogether, these results suggested that Accell siRNA Delivery Media fails to support optimal growth and viability of Mm6 monocytes during incubation periods greater than 24 h, which may be associated with the lack of serum in this medium. As such, in its current formulation, this delivery medium would not be suitable for transfection assays in this cell line.

Optimization of siRNA delivery media to achieve optimal transfection while maintaining cell health

A series of experiments were subsequently undertaken to test the capacity for n=4 distinct delivery media to promote optimal transfection, while maintaining cell viability. Among the tested media **(Figure 3.1)**, we included modified Accell siRNA Delivery Media **(A, B)** and complete medium with

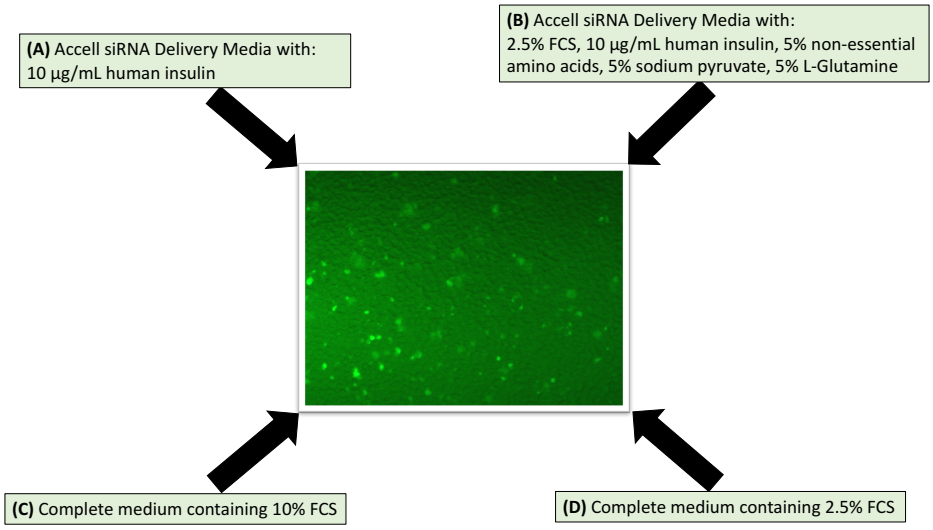

Figure 3.1. Mm6 monocytes transfected with FAM-labeled siRNA using n=4 distinct delivery media. Human Mm6 monocytes seeded at $4x10^5$ cells/500 µL or $6x10^5$ cells/500 µL were subjected to delivery media conditions **(A-D)** to assess for differential siRNA uptake and cell viability. Complete medium, RPMI 1640 with 25 mM HEPES, 10% fetal calf serum, 1% L-Glutamine, 1% non-essential amino acids, 1% sodium pyruvate, and 10 µg/mL human insulin.

varying concentrations of FCS (fetal calf serum) **(C, D)**. In n=2 independent assays, human Mm6 monocytes seeded at $4x10^5$ cells/500 µL or $6x10^5$ cells/500 µL were incubated with 0.25 µM of FAM-labeled, Accell Green NT siRNA, for 72 h, under the four distinct delivery media conditions **(A-D)**. For all conditions, 500 µL of complete medium was added to monocyte cultures at either 24 h or 48 h post-transfection for a final volume of 1 mL/well, to maintain cell health.

Following 72 h of transfection, cellular viability and fluorescence were assessed using BD LSRII flow cytometer and analyzed with FACSDiva v.6 software to identify optimal transfection condition(s) for siRNA delivery. Propidium iodide (PI) (1 µg/mL) was used as the viability exclusion dye, and FAM fluorescence was assumed to correlate with siRNA transfection. The flow cytometry data (not shown) demonstrated that the highest percentage of viable and FAM-fluorescent Mm6 monocytes was obtained when siRNAs were introduced using delivery conditions **(A)** or **(C)**. Specifically, of the $4x10^5$ or $6x10^5$ monocytes/500 µL exposed to 0.25 µM of FAM-labeled siRNA and supplemented with 500 µL of complete medium at 48 h post-transfection, 83% **(A)** or 78% **(C)** were observed to be viable and fluorescent at 72 h post-transfection.

Assessing for evidence of Accell siRNA-mediated gene silencing in human Mm6 monocytes

These experiments were designed to evaluate Accell siRNA-mediated gene silencing in human Mm6 monocytes subjected to transfection conditions determined to be optimal based on the previously described flow cytometry data. Accordingly, Mm6 monocytes were transfected with 0.25 µM of Accell Human GADPH-targeting siRNA in the presence of **(A)** Accell siRNA Delivery Media supplemented with 10 µg/mL human insulin or **(C)** complete medium, followed by supplementation with complete medium at 48 h post-transfection. Non-transfected Mm6 monocytes and monocytes transfected with either Accell Non-targeting Control (NTC) siRNA or cyclophilin B-targeting siRNA SMARTpool were included as controls. Numerous permutations of this experimental approach were undertaken, such as performing initial transfections in small volumes (200 µL) of Accell Delivery Media (to promote monocyte-siRNA

contact) followed by supplementation with complete medium 3 h post-transfection (for a 1 mL final volume). Alongside these assays in Mm6 monocytes, the HEK 293 (adherent) human epithelial cell line and THP-1 (suspension) human monocytic cell line were included as controls and subjected to similar transfection conditions and siRNAs (excluding serum-free conditions). After 72 h of transfection, total RNA was extracted from Mm6, HEK 293, or THP-1 cells using Isolate II RNA Minikit (Bioline), and cDNA was subsequently synthesized using SensiFAST™ cDNA synthesis kit (Bioline) according to manufacturer's recommendations. Absolute mRNA expression of GADPH or cyclophilin B was measured on the StepOne Real-Time PCR system (ThermoFisher Scientific; the use of this device was kindly permitted by Dr. Monique França) using corresponding IDT primer-probe sets **(Table 3.1)** and TaqMan™ Gene Expression Master Mix (4369016 ThermoFisher Scientific). Collectively, neither Mm6, HEK 293, nor THP-1 cells displayed demonstrable GAPDH or cyclophilin B knockdown by real-time quantitative PCR (qPCR) under any of the assayed conditions (data not shown). Although preliminary flow cytometry data with Mm6 monocytes had suggested the potential feasibility of transfection with Accell siRNAs under serum-inclusive conditions, the lack of gene silencing in these three distinct cell lines supported the notion that the incorporation of serum into the delivery medium may interfere with Accell siRNA uptake into cells, thereby precluding knockdown of targeted genes.

Table 3.1 IDT primer-probe sets

Gene	Assay ID	Probe	Primer 1	Primer 2
GAPDH	Hs.PT.39a.22214836	5'-/56-FAM/AAGGTCGGA/ZEN/GTCAACGGATTTGGTC/3IABkFQ/-3'	5'-TGTAGTTGAGGTCAATGAAGGG-3'	5'-ACATCGCTCAGACACCATG-3'
Cyclophilin B	*	*	*	*

*Proprietary. Assay IDs: Hs.PT.58.40291667 and Hs.PT.58.40006718.

Assessment of Accell siRNA-mediated gene silencing in HEK 293 cells under serum-free conditions

Additional transfections were performed in the easy-to-transfect cell line, HEK 293, to further investigate the potential for serum to interfere with the cellular uptake of Accell siRNAs and to validate the activity of these siRNAs. In following technical suggestions from GE Dharmacon, 1 µM (rather than 0.25 µM) of siRNA was used to transfect HEK 293 cells under strictly serum-free conditions. Briefly, HEK 293 cells at 40-50% confluency were transfected with 1 µM of Accell GADPH-targeting siRNA, Accell NTC siRNA, or Accell Green NT siRNA, in serum-free Accell Delivery Media for the entire duration of the transfection process. Non-transfected HEK 293 cells were included as an additional control. Following 72 h of transfection, qPCR revealed 94.4% knockdown of GAPDH in this cell line; these preliminary data are presented in **Figure 3.2**.

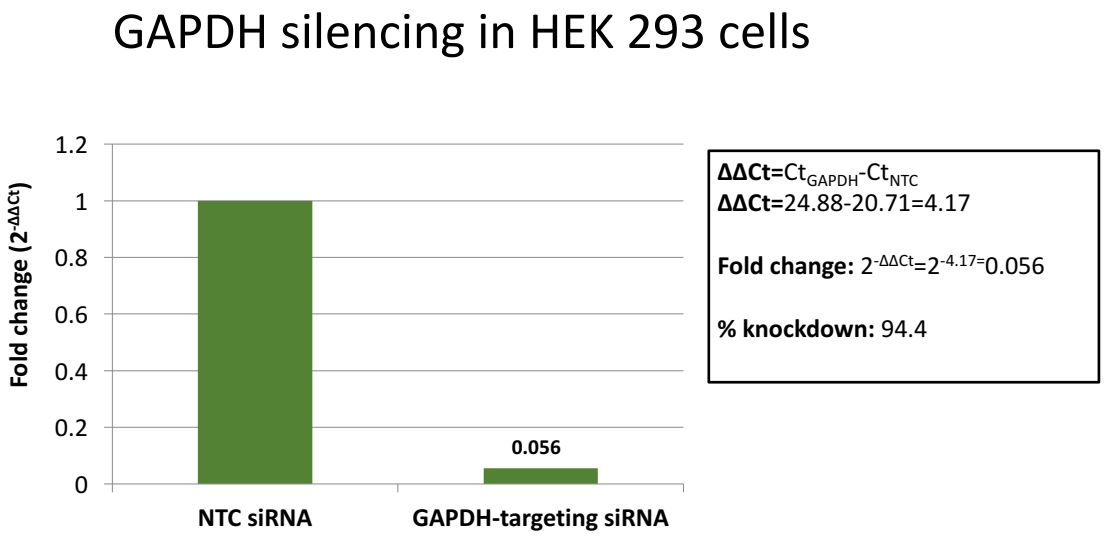

Figure 3.2. Accell siRNA-mediated gene silencing in HEK 293 cells under serum-free conditions. The fold change represents the GAPDH mRNA expression in HEK 293 cells treated with GAPDH-targeting siRNA relative to cells treated with NTC siRNA.

These data effectively validated this siRNA delivery system in an adherent epithelial cell line and demonstrated that Accell siRNA-mediated gene silencing can be achieved when high siRNA concentrations are used (1 µM) in the absence of serum. Following these results, we performed additional transfections in Mm6 monocytes that were modified to include higher siRNA concentrations,

while maintaining the incorporation of serum in the transfection process to prevent loss of cellular viability. Briefly, Mm6 monocytes were transfected with 1 µM of GADPH-targeting siRNA in the presence of n=3 delivery conditions: 1) Accell Delivery Media supplemented with complete medium at 48 h post-transfection, 2) Accell Delivery Media supplemented with complete medium at 3 h post-transfection, or 3) complete medium. Under these three conditions, GAPDH gene knockdown was not evident by qPCR 72 h following transfection (data not shown). The lack of GAPDH silencing observed in Mm6 monocytes transfected under serum-inclusive conditions, even at this high siRNA concentration, provided further evidence for the interference of serum in the siRNA delivery process. However, the incompatibility of Mm6 monocytes with this siRNA delivery system could not be excluded, and additional assays were hereafter conducted to further clarify these data.

Microscopic visualization of Mm6 monocytes following transfection with Accell siRNAs

To better understand this inability to achieve silencing of GAPDH or cyclophilin B in Mm6 monocytes using 0.25-1 µM of siRNA and several distinct delivery media, we aimed to visualize, by confocal microscopy, the fate of FAM-labeled siRNAs introduced under both serum-free or serum-inclusive conditions. Briefly, human Mm6 monocytes seeded at $3x10^5$ to $6x10^5$ cells/500 µL were incubated with 0.25 µM of FAM-labeled Accell Green NT siRNA for 24 h, 48 h, or 72 h, in Accell siRNA Delivery Media. Cultures were supplemented with complete medium at 48 h post-transfection to maintain cell health. At the indicated time points post-transfection, DAPI was added to monocyte cultures at a concentration of 1:1000, for nuclear visualization, and FAM-labeled siRNA uptake was assessed using Nikon Eclipse Ti inverted laser-scanning confocal microscope **(Figure 3.3)**.

The identification of FAM-fluorescent siRNAs closely apposed and restricted to the monocyte surface suggested that siRNA uptake into Mm6 monocytes was impaired. Given the need to incorporate serum into the delivery medium at some point during the 72 h transfection process to maintain Mm6

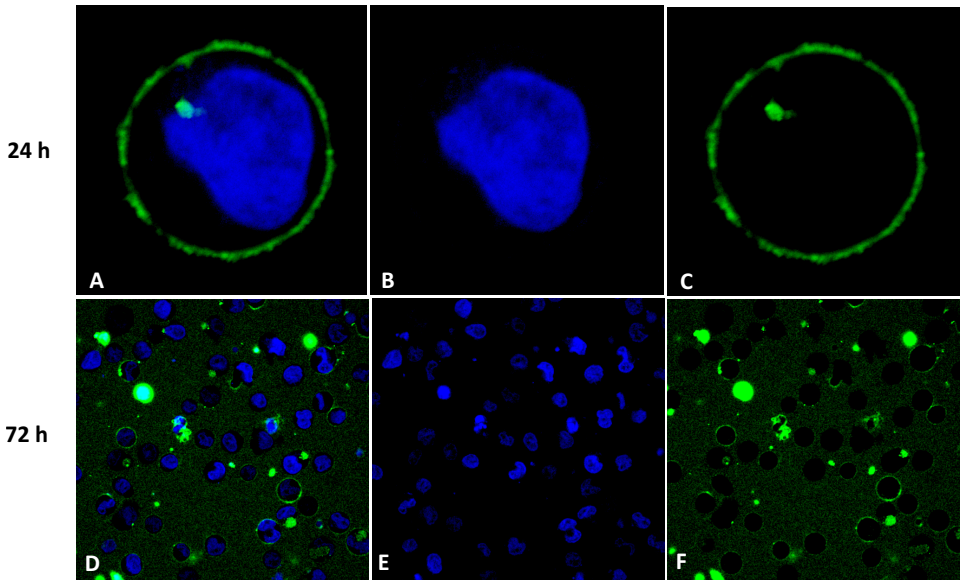

Figure 3.3. Localization of FAM-labeled Accell siRNAs during transfection of human Mm6 monocytes. Monocytes incubated with 0.25 µM of FAM-labeled Accell NT siRNA for 24 h **(A,C)** or 72 h **(D,F)**. **A** and **D** represent overlays of DAPI and FAM fluorescent signals showing extracellular or pericellular locations of siRNAs, with no evidence of intracellular uptake at any assayed time point [24 h, 48 h (data not shown), 72 h]. **B** and **E** represent DAPI-stained monocytes in the absence of FAM-labeled NT siRNA.

monocyte viability, it is possible that serum proteins may be preventing these siRNAs from accessing critical monocyte receptors necessary for successful uptake. This explanation has relevance for the 72 h condition, which incorporates serum in the late transfection process. However, the lack of siRNA uptake, even under serum-free conditions (24 or 48 h of transfection), may be related to the structural conformation of the siRNA. These Accell siRNAs are reportedly specially modified (by an undisclosed, proprietary mechanism) to allow for cellular uptake in the absence of additional transfection reagents. As such, Accell siRNAs may have structural conformation incompatibility with Mm6 monocyte surface receptors responsible for endocytic uptake. In light of these data, we sought an alternative means of siRNA delivery into this difficult-to-transfect cell line. The Amaxa Nucleofector (modified electroporator) has been consistently reported to successfully deliver nucleic acids into Mm6 cells and was therefore subsequently employed.[9-13]

3.3 Nucleofector technology for nucleic acid delivery into human Mm6 monocytes.

Nucleofector-mediated uptake of fluorescent RNA duplexes into human Mm6 monocytes

These initial assays were designed to optimize nucleic acid delivery conditions using Nucleofector technology. Human Mm6 monocytes were seeded at $3x10^5$ cells/mL on day 1, and nucleofection was performed on day 3 post-passage. Nucleofection was performed using the Amaxa Nucleofector Cell Line Kit V (VCA-1003 Lonza) using nucleofection programs T-030, T-027, U-001, V-001, T-001 (Nucleofector 2b Device Lonza; the use of this device was kindly permitted by Dr. Kojo Mensa-Wilmot). Under these conditions, a series of nucleofections were conducted with $2x10^6$ Mm6 cells per 100 µL of Cell Line Nucleofector Solution V (Lonza) and 5-50 nM of a DY-547-labeled, RNA oligonucleotide duplex (siGLO Red Transfection Indicator D-001630-02 GE Dharmacon). Flow cytometry and confocal microscopy were performed 48 h post-nucleofection. Flow cytometry data (not shown) suggested that among the tested Nucleofector programs, U-001, V-001, and T-001 promoted the most acceptable transfection efficiency and cellular viability, with an average of ~80% DY-547 fluorescent cells and ~50% viability (the latter determined by PI fluorescence). Viability was also assessed via Trypan blue staining and ranged from 30% to 50% in siRNA-treated, nucleofected cells. Confocal microscopy was performed, in-parallel, to confirm the intracellular uptake of the introduced siRNA duplexes **(Figure 3.4)**. Overall, these observations indicated that, while Nucleofector technology is very efficient at delivering oligonucleotides into Mm6 monocytes, there is considerable cell death associated with introducing nucleic acids (at these concentrations) using this technology. The exact contribution of the nucleofection process itself toward cellular viability was, however, unclear (ie., the cellular viability of non-siRNA treated, nucleofected cells was not assessed).

Evaluation of gene silencing in human Mm6 monocytes using Amaxa Nucleofector technology

Following the identification of optimal nucleofection conditions, gene silencing experiments were undertaken. At 3 days post-passage, $2x10^6$ Mm6 monocytes/100 µL were nucleofected with 50 or

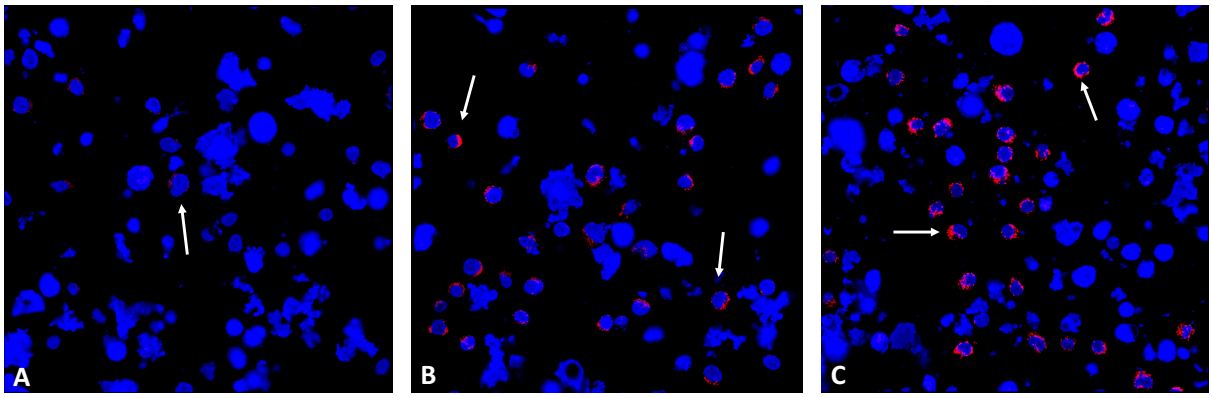

Figure 3.4. Amaxa Nucleofector technology enables delivery of RNA duplexes into Mm6 monocytes. DAPI-stained monocytes at 48 h post-nucleofection with 5 nM **(A)**, 25 nM **(B)**, or 50 nM **(C)** of DY-547-labeled RNA duplexes (program T-027). Intracellular, perinuclear, fluorescent RNA duplexes (white arrows). Similar results were noted for other nucleofection programs (data not shown).

250 nM of SiGENOME GAPDH-targeting siRNA (D-001140-01 GE Dharmacon) using programs T-001, V-001, or U-001. At 48 h post-nucleofection, GAPDH knockdown could not be demonstrated by qPCR under these conditions. Evidence of GAPDH knockdown was, however, observed for an early time point (24 h post-nucleofection) in Mm6 monocytes treated with 250 nM of GADPH-targeting siRNA using programs U-001 (~62% knockdown) or V-001 (~67% knockdown). In light of these data, subsequent GAPDH silencing assays were performed over a 24 h period, using V-001 as the optimal nucleofection program for transfection efficiency. The level of silencing of the GADPH gene was further enhanced from 60% to 90% by increasing the siRNA concentration from 250 nM to 1 µM. As a negative control, siGENOME NTC siRNA (D-001210-01 GE Dharmacon) was used for all assays. Collectively, these findings suggested that high siRNA concentrations are required to achieve a high level of gene knockdown in this cell line, and that silencing of GAPDH beyond 24 h likely results in considerable cell death, thus precluding identifiable knockdown at later assayed time points.

3.4 Transfection reagents as an alternative to Nucleofector technology in nucleic acid delivery.

Preliminary assessment of transfection efficiency using n=16 distinct transfection reagents

Given the suboptimal Mm6 monocyte viability (~50%) associated with delivering nucleic acids using Amaxa Nucleofector technology, a multitude of transfection reagents were tested as a potential low cellular toxicity alternative. The capacity for n=16 tested transfection reagents **(Table 3.2)** to promote successful delivery of nucleic acids into Mm6 monocytes was determined in the context of a GFP-expressing Plasmid (pmaxGFP Lonza). Briefly, 2 µL of pmaxGFP was added to 25 µL of serum-free Opti-MEM I (31985062 Thermo Fisher Scientific) for 5 min at room temperature, prior to the addition of 2 µL or 6.25 µL (PEI) of transfection reagent. Plasmid-transfection reagent complexes were incubated at room temperature for 20 min and then added dropwise to Mm6 monocytes seeded at $2.5x10^5$ cells/mL, $3.5x10^5$ cells/mL, or $1x10^6$ cells/mL in 1 mL of complete medium. RAW 264.7 murine macrophages (adherent cell line) were transfected, in-parallel, as a positive control. Intracellular GFP fluorescence was evaluated 72 h post-transfection, via microscopy, as an indication of plasmid uptake and expression. Fluorescence data were subjectively scored according to percentage of fluorescent cells and fluorescence intensity per cell. According to these criteria, X-tremeGENE HP DNA transfection reagent was found to promote the highest percentage of fluorescent cells with the brightest fluorescence intensity per cell, across all tested transfection reagents. It should be noted that although use of this transfection reagent resulted in the highest transfection efficiency, the number of fluorescent cells was still subjectively low, with an average of 10-15 GFP-expressing Mm6 monocytes out of $1x10^6$ total cells exposed to plasmid DNA-transfection reagent complexes. Subsequent experiments aimed at optimizing transfection conditions determined that increasing X-tremeGENE HP DNA volumes from 2 µL to 6 µL considerably improved the percentage of fluorescent cells and fluorescence intensity per cell. These findings further illustrate the refractory nature of these human Mm6 monocytes towards the transfection process.

Table 3.2. Human Mm6 monocytes subjected to n=16 transfection reagents for plasmid delivery.

Transfection reagents	Degree of cellular fluorescence as a correlate of transfection (percentage of fluorescent cells, fluorescence intensity per cell)
Polyethylenimine (PEI)	+
Dharmafect 1-3 (GE Dharmacon)	+
Lipofectamine 2000 and 3000 (Thermo Fisher Scientific)	+
Lipofectamine RNAiMAX (Thermo Fisher Scientific)	+
TransIT-LT-1 (Mirus Bio LLC)	+
Turbofect (Thermo Fisher Scientific)	+
Avalanche (EZ Biosystems)	+
Metafectene (Biontex)	+
TransIT (Mirus Bio LLC)	+
Transit 293 (Mirus Bio LLC)	+
TransIT-2020 (Mirus Bio LLC)	+
GeneJuice (Millpore Sigma)	+
X-tremeGENE HP DNA (Sigma-Aldrich)	+++

+=very low percentage of fluorescent cells with low fluorescent signal; +++=higher percentage of fluorescent cells with higher fluorescent signal.

The potential for X-tremeGENE siRNA transfection reagent to deliver RNA duplexes into Mm6 monocytes

The encouraging data observed with X-tremeGENE HP DNA transfection reagent and pmaxGFP plasmid prompted a series of experiments using X-tremeGENE transfection reagent specific for siRNA delivery (X-tremeGENE siRNA) in the context of fluorescent RNA duplexes. DY-547-labeled RNA duplex-transfection reagent complexes were prepared as previously described using 250 nM, 500 nM, or 1 µM of DY-547-labeled, siGLO Red Transfection Indicator and 6 µL of X-tremeGENE siRNA transfection reagent. These complexes were added dropwise to $3.0x10^5$ Mm6 monocytes/mL, and confocal microscopy was performed 72 h post-transfection. As determined by confocal microscopy, the highest proportion of Mm6 monocytes with intracellular DY-547 fluorescence was noted for cells transfected with 1 µM of DY-547-labeled, siGLO Red Transfection Indicator and 6 µL of X-tremeGENE siRNA

transfection reagent. **Figure 3.5** illustrates the intracellular uptake of fluorescent RNA duplexes, into Mm6 monocytes, using X-tremeGENE siRNA transfection reagent, under these conditions. As noted in prior assays, while transfection of Mm6 monocytes with siRNAs and plasmids is feasible, the necessity for high concentrations of nucleic acids and/or transfection reagent further illustrate the inefficiency of the transfection process in this cell line.

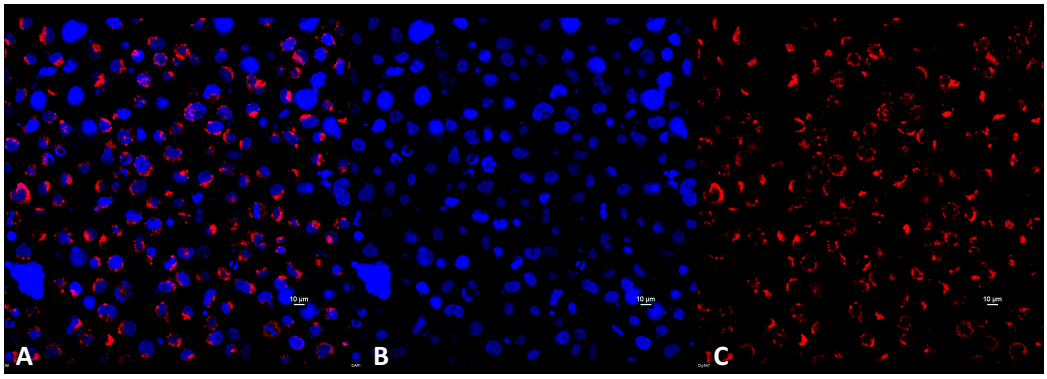

Figure 3.5. Human monocyte uptake of RNA duplexes using X-tremeGENE siRNA transfection reagent. **A,B.** DAPI-stained Mm6 monocytes 72 h post-transfection with 1 µM of DY-547-labeled SiGLO Red Transfection Indicator and 6 µL of X-tremeGENE siRNA transfection reagent **(A)** or untreated **(B)**. **(C)** Non-DAPI stained, transfected monocytes showing intracellular DY-547 fluorescence.

Evaluating X-tremeGENE siRNA transfection reagent-mediated gene silencing in Mm6 monocytes

The confocal microscopy data provided evidence for intracellular delivery of RNA duplexes into Mm6 monocytes using X-tremeGENE siRNA transfection reagent, suggesting that gene silencing may be achieved in this system. To determine this, SiGENOME GAPDH-targeting- and NTC siRNA-transfection reagent complexes were prepared using 1 µM of siRNA and 6 µL of X-tremeGENE siRNA transfection reagent and added to $3.0x10^5$ Mm6 monocytes/mL in a dropwise manner. Following 24 h of transfection, GAPDH knockdown could not be demonstrated by qPCR. Towards achieving demonstrable GADPH knockdown, permutations of this protocol included testing a range of Mm6 monocyte seeding densities ($3.0x10^5$-$5.0x10^5$ cells/mL), siRNA concentrations (75 nM, 150 nM, 225 nM), transfection reagent volumes (2.5 µL, 5 µL, 7.5 µL, 10 µL), and total volumes of cell culture medium per well (500 µL to 1 mL). Additional tested modifications included: 1) post-transfection centrifugation (400 x g, 1 min) to

promote contact between monocytes and siRNA-transfection reagent complexes and 2) reverse transfection (adding monocytes onto pre-formed siRNA-transfection reagent complexes) in small volumes, as outlined by Troegler *et al*. 2014.[5] Among these tested conditions, complexes of 225 nM of siRNA and 10 µL of X-tremeGENE siRNA introduced onto Mm6 monocytes promoted 40-70% GAPDH knockdown 24 h or 48 h post-transfection as noted by qPCR. These results indicated that knockdown of this reference gene could only be achieved using high X-tremeGENE siRNA transfection reagent concentrations in this cell line. Furthermore, given the highly variable level of knockdown observed under these conditions, we did not further pursue this silencing approach to evaluate the role of genes of interest in the intracellular replication of *Bm* or *Bt* in human monocytes.

3.5 Lentiviral vectors as an alternative to siRNA-mediated gene silencing in human Mm6 monocytes.

The drawbacks associated with each of the tested methodologies (Accell siRNA delivery systems, nucleofection-based siRNA delivery, lipid-based transfection of plasmids or siRNAs) prompted us to test an additional approach towards achieving gene silencing: lentiviral vector-based stable transfection. As part of a collaboration with the Starai laboratory, we aimed to create a line of stably-transfected Mm6 monocytes exhibiting knockdown of host genes of interest. In preliminary assays, we tested the feasibility of stable transfection in Mm6 monocytes using the lentiviral plasmid vector control, MISSION pLKO.1-puro-CMV-TurboGFP Positive Control Plasmid DNA (SHC003 SIGMA; **Figure 3.6**), provided by Dr. Vinny Starai. We selected nucleofection as a means to deliver this plasmid, given that it was determined, through our studies, to be the most reliable system for achieving nucleic acid delivery, with the potential to induce a high level of gene silencing of select gene targets. In the context of generating stable transfectants, the suboptimal viability associated with nucleofection of these monocytes may not represent a significant limitation, given that the potentially few transfectants generated post-nucleofection could be further expanded during the selection process.

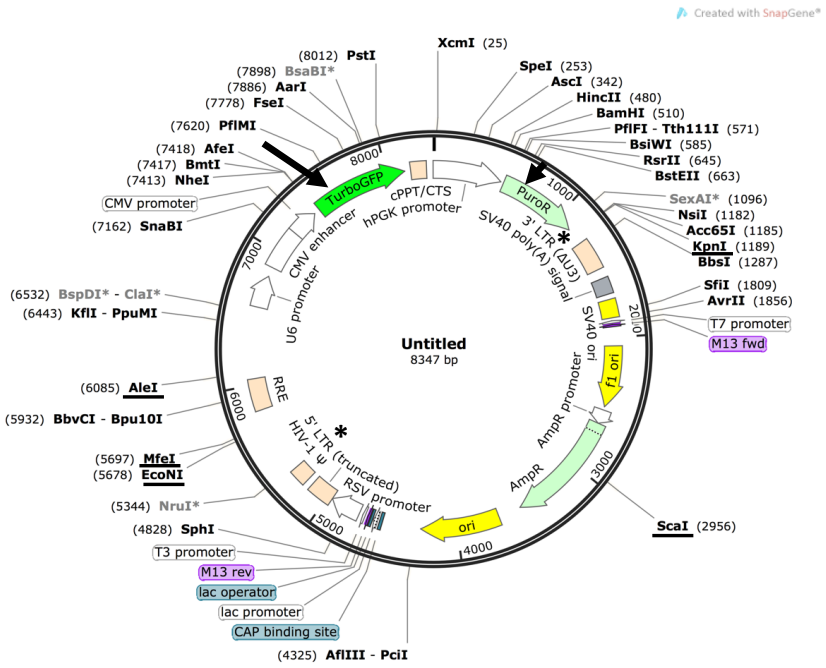

Figure 3.6. MISSION pLKO.1-puro-CMV-TurboGFP lentiviral plasmid vector positive control. Intracellular expression of TurboGFP **(long arrow)** enables microscopic confirmation of transfection, PuroR **(short arrow)** directs the production of the puromycin resistance protein necessary for puromycin selection of transfectants, and the lentiviral long-tandem repeats (LTRs) demarcate the insertion site of the lentiviral vector **(asterisks)**. Restriction enzymes are underlined in **black**.

Given that plasmid SHC003 does not possess the reverse transcriptase and integrase genes to drive plasmid integration, we sought to determine whether integration of linearized plasmid into the Mm6 genome could be achieved during the mutation-prone cellular division of this immortalized cell line. In this line of endeavor, 5 µg, 10 µg, or 20 µg of plasmid was linearized using AleI, EcoNI, MfeI, KpnI or ScaI-HF restriction enzymes (NEB), and the resulting linearized plasmid DNA was precipitated with 3 M sodium acetate and 100% ethanol, and resuspended in 10 µL of TE buffer. Subsequently, Mm6 monocytes seeded at $3.0x10^5$ cells/mL on day 1 were nucleofected on day 2 (corresponding to cellular exponential growth phase), using $2x10^6$ cells/100 µL and a range of plasmid DNA concentrations (program V-001 and Cell Line Kit V, as previously described). Following nucleofection, a number of recovery conditions were evaluated, such as supplementing nucleofected Mm6 monocytes with 500 µL of warm complete medium prior to dropwise transfer into: 1) a single well of a 24-well plate containing

900 µL of complete medium (for a total volume of 1.5 mL/well), or 2) a conical tube containing 2.7 mL of warm complete medium before adding the suspension of nucleofected cells into 3 separate wells of a 24-well plate at 1 mL/well. Twenty-four hours post-nucleofection, monocyte cultures were subjected to puromycin selection (0.1 µg/mL) and replenished with this concentration of puromycin every 2-3 days to select for monocytes carrying the plasmid and, therefore, expressing the puromycin resistance protein. An initial microscopic assessment of transfection was also conducted beginning at 24 h post-nucleofection, by examining the level of intracellular GFP fluorescence, which would support intracellular expression of the GFP protein and therefore confirm plasmid uptake. At this time point, monocytes nucleofected with 5 µg or 10 µg of linearized plasmid DNA displayed no GFP fluorescence, while cells exposed to 20 µg exhibited scattered, bright, intracellular, GFP fluorescence, as noted by fluorescence microscopy (EVOS FL ThermoFisher Scientific). At 72 h post-nucleofection, rare intracellular GFP fluorescence was noted in cells exposed to 10 µg of plasmid, in addition to those treated with 20 µg of plasmid; however, cells treated with 5 µg of plasmid remained non-fluorescent. By day 6 post-nucleofection, only cells nucleofected with 20 µg of plasmid exhibited intracellular GFP fluorescence; however, under post-nucleofection puromycin selection, these cells exhibited progressively worsening growth impairments to cell death (as noted by Trypan blue counting). Approximately 2 weeks post-puromycin selection, there was extensive death of Mm6 monocytes nucleofected with 20 µg of plasmid, as demonstrated by widespread Trypan blue staining. Therefore, outgrowth of Mm6 human monocytes presumed to be stably transfected with SHC003 plasmid could not be achieved.

Although stable transfectants could not be obtained using this plasmid and the employed methodology, the difficulties encountered during these experiments helped generate a better understanding of both nucleofection and the optimal generation of stable transfectants using lentiviral vector-based plasmids, in this cell line. With regards to the nucleofection process, Mm6 monocytes nucleofected with 5 µg of plasmid DNA under the described conditions were noted to exhibit minimal

cell death and resumed normal growth patterns 72 h post-nucleofection. These results suggested that cellular toxicity associated with nucleofection may be primarily related to the need to use high RNA or DNA concentrations to achieve delivery in this human monocytic cell line, rather than the result of the nucleofection process itself. In terms of generating stable transfectants, the widespread cell death noted in Mm6 monocytes 2 weeks post-nucleofection and puromycin selection suggested that the incorporation of plasmid DNA into the genome of this cell line may not be feasible in the absence of reverse transcriptase and integrase genes.

Altogether, it is clear that high concentrations of plasmid DNA can be successfully delivered into Mm6 monocytes via nucleofection. However, the generation of stable transfectants requires post-uptake plasmid integration, with selection and outgrowth of plasmid-carrying cells. In our case, impaired growth of nucleofected Mm6 cells following puromycin selection is most likely attributable to a lack of integration of the plasmid (and therefore puromycin resistance gene (PuroR)) into the Mm6 genome. Specifically, post-puromycin selection growth impairments that progressed to widespread cell death likely represented a manifestation of puromycin sensitivity by Mm6 monocytes, which were likely not episomally expressing the PuroR protein (or to a very limited extent). Although episomal expression of the PuroR protein was evidently low to absent, some degree of long-term (approximately 2 weeks) episomal plasmid replication was, however, evident through GFP fluorescence. Therefore, when generating stable transfectants using the difficult-to-transfect human Mm6 monocytic cell line, the selection of a plasmid with reverse transcriptase and integrase genes is likely critical to ensure plasmid integration.

3.6 Summary.

We have explored a wide range of silencing modalities in human Mm6 monocytes. We have found transfection of siRNAs and plasmids (including lentiviral vectors) to be feasible but overall inefficient in this cell line, with requirements for high nucleic acid and/or transfection reagent

concentrations to achieve delivery and gene silencing, suggesting that, overall, this cell line tends to be refractory to the transfection process. Among the tested transfection modalities, we have shown that Accell siRNAs, which are reportedly modified to ensure silencing in difficult-to-transfect cells, such as monocyte-macrophages, are not taken up by human Mm6 monocytes and, rather, remain in the extracellular milieu or closely apposed to the cell surface. In contrast to our findings in human monocytes, the Accell siRNA silencing platform promoted robust gene silencing in an epithelial, adherent cell line (HEK 293) when used at high siRNA concentrations. These results suggested that undifferentiated, non-adherent monocytes may, in fact, not be compatible with Accell siRNA delivery systems. Unlike our findings with Accell siRNA delivery systems, we found that transfection reagent-based plasmid or siGENOME siRNA delivery was achievable in this cell line, albeit with very few transfected cells or only inconsistent, low-level, gene knockdown. Altogether, among all of the tested platforms in our studies, modified electroporation via the Amaxa Nucleofector proved to be the most reliable platform to achieve consistent, robust levels of knockdown in the human Mm6 monocyte cell line. As a result, this platform was employed as part of our studies aimed at understanding the role of negative regulators of inflammation in the role of *Bm* pathogenesis (initial studies conducted with *Bt*) **(Chapter 4)**. Despite the efficacy of this platform, further optimizations are warranted to mitigate the detrimental effects of the nucleofection process on the cellular viability of human Mm6 monocytes, and a number of post-nucleofection recovery methods are proposed in **Chapter 7**.

3.7 References.

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CHAPTER 4

NEGATIVE REGULATORS OF INFLAMMATION:

GENE EXPRESSION, SILENCING, AND FUNCTIONAL INHIBITION IN HUMAN MONOCYTES

4.1 Introduction.

There is published evidence supporting a link between pathogenic members of the *Burkholderia* genus, such as *Bp*, and negative regulators of inflammation **(Chapter 1.5)**. *Burkholderia pseudomallei* has been reported to induce increased expression of several negative regulators of inflammation both at the cellular and tissue level, in human patients, as well as in murine models of infection.[1-14] For some of these negative regulators of inflammation, *Bp*-induced expression directly correlated with enhanced replication within host phagocytes, suggesting that this bacterium may be modulating the expression of these genes as an immunoevasive strategy to support intracellular persistence.[1-3, 8] With this in mind, one of the aims of this research project has been to determine whether such immunomodulatory mechanisms are also employed by the closely-related pathogen, *Bm*. To determine this, small-scale, preliminary studies (**Chapter 4.2**) conducted prior to the global transcriptome analysis (detailed in **Chapter 2**) had initially focused on determining the role of select negative regulators of inflammation, SARM-1 and SENP6, in the intracellular pathogenesis of *Bt* (as a model for the potential role of these genes in *Bm* pathogenesis) in human monocytes. These genes were selected for study based on the described link between SARM-1 and *Bp*[3, 8] (and therefore, the possibility of a similar link with *Bm*) and investigating the relationship between a novel negative regulator of inflammation (SENP6)[15] and members of the *Burkholderia* genus.

The results of these limited preliminary studies prompted us to adopt a global, genome-wide approach to elucidating host monocyte gene modulation by *Bm* or *Bt*. The transcriptome analysis of *Bm*-

or *Bt*-infected human monocytes broadened our understanding of the possible infection-driven modulation of negative regulators of inflammation (among other immune system-related pathways), with additional, yet unexplored, potential links to several other negative regulators of inflammation. A subset of these, SOCS3, PTGS2 (COX-2), and TNFAIP3 (A20), was selected for further study **(Chapter 4.3)** based on known links to *Bp* infection[1, 4-7] and evidence of significantly increased expression during *Bm* or *Bt* infection of human monocytes in our transcriptome dataset (**Figure 2.2**).

4.2 Understanding the role of SARM-1 and SENP6 in the *Bt*-human monocyte interface.

Negative regulators of TLR signaling: SARM-1 and SENP6

As part of a regulatory mechanism that fine-tunes the immune response, Sterile-α-and Armadillo Motif-Containing Protein (SARM-1) and SUMO-specific protease 6 (SENP6) negatively regulate TLR signaling pathways, leading to dampened cytokine responses.[3, 8, 15, 16] Specifically, SARM-1 is a TLR adaptor that inhibits MyD88-independent TLR signaling via direct interaction with TRIF **(Figure 4.1A)** and represents one of the negative regulators exploited by *Bp* during infection of murine macrophages, with *Bp*-induced expression of this gene correlating with enhanced intracellular survival [3, 8] (as discussed in **Chapter 1.5**). On the other hand, SENP6 inhibits TLR signaling at the level of the inhibitor of NF-κB (IκB) kinase (IKK) complex, resulting in NF-κB attenuation **(Figure 4.1B)**[3, 8, 15] and has, to date, not been examined during infection with *Burkholderia* sp. To investigate the potential role of SARM-1 and SENP6 in the intracellular survival of *Bm,* we conducted initial studies with *Bt* in the context of human monocytes exhibiting silencing of these genes.

Human Mm6 monocyte basal expression of SARM-1 and SENP6

The basal expression of negative regulators of inflammation, SARM-1 and SENP6, in human monocytes, was examined by qPCR (according to methods described in **Chapter 3**). Briefly, total RNA was extracted from $1x10^6$ Mm6 monocytes, and cDNA was subsequently synthesized and assayed by

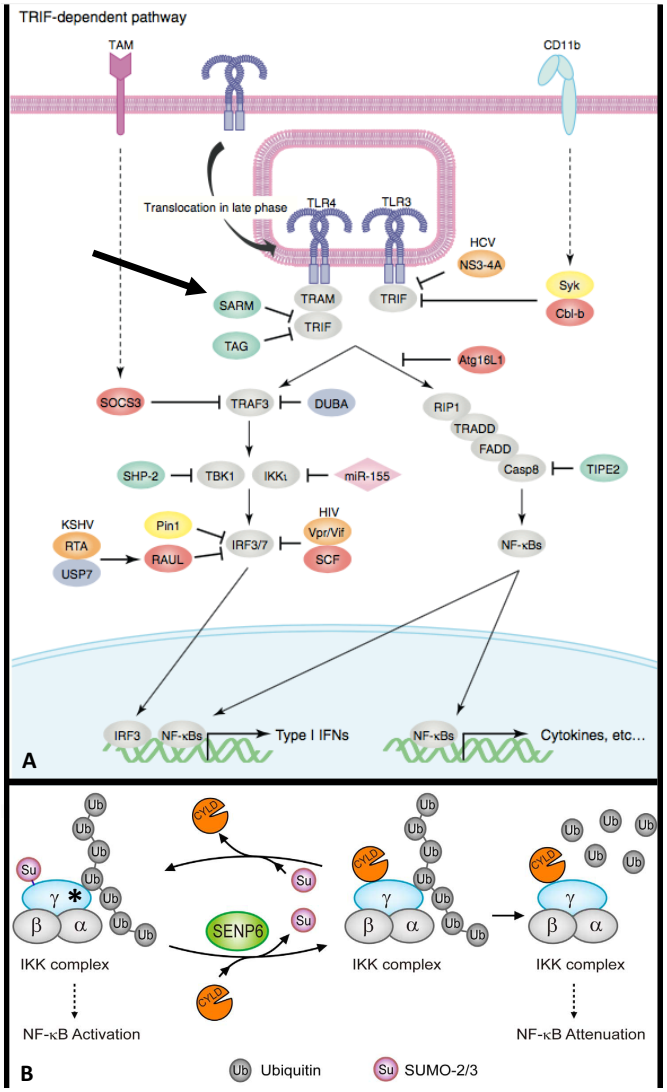

Figure 4.1. Negative regulation of TLR pathways by SARM-1 and SENP6.
(A) TRIF inhibition by SARM-1 **(arrow)** results in downregulation of TLR-dependent inflammatory responses. Modified from Kondo, Takeshi, Taro Kawai, and Shizuo Akira. "Dissecting negative regulation of Toll-like receptor signaling." *Trends in immunology* 33.9 (2012): 449-458.
(B) SENP6-mediated de-SUMOylation of IKKγ **(*)** allows CYLD binding and deubiquitination of IKKγ, resulting in IKK complex inhibition and attenuation of NF-κB signaling. Modified from Liu, Xing, *et al*. "Negative regulation of TLR inflammatory signaling by the SUMO-deconjugating enzyme SENP6." *PLoS pathogens* 9.6 (2013): e1003480.

qPCR to measure mRNA expression of SARM-1 and SENP6 host genes using IDT primer-probe sets **(Table 4.1). Figure 4.2** demonstrates that SARM-1 and SENP6 are expressed at a very low transcriptional level compared to the GADPH reference gene.

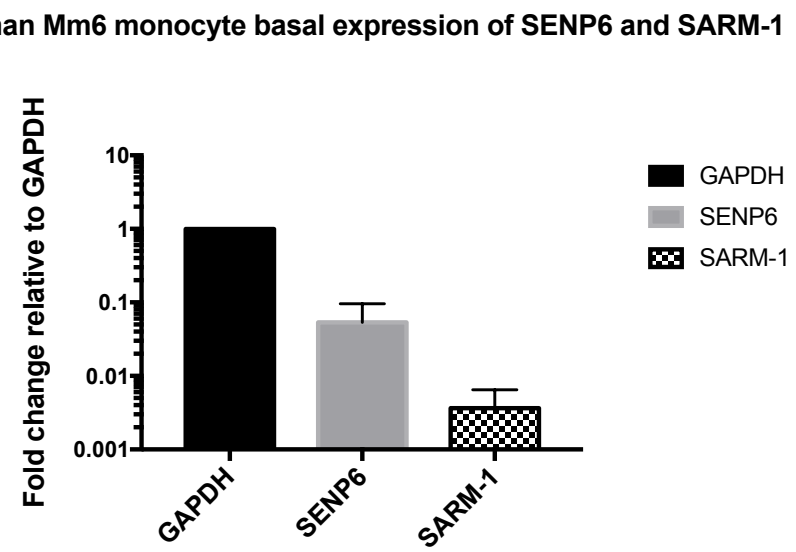

Figure 4.2. Human Mm6 monocytes express low endogenous levels of SARM-1 and SENP6. The absolute mRNA expression of SENP6 and SARM-1 is normalized to GAPDH, which is set at 1.

Human Mm6 monocyte expression of SARM-1 and SENP6 during infection

Following the determination of the endogenous mRNA expression of SARM-1 and SENP6 in uninfected human monocytes, we sought to evaluate transcriptional changes that these negative regulators of inflammation may undergo during infection with *Bt*, to uncover evidence for potential infection-induced modulation of these genes. Towards this end, triplicate subsets of Mm6 monocytes seeded at $1x10^6$ cells/mL were incubated with 25 µL of a PBS suspension of *Bt* DW503 at an OD_{580} of ~1.4 (corresponding to an MOI of 1:40), for 1 h, 2 h, 4 h, or 6 h. In-parallel, additional triplicate subsets of Mm6 monocytes were treated with 10 ng/mL of *E.coli* 0111:B4 LPS (L4391 SIGMA) for 1 h, 2 h, 4 h, or 6 h, as an internal control. Additional controls for this study included human monocyte subsets seeded to perform an in-parallel intracellular survival assay (described in **Chapter 2**) to validate the Mm6 monocyte-*Bt* interaction in these experiments (data not shown). Three, independent, co-incubation assays were performed, and the resulting SARM-1 and SENP6 gene expression data are represented in **Figure 4.3**.

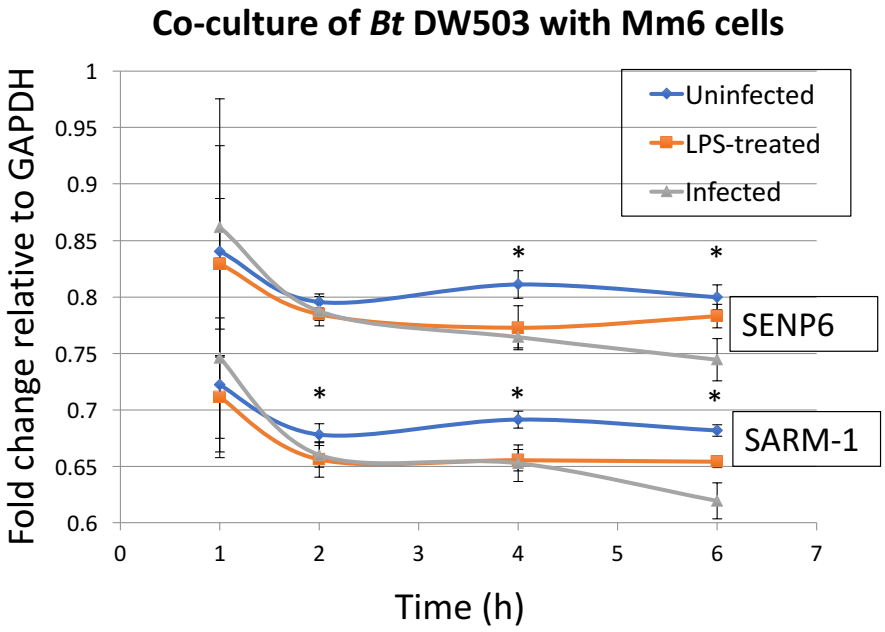

Figure 4.3. Modulation of human monocyte SARM-1 and SENP6 expression during *Bt* infection. The mRNA expression of SARM-1 and SENP6 is normalized to GAPDH, which is set at 1. The asterisks denote statistically significant gene expression changes between uninfected and *Bt*-infected monocytes at *p<0.05 (unpaired t-test).

Collectively, these data demonstrate time-dependent modulation of negative regulators of inflammation, SARM-1 and SENP6, during *Bt* infection of human monocytes. At 1 h postinfection, mRNA expression of SARM-1 and SENP6 was increased (though not significantly) in infected monocytes, relative to uninfected cells. Given that *Burkholderia* sp., such as *Bt*, are rapidly phagocytized and quickly evade the phagocytic vacuole to replicate intracellularly,[17, 18] an early increase in the expression of genes capable of dampening inflammatory responses and host defense mechanisms most likely represents bacteria-mediated modulation of the immune response. As a result, this early downregulation of immune responses would then enable further, unabated, bacterial replication and rapid spread to adjacent cells. In time points beyond 1 h of infection, there is a switch to a progressive and continuous decrease in SARM-1 and SENP6 expression in *Bt*-infected human monocytes, as compared to uninfected cells, which is most pronounced at 6 h and reaches significance for all but the 2 h postinfection time point for SENP6 (*p<0.05). Given that decreased expression of SARM-1 and SENP6 would act to promote

an enhanced inflammatory state, this delayed decrease in the expression of these genes, in the context of *Bt* infection, likely represents a reactive, host-related response to overwhelming bacterial replication, rather than a bacteria-mediated effect. This progressive, time-dependent decrease in the expression of these negative regulators differs from the relationship noted for select negative regulators of inflammation and pathogenic *Burkholderia* sp. (such as *Bp*), which reportedly upregulate the expression of these genes, in association with enhanced intracellular replication. The differences between our findings in this co-incubation study and those with *Bp* may be related to the *Burkholderia* sp. (relatively non-pathogenic vs. pathogenic) used in the studies and, therefore, may shed light on molecular mechanisms specific to pathogenic *Burkholderia* strains. Alternatively, these differences may be associated with the specific *Bt* isolate used, the inoculum dosage, or the cell culture system (murine, human, primary vs immortalized cells, epithelial, macrophage, etc).

Next, to validate the *E.coli* LPS internal control used in this study, TNF-α expression of LPS-stimulated human Mm6 monocytes was measured by qPCR using IDT primer-probe sets **(Table 4.1)** to confirm LPS-induced monocyte activation responses. For these experiments, *Bt*-infected monocytes were used as controls and are included in the data representative of n=3 independent assays **(Figure 4.4)**. These data indicate that as compared to untreated Mm6 monocytes, LPS-treated cells exhibited marked increases in TNF-α mRNA expression at all assayed time points, thereby validating LPS as an activation control. This increased expression was, however, noted to decline over time, indicating time-dependent LPS resistance in this cell line.

Silencing of SARM-1 and SENP6 genes in human Mm6 monocytes via siRNAs

Although prior experiments revealed that SARM-1 and SENP6 expression were overall decreased during *Bt* infection, we still had interests in clarifying the effect that SARM-1 or SENP6 deficiency may play in the intracellular survival of *Bm*. To investigate this, we aimed to first achieve silencing of these genes via siRNAs and then, subsequently, determine the impact of SARM-1 or SENP6 deficiency on *Bt*

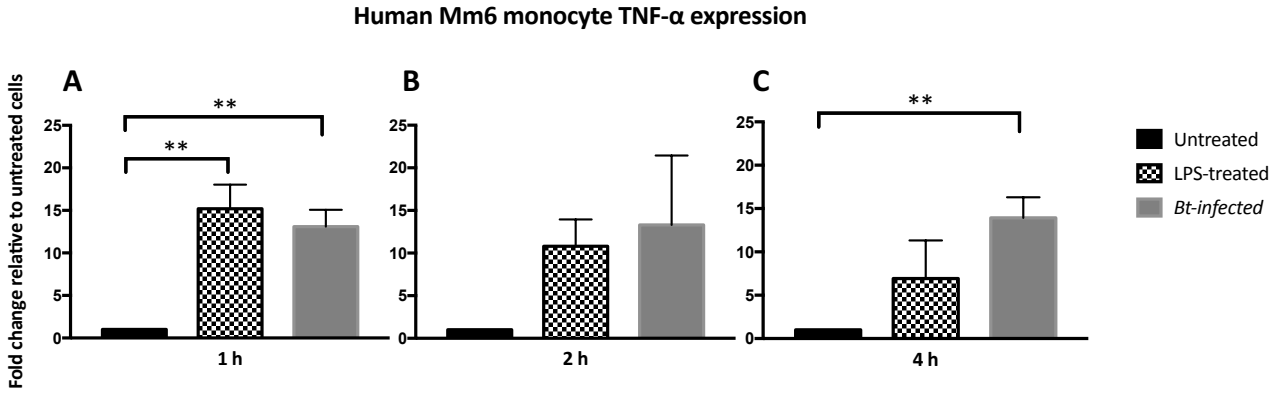

Figure 4.4. Human Mm6 monocytes activated by *E.coli* LPS enhance expression of TNF-α.
(A) Stimulation of monocytes with 10 ng/mL of LPS for 1 h resulted in highly significant increases in TNF-α expression (***p*=0.0019). Increased expression of TNF-α was also noted for 2 h **(B)** or 4 h **(C)** LPS-stimulated monocytes, but this did not reach statistical significance. Significance was determined by one-way ANOVA and Tukey's multiple comparisons.

Table 4.1 IDT primer-probe sets

Gene	Assay ID	Probe	Primer 1	Primer 2
SARM-1	ACC#NM_015077.3 Set 2	5'-56-FAM/TGTCTCCAA/ZEN/TCAGTCCCACTCCCT/3IBkFQ/-3'	5'-CACAGGACAGCGCCAATAA-3'	5'-CGCAGGAAACCTCTCTGAATAC-3'
SENP6	Hs.PT.58.26151593	5'-/56-FAM/ATTGCCAAA/ZEN/CTGGTCTTTCATTCTTGCTT/3IABkFQ/-3'	5'-GACGTTTCAGAGGTGTGTTGA-3'	5'-GTGAACTACGAAGCATTCCAGA-3'
TNF	Hs.PT.58.45380900	5'-/56-FAM/AGATGATCT/ZEN/GACTGCCTGGGCC/3IABkFQ/3'	5'-TCAGCTTGAGGGTTTGCTAC-3'	5'-TGCACTTTGGAGTGATCGG-3'
NOS2	Hs.PT.58.14740388	5'-/56-FAM/TATTCAGCTGTGCCTTCAACCCCA/3IABkFQ/3'	5'-GCAGCTCAGCCTGTACT-3'	5'-CACCATCCTCTTTGCGACA-3'

intracellular growth indices in the context of intracellular survival assays. Towards achieving silencing, we followed methodology outlined in **Chapter 3** for the successful delivery of GAPDH-targeting siRNAs into human Mm6 monocytes.

Briefly, $2x10^6$ monocytes per 100 µL of nucleofection solution were exposed to 500 nM of siGENOME SENP6- (M-006044-01) or SARM-1- (M-008076-01) targeting siRNAs using program V-001. Human monocyte cDNA were subsequently assayed by qPCR at 24 h, 48 h, or 72 h post-nucleofection, to confirm silencing of SENP6 or SARM-1; however, knockdown of these genes was not demonstrated under these conditions. After increasing the siRNA concentration during transfection from 500 nM to 1 µM, approximately 50-70% knockdown of SENP6 could be achieved at 24, 48, or 72 h post-nucleofection. However, only incremental (~39%) knockdown of SARM-1 could be achieved at 24 h, with no evidence of knockdown at 48 or 72 h. Non-targeting control (NTC) siRNA was used as a negative control for all assays. The inability to obtain reliable and demonstrable knockdown of SARM-1 may be related to the inherently, very low, endogenous expression of this gene, with siRNA-mediated silencing further reducing this expression and potentially resulting in loss of cellular viability and therefore, few monocytes with detectable levels of knockdown. Given that consistent knockdown could only be achieved for the SENP6 gene, further studies were dedicated to understanding the role of this negative regulator of inflammation in the intracellular survival of *Bt*.

Intracellular survival assays with SENP6-deficient human Mm6 monocytes and Bt

Two, triplicate subsets of Mm6 monocytes were nucleofected ($2x10^6$ cells per 100 µL of nucleofection solution) with 1 µM of SENP6-targeting or NTC siRNA. In-parallel positive controls for the subsequent intracellular survival assays included two, duplicate subsets of non-nucleofected, non-siRNA-treated Mm6 monocytes. Additional controls for this assay included Mm6 monocytes nucleofected with 1 µM of GAPDH- or SENP6-targeting siRNA, or NTC siRNA, to be assayed by qPCR at 24 or 48 h post-nucleofection to confirm gene knockdown in our study.

At 48 h post-nucleofection, Mm6 monocytes nucleofected with SENP6-targeting or NTC siRNA and non-nucleofected, non-siRNA-treated Mm6 monocytes were infected with *Bt* DW503 as previously described (**Chapter 2)**. Briefly, monocytes seeded at $1x10^6$ cells/mL were infected with 25 µL of a *Bt* DW503 suspension in PBS at an OD_{580} of ~1.4 (corresponding to an MOI of 1:40), and *Bt* intracellular growth indices were evaluated by comparing CFUs representative of the phagocytosis index (4 h postinfection) to those representing the replication index (10 h postinfection). In n=3 independent assays, a consistent difference in *Bt* intracellular growth indices was not observed between Mm6 monocytes exhibiting SENP6-knockdown, monocytes receiving NTC siRNA, and non-nucleofected, non-siRNA-treated monocytes. These data indicate that SENP6 may not play a critical role in the intracellular survival of *Bt* DW503 in human Mm6 monocytes. Alternatively, given the incomplete (50-70%) level of knockdown of SENP6 gene expression, it is possible that the remaining, low expression of this gene may still be sufficient to allow for normal biological function, thereby preventing a detectable reduction in bacterial replication in the context of this assay. In light of these findings, we opted not to pursue further studies with *Bm*.

4.3 Understanding the role of SOCS3, PTGS2, and TNFAIP3 in the *Bt*-human monocyte interface.

Negative regulators of inflammation: SOCS3, PTGS2 (COX-2), and TNFAIP3 (A20)

Similar to other negative regulators of inflammation, SOCS3, PTGS2 (COX-2), and TNFAIP3 (A20) function to maintain a homeostatic balance between immune activation and inhibition and, for this reason, can be exploited by pathogens during infection. Suppressor of cytokine signaling 3 (SOCS3) mediates suppression of TLR, IL-1, and cytokine signaling pathways, resulting in dampened pro-inflammatory cytokine production **(Figure 4.5A)**.[19, 20] Similarly, TNFAIP3 (A20) inhibits NF-κB-dependent transcription of pro-inflammatory cytokines by negatively regulating TLR and TNF receptor pathways **(Figure 4.5B)**.[16, 21, 22] Cyclooxygenase 2 (COX-2/PTGS2) indirectly suppresses NO production in macrophages by upregulating PGE_2 and Arg2 **(Figure 4.5C)**.[1] Upregulation of the COX-2, PGE_2, and Arg2

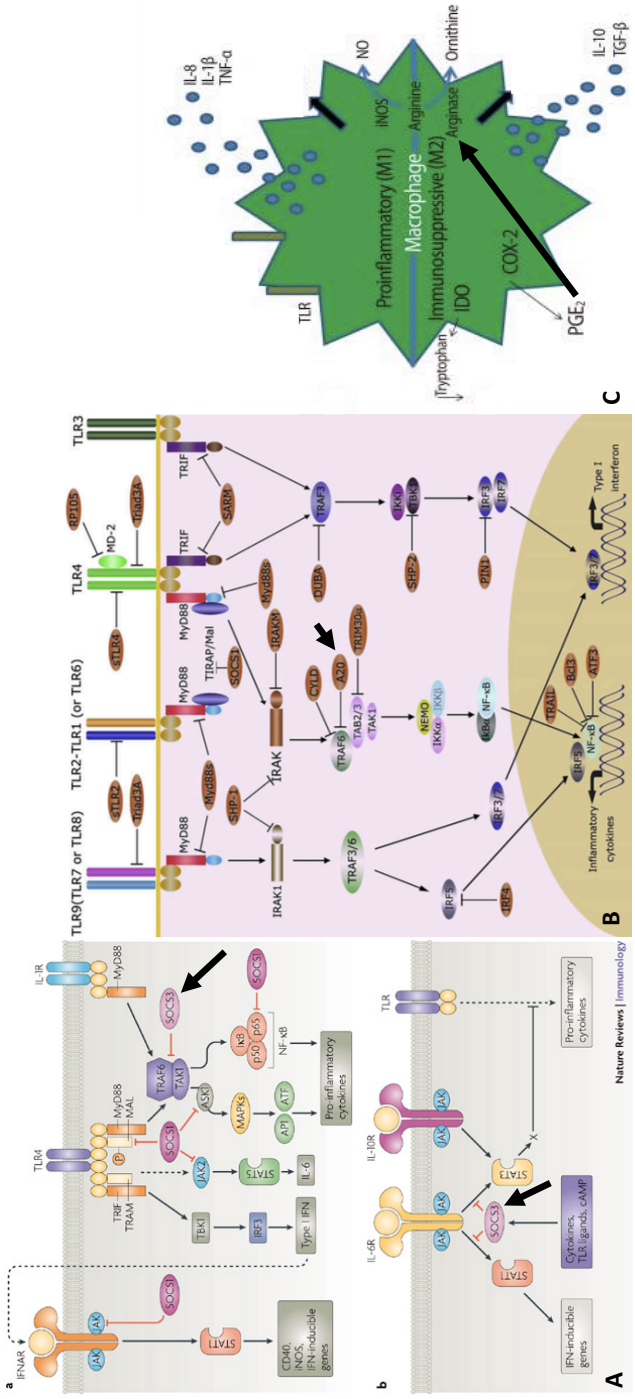

Figure 4.5. Negative regulators of inflammation upregulated in *Bm*- or *Bt*-infected monocytes.
(A) SOCS3-mediated suppression of both NF-κB-dependent transcription of pro-inflammatory cytokines and cytokine signaling at the level of the TRAF6-TAK1 complex and JAK/STAT pathway, respectively **(arrows)**. Modified from Yoshimura, Akihiko, Tetsuji Naka, and Masato Kubo. "SOCS proteins, cytokine signalling and immune regulation." *Nature Reviews Immunology* 7.6 (2007): 454-465.
(B) A20-mediated attenuation of TLR signaling at the level of TRAF6, with suppression of NF-κB signaling (**short arrow**). Modified from Wang, Jie, *et al.* "Negative regulation of Toll-like receptor signaling pathway." *Microbes and infection* 11.3 (2009): 321-327. **(C)** COX-2- and PGE_2-mediated upregulation of Arg2. Competition of Arg2 with iNOS interferes with macrophage NO production and bactericidal activity. Modified from Lee, Ji Yeong, Millina Lee, and Sung Ki Lee. "Role of endometrial immune cells in implantation." *Clinical and experimental reproductive medicine* 38.3 (2011): 119-125.

pathways and resulting decreases in NO production has been shown to contribute to the intracellular survival of multiple pathogens, including *Bp*.[1, 19, 20] Altogether, as noted with *Bp*, an increased expression of SOCS3, TNFAIP3(A20), and PTGS2 (COX-2), was likewise observed in *Bm*- or *Bt*-infected human Mm6

monocytes (Chapter 2). Subsequent gene functional inhibition studies are aimed at understanding whether modulation of these genes during infection represents a host-mediated effect or a strategy utilized by these bacteria to promote intracellular replication.

Chemical inhibition of SOCS3, PTGS2 (COX-2), or TNFAIP3 (A20) gene function

Given the suboptimal viability noted in human Mm6 monocytes nucleofected with high concentrations of siRNAs and the cost-ineffective nature of the nucleofection approach, we explored chemical inhibition of gene function as an alternative approach to gene silencing. Using chemical inhibitors, we sought to evaluate the role of additional negative regulators of inflammation in the intracellular pathogenesis of *Bm* (as before, with initial studies in *Bt*). **Table 4.2** represents a list of the chemical inhibitors used and their respective target(s).

Table 4.2. Chemical inhibition of genes representing negative regulators of inflammation.

Chemical inhibitors	Gene target(s)	Mechanism of action	Effect(s)	Reference
Zoledronic acid	SOCS3, SOCS1, IRAK-M	Decreases phosphorylation of Stat-3 in a mevalonate pathway-dependent manner Enhances IKBα degradation	Potentiation of macrophage NF-κB activation, cytokine, and NO production, in response to TLR ligands, IL-1, and exogenous antigens	[23-25]
NS398	COX-2 (and downstream signaling)	Inhibition of COX-2 activity (non-selective)	Enhanced macrophage intracellular killing of *Bp* and NO release Enhanced clearance of *Bp* and improved survival in murine melioidosis	[1]
COX-2 inhibitor I	COX-2 (and downstream signaling)	Inhibition of COX-2 activity (selective) in human monocytes and whole blood	Potential to enhance macrophage microbicidal defense mechanisms	[26]
NSC11200	TNFAIP3	Inhibition of	Potential to enhance macrophage	[27, 28]

	(A20)	deubiquitinase activity	microbicidal defense mechanisms and reduce growth of intracellular pathogens	
NSC267309	TNFAIP3 (A20)	Inhibition of deubiquitinase activity	Potential to enhance macrophage microbicidal defense mechanisms and reduce growth of intracellular pathogens	[27, 28]

Chemical inhibition of SOCS3 function in human Mm6 monocytes using zoledronic acid

To understand the role that SOCS3 may play in the intracellular survival of *Bt*, chemical inhibition of this negative regulator of inflammation was attempted via the amino-bisphosphonate, zoledronic acid, in lieu of nucleofection.[23, 25] The zoledronic acid (ZA) inhibitor was selected based on its potential applicability as an immunotherapeutic.[23] Although we were specifically interested in the inhibition of SOCS3 given the potential link of this gene to *Bp*, [4-7] *Bt*, or *Bm* infection, it is important to note that ZA additionally targets other negative regulators of inflammation, such as SOCS1 and IRAK-M **(Table 4.2)**.[23-25] Zoledronic acid inhibition of SOCS3, SOCS1, and IRAK-M altogether acts to enhance NF-κB activation, as well as cytokine and NO production, in primary human peripheral blood mononuclear cells (PBMCs), murine bone marrow-derived macrophages (BMDMs), and murine macrophage cell lines (RAW 264.7) when used at a 10 μM concentration.[23-25] With the exception of the study by Scheller *et al.* (2011) in which ZA treatment alone could induce enhanced macrophage expression of IL-6,[23] two studies demonstrate that ZA does not possess direct immune-stimulating or pro-inflammatory activity by itself; rather, following a pre-incubation period, its role is to prime and enhance the phagocyte response to TLR ligands (eg., LPS), IL-1, or exogenous antigens.[24, 25]

Intracellular survival assays in human Mm6 monocytes with functional inhibition of SOCS3

In these assays, we sought to determine the effect of ZA-mediated SOCS3 functional inhibition on human Mm6 monocytes in the context of potential priming of antimicrobial defense mechanisms to achieve reduced replication of intracellular bacteria, such as *Bm* or *Bt*. Prior to conducting intracellular

survival assays, Mm6 monocytes were first exposed to a range of zoledronic acid (H683 AK Scientific) concentrations (0.1-100 µM) overnight and determined to exhibit no inhibitor-associated cytotoxicity by Trypan blue staining. Then, Mm6 monocytes seeded at $6x10^5$ cells/mL were exposed to 10 µM of zoledronic acid for 24 h (to achieve the priming effect described in the literature) or untreated (control) prior to infection with *Bt* DW503 as previously described. For ZA-primed monocytes, exposure to this inhibitor was maintained throughout the duration of each intracellular survival assay. Collectively, there were no consistent differences in *Bt* intracellular growth indices between ZA-primed Mm6 monocytes or untreated monocytes in n=4 independent, intracellular survival assays (data not shown). These results prompted us to gain a better understanding of the potential mechanisms of action of ZA in human Mm6 monocytes and to validate its reported effects in potentiating macrophage pro-inflammatory and NO responses.

Evaluating the potential effect of ZA in priming human Mm6 monocyte activation responses

Since ZA reportedly potentiates LPS-induced inflammatory responses in murine macrophages and human PBMCs,[24, 25] we sought to determine whether similar immunostimulatory effects can be achieved in human Mm6 monocytes. Briefly, monocytes were treated with 10 or 100 µM of ZA for 24 h prior to exposure to 100 ng/mL of *E.coli* LPS for an additional 2 or 4 h, as previously described.[24] Subsets of LPS-only stimulated monocytes, ZA-only treated monocytes, and non-ZA, non-LPS treated (untreated) monocytes were included as controls. Following 2 and 4 h of LPS stimulation, Mm6 monocytes were lysed, total RNA was extracted, and cDNA were assayed by qPCR using IDT primer-probe sets **(Table 4.1)** for changes in TNF-α or NOS2 mRNA expression that would correlate with monocyte activation.

In n=3, independent assays, when compared to untreated human Mm6 monocytes, LPS-stimulation alone induced ~15-fold or ~4-fold increases in TNF-α mRNA expression at 2 h or 4 h, respectively. However, this enhanced expression was not observed to be significantly potentiated by ZA **(Figure 4.6)**. Zoledronic acid treatment of human monocytes likewise did not result in enhanced LPS-

mediated NOS2 (iNOS) gene expression (data not shown). In fact, for all tested conditions, NOS2 (iNOS) mRNA expression was either below the threshold of qPCR detection or registered at a Ct value of >35. Furthermore, as was noted by Norton *et al.* (2011)[25] and Muratsu *et al.* (2013)[24] in studies with murine macrophages or human PBMCs, ZA alone was not observed to promote inflammatory responses in Mm6 monocytes (ie., TNF-α expression in ZA-treated cells was similar to that of untreated cells).

Collectively, data in **Figure 4.6** demonstrate that Mm6 monocytes are highly responsive to LPS stimulation at 100 ng/mL at earlier time points (2 h), as evidenced by significantly marked upregulation of TNF-α expression. The less substantial increase in TNF-α mRNA expression observed after 4 h of LPS exposure denotes time-dependent LPS resistance (noted in prior assays, **Figure 4.4**). Overall, the notable responsiveness of Mm6 monocytes to LPS stimulation (even at 4 h) may help explain the lack of a detectable potentiating effect by ZA, since LPS alone induces such an inherently high level of pro-inflammatory gene (eg., TNF-α) expression.

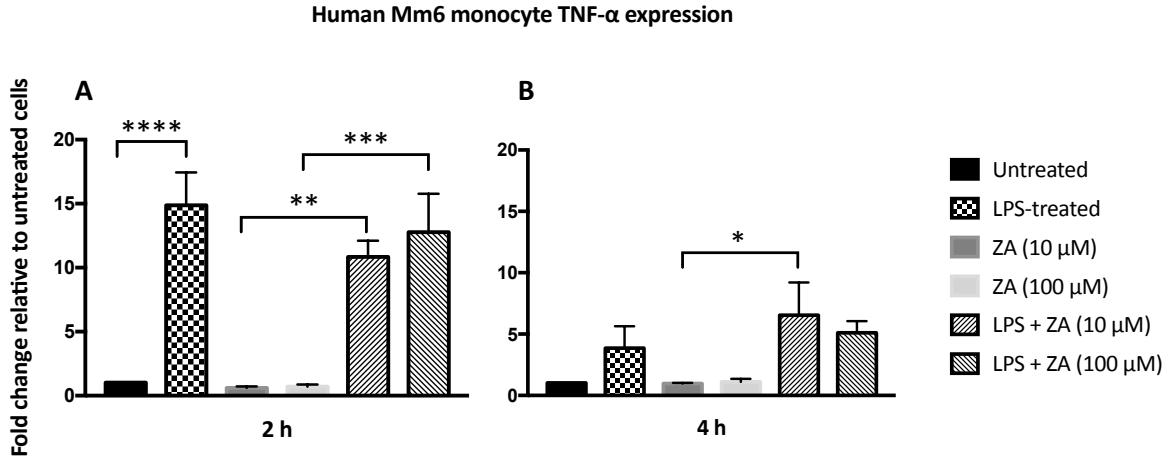

Figure 4.6. ZA fails to significantly enhance LPS-mediated TNF-α expression in Mm6 monocytes. **(A)** Human Mm6 monocytes stimulated with 100 ng/mL of *E.coli* LPS for 2 h exhibited significantly higher TNF-α expression compared to untreated (****p<0.0001) or ZA-only treated monocytes (**p=0.0013, ***p=0.0003). **(B)** Monocytes stimulated with LPS for 4 h exhibited increased TNF-α expression, which reached statistical significance for one of the monocyte subsets (*p=0.0209).

Given that Mm6 monocyte NOS2 (iNOS) expression was often too low to be detectable by qPCR (even 2 or 4 h post-LPS stimulation), the Griess assay was subsequently employed to quantify supernatant levels of nitrite, a stable end-product used as an indicator of NO production, as an alternative correlate of iNOS activity. However, following 24 h of ZA pre-treatment and 2 or 4 h of LPS exposure, Mm6 monocytes failed to release detectable levels of nitrite within the supernatant. Human Mm6 monocytes treated with 30 ng/mL of PMA +/- 1 µg/mL ionomycin for 1 or 5 h prior to ZA and/or LPS treatment, with the aim to induce differentiation into macrophages, likewise did not produce detectable levels of nitrite (data not shown).

These results prompted us to conduct additional experiments in an alternative cell culture system to validate the Griess assay. In n=2 independent assays, primary differentiated and adherent murine dendritic cells (DCs) were seeded at $5x10^5$ cells/mL, treated with 10 or 100 µM of ZA for 24 h, and subsequently exposed to 100 ng/mL of LPS for 1 h, 2 h, 4 h, or 24 h prior to supernatant collection. Untreated, ZA-only treated, and LPS-only treated cells were included as controls. Murine DCs treated with LPS for 24 h represented the only subset to produce detectable nitrite levels in the supernatant, regardless of ZA pre-treatment. This finding indicated that mononuclear phagocytes release detectable nitrite in supernatant following a prolonged period of LPS treatment (24 h). However, ZA was not observed to enhance LPS-mediated nitrite (and therefore NO) responses in murine DCs (data not shown). Therefore, although these assays provided validation for the established Griess assay, ZA-mediated immunostimulatory effects were likewise not discernible in this alternative primary cell culture system.

Having established optimal conditions for the detection of nitrite in the context of the Griess assay, we next conducted similar experiments in Mm6 monocytes. Briefly, $5x10^4$ monocytes in 300 µL (48-well plate) were subjected to ZA (1 µM, 10 µM, 100 µM) for 24 h, prior to stimulation with 10 or 100 ng/mL of LPS for 24 h. As before, untreated, ZA-only treated, or LPS-only treated cells were included as

controls. However, nitrite levels remained undetectable in the supernatant, even following this prolonged period of LPS stimulation. Together with the low to absent NOS2 (iNOS) expression previously noted by qPCR, these findings suggested that expression of this gene may be inherently low in this cell line or that induced expression may require exposure to antigens other than TLR4 ligands. In this case, any priming effect of ZA on iNOS expression or activity would not be detectable in this cell line.

These unexpected findings in both human and murine cell culture systems prompted us to utilize RAW 264.7 murine macrophages as a positive control to validate ZA activity in the context of the Griess assay.[24] These murine macrophages reportedly respond to ZA pre-treatment and LPS stimulation with enhanced LPS-mediated inflammatory responses and NO production.[24] Briefly, a range of RAW 264.7 cell densities ($2x10^3$ to $2x10^4$ cells in 200-300 µL volumes) were seeded on day 1 and treated with 1, 10, or 100 µM of ZA on day 2. Following 24 h of ZA treatment, 2, 10, or 100 ng/mL of LPS were added to macrophage cultures, and the supernatant was collected 24 h post-LPS exposure. Untreated macrophages, ZA-only treated macrophages, and LPS-only treated macrophages were included as controls. Among n=6, independent, Griess assays, $2x10^4$ RAW 264.7 macrophages exposed to LPS (or a combination of ZA and LPS) produced detectable nitrite in the supernatant at concentrations of up to 60 µM. However, as noted in other cell culture systems in our studies, ZA priming of RAW 264.7 macrophages failed to further enhance LPS-induced nitrite production (data not shown), which contradicted the findings by Muratsu *et al.* (2013). Possible explanations for the discrepant findings between these studies and our findings in three tested cell culture systems may be related to different LPS preparations or ZA formulations (Novartis Pharma AG zoledronic acid hydrated disodium salt vs. AK Scientific zoledronic monohydrate) used for the assays. The potential impact of formulation on ZA activity and the lack of expected priming activity as reported in the literature may help explain the lack of differences in *Bt* intracellular replication between ZA-treated and untreated Mm6 monocytes. As a

result of this, we were unable to conclusively determine the role of SOCS3 in the intracellular survival of *Bt* (or *Bm*).

Chemical inhibition of COX-2 (PTGS2) in human Mm6 monocytes via NS398 or COX-2 Inhibitor I

Through these chemical inhibition studies, we were interested in determining the role of COX-2 in *Bt* or *Bm* infection, given the evidence of significantly increased expression of this gene in *Bm*- or *Bt*-infected human monocytes **(Chapter 2)** and the described role of COX-2 in *Bp* infection. The chemical inhibitors used for this study were selected based on potential immunotherapeutic applications (COX-2 Inhibitor I) or previously reported therapeutic activity in the context of murine melioidosis (NS398).[1] Of note, COX-2 inhibition via these inhibitors is not selective and additionally results in suppression of downstream effectors, such as prostaglandins (eg. PGE_2), well as some enzymes (Arg2).[1] In the context of *Bp* infection of murine BMDMs, previous studies reported that BMDMs treated with 100 µM of NS398 exhibited killing of intracellular *Bp*, with enhanced nitrite production.[1] This effect was not specific to NS398 as indomethacin-mediated COX inhibition yielded similar results.[1] Given these findings, our aim was to determine whether COX-2 pathway inhibition would similarly impair *Bm* replication within human monocytes.

Intracellular survival assays in human monocytes with functional inhibition of COX-2 (PTGS2)

The cyclooxygenase 2 (COX-2) inhibitor, NS398, (Cayman Chemical) was reconstituted in DMSO and added to human Mm6 monocytes at concentrations ranging from 20-100 µM. Following 24 h of exposure, cellular cytoxicity was determined to be negligible by Trypan blue staining. To determine the effect of COX-2 inhibition on the intracellular replication of *Bt* in human monocytes as a model for potential effects on *Bm* intracellular replication, intracellular survival assays were performed as previously described. Briefly, $1x10^6$ Mm6 monocytes/mL were either exposed to 100 µM of NS398 and immediately infected or pre-exposed to NS398 for 4 h prior to infection with 25 µL of a *Bt* suspension in

PBS at an OD_{580} of ~1.4 (corresponding to an MOI of 1:40). Exposure to 100 μM of NS398 was maintained throughout the duration of each assay. Untreated human monocytes and monocytes exposed to DMSO (vehicle control) alone were included as controls. In n=7 independent assays, there were no significant differences in the intracellular growth of *Bt* in monocytes treated with NS398, DMSO-alone, or untreated. Three additional sets of similar assays were performed with human Mm6 monocytes treated with 10 or 50 μM of NS398, DMSO-alone, or untreated, and likewise, did not reveal significant differences in the intracellular growth of *Bt*.

These results suggested that the lack of a discernible effect may be related to poor inhibitor uptake within human Mm6 monocytes. Thus, we investigated a different COX-2 inhibitor, COX-2 Inhibitor I (CAS 416901-58-1 Calbiochem), reported to be cell-permeable and specifically target and have high activity against human monocyte COX-2. This COX-2 Inhibitor I was reconstituted in DMSO and similarly well-tolerated by Mm6 monocytes, with no evidence of cytotoxicity by Trypan blue staining following overnight exposure to 20-100 μM concentrations. Next, n=2 independent intracellular survival assays showed no significant differences in the intracellular growth of *Bt* between Mm6 monocytes treated with 10 or 100 μM of COX-2 inhibitor I, DMSO-treated, or untreated monocytes. Collectively, studies with NS398 or COX-2 inhibitor I suggested that Mm6 monocytes may not be responsive to COX-2 inhibition. Alternatively, these particular COX-2 inhibitors may not function in Mm6 monocytes. Lastly, it is possible that COX-2 inhibition does not affect the intracellular growth of *Bt* in this cell line.

Chemical inhibition of TNFAIP3 (A20) in human Mm6 monocytes using NSC11200 and NSC267309

Published reports have described links between TNFAIP3 (A20) and multiple intracellular bacterial pathogens, such as *Bp* and *Mycobacterium tuberculosis* (*Mtb*).[4, 7, 28] In addition, our transcriptome dataset showed enhanced expression of this negative regulator of inflammation in *Bm*- or *Bt*-infected human monocytes **(Chapter 2)**. Based on this, we sought to understand the potential role of A20 in the intracellular survival of these *Burkholderia* sp. While we were primarily interested in A20

inhibition, it is important to note that NSC11200 and NSC267309 inhibitors can also target deubiquitinases other than A20.[27] In a study by Shi and colleagues, A20 functional inhibition was achieved at a concentration of 100 μM.[27] However, this concentration can inhibit the growth of some tumor cell lines and therefore, has the potential to inhibit the growth of the human Mm6 monocytic leukemia cell line.[27] In the context of bacterial infection, macrophages from A20-deficient mice displayed attenuated *Mtb* intracellular survival and enhanced production of TNF-α, IL-1β, and nitrite.[28] Given these findings, we first sought to determine if chemical inhibition of A20 may have similar pro-inflammatory effects in the human Mm6 monocytic cell line.

Evaluating the effect of NSC11200 and NSC267309 on human monocyte activation responses

Chemical inhibitors, NSC11200 and NSC267309, were obtained from the NIH NCI Developmental Therapeutic Program's Open Compound Repository. Human Mm6 monocytes exposed to 100 μM of NSC267309 displayed marked cytotoxicity as demonstrated by Trypan blue staining. As previously mentioned, this finding was a potential outcome and is supported in the literature (inhibition of tumor cell line growth).[27] Given this result, NSC267309 was excluded from further study. Chemical inhibitor NSC11200 did not compromise cellular viability at the same concentration and was, therefore, further investigated for its potential to enhance cellular activation and/or inflammatory responses in human Mm6 monocytes. Briefly, $5x10^5$ Mm6 monocytes/mL were treated with 100 to 200 μM of NSC11200 for 24 h and then subsequently exposed to 10 or 100 ng/mL of LPS for 24 h. Untreated Mm6 monocytes, NSC11200-only treated monocytes, or monocytes treated with LPS alone were included as controls. Human Mm6 monocyte cDNA were assayed by qPCR for changes in TNF-α or NOS2 (iNOS) expression indicative of monocytic activation. However, NSC11200 did not appear to modulate (or enhance) Mm6 monocyte responses to LPS. Similar findings were noted in an alternative tested cell culture system (RAW 264.7 murine macrophages) and included: 1) marked cytotoxicity associated with exposure to 100 μM of NSC267309 and 2) lack of observable enhancement of LPS-mediated nitrite production in

macrophages treated with 100 µM of NSC11200 for 24 h prior to stimulation with 10 or 100 ng/mL of LPS for 24 h, in n=2 independent Griess assays. Collectively, the lack of an observable pro-inflammatory effect associated with A20 inhibition in these human and murine systems may suggest: 1) the lack of efficacy of NSC11200 in achieving A20 inhibition in these two cell lines or in completely inhibiting the function of this negative regulator of inflammation or 2) that non-selective A20 inhibition by NSC11200 results in alterations in the expression of interconnected pathways, which potentially counteract any pro-inflammatory effects. In light of these findings, we did not pursue intracellular survival assays with NSC11200-treated human monocytes and *Bt*.

4.4 Summary.

We investigated the human monocyte expression dynamics of negative regulators of inflammation during infection with *Bm* or *Bt* and demonstrated time-dependent, differential expression of these genes during infection. Specifically, we observed that monocyte expression of SARM-1 and SENP6 decreased as infection progressed, suggesting that this response may represent an inflammatory, host-mediated reaction to intracellular infection. On the other hand, time-dependent, enhanced monocyte expression of other negative regulators of inflammation, such as SOCS3, PTGS2, or TNFAIP3, would suggest direct induction by the bacterium, given that upregulation of these genes would overall contribute to dampened antimicrobial defense mechanisms and, thus, promote survival of intracellular bacteria. To test these hypotheses and elucidate the role that these negative regulators may play in the intracellular survival of *Bm* within human monocytes, we conducted initial studies with *Bt*. In the first part of our studies, we utilized the Amaxa Nucleofector to silence human Mm6 monocyte SENP6 expression but did not identify a discernible role for this gene in the intracellular survival of *Bt* in these monocytes. In our subsequent studies, we employed a wide range of chemical inhibitors (zoledronic acid, NS398, COX-2 inhibitor I, NSC11200, NSC267309) to target the gene function of SOCS3, PTGS2, or TNFAIP3, respectively, and likewise did not identify a detectable effect of gene functional inhibition on

the intracellular survival of *Bt* in human monocytes or on monocyte inflammatory responses. Although the role of these negative regulators of inflammation in the intracellular survival of *Bt* (and therefore *Bm*) could not be elucidated via gene silencing or chemical inhibition, these studies allowed us to gain a more thorough understanding of the human Mm6 monocytic cell line. Through our studies, we observed these monocytes to be highly responsive to *E. coli* LPS stimulation, with enhanced TNF-α expression at early time points accompanied by a time-dependent decrease in gene expression, indicating resistance to LPS stimulation over time. We also noted that this cell line expresses virtually undetectable levels of NOS2 mRNA endogenously or post-LPS stimulation, and these findings are in agreement with the lack of NO detected in Mm6 monocyte supernatant following LPS stimulation. These observations may suggest that this cell line does not readily express NOS2 or that antigens other than LPS may be required to stimulate the expression of this gene to allow for detectable quantification of NO release. Further discussions of these global results are undertaken in **Chapter 7**.

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CHAPTER 5

THE ROLE OF THE IL-17A PATHWAY IN THE *BURKHOLDERIA* SP.-HUMAN MONOCYTE INTERFACE

5.1 Introduction.

IL-17 signaling pathways were found to be significantly enriched in the transcriptome of *Bm*- or *Bt*-infected human monocytes, with evidence of differential regulation of the IL-17A pathway across these two infections **(Chapter 2).** These findings prompted us to explore the molecular mechanisms underlying a potential relationship between the IL-17A signaling pathway and the intracellular pathogenesis of *Bm* or *Bt*. While the role of IL-17 pathways has, to date, not been previously investigated in the context of any *Burkholderia* sp., a potential link to these pathways has been described for *Bp* in both human and murine systems. Specifically, human peripheral blood mononuclear cells (PBMCs) from melioidosis patients or from healthy patients stimulated *ex vivo* with *Bp* displayed significantly increased IL-17A expression.[1, 2] Likewise, the transcriptome of *Bp*-infected human olfactory ensheathing cells (primary olfactory glia) revealed an enrichment for the IL-17 signaling canonical pathway, among other modulated pathways.[3] Furthermore, significantly enhanced IL-17 levels were noted in murine tissues and/or serum in two different models of inhalational melioidosis.[3, 4]

Among the IL-17 cytokine family members, IL-17A has been reported to play a crucial role in host resistance to several intracellular and extracellular bacterial pathogens (**Table 5.1),** with demonstrated applicability as an immunotherapeutic in some studies.[5-16] In fact, there is a large body of work detailing the specific immunomodulatory effects of IL-17A that underlie its critical role in mediating host defense.[5-11, 13-23] In particular, IL-17A is responsible for potently upregulating cytokine production in a wide range of cell types, as well as priming and recruiting critical immune cells to sites of infection.[16-18, 20, 21] Given what is known of these immunostimulatory effects and specifically, of the

capacity for this cytokine to prime phagocytic cells towards enhanced intracellular killing of bacterial pathogens **(Table 5.1)**,[8, 9, 12] we sought to determine if IL-17A may similarly prime human Mm6 monocytes to restrict the intracellular survival of *Bt* in our initial studies **(Chapter 5.2)**. We then further analyzed human Mm6 monocyte responses to IL-17A by assessing the surface expression of the IL-17 receptor complex (IL-17RA/RC) in undifferentiated and PMA-stimulated monocytes **(Chapter 5.3)**.

Table 5.1. Documented relationships between IL-17A and select bacterial pathogens.

Bacterial agent	IL-17A-mediated effects	Reference
Francisella tularensis (Ft)	***In vitro*** • IL-17A priming of *Ft*-infected or uninfected murine BMDMs induced significant increases in IL-12 and IFN-γ • Enhanced *Ft* clearance in IL-17A primed murine BMDMs ***In vivo***: • IL-17A$^{-/-}$ and IL-17R$^{-/-}$ mice had significantly higher tissue colonization and decreased survival • rmIL-17A treatment incrementally delayed onset of death	9, 13, 15
Listeria monocytogenes	IL-17A$^{-/-}$ mice exhibited enhanced liver bacterial burdens	7
***Mycobacterium tuberculosis* HN878**	Aerosol-infected IL-17A$^{-/-}$ mice exhibited enhanced lung bacterial burdens	14
Klebsiella pneumoniae	AdIL-17A pre-treatment significantly enhanced lung bacterial clearance following intranasal infection	16
Bordetella pertussis (Bop)	***In vitro*** IL-17A priming of murine peritoneal, MHS, and J774 macrophages enhanced intracellular killing of *Bop* ***In vivo*** Enhanced bacterial burdens in *Bop*-infected IL-17A$^{-/-}$ mice	6, 8
Pseudomonas aeruginosa	• IL-17RA$^{-/-}$ mice exhibited significantly increased pulmonary CFUs and enhanced mortality (NH57388A and YH5 strains) • Enhanced incidence of bacterial colonization in IL-17A$^{-/-}$ and IL-17RA$^{-/-}$ mice (AA43 strain)	5, 10, 11
Streptococcus pneumoniae	rhIL-17A pre-treatment of human neutrophils significantly enhanced killing of intracellular bacteria in the presence or absence of antibodies/complement	12

BMDMs, bone marrow-derived macrophages; rmIL-17A, recombinant murine IL-17; AdIL-17A, recombinant adenovirus encoding murine IL-17; rhIL-17A, recombinant human IL-17A.

5.2 Elucidating the role of IL-17A in the intracellular survival of *Bt* in human Mm6 monocytes.

Human Mm6 monocyte response to IL-17A stimulation

Recombinant human IL-17A (rhIL-17A) (317-ILB-050 R&D Systems) was reconstituted in 4 mM HCl (320331 Sigma Aldrich), and a series of dose-response experiments was performed with $1x10^6$ Mm6 monocytes exposed to a range of rhIL-17A concentrations (10, 20, 50,100, 200 ng/mL). Human Mm6 monocytes treated with HCl at the corresponding concentrations were included as a vehicle-control. In agreement with prior studies in primary human monocytes,[24] rhIL-17A was not found to impact monocyte viability or differentiation (ie., there were no morphologic changes suggestive of monocyte-to-macrophage differentiation, such as evidence of cellular adherence to cell culture dishes), in a 7-day co-incubation period.

Examining the effect of IL-17A priming of human Mm6 monocytes on the intracellular survival of Bt

Interleukin-17A can induce a pro-inflammatory transcriptome in phagocytic cells and prime macrophages and neutrophils towards enhanced intracellular killing of bacterial pathogens, in both human and murine systems.[8, 9, 12, 22, 24-27] Specifically, macrophages incubated with 10 to 200 ng/mL of IL-17A for a period of time (18 to 72 h) displayed upregulation of pro-inflammatory cytokines (eg., IL-1β, IL-1α, TNF-α, IL-6, IL-12, and IFN-γ) with a Th1 bias, chemokines (as well as respective cytokine and chemokine receptors that allow for signal transduction), genes involved in inflammatory responses, and cell adhesion molecules.[24-27] In the context of bacterial infection, two studies have shown that priming or co-culture of macrophages with 50 to 100 ng/mL of IL-17A resulted in enhanced intracellular killing of targeted pathogens, as compared to unprimed cells.[8, 9]

With this in mind, human Mm6 monocytes seeded at $1x10^6$ cells/mL were primed with 125 ng/mL of rhIL-17A (an intermediate concentration based on published IL-17A studies) for 2 h prior to infection with 25 µL of a *Bt* DW503 suspension in PBS at an OD_{580} of ~1.4 (corresponding to an MOI of 1:40), in the context of the intracellular survival assay outlined in **Chapter 2**. Vehicle-treated and

untreated (unprimed) Mm6 monocytes were included as controls for all assays. Three independent intracellular survival assays failed to demonstrate a difference in *Bt* intracellular growth indices between IL-17A-primed, unprimed, or vehicle-treated human monocytes under these conditions **(Table 5.2)**. These results prompted further experiments that included prolonged periods of IL-17A priming (24 h) and increased cytokine concentrations (200 to 500 ng/mL), with each condition assayed in n=4 independent experiments **(Table 5.2)**.

Table 5.2. IL-17A priming of monocytes has no observable effect on the intracellular replication of *Bt.*

IL-17A concentration	Human monocyte priming period	Effect
125 ng/mL	2 h	No difference in *Bt* intracellular growth indices
200 ng/mL	24 h	No difference in *Bt* intracellular growth indices
500 ng/mL	24 h	No difference in *Bt* intracellular growth indices

The findings presented in **Table 5.2** may suggest that rhIL-17A has no effect on the intracellular growth of *Bt* in human Mm6 monocytes. Alternatively, the lack of a discernible IL-17A-mediated effect on *Bt* phagocytosis and replication in human Mm6 monocytes may be related to the capacity for these monocytes to respond to cytokine stimulation. Specifically, human Mm6 monocytes may be less responsive to rhIL-17A priming in the undifferentiated state. Furthermore, this lack of responsiveness may be related to low surface expression of the IL-17 receptor (IL-17RA/RC) in undifferentiated monocytes, thus precluding signal transduction and observable downstream effects. We therefore surmised that human Mm6 monocyte IL-17RA/RC expression may be enhanced following differentiation to the macrophage state. With this in mind, subsequent experiments were aimed at optimizing PMA-induced differentiation of Mm6 monocytes to macrophages and determining the IL-17RA/RC surface expression in PMA-stimulated (differentiated) and undifferentiated Mm6 monocytes.

5.3 Determining the IL-17 receptor complex (RA/RC) surface expression of human Mm6 monocytes.

Differentiation of human Mm6 monocytes

Prior to assessing the IL-17RA/IL-17RC complex surface expression in PMA-differentiated human Mm6 monocytes, PMA differentiation conditions were optimized according to specific phenotypic criteria (ie., monocyte aggregate formation and adherence to cell culture dishes), as previously described.[28] For these assays, morphologic evidence of adherence was used as a preliminary correlate of PMA-induced differentiation. Mm6 monocytes were seeded at different cell densities and exposed to a wide range of PMA concentrations (0.1 ng/mL to 62.5 ng/mL) for 3, 24, or 48 h, to identify the optimal monocyte-to-macrophage differentiation condition(s) **(Table 5.3)**. For PMA concentrations ≥ 2.5 ng/mL, Mm6 monocytes were found to form dense cellular aggregates that exhibited loose adherence as early as 30 min post-stimulation; however, this adherent phenotype was found to dissipate over time for conditions that included high Mm6 monocyte seeding densities. For conditions that promoted monocyte adherence beyond early time points, a post-PMA resting period (16-18 h) wherein monocytes were washed (1X PBS, 1 mL) and exposed to complete medium devoid of PMA, similarly resulted in dissipation of loosely-adherent cellular aggregates, with reversion to non-adherent, individual cells (giving the appearance of reversion to the undifferentiated state). Based on the data presented in **Table 5.3**, low cell densities ($3.5x10^5$ cells/mL) exposed to 10 ng/mL of PMA for 48 h resulted in the highest level of cellular adherence (ie., suggesting the highest level of differentiation).[28] Given that formation of adherent cellular aggregates is merely suggestive of differentiation, we sought to confirm this phenotype via assessing changes in the surface expression of CD11b, which would indicate differentiation to the macrophage state.[29]

Table 5.3. Optimizing PMA-induced human Mm6 monocyte-to-macrophage differentiation conditions.

PMA concentration	Cell density	Time course	Results
10 ng/mL	$1x10^6$ cells/mL	24 or 48 h	Non-adherent, large dense cellular aggregates
12 ng/mL or 62.5 ng/mL	$1x10^6$ cells/mL	3 h	Non-adherent, large dense cellular aggregates
2.5, 5, 10 ng/mL	$9.5x10^5$ cells/mL	24 h	Non-adherent, large dense cellular aggregates
0.1 ng/mL, 0.5 ng/mL, 1 ng/mL	$9.5x10^5$ cells/mL	24 h	Only rare dense cellular aggregates, with a predominance of single, scattered cells
2.5 ng/mL 5 ng/mL 10 ng/mL	$8.5x10^5$ cells/mL	48 h	• Non-adherent, large dense cellular aggregates • Non-adherent, large dense cellular aggregates • Some adherent aggregates
5 ng/mL	$5.5x10^5$ cells/mL $3.5x10^5$ cells/mL	48 h	• Few adherent cells, abundant large aggregates • Some adherent cellular aggregates
10 ng/mL	$5.5x10^5$ cells/mL $3.5x10^5$ cells/mL	48 h	• Some adherent cellular aggregates • Numerous adherent cellular aggregates

Determination of human Mm6 monocyte differentiation by assessing surface receptor changes

To confirm monocyte-to-macrophage differentiation, changes in CD11b surface receptor expression were assessed, and CD14 was utilized alongside as a control, given its widespread expression in both monocytes and macrophages.[30] Human Mm6 monocytes were seeded at $3.5x10^5$ cells/mL, $5.5x10^5$ cells/mL, or $8.5x10^5$ cells/mL in 24-well plates and exposed to 10 ng/mL of PMA for 48 h to achieve monocytic adherence. After 48 h of PMA stimulation, adherent cultures were centrifuged at 400 x g for 4 min and gently washed with 1 mL of PBS prior to incubation with 100 µL of cold stain wash buffer (SWB) containing heat-inactivated (HI) pooled normal human serum (NHS) (IPLA-SER, Innovative Research) at a concentration of 1:100 for Fc receptor (FcR) blocking (10 min on ice). Following FcR blocking, monocytes were incubated with a total of 110 µL SWB containing 5 µL of anti-human, APC-conjugated, CD11b antibody (Clone ICRF44, STEMCELL Technologies) or 5 µL of anti-human, FITC-conjugated, CD14 antibody (Clone M5E2, STEMCELL technologies) for 30 min at 4°C. Subsequent to washing with 1 mL of SWB, cells were then fixed in PBS containing 2% paraformaldehyde for 20 min at

room temperature. After further washing with 1 mL of PBS, cell pellets were resuspended in 250 µL of PBS to be assayed on the BD Accuri C6 flow cytometer (BD Biosciences). Controls for this assay included a subset of unstimulated Mm6 monocytes that were also exposed to CD11b or CD14 antibodies and an unstimulated, unstained subset of cells (negative control).

The results presented in **Figure 5.1** are preliminary but suggest that while Mm6 monocytes express CD14 (as previously described),[30, 31] these cells do not appear to express detectable levels of the macrophage marker, CD11b, prior to or following PMA-stimulation. However, it was subsequently

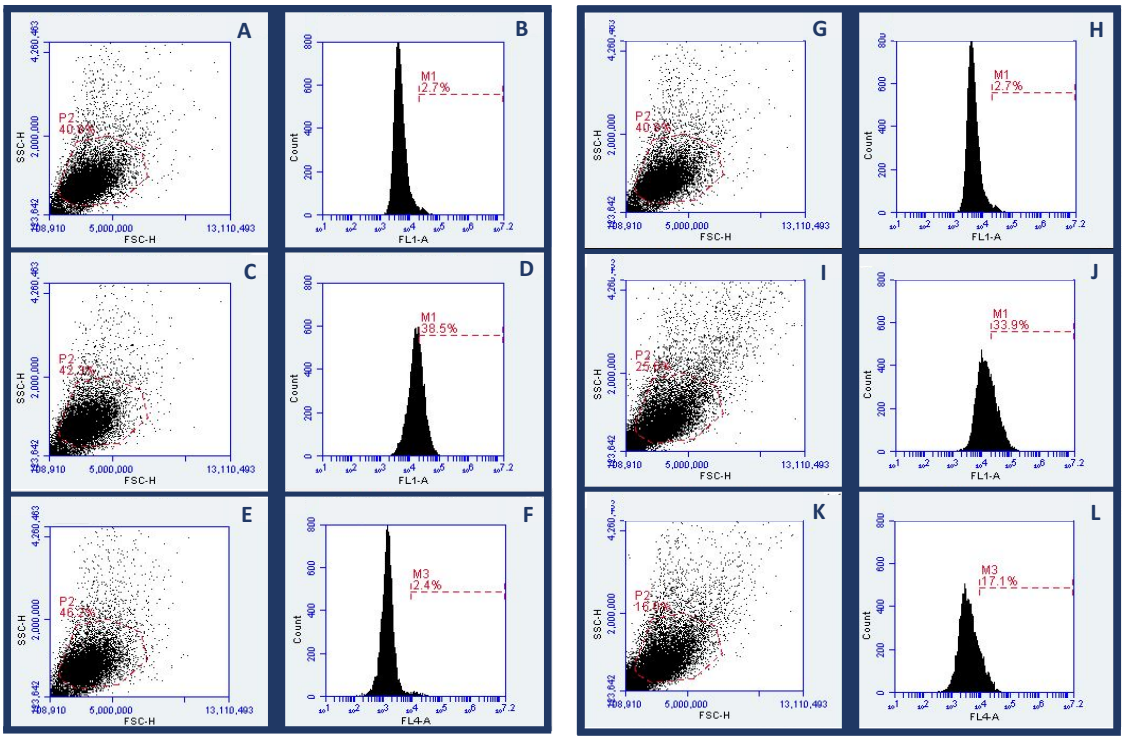

Figure 5.1. Surface expression of CD11b in resting or PMA-stimulated human Mm6 monocytes. Resting human monocytes were unstained **(A-B),** stained with anti-CD14 (Clone M5E2) **(C-D)**, or anti-CD11b (Clone ICRF44) **(E-F)**. PMA-treated monocytes were unstained **(G-H)**, stained with anti-CD14 (Clone M5E2) **(I-J)**, or anti-CD11b (Clone ICRF44) **(K-L)**. Resting or PMA-stimulated human monocytes express CD14 **(C-D, I-J)**, but CD11b expression is not discernible using anti-CD11b ICRF44 clone **(E-F, K-L)**.

determined that the anti-CD11b antibody clone (Clone ICRF44) utilized in our assays may not have been optimal for the assessment of CD11b expression in human Mm6 monocytes, as described by Weissgerber and colleagues.[32] A different CD11b antibody clone, Bear-1, has, on the other hand, been

reported to successfully highlight CD11b expression in human Mm6 monocytes, which were unexpectedly shown to express this macrophage marker at a basal level (as undifferentiated monocytes).[32] Therefore, a more valid approach to evaluate the differentiation of this cell line may be to assess for changes in CD11c surface expression, as previously performed to confirm human Mm6 monocyte-to-macrophage differentiation.[28]

IL-17 receptor (IL-17RA/RC) expression of PMA-stimulated and resting human Mm6 monocytes

Although PMA-induced differentiation of Mm6 monocytes could not be confirmed using the anti-CD11b antibody (Clone ICRF44), morphologic changes were strongly supportive of a differentiated state. With the assumption that PMA-treated, adherent Mm6 monocytes may be differentiated, we sought to determine the IL-17RA/RC surface expression of these presumably differentiated monocytes as it compared to resting (non-PMA-exposed) monocytes. To investigate this, $1x10^6$ Mm6 monocytes were resuspended in cold SWB with HI pooled NHS at a concentration of 1:100 for FcR blocking (10 min on ice). Subsequently, Mm6 monocytes were centrifuged at 400 x g for 4 min and resuspended in SWB containing either 0.25 µg of mouse anti-human monoclonal IL-17RC antibody (MAB22691-SP R&D Systems) or 2.5 µg of mouse anti-human monoclonal IL-17RA antibody (MAB177-SP R&D Systems) for 30 min at 4°C. Following two washes with 1 mL of SWB, cells were resuspended in SWB containing goat anti-mouse Alexa Fluor 647 secondary antibody (A-21235 ThermoFisher Scientific), incubated for 30 min at 4°C, and again washed twice with 1 mL of SWB. Cells were then fixed in PBS containing 2% paraformaldehyde for 20 min at room temperature. Finally, following 2 washes with 1 mL of PBS, cell pellets were resuspended in 250 µL of PBS to be assayed on the BD Accuri C6 flow cytometer.

Data from n=2 independent assays are represented in **Figure 5.2** and illustrate IL-17RA subunit expression in both PMA-stimulated and resting human Mm6 monocytes. Surface expression of the IL-17RC subunit was, however, not detected in Mm6 monocytes, which is contrary to what has been previously reported for primary human monocytes.[33, 34] Given this, it is possible that the lack of

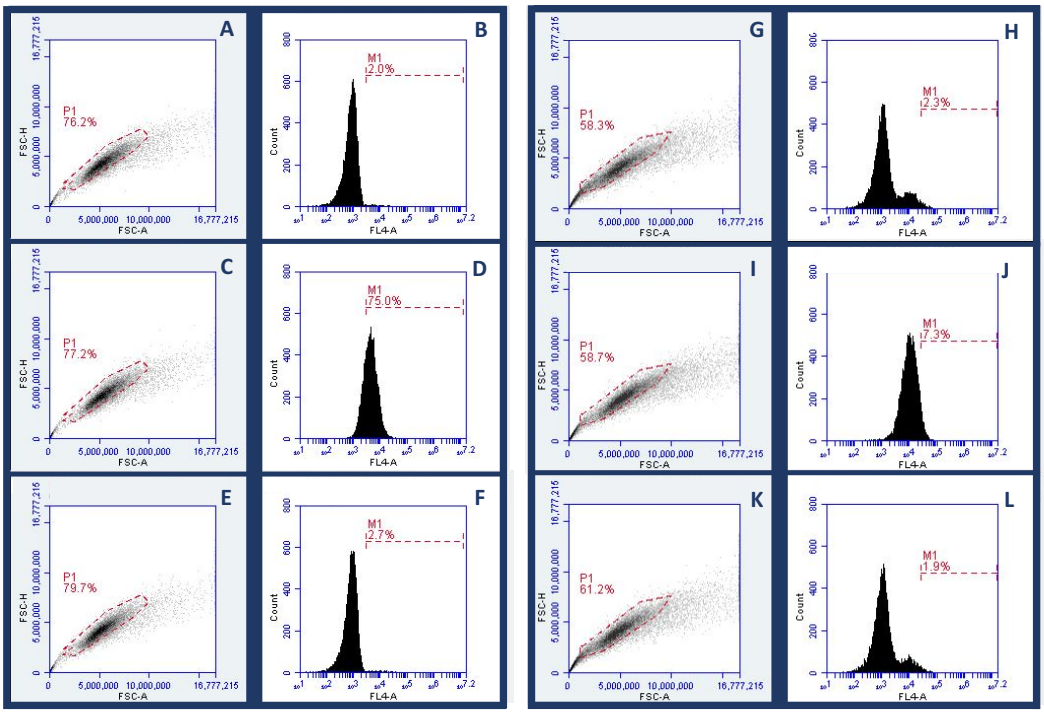

Figure 5.2. IL-17 receptor complex expression in resting or PMA-stimulated human Mm6 monocytes. Resting human monocytes were unstained **(A-B),** stained with anti-IL-17RA **(C-D)** or anti-IL-17RC **(E-F)** antibodies. PMA-stimulated monocytes were unstained **(G-H)**, stained with anti-IL-17RA **(I-J)** or anti-IL-17RC **(K-L)** antibodies. Resting or PMA-stimulated monocytes express IL-17RA **(C-D, I-J)**, but expression of IL-17RC **(E-F, K-L)** is not detectable by anti-human IL-17RC antibody (MAB22691-SP).

detectable IL-17RC subunit expression in our assays is related to the detection sensitivity of the anti-IL-17RC monoclonal antibody in the context of a lowly expressed subunit, or less likely, because of impaired antibody binding activity. Alternatively, it is possible that the human Mm6 monocytic leukemia cell line truly does not express the IL-17RC subunit. In this case, lack of expression of this critical subunit would preclude signal transduction[35, 36] in IL-17A-primed monocytes and therefore, potentially help explain the lack of an observable effect in the context of intracellular survival assays with *Bt*.

The lack of detectable IL-17RC subunit expression in Mm6 monocytes prompted us to conduct two additional experiments aimed at validating the activity of the monoclonal anti-IL-17RC antibody (MAB22691-SP). For these assays, primary human PBMCs were used as a positive control, as these cells have been reported to express both IL-17RA and IL-17RC subunits of the IL-17 receptor complex.[33, 34]

However, data (not shown) obtained with human PBMCs likewise illustrated IL-17RA surface expression with an apparent lack of IL-17RC expression. Four-fold increases in anti-IL-17RC antibody concentration similarly failed to identify surface expression of this subunit in PBMCs. Altogether, the lack of discernible surface IL-17RC expression in cells known to express this subunit (though not at a high level) may, in fact, be related to the monoclonal nature of the anti-IL-17RC antibody used. We surmised that a polyclonal, biotinylated, anti-IL-17RC antibody (BAF2269 R&D Systems) may be more appropriate in confirming expression of a potentially lowly-expressed subunit and plan to test Mm6 monocytes in conjunction with PBMCs and the positive control cell line used by R&D Systems (K562 human chronic myelogenous leukemia cell line) with this antibody, in future studies.

5.4 Summary.

Given that the transcriptome of human monocytes infected with *Bm* or *Bt* exhibited significant enrichment for IL-17 signaling pathways and that, among these canonical pathways, IL-17A signaling appeared to be differentially modulated across these two infections, we sought to elucidate the role that this pathway may play in the intracellular pathogenesis of *Bt* (as a model for its potential role in *Bm* infection). We focused on the potential impact of IL-17A in activating and/or priming the bactericidal function of human monocytes towards reducing intracellular bacterial replication. However, our studies did not identify morphologic changes in IL-17A-stimulated human Mm6 monocytes suggestive of an activated phenotype (a finding consistent with the literature), nor did priming of these monocytes result in differential intracellular replication of *Bt* across multiple cytokine concentrations and priming conditions. Therefore, these results led us to investigate the capacity for this cell line to respond to IL-17A stimulation via a thorough analysis of the surface expression of the IL-17RA/RC complex required to initiate the downstream IL-17 signaling cascade. In particular, we sought to determine the possibility for differential expression of IL-17RA/RC in undifferentiated or differentiated monocytes and whether the lack of apparent response to IL-17A stimulation may be correlated with poor IL-17RA/RC expression in

the undifferentiated monocytic state. As part of these additional studies, we identified morphologically optimal differentiation conditions for human Mm6 monocytes using PMA and determined that evaluating for differentiation of this cell line should be conducted via an assessment of CD11c, rather than CD11b surface expression. We found that PMA-stimulated (differentiated) and undifferentiated Mm6 human monocytes both exhibited IL-17RA expression but appeared to lack expression of the IL17RC subunit necessary for signal transduction. Additional assays performed with human PBMCs, as a positive control, likewise demonstrated expression of IL-17RA but lack of RC subunit expression, in contrast to published data, suggesting that the monoclonal antibody used may not be capable of detecting the RC subunit. Given these results, future studies are warranted to conclusively determine the surface expression of the entire IL-17RA/RC complex in human Mm6 monocytes in the context of a polyclonal anti-IL-17RC antibody capable of detecting a potentially lowly-expressed subunit.

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CHAPTER 6

ELUCIDATING THE ROLE OF HUMAN PTX3 IN THE PATHOGENESIS OF *BURKHOLDERIA* SP.

6.1 Introduction.

Transcriptome profiling of *Bm*- or *Bt*-infected human monocytes also revealed significant enrichment for the Role of Pattern Recognition Receptors canonical pathway, with significant activation of this pathway over time for *Bm*-infected monocytes **(Chapter 2)**. Among the members of this pathway, the soluble pattern recognition receptor (PRR), PTX3, was found to be significantly upregulated at the gene-level for both *Bm*- or *Bt*-infected human monocytes **(Chapter 2)**. Given that PTX3 has been shown to be involved in microbial recognition, complement activation and modulation, opsonophagocytosis, and host resistance against several bacterial, fungal, and viral agents[1-11] (as reviewed in **Chapter 1.6**), we were interested in elucidating the previously unexplored role of PTX3 in the pathogenesis of *Bm* infection.

Towards further defining this identified link between *Bm*, *Bt*, and PTX3, we first pursued binding assays to determine the capacity for host PTX3 to target and opsonize *Bt* initially **(Chapter 6.2)**. Following the novel identification of a binding interaction between PTX3 and *Bt*, we then sought to address the specific biological function of PTX3 as part of the soluble innate immune response against intravascular bacteria, prior to the establishment of intracellular infection. Specifically, we had interests in defining the impact of the well-documented, synergistic, PTX3-complement system relationship on the survival kinetics of *Bt* in serum **(Chapter 6.3)**. Through serum bactericidal assays, we aimed to determine if PTX3 pre-opsonization of *Bt* may prime serum-based innate immune defenses to enhance complement-mediated bacterial lysis, or, conversely, if *Bt* may benefit from coating with PTX3 via masking of critical surface components to evade complement deposition and lysis. Beyond the interactions

of soluble innate immune response components with *Bt*, we were further interested in examining the impact of PTX3-complement complex formation on the host monocyte-bacteria interface. In this line of endeavor, we employed opsonophagocytic and intracellular survival assays to determine if PTX3 pre-opsonization may enhance phagocytic uptake and killing of phagocytized bacteria, or, conversely, if binding of PTX3 to bacterial surfaces would promote cellular invasion and increased intracellular growth of opsonized bacteria. These studies with *Bt* were followed-up with binding assays with the pathogenic *Burkholderia* sp., *Bm* and *Bp*, and the resulting preliminary data are presented in **Chapter 6.4**.

6.2 Human PTX3 binds to *Burkholderia thailandensis.*

Binding assays

Given that *Bm*- and *Bt*-infected human monocytes exhibited progressive, time-dependent, enhanced expression of the soluble PRR, PTX3, these assays were conducted to first characterize a potential binding interaction between PTX3 and *Bt* (see **Chapter 6.4** for binding assays with *Bm* and *Bp*). Plate-grown *Bt* DW503 bacteria were resuspended in PBS to an OD_{580} of ~1.4. Ten microliters of this bacterial suspension were added to 1.7 mL microcentrifuge tubes containing HBSS (10-508F, Lonza) with 10 µg/mL of carrier-free rhPTX3 (1826-TS/CF, R&D Systems) in a total volume of 100 µL. Following brief vortexing and centrifugation, tubes were incubated for 1 h in 5% CO_2 at 37°C to promote *Bt*-PTX3 binding. Thereafter, tubes were centrifuged at 1500 x g for 10 min to remove unbound rhPTX3. Bacterial pellets were then resuspended in HBSS containing 1% BSA Fraction V (Fisher Bioreagents) and 0.5 µg/mL of biotinylated, polyclonal, anti-human PTX3 antibody (BAF1826, R&D Systems). Tubes were briefly mixed, centrifuged, and placed at 4°C for 30 min. Following washing with HBSS, bacterial pellets were resuspended in HBSS with 1% BSA and 10 µL of phycoerythrin (PE)-labeled streptavidin (F0040, R&D Systems). After brief mixing and centrifugation, tubes were subsequently placed at 4°C for 30 min. Next, bacterial pellets were washed with HBSS, resuspended in PBS containing 2% paraformaldehyde, and incubated at room temperature for 20 min. Following a final wash, bacterial pellets were resuspended in

200 µL of PBS, and samples were assayed on the BD LSRII flow cytometer. Three controls were included for each assay and consisted of HBSS containing: 1) bacteria only, 2) bacteria with anti-human PTX3 antibody and streptavidin-PE, and 3) bacteria with rhPTX3 and streptavidin-PE. In parallel, *Pseudomonas aeruginosa* PA01 (a generous gift from Dr. Balazs Rada) was included as a positive control, as this strain has been previously reported to bind to rhPTX3.[9] The data representative of n=5 independent assays are depicted in the histogram below (**Figure 6.1)** and demonstrate binding of the soluble PRR, PTX3, to *Burkholderia thailandensis*, presumably as part of the innate immune response against intravascular bacteria.

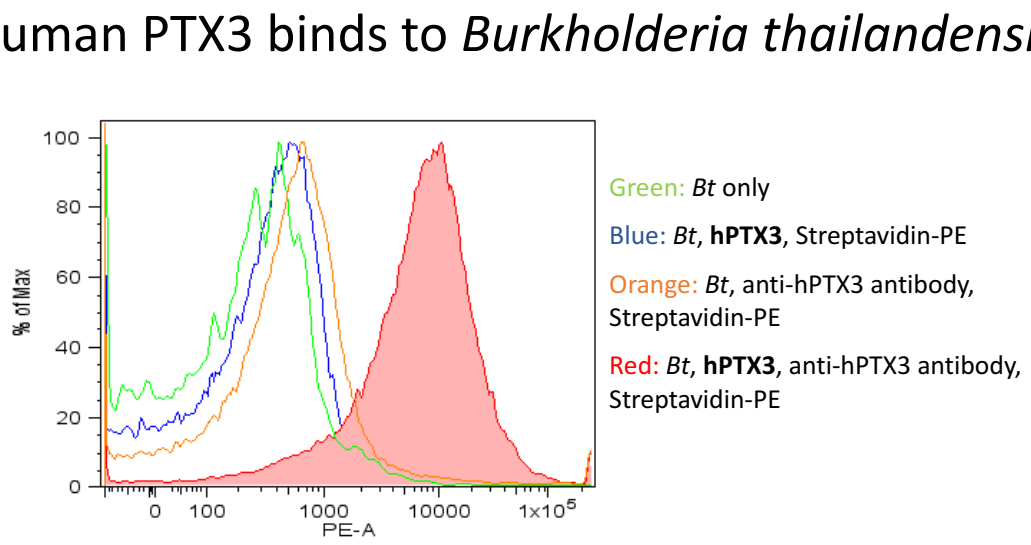


Figure 6.1. Interaction between human PTX3 and *Burkholderia thailandensis* DW503.
Binding between rhPTX3 and 10^7 bacteria was demonstrated via biotinylated anti-hPTX3 antibody and PE-labeled streptavidin. Green, blue, and orange curves show control fluorescence. The data across all treatment groups are normalized to the number of cells (% of Max).

6.3 Characterizing the role of PTX3 binding in the innate immune response against *Bt.*

Bactericidal assays with PTX3 and normal human serum

Following the novel characterization of a binding interaction between PTX3 and *Bt*, we employed serum bactericidal assays to explore the nature of this interaction and assess whether binding may represent a host-related defense mechanism or may be promoted by the bacterium as an

immunoevasive strategy. More specifically, these assays were designed to identify whether a cooperative relationship may exist between the serum-based host complement system and PTX3 in potentially enhancing complement-directed bacterial lysis, or, conversely, if *Bt* may benefit from this binding interaction via masking of critical surface components and evasion of complement binding and resulting lysis. Towards this end, the initial phase of these assays models binding conditions established in our binding assays prior to the addition of serum as the source of active complement.

As such, plate-grown *Bt* DW503 bacteria were resuspended in PBS to an OD_{580} of ~1.4, and 10 µL of this suspension were added to microcentrifuge tubes containing HBSS with 10 µg/mL of rhPTX3 (opsonized bacteria) or HBSS alone (unopsonized control) in a total volume of 100 µL. Following brief vortexing and centrifugation, microcentrifuge tubes were incubated for 1 h in 5% CO_2 at 37°C to allow binding between *Bt* and PTX3. Tubes were subsequently centrifuged at 1500 x g for 10 min to remove unbound rhPTX3 from opsonized bacteria. Pooled normal human serum (NHS) was then added as the source of complement at different concentrations (5%, 10%, 25%, 50%) to HBSS containing rhPTX3-opsonized bacteria or unopsonized bacteria, in a total volume of 100 µL. A negative control consisting of HBSS and bacteria in the absence of NHS was also included. Following the addition of pooled NHS, all tubes (including no serum control) were subsequently incubated in 5% CO_2 at 37°C for 0 h, 2 h, or 6 h to assess for changes in bacterial CFUs reflective of differential complement-mediated lysis over time. At the indicated time points, PTX3-opsonized bacteria with serum, unopsonized bacteria with serum, and a *Bt*-no serum control were subjected to serial dilutions and plating onto LB-agar plates for enumeration of bacterial CFUs after 48 h of incubation in 5% CO_2 at 37°C. For all assays, the bactericidal activity of complement in NHS was validated by subjecting the serum-sensitive *Moraxella catarrhalis* O35EΔ2 mutant strain[12] (a generous gift from Dr. Eric Lafontaine) to 10% NHS for 2 h and demonstrating consistent killing of this strain under these conditions (data not shown).

Four, independent, serum bactericidal assays were performed with *Bt* for each serum concentration (5%, 10%, 25%, 50%), and resulting data are depicted in **Figure 6.2**. Collectively, these

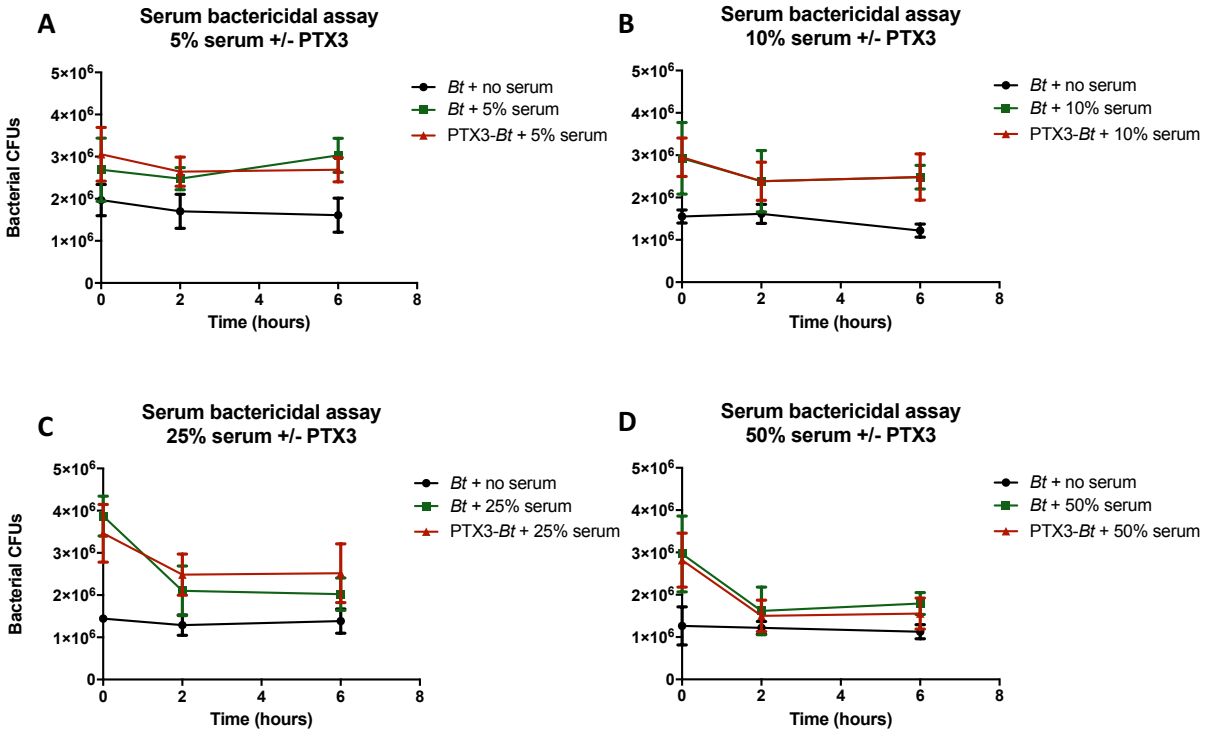


Figure 6.2. Human PTX3 does not potentiate the lytic activity of complement in human serum. Complexes of PTX3 and 10^7 *Bt* were subjected to **(A)** 5% NHS, **(B)** 10% NHS, **(C)** 25% NHS, or **(D)** 50% NHS, and bacterial CFUs were enumerated at 0, 2, or 6 h post-serum exposure. Two-way ANOVA and Bonferroni multiple comparisons revealed no significant differences in CFUs between PTX3-opsonized or unopsonized bacteria.

data demonstrate that unopsonized and PTX3-opsonized *Bt* are similarly resistant to complement-mediated lysis in a 6 h period (even at 50% NHS), which is consistent with prior studies with *Bt*.[13, 14] In other words, PTX3 pre-opsonization of *Bt* does not alter the complement resistance of *Bt*, as there are no further reductions in CFUs for pre-opsonized bacteria across the tested NHS concentrations. Altogether, the similar growth kinetics of unopsonized or PTX3-opsonized *Bt* in serum may suggest that PTX3-mediated potentiation of complement-directed lysis may be masked by the high level of serum resistance inherent to this bacterium. It is also possible that while PTX3 binds to *Bt* as part of the host

effort to recruit complement proteins and target bacteria for lysis, *Bt* is capable of evading PTX3-mediated enhancement of the lytic phase of complement. Alternatively, these data may instead reflect insights on PTX3 mechanisms of action in suggesting that the properties of this PRR are restricted to the recruitment of complement proteins to microbial surfaces,[5, 6] rather than also regulating the activation of the terminal lytic phase of complement.

In addition to the aforementioned experiments, it should be noted that a subset of serum bactericidal assays was performed prior to the identification of a binding interaction between *Bt* and PTX3. An overview of the data obtained from these assays is presented in **Table 6.1**, and results for each assay condition are representative of three to five, independent experiments. As in prior assays,

Table 6.1. Initial assays investigating a potential host complement-PTX3 synergy in bacterial lysis.

Assay	Serum concentration	PTX3 concentration	Experimental design	Results
1	50%	0.5 µg/mL	Co-incubation of serum, PTX3, and *Bt* (no priming period)	No difference in *Bt* CFUs in the presence or absence of PTX3
2	50%	1 µg/mL	Co-incubation of serum, PTX3, and *Bt* (no priming period)	No difference in *Bt* CFUs in the presence or absence of PTX3
3	50%	5 µg/mL	Co-incubation of serum, PTX3, and *Bt* (no priming period)	No difference in *Bt* CFUs in the presence or absence of PTX3
4	50%	10 µg/mL	Co-incubation of serum, PTX3, and *Bt* (no priming period)	No difference in *Bt* CFUs in the presence or absence of PTX3
5	50%	10 µg/mL	PTX3 pre-opsonized *Bt* +/- centrifugation prior to serum treatment	No difference in *Bt* CFUs in the presence or absence of PTX3

the growth kinetics of PTX3-opsonized or unopsonized *Bt* were assessed in NHS across a 6 h time course. In-parallel controls included *Bt* only (no serum control), in addition to a validation of the NHS bactericidal activity using the serum-sensitive *M. catarrhalis* O35EΔ2 strain, as previously described. In contrast to prior serum bactericidal assays, a gelatin-based, Ca^{+2}-Mg^{+2}-$NaCl^{-}$ buffer (rather than HBSS)

was used as source of calcium and magnesium for complement activity. In these assays, *Bt* was also shown to exhibit resistance to 50% NHS across a 6 h time course, and this serum resistance was unaltered (ie., not attenuated) by PTX3 co-incubation or pre-opsonization. These results corroborated those displayed in **Figure 6.2**.

The lack of detectable synergy between human PTX3 and the complement system in amplifying lysis of *Bt* in this initial study **(Table 6.1)** prompted additional studies with a distinct, unencapsulated, gram-negative bacterium, *M. catarrhalis.* Serum-sensitive (*M. catarrhalis* O35EΔ2) and serum-resistant (wild-type (WT) *M. catarrhalis* O35E)[12] (also obtained from Dr. Lafontaine) strains were included in these additional studies given a reported possible link between *M. catarrhalis* and PTX3.[15] Mutant and WT strains were each subjected to 10% NHS and the highest tested PTX3 concentration (10 μg/mL), under different experimental conditions. The results (not shown) demonstrated that PTX3 pre-opsonization neither potentiated the bactericidal activity of 10% NHS against the serum-sensitive strain, nor did it act synergistically with complement to induce bactericidal activity against the WT strain, in a 2 h period. In fact, while 10% NHS alone promoted exponential growth of WT *M. catarrhalis* over time, PTX3 pre-opsonization resulted in an even more pronounced enhancement of bacterial growth in NHS, a notable effect that was not seen with *Bt*. Based on the known metabolic characteristics of *M. catarrhalis*, it is plausible that this bacterium utilizes the PTX3 protein as a source of amino acids for increased growth.[16] Further studies would need to be pursued to investigate PTX3 degradation by *M. catarrhalis*.

In summary, PTX3 was not observed to potentiate the terminal lytic phase of complement across two, different, bacterial species that included both serum-resistant (*Bt* DW503, *M. catarrhalis* O35E) and serum-sensitive (*M. catarrhalis* O35EΔ2) strains. These findings suggest that the serum resistance or sensitivity profile of a microorganism may not have an impact on the role of PTX3 in the complement-pathogen interface. Thus, lack of detectable synergy between PTX3 and the lytic phase of complement towards *Bt* may, in fact, be related to the mechanism of action of PTX3, rather than

represent a false negative result associated with high serum resistance. In other words, our data, along with those generated by Moalli *et al.* 2011 with *Pseudomonas aeruginosa*,[9] may suggest that the well-described synergistic relationship between PTX3 and complement is confined to the initiation of the complement pathway, rather than also involving activation of the terminal lytic phase of this innate immune response defense system. That being said, we cannot exclude the possibility that all three bacterial species may be capable of evading PTX3-mediated amplification of innate immune responses, with PTX3 production representing a futile host response to amplify complement-directed lysis and limit infection.

Bactericidal assays with PTX3, normal human serum, and anti-Bm antibodies

These additional bactericidal assays were conducted to further investigate the possibility that serum resistance displayed by *Bt* may be masking a synergistic effect between complement and PTX3 towards bacterial lysis. To accomplish this, we aimed to bolster the bactericidal activity of complement in the NHS by supplementing with hyperimmune murine serum (another contribution by Dr. Lafontaine) generated by vaccinating mice with *B. mallei* ATCC 23344 *batA* knock-out strain.[17] The hyperimmune serum and purified IgG antibodies were shown to bind to WT *Bm* as well as promote enhanced phagocytosis and intracellular killing of antibody-opsonized *Bm* by murine macrophages.[17] In light of these findings, we surmised that antibodies in the hyperimmune serum would also recognize and bind to *Bt* and that resulting amplification of antigen-antibody complex formation would augment the activation of the classical complement pathway and, potentially, the downstream lytic pathway.

To test our hypothesis, serum bactericidal assays were modified to assess the role of PTX3 in *Bt* infection in the context of an amplified classical complement pathway that could potentially, potently drive bacterial lysis. To accomplish this, differential survival kinetics of PTX3-opsonized or unopsonized *Bt* were assessed in NHS supplemented with 1 µL of hyperimmune serum (1:100 concentration) containing anti-*Bm* antibodies. A no serum control (*Bt* no serum) was also included. These assays were

conducted using 10% and 25% NHS concentrations, and the data representative of four to five assays for each serum concentration are depicted in **Figure 6.3**.

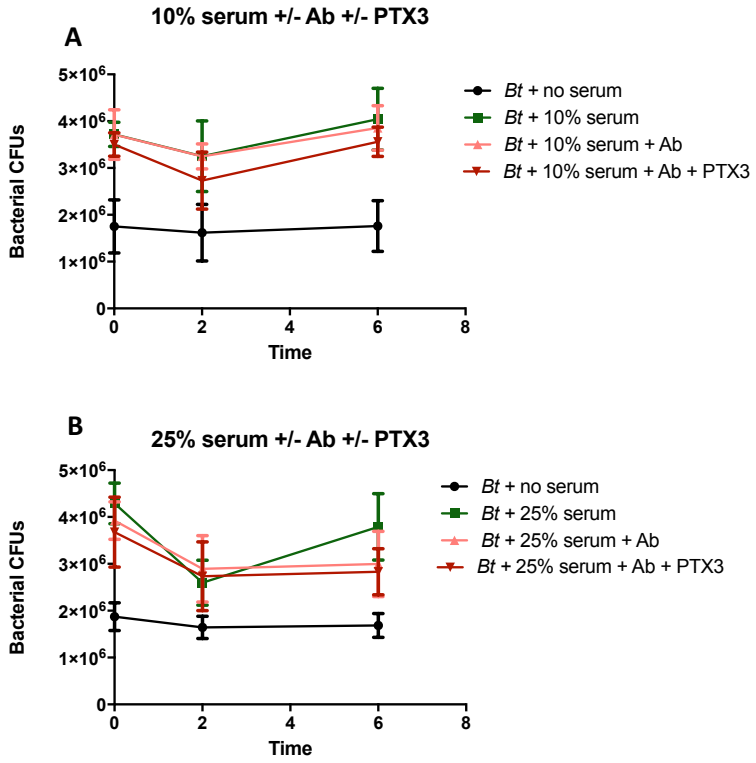


Figure 6.3. Anti-*Bm* antibodies fail to bolster the lytic activity of complement in NHS towards *Bt*. Complexes of PTX3 and 10^7 *Bt* were subjected to **(A)** 10% NHS or **(B)** 25% NHS +/- hyperimmune serum containing anti-*Bm* antibodies (Ab). Bacterial CFUs were enumerated at 0, 2, or 6 h post-serum exposure. Two-way ANOVA and Bonferroni multiple comparisons revealed no significant differences in CFUs between PTX3-opsonized or unopsonized bacteria.

While anti-*Bm* antibodies within the hyperimmune serum are likely to be opsonic to *Bt* (given opsonic properties towards *Bm*)[17] and may amplify the initiation of the classical complement pathway, the presence of these opsonic antibodies did not significantly impact the terminal complement cascade, with no significant decrease in the serum resistance of PTX3-opsonized or non-PTX3 opsonized *Bt* in our assays **(Figure 6.3)**. However, these data may be explained by the fact that hyperimmune serum at a concentration of 1:100 may not be sufficient to overcome the inherently high level of serum resistance exhibited by *Bt* at 10% or 25% NHS. Additionally, it is possible that PTX3 bound to bacterial surfaces does

not cooperate with antibodies specific to target bacteria to promote detectable potentiation of the terminal lytic pathway of complement. Further assays, such as the evaluation of *Bt* binding to antibodies in this murine hyperimmune serum would help in the clarification of these data and in the development of additional, more informative conclusions.

Intracellular survival assays in human Mm6 monocytes with unopsonized or PTX3-opsonized Bt

Overall, serum bactericidal assays did not suggest a role for PTX3 in promoting complement-mediated lysis of *Bt*. Based on these data and on the known opsonophagocytic properties of PTX3 towards several bacterial and fungal agents,[2-4, 6, 8, 9] we sought to determine if PTX3 opsonization of *Bt* may instead be involved in facilitating uptake by host phagocytic cells. To achieve this, we employed modified intracellular survival assays to assess the effect of PTX3 pre-opsonization on phagocytosis, as well as replication and overall intracellular growth of *Bt* within human monocytes.

Intracellular survival assays were modified to include a period of pre-opsonization of *Bt* with PTX3 prior to infection of human monocytes. Accordingly, 10 µL of a *Bt* suspension at an OD_{580} of ~1.4 were added to HBSS containing 10 µg/mL of rhPTX3 (opsonized bacteria) or HBSS alone (unopsonized *Bt* control) in a total volume of 100 µL. Binding of *Bt* to rhPTX3 was promoted via incubation in 5% CO_2 at 37°C for 1 h. Following incubation, *Bt*-hPTX3 complexes or unopsonized *Bt* in HBSS were then subjected to centrifugation at 1500 x g for 10 min. PTX3 pre-opsonized or unopsonized *Bt* were then resuspended in 25 µL of complete medium and added to two, quadruplicate sets of $1x10^6$ human Mm6 monocytes/mL, respectively. The remainder of the intracellular survival assays were conducted as described in **Chapter 2**. Three independent assays were undertaken, and intracellular bacteria enumerated at 4 h (phagocytosis) or 10 h postinfection (replication) were compared between PTX3 pre-opsonized and unopsonized conditions **(Figure 6.4)**. Collectively, these data failed to reveal a significant difference in phagocytosis or replication between PTX3 pre-opsonized or unopsonized *Bt*, in human monocytes. In fact, similar intracellular growth indices were noted for PTX3 pre-opsonized or

unopsonized bacteria. Given the reported complement dependence of PTX3-mediated opsonophagocytosis of select organisms,[4, 8, 9] we surmised that the lack of an observable effect of PTX3

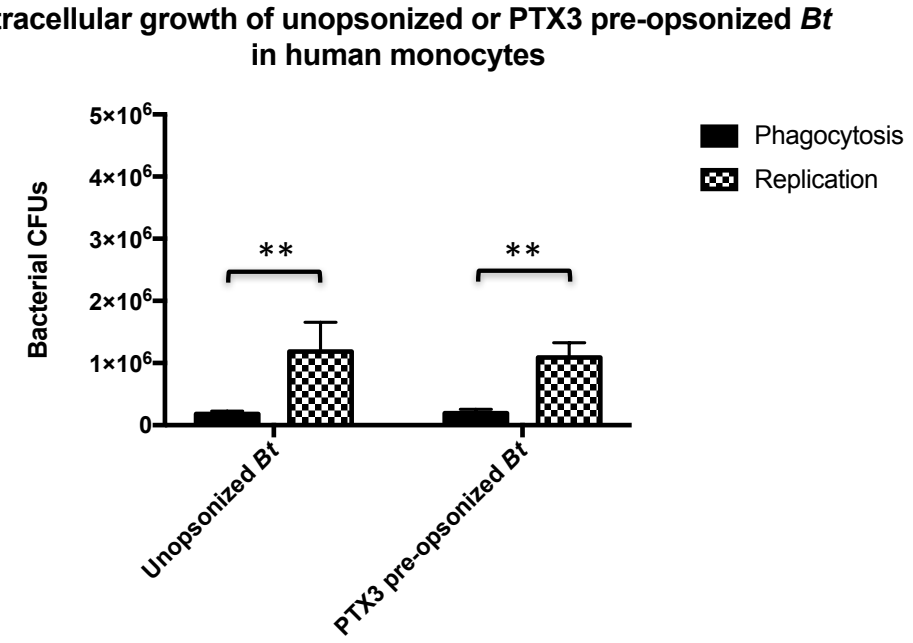


Figure 6.4. The impact of PTX3 on bacterial phagocytosis and replication within human monocytes. Pre-infection heterocomplexes of 10 μg/mL of PTX3 and 10^7 *Bt* were used to infect $1x10^6$ human monocytes in the absence of complement. Intracellular bacterial CFUs were enumerated 4 h postinfection (phagocytosis) and 10 h postinfection (replication). PTX3-opsonized or unopsonized *Bt* did not exhibit significant differences in phagocytic uptake or replication within human Mm6 monocytes (unpaired t-test). Overall, both PTX3 pre-opsonized and unopsonized *Bt* displayed significant intracellular growth within human Mm6 monocytes (**p=0.0026, unpaired t-test).

on phagocytosis or replication of *Bt* may be directly related to the absence of serum in this *in vitro* infection system.

With this in mind, subsequent assays were aimed at identifying a synergy between PTX3 pre-opsonization of bacterial surfaces and complement, on the outcome of *Bt* infection of human monocytes. These intracellular survival assays were performed as previously described, and two, quadruplicate sets of $1x10^6$ human monocytes/mL were infected with PTX3 pre-opsonized or unopsonized *Bt*, respectively, in the presence of 10% NHS (maintained as part of the medium until 4 h postinfection). The remainder of these intracellular survival assays were performed as described in

Chapter 2. As in prior assays, the bactericidal activity of complement in NHS was validated using the serum-sensitive *Moraxella catarrhalis* O35EΔ2 strain. For these assays, we also addressed the possibility of an additional source of active complement within the complete cell culture medium. We demonstrated that 10% FCS present within the cell culture medium possessed no inherent complement activity, as evidenced by lack of bactericidal activity against *Moraxella catarrhalis* O35EΔ2 for a 6 h period (data not shown). Four, independent, intracellular survival assays were conducted, in which bacterial phagocytosis and replication were compared between unopsonized and PTX3-opsonized *Bt*, in human monocytes, in the presence of serum **(Figure 6.5)**.

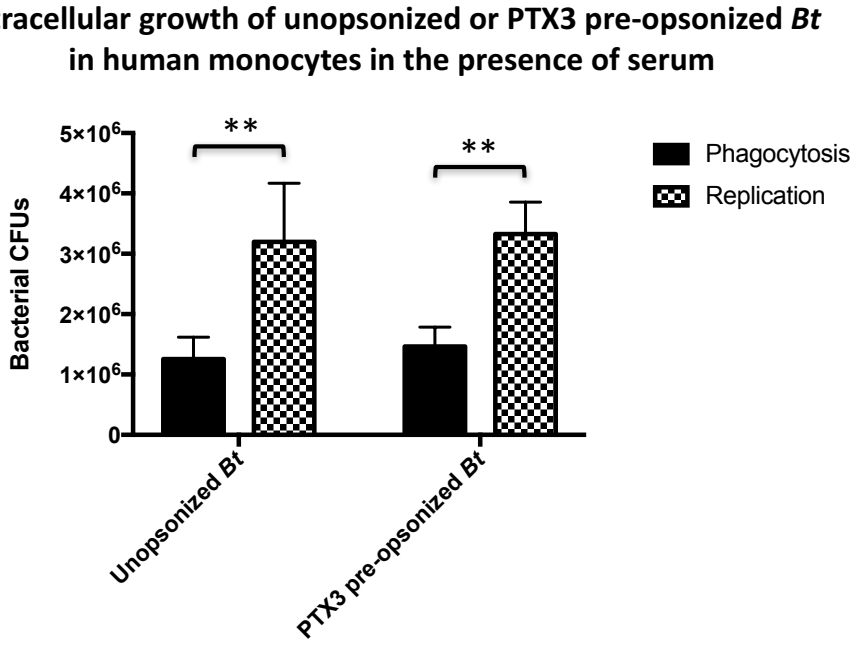


Figure 6.5. The role of PTX3-complement complexes in *Bt* intracellular growth within monocytes. Pre-infection complexes between 10 µg/mL of PTX3 and 10^7 *Bt* were used to infect $1x10^6$ human monocytes in the presence of active complement. Intracellular bacterial CFUs were enumerated 4 h postinfection (phagocytosis) and 10 h postinfection (replication). PTX3-opsonized or unopsonized *Bt* did not exhibit significant differences in phagocytic uptake or replication in human Mm6 monocytes (unpaired t-test). Overall, both PTX3 pre-opsonized and unopsonized *Bt* displayed significant intracellular growth within human Mm6 monocytes (**p=0.0042, unpaired t-test).

Altogether, the data presented in **Figure 6.5** failed to reveal a complement-dependent effect of PTX3 on phagocytosis, replication, or overall growth of *Bt* within Mm6 monocytes. Although it is possible

that PTX3 pre-opsonization of *Bt* may not play a significant role in the phagocytic uptake or replication of this bacterium within human monocytes, we cannot exclude the possibility that the lack of a discernible effect may be related to the time points at which phagocytic or replication indices were assessed. Specifically, the established intracellular survival assay assesses phagocytic uptake and replication of *Bt* within human monocytes at 4 h and 10 h postinfection, respectively. Given that phagocytosis of *Burkholderia* sp. can occur as early as 10-15 min postinfection,[14, 18] and PTX3-mediated effects on phagocytosis are predominantly observed within 15-30 min and up to 2 h postinfection,[2-4, 8, 9] it is possible that PTX3-driven effects may not be observed for time points that are far beyond the phagocytic process. Therefore, we next aimed to identify a possible PTX3-mediated opsonophagocytic effect on *Bt* at earlier time points more representative of phagocytosis.

Opsonophagocytic assays

These assays were designed to directly assess the impact of PTX3 pre-opsonization of *Bt* on phagocytic uptake by human Mm6 monocytes. The initial phase of these assays modeled that of other assays in this Chapter, with 10 µL of a *Bt* suspension at an OD_{580} of ~1.4 added to HBSS containing 10 µg/mL or 30 µg/mL of rhPTX3 (opsonized *Bt*) or HBSS alone (unopsonized *Bt* control) in a total volume of 100 µL. Binding of *Bt* to rhPTX3 was promoted via incubation in 5% CO_2 at 37°C for 1 h. Following incubation, *Bt*-hPTX3 complexes or unopsonized *Bt* in HBSS were then subjected to centrifugation at 1500 x g for 10 min. PTX3 pre-opsonized or unopsonized *Bt* were then resuspended in 25 µL of complete medium and used to respectively infect two, duplicate or triplicate sets of $1x10^6$ human Mm6 monocytes/mL seeded in the presence of 10% NHS. Following 30 min of infection, monocytes were subjected to centrifugation at 400 x g for 4 min, washed with 1.7 mL of complete medium, and resuspended in 0.5% saponin for 5 min in 5% CO_2 at 37°C. Resulting monocyte lysates were serially diluted and plated onto LB-agar plates to assess the phagocytic uptake of PTX3-*Bt* or *Bt* by human monocytes in a 30 min time course. Three, independent assays were conducted using 10 µg/mL **(A)** or

30 μg/mL **(B)** of PTX3 **(Figure 6.6)**. The data presented in **Figure 6.6** suggest that there is no clearly discernible role for PTX3 in the phagocytic uptake of *Bt*, even at this early time point.

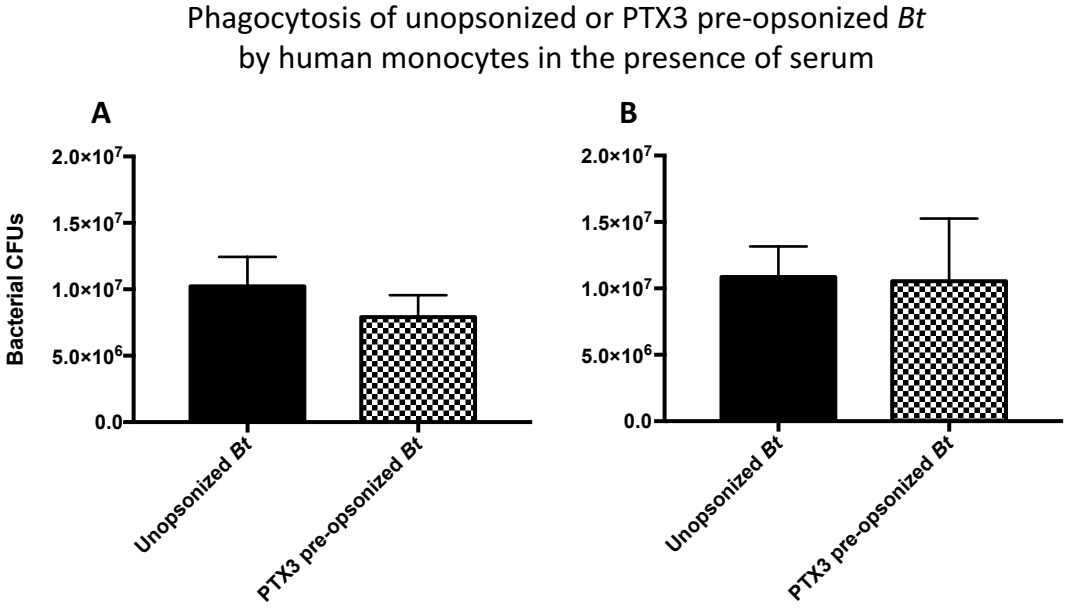


Figure 6.6. The role of PTX3-complement complexes in *Bt* phagocytosis by human monocytes. Pre-infection complexes between 10 μg/mL **(A)** or 30 μg/mL **(B)** of PTX3 and 10^7 *Bt* were used to infect $1x10^6$ human monocytes in the presence of active complement. Intracellular bacterial CFUs representative of phagocytosis were enumerated following 30 min of infection. An unpaired t-test did not reveal significant differences between phagocytosis for PTX3-opsonized or unopsonized *Bt*.

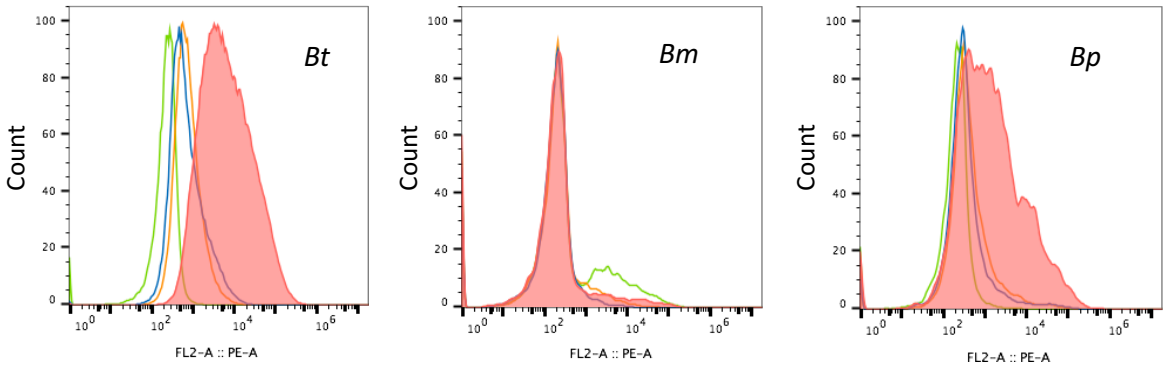
Collectively, the data presented in Chapter 6, thus far, demonstrate that the host cell-derived soluble PRR, PTX3, targets and binds *Bt* as part of the innate immune response to intravascular bacteria. However, what remains to be determined is whether the lack of cooperation between *Bt*-bound PTX3 and the terminal lytic pathway of complement represents a limitation in the mechanism of action of this PRR or whether *Bt* is capable of evading PTX3-mediated activation of this downstream effector phase of complement. Furthermore, beyond interactions with complement, *Bt*-bound PTX3 does not appear to impact uptake or intracellular replication of this bacterium in human monocytes. These findings may suggest that human Mm6 monocytes do not express sufficiently high levels of critical phagocytic receptors needed to enhance the phagocytosis of *Bt*-bound PTX3, such as specific FcγRs[8] (or that PTX3 does not bind to Mm6 monocyte surface receptors and, therefore, cannot amplify phagocytosis) and

that PTX3 does not activate human Mm6 monocyte bactericidal mechanisms. Although PTX3 was not found to represent a critical PRR in the opsonophagocytosis or intracellular survival of *Bt* in human Mm6 monocytes, NHS was identified as playing a notable role in enhancing, greater than 10-fold, the phagocytosis of *Bt* by human monocytes, as compared to serum-free conditions **(Figure 6.5)**. This NHS-mediated, marked increase in phagocytosis, was noted both in the presence or absence of PTX3 pre-opsonization. This finding may suggest that cooperation between multiple, diverse opsonins capable of binding to several distinct bacterial surface components and corresponding phagocytic receptors may be necessary to amplify uptake of *Bt* by human monocytes and that PTX3 does not further potentiate the opsonic activity of these NHS-derived opsonins.

6.4 Exploring human PTX3 as a potential opsonin for pathogenic *Burkholderia* sp.

The highly-induced expression of PTX3 in *Bm*- or *Bt*-infected human monocytes had led to initial investigations of the relationship between *Bt* and this soluble PRR. Following the characterization of the *Bt*-PTX3 relationship **(Chapters 6.2-6.3)**, additional binding assays were undertaken to identify whether a similar binding interaction may exist between PTX3 and *Bm* or the closely genetically-related, pathogenic, *Bp*. Previously established binding assays were adapted to detect binding between PTX3 and *Bm* ATCC23344 or *Bp* K96243. *Burkholderia thailandensis* DW503 was also included as a positive control to validate these binding assays. Modifications to the previously described experimental approach **(Chapter 6.2)** included detecting binding between 10 μg/mL of human PTX3 and ~10^6 bacteria following incubation with biotinylated anti-hPTX3 antibody for 1 h at room temperature and phycoerythrin-labeled streptavidin for 1 h at room temperature, with all centrifugation steps conducted in a microcentrifuge at 13,000 rpm for 5 min. Preliminary flow cytometry data are displayed in the subsequent histograms **(Figure 6.7)**.

The conditions of the assay were validated by demonstrating binding of human PTX3 to *Bt* **(Figure 6.7)**, as previously described **(Chapter 6.2)**. The additional flow cytometry data generated with *Bm* or *Bp* and PTX3 showed lack of binding to *Bm*, but some binding of this PRR to *Bp*. The recognition



Green: bacteria only
Blue: bacteria, **hPTX3**, Streptavidin-PE
Orange: bacteria, anti-hPTX3 antibody, Streptavidin-PE
Red: bacteria, **hPTX3**, anti-hPTX3 antibody, Streptavidin-PE

Figure 6.7. Interaction of human PTX3 with *Burkholderia mallei* or *Burkholderia pseudomallei*. Binding between rhPTX3 and ~10^6 bacteria was demonstrated via biotinylated anti-hPTX3 antibody and PE-labeled streptavidin. *Burkholderia thailandensis* was used as a positive control. Green, blue, and orange curves show control fluorescence. The data across all treatment groups are normalized to the number of cells.

and binding of PTX3 to *Bt* or *Bp* (but not *Bm*) could be related to the fact that these two species are more closely genetically related **(Figure 6.8)**.[19, 20] However, this explanation is not very likely given that despite phylogenetic divergence, there is still 99% sequence homology among conserved regions of *Bm* and *Bp* or *Bt* (as reviewed by [21]). Thus, the inability of PTX3 to recognize and target *Bm* for opsonization may, rather, be related to the fact that *Bt* and *Bp* represent environmental saprophytes, while *Bm* is a host-adapted bacterium.[19, 22, 23] In other words, *Bm* has adapted to evade host immune responses to allow for intracellular replication and long-term persistence within host tissues. Therefore, evading recognition and binding by a soluble PRR, such as PTX3, may constitute a survival advantage for this bacterium, which would then suggest that there may be selective pressure in evading opsonization by

this PRR. This would then further suggest that PTX3 may, in fact, represent a critical part of the host defense against *Bm* infection and that further investigations focused on understanding the role of PTX3 in host defense against *Bm* are warranted.

Coenye, Tom, and Peter Vandamme. "Diversity and significance of Burkholderia species occupying diverse ecological niches." *Environmental Microbiology* 5.9 (2003): 719-729.

Figure 6.8. Diversity of species within the *Burkholderia* genus occupying diverse ecological niches. Phylogenetic tree constructed based on 16S rDNA sequences showing the positions of all *Burkholderia* sp. and representatives of related genera. Scale bar indicates 5% sequence dissimilarity. The **black arrow** highlights the high level of genetic relatedness between *Burkholderia thailandensis* and *Burkholderia pseudomallei*. Adapted from: Coenye, Tom, and Peter Vandamme. "Diversity and significance of Burkholderia species occupying diverse ecological niches." *Environmental Microbiology* 5.9 (2003): 719-729.

6.5 Summary.

The pattern recognition receptor pathway was represented among the enriched canonical pathways in the transcriptome of *Bm*- or *Bt*-infected human monocytes, with evidence of pathway

activation during both infections. Among the members of the PRR pathway, PTX3, was found to be upregulated by *Bm*- or *Bt*-infected human monocytes in a time-dependent manner. As such, we explored the interplay between the soluble PRR, PTX3, and *Bm*, *Bt*, or, *Bp*, as it pertains to intravascular infection and critical, blood-associated, innate immune response components, such as the complement system and monocytes. These investigations led us to identify PTX3 as a novel opsonin capable of recognizing environmental *Burkholderia* sp., such as *Bt* and *Bp*, but unable to target or opsonize the host-adapted pathogen, *Bm*. Given that *Bm* has adapted to evade innate immune responses to persist within the host, we surmised that evading recognition by a soluble PRR, such as PTX3, may constitute a survival advantage for this pathogen. Further studies are warranted to elucidate the biological implications for evasion of PTX3 binding by *Bm* **(Chapter 7)**. In the case of PTX3 recognition of *Bt* or *Bp*, follow-up studies were conducted with *Bt*, to address potential downstream effector mechanisms associated with PTX3 opsonization. These studies did not identify a significant role for PTX3 in the amplification of the terminal lytic phase of complement towards this complement resistant bacterium. An apparent lack of involvement of PTX3 in complement-mediated lysis was also noted for two *Moraxella catarrhalis* strains in our study, as well as in published data with *Pseudomonas aeruginosa*, suggesting that, perhaps, the role of PTX3 may truly be confined to the recruitment of complement to microbial surfaces, rather than also activating the terminal lytic phase of this cascade. Pentraxin-3 opsonization of *Bt* was likewise not demonstrated to enhance the uptake or influence the intracellular survival of this bacterium in human monocytes, in the absence or presence of complement. These results warrant further investigations of the PTX3-human Mm6 monocyte interface, particularly regarding the capacity for PTX3 to bind to Mm6 monocyte surface receptors to achieve an opsonophagocytic effect **(Chapter 7)**.

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CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions.

The overarching aim of this research project has been to help fill the knowledge gap regarding the interaction between *Bm* and host immune cells and the potential for *Bm*-directed modulation of immune response genes and pathways to drive intracellular replication. To accomplish this, we elected to study the interaction between *Bm* and human Mm6 monocytes based on relevance to the susceptible human host and to the blood-monocyte-bacteria interface. Given that the closely genetically-related, relatively avirulent, *Burkholderia* sp., *Bt*, has been shown to exhibit a similar intracellular lifestyle as *Bm in vitro*,[1] we conducted initial studies in the BSL-2 using *Bt* as our surrogate model organism.

As a first step towards identifying immunomodulatory mechanisms exploited by and specific to *Bm* to promote intracellular replication, our laboratory initially developed an intracellular survival assay system with human Mm6 monocytes and *Bt*, which was then adapted to study the intracellular pathogenesis of *Bm* **(Chapter 2)**. In this system, these *Burkholderia* species replicated significantly within human Mm6 monocytes. Furthermore, a ten-fold dilution of *Bm* suspension replicated within human monocytes to the same extent as an undiluted suspension of *Bt*, suggesting differential modulation of monocyte gene expression. To investigate this potential for differential gene modulation during infection with *Bm* or *Bt*, we used this cell culture system to interrogate the transcriptome of *Bm*- or *Bt*-infected human monocytes on a global, genome-wide scale. Mining of the transcriptome dataset identified negative regulators of inflammation as well as IL-17 receptor signaling and pattern recognition receptor pathways as significantly modulated genes and pathways during *Bm* or *Bt* infection.

In the case of negative regulators of inflammation, the majority of these immunoregulatory genes [eg., PTGS2 (COX2), SOCS3, TNFAIP3 (A20)] were upregulated by *Bm*- or *Bt*-infected human monocytes **(Chapter 2)**, with the exception of SENP6 and SARM-1 (which were downregulated at time points later than 1 h postinfection) **(Chapter 4)**. A progressive, time-dependent increase in the expression of several negative regulators of inflammation in *Bm*- or *Bt*-infected monocytes would then act to dampen immune responses in favor of intracellular replication and, therefore, most likely represents a bacteria-mediated effect. In contrast, the delayed but progressive decrease in the expression of other negative regulators of inflammation (SENP6 and SARM-1) is more reflective of a reactive, enhanced, postinfection, host inflammatory response to bacterial replication. Overall, the finding that most negative regulators of inflammation are highly induced following infection with *Bm* or *Bt* is in agreement with data from studies with *Bp* showing that infection of host macrophages also results in enhanced expression of select negative regulators.[2-8] As noted for *Bp*, increased expression of negative regulators of inflammation during *Bm* intracellular infection may also reflect a strategy by which this bacterium promotes replication within host cells. Although *Bm* and *Bt* both induced the expression of these genes during infection of human monocytes, the magnitude (fold change) of gene expression was lower in *Bm*-infected monocytes as compared to *Bt*-infected monocytes. This finding may be related to the ten-fold lower inoculum concentration used for *Bm* infections, thereby precluding direct comparisons between the magnitude of gene expression changes induced by these two *Burkholderia* sp.

To explore the potential role of select negative regulators of inflammation [SOCS3, COX-2 (PTGS2), TNFAIP3 (A20), SENP6, SARM-1] in the intracellular pathogenesis of *Bm*, our aim was to utilize gene silencing and chemical inhibition approaches in human Mm6 monocytes **(Chapters 3,4)**. However, the limitations of these approaches precluded gaining insights into the role of these genes in *Bt* infection of human monocytes (and therefore, future studies were not pursued with *Bm*). Nonetheless, these

studies helped generate a better understanding of the feasibility of gene silencing **(Chapter 3)** and functional inhibition **(Chapter 4)** approaches in the human Mm6 monocytic cell line.

Among the tested methodologies, Amaxa Nucleofector technology represented the most suitable platform for the successful delivery of nucleic acids into human Mm6 monocytes. **Chapter 3** detailed the development of a highly reproducible nucleofection system with this cell line, through which we achieved consistently robust levels of gene silencing. However, associated drawbacks included the necessity for high concentrations of siRNAs to achieve silencing (potentially due to the difficult-to-transfect nature of this cell line and/or the siRNA platform used) and the suboptimal viability of nucleofected cells. To mitigate this, future modifications to the experimental approach would include using the Human Monocyte Nucleofector Kit (rather than Amaxa Cell Line Nucleofector Kit V used in our studies) and supplementing the post-nucleofection Human Monocyte Nucleofector Medium with 20% human serum, to maintain high cell viability and functionality as demonstrated by Scherer *et al.* and Schnoor *et al.* in both primary human monocytes and THP-1 cells.[9, 10] Furthermore, using siRNAs that can be efficiently delivered at lower concentrations (eg., ON-TARGETplus siRNA) would likewise minimize the loss of cellular viability as well as off-target effects. As another alternative, achieving knockout phenotypes through the use of CRISPR-Cas9 technology may circumvent the need to use high siRNA concentrations to elicit and maintain gene silencing. However, detrimental effects on cell viability may still be encountered depending on the concentration of guide RNA complex used in the assays.

While the limitations of the nucleofection approach were primarily restricted to poor cell viability, functional inhibition studies using chemical inhibitors were limited by the lack of observable effects in multiple target cell lines **(Chapter 4)**. The repeated lack of a discernible zoledronic acid (ZA)-mediated effect in priming inflammatory responses in three different cell culture systems, and in the intracellular growth of *Bt* in ZA-primed human Mm6 monocytes, suggested that future studies investigating the role of its gene target, SOCS3, in the intracellular pathogenesis of *Bm*, should be

pursued in the context of gene silencing, rather than chemical inhibition. When further investigating the potential role of COX-2 in *Bm* pathogenesis, future directions should include measuring human Mm6 monocyte supernatant levels of PGE_2 (a lipid mediator derived from COX metabolism),[2] prior to and post-COX-2 inhibition, to confirm the feasibility of COX-2 inhibition via the selected inhibitors in this cell line. If COX-2 inhibition is shown to reduce PGE_2 levels but does not affect the intracellular replication of *Bt* in human Mm6 monocytes, this would suggest that COX-2 does not play a crucial role in the *in vitro* pathogenesis of *Bt*. On the contrary, if COX-2 inhibition is not accompanied by decreased PGE_2 levels, this would indicate that Mm6 monocytes are either: 1) not responsive to the tested inhibitors or 2) not responsive to COX-2 inhibition. To address these possibilities, additional inhibitors can be tested in Mm6 monocytes or similar functional inhibition assays can be conducted in cell culture systems reported to respond to COX-2 inhibition (eg., murine BMDMs).[2] For studies aimed at understanding the role of TNFAIP3 (A20) in the intracellular pathogenesis of *Bm*, gene silencing should be pursued in lieu of chemical inhibition given both the lack of available, selective, A20 inhibitors and our findings showing that priming of inflammatory responses could not be achieved in two different cell culture systems with a non-specific deubiquitinase inhibitor (NSC11200).

The described limitations of gene silencing and functional inhibition studies in human Mm6 monocytes prompted us to study the IL-17 signaling pathway in the context of cytokine-primed monocytes infected with *Bt* **(Chapter 5)**. This Chapter describes the lack of responsiveness of Mm6 monocytes to a wide range of IL-17A concentrations and incubation periods in the context of intracellular survival assays with *Bt*, with no apparent effect on *Bt* intracellular growth. As such, future assays would be aimed at addressing whether apparent lack of signal transduction may be associated with the undifferentiated state of these Mm6 monocytes and/or with low IL-17RA/RC receptor complex expression. First, PMA-differentiation of Mm6 monocytes should be confirmed by assessing for changes in CD11c surface expression, and second, a monoclonal IL-17RA antibody and a biotinylated, polyclonal

IL-17RC antibody (able to detect potentially low levels of this subunit) should be used to determine the surface expression of IL-17RA/RC in resting or PMA-treated (differentiated) Mm6 monocytes. In addition to studies in human Mm6 monocytes, further investigations of the interplay between the IL-17 pathway and *Bm* may be pursued with murine alveolar macrophages (MHS cells), based on the reported responsiveness of this cell line to IL-17A stimulation[11] and the relevance to pulmonary glanders.

Studies conducted in **Chapter 6** were similarly focused on determining the potential for genes modulated by *Bm* or *Bt* to prime innate immune responses when expressed at high enough levels prior to infection. Given that the soluble PRR, PTX3, was shown to be significantly upregulated by both *Bm*- or *Bt*-infected monocytes **(Chapter 2)**, we explored the relationship between this PRR and *Bt*, *Bm*, and the closely genetically-related *Bp*. In this Chapter, we first demonstrated that although PTX3 could bind to *Bt*, this PRR did not play an apparent role in amplifying complement-mediated lysis of this bacterium, nor did it affect phagocytosis (30 min or 4 h postinfection) or replication (10 postinfection) of this bacterium within human monocytes. Beyond the *Bt*-PTX3 relationship, we subsequently demonstrated that PTX3 also bound to *Bp* but could not bind to *Bm*. The differential binding of PTX3 to *Bt*, *Bm*, or *Bp* suggested that, unlike its environmental, saprophytic counterparts, *Bm* may have adapted to evade recognition by PTX3 and, therefore, this PRR may represent a critical component of host defense against this pathogen. As such, future directions would entail better defining the role(s) of PTX3 in the pathogenesis of *Bt*, *Bm*, or *Bp* **(Chapter 7.2)**.

7.2 Future directions.

Investigating the binding of PTX3 to human Mm6 monocyte surface receptors

To provide clarification on the opsonophagocytosis and intracellular survival assay data presented in **Chapter 6**, follow-up assays should investigate the capacity for PTX3 to bind to the surface of human Mm6 monocytes. Binding may be detected between human Mm6 monocytes and fluorescently-labeled PTX3, in the presence or absence of NHS, by confocal microscopy or flow

cytometry. These binding assays would allow us to determine whether the lack of a PTX3-mediated effect on the opsonophagocytosis of *Bt* by human Mm6 monocytes may be related to the inability of *Bt*-bound PTX3 to bind to monocyte receptors, thereby precluding this PRR from enhancing uptake of *Bt* over the background level of phagocytosis. If binding is detected through these assays, then either human Mm6 monocytes possess insufficiently high numbers of PTX3-specific receptors to amplify the overall phagocytosis of *Bt*, or PTX3 does not play a role in the opsonophagocytosis of *Bt* in human monocytes, as previously discussed. On the other hand, if PTX3 does not bind to Mm6 monocytes, then this would explain the lack of an observable amplification of phagocytosis. In this case, additional opsonophagocytic assays should be pursued with *Bt* in alternative cell culture systems known to respond to PTX3, such as murine or human neutrophils[12-14] and murine macrophages.[15]

Investigating the immunotherapeutic applications of PTX3 in an aerosol murine model of glanders

Preliminary binding assays had demonstrated binding of PTX3 to *Bt* and *Bp*, but not *Bm*, suggesting that *Bm* may have evolved to evade recognition by this PRR as a potential strategy to persist within the host. Although PTX3 fails to target *Bm*, this PRR may still play a role in *Bm* infection *in vivo* through binding-independent mechanisms, as was shown in the case of PTX3 and infection due to *Klebsiella pneumoniae (Kp)* ATCC27736.[16] In this murine model of *Kp* pulmonary infection, PTX3 contributed to balanced inflammatory responses, with greater neutrophil influx and bacterial phagocytosis by pulmonary neutrophils.[16] These findings suggest that the potential for binding-independent therapeutic activity of PTX3 in *Bm* infection warrants investigation, and possible future *in vivo* experiments would employ the aerosol murine glanders model established by Lafontaine *et al.* 2013.[17] In this model, the potential immunotherapeutic application of recombinant murine PTX3 in priming innate immune responses prior to infection (as compared to PTX3 administration at the time of infection or postinfection), would be determined by evaluating survival, organ bacterial loads, and histopathologic lesions, as well as potential effects on modulating cytokine profiles, inflammatory cell

recruitment, and bacterial phagocytosis. Based on the demonstrated therapeutic activity of PTX3 in several infection models (discussed in **Chapter 1**), results expected from these *in vivo* studies may include enhanced survival, decreased CFUs in target organs, and enhanced Th1-biased inflammatory responses (increased levels of IFN-γ and IL-12) as part of a balanced immune response with early recruitment of inflammatory cells (particularly neutrophils) to sites of infection.

Evaluating the relevance of PTX3 as a potential biomarker for disease due to Burkholderia sp.

The use of PTX3 as a biomarker has been described for various inflammatory conditions and diseases in human medicine (for example,[18-20]); however, its use as a potential marker, has, to date, not been explored in the context of any *Burkholderia* sp. or veterinary infectious diseases. Therefore, additional *in vivo* studies could evaluate the relevance of PTX3, among other immune response-related genes, as a potential biomarker for disease due to *Burkholderia* sp. These future *in vivo* studies would employ murine models of *Burkholderia* sp. infection to identify putative biomarkers, which may then be further evaluated for correlations with disease progression and prognosis in *Burkholderia* sp.-infected human patients in a clinical setting. In these proposed *in vivo* studies, potential biomarkers specific to *Bm* infection may be identified by assessing the transcriptome and proteome profile of blood and lung from *Bm* aerosol-infected mice, as compared to that of *Bp*-infected or uninfected mice, across early and late time points postinfection. By interrogating the blood and pulmonary transcriptome of infected mice, the expression patterns of additional, soluble PRRs produced systemically or locally within the lung can also be determined. While comparing the transcriptome of the blood and lung of *Bm*- and *Bp*-infected mice to that of *Bt*-infected mice would also be informative, these investigations would require the development of an additional, adapted aerosol model with *Bt*. Collectively, the findings from these transcriptome profile studies would help further build upon the work by Glaros *et al.* (2015)[21] in the identification of serum biomarkers in *Bm*-infected non-human primates as well as in other biomarker studies in the context of melioidosis.[22-24]

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