TIFA-Mediated Innate Immune Recognition of the Bacterial Metabolite HBP and its Role in Host Defense

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Host recognition of pathogen-associated molecular patterns (PAMPs) initiates an innate immune response that is critical for pathogen elimination and engagement of adaptive immunity. Here I show that mammalian immune and nonimmune cells can detect and respond to the bacterial-derived monosaccharide heptose-1,7-bisphosphate (HBP). A metabolic intermediate in lipopolysaccharide (LPS) biosynthesis, HBP is highly conserved in Gram-negative bacteria, yet absent from eukaryotic cells. Detection of HBP within the host cytosol activated the NF-κB pathway in vitro, and induced innate and adaptive immune responses in vivo. HBP-induced signaling was independent of known pattern recognition receptor pathways. Therefore, I used a genome-wide RNAi screen to uncover an innate immune signaling axis, mediated by the TRAF-interacting protein with forkhead-associated domain (TIFA). Contamination of the host cytosol with HBP induced TIFA phosphorylation-dependent oligomerization and concomitant activation of the ubiquitin ligase TRAF6. In addition to recognizing HBP released upon bacterial lysis extracellularly or within the phagolysosome, I show that TIFA is an innate immune sensor for cytosol-invasive Gram-negative bacteria, as invasive Shigella flexneri, or a vacuole-escaping Salmonella mutant, released the HBP during cytosolic growth, triggering a sustained wave of TIFA activation that followed transient activation of the NOD1 pathway. Bacterial growth within
the cytosol was essential for HBP release and concomitant TIFA activation, since prolonged infection with wild-type *Shigella*, but not metabolically attenuated invasive mutants, triggered TIFA-oligomerization and signaling to drive a massive NF-κB response from human epithelial cells. My findings identify HBP as a novel PAMP, and as a corollary, TIFA as a new innate immune signalling effector that is the keystone of an immunosurveillance system that detects Gram-negative bacterial infection, and which escalates the immune response to invasive bacteria that exploit the host cytosol as a niche for growth and replication.
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Foremost I would like to thank Scott for being a fantastic supervisor. His drive to answer the fundamental questions of basic science were a continued motivation in the completion of this thesis. This project started 10 years before my arrival as more of a “trial and error approach” to explain a peculiar phenomenon observed in the lab. I have no doubt that in those 10 years there were many moments of frustration in which the motivation to find the answer to this question waned. The vast majority of professors would have swept this under the rug, explaining it away as a “mystery of science”. It is to Scotts great credit that he kept this project alive, and along with a postdoctoral fellow Dr. Rebecca Malott, laid the ground work for the discoveries outlined in this thesis.

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1 Chapter 1: Introduction

1.1 Immunity to infectious disease

The mammalian host exists in close proximity with the immense numbers of microorganisms that colonize it. Whether this colonization is beneficial or detrimental to the host depends both on what niche is colonized, and the adaptation strategy employed by the microbe. Whereas colonization of normally sterile sites like internal organs is a precursor for infectious disease, colonization of the gastrointestinal tract with commensal microbiota helps in digestion and plays an essential role in the development and function of the mucosal immune system. The specific strategy employed by microbes to colonize their niche also has a significant impact on host-fitness. For pathogens, the set of factors that differentiate them from non-pathogenic commensal microbes are termed virulence factors, and are often encoded on mobile genetic elements that can be transferred between bacterial species (Hacker and Carniel, 2001; Medzhitov, 2007). Adaptations endowed by virulence factors are multiple, and include: attachment and penetration of epithelial surfaces, invasion and exploitation of the host cytosol, nutrient acquisition, and immune evasion (Medzhitov, 2007). The difficult task of the immune system is to protect the host from the pathogenic effect of virulent microbes while not responding to the normal commensal flora that remain in their appropriate niche.

1.1.1 Innate and Adaptive Immunity

Protective immunity to infection with pathogenic microorganisms in jawed vertebrates depends upon the successful engagement of both the innate and adaptive immune systems. The innate and adaptive immune systems differ in four fundamental areas: First, the kinetics of the response; second, the cell types involved; third, the types of receptors employed; fourth, the type of effector response. The innate response is the first line of defense against infection, and occurs almost instantaneously upon contact with a microorganism. In contrast, an adaptive immune response can take days, and generally intensifies upon multiple contacts with the same organism. Considering the importance of monitoring all niches that could potentially come into contact with pathogens, virtually all cell types within the host can contribute to the innate immune response. This is in contrast with adaptive immunity, which is mediated by T and B lymphocytes from the hematopoietic system. Innate immune recognition is mediated by a limited number of germline encoded pattern recognition receptors (PRRs) that can recognize broadly
possessed invariant features of microorganisms (Janeway, 1989). By contrast, adaptive immune recognition by B and T lymphocytes is mediated by antigen receptors assembled from somatic recombination of germline-encoded gene segments resulting in a nearly infinite collection of clonally-expressed receptors with narrow specificities (Schatz et al., 1992). Finally, whereas an adaptive response is composed of both antibody and cell-mediated effector mechanisms designed to provide long lasting protection from infection, an innate immune response, while more broadly applicable, is short lived and based on cell-autonomous responses including secretion of cytokines, chemokines, antimicrobial peptides, and an intracellular degradation system termed autophagy or xenophagy (Levine, 2005). The nature of the response elicited is largely dictated by a combination of which PRRs are engaged and the cell types involved. Finally, while it is true that the innate and adaptive immune systems evolved at different stages in phylogeny and are fundamentally different in many ways, they are inexorably linked, as a protective immune response typically requires intimate cooperation between both modules. Indeed, innate immune recognition must precede activation of antigen receptors on T and B lymphocytes for an adaptive response to occur (Iwasaki and Medzhitov, 2010). The details of this relationship will be addressed in subsequent sections.

1.2 Pattern recognition and the innate immune system

Originally proposed by Charles Janeway Jr. in 1989, pattern recognition provides the theoretical framework for much of our understanding of the function of the innate immune system and its engagement of adaptive immunity (Janeway, 1989). His revolutionary idea was that the innate immune system can determine the origin of foreign antigens and instruct T and B cells in the development of an appropriate response to those antigens that are microbial in origin (Medzhitov, 2009). In the years that followed, many studies have validated those original ideas and it is those I will summarize in the following sections.

1.2.1 Pathogen-associated molecular patterns (PAMPs)

As described above, the innate immune system relies on PRRs that broadly recognize molecules that possess motifs or patterns common to microorganisms. The molecules recognized by PRRs are termed pathogen-associated molecular patterns (PAMPs); despite the fact that many of these same structures can be found on non-pathogenic organisms. There are three primary characteristics of PAMPs that have lead to their evolutionary selection for use by the innate
immune system: First, they are common among broad classes of microbes, meaning that a limited set of germline-encoded PRRs can obtain information about the identity of the microbe in question; second, they are products or intermediates in biological pathways unique to microorganisms and absent from the host, thereby permitting discrimination of molecules of self from nonself origin; third, they have an essential role in some aspect of the microorganisms physiology or virulence preventing microbial adaptation to hide from cellular receptors (Medzhitov, 2007). Because of differences in eukaryotic versus microbial cell structure, many PAMPs are essential cell wall components like lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids, lipoproteins or fungal β-glucan, however other features unique to microbial life are also detected. A summary of pathogen associated molecular patterns common to classes of microbes is presented in Figure 1A.

1.2.2 Pattern recognition receptors (PRRs)

Several types of pattern recognition receptors have been discovered and characterized to date including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and cytosolic DNA receptors. These receptors differ in their cellular expression patterns, their cognate PAMPs recognized, their distribution among subcellular compartments, and what signaling cascades are initiated upon ligand recognition. A summary of the major classes of PRRs described to date and there subcellular distribution is presented in Figure 1B.

1.2.2.1 Toll-like receptors (TLRs)

TLRs were the first PRRs discovered and are the most widely studied pathogen sensors. Originally identified in Drosophila (Lemaitre et al., 1996), 10 TLRs have been found encoded in the human genome and 12 have been identified in the murine genome. Among the most important factors that distinguish the function of the different TLRs is in what subcellular compartment the protein is expressed. TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface, while TLRs 3, 7, 8 and 9 are exclusively expressed within the endocytic pathway (Kumar et al., 2011). TLRs are membrane glycoproteins that are made up of extracellular leucine rich repeats (LRRs) that mediate ligand recognition, and cytoplasmic toll/interleukin-1 receptor (TIR) domain that mediates downstream signaling. Bacterial PAMPs sensed by TLRs include: the endotoxin of LPS, which is the major component of the Gram-negative cell, is recognized by
TLR4 in conjunction with the co-receptor MD-2 and CD14 (Shimazu et al., 1999); the cell-wall component peptidoglycan is sensed by TLR2 (Schwandner et al., 1999), while diacyl and

Figure 1  Recognition of PAMPs by PRRs. (A) Shown is some of the pathogen associated molecular patterns (PAMPs) that collectively define various classes of microorganisms. (B) Shown are major types of pattern recognition receptors, their subcellular distribution, and representative microbial ligands. B is Adapted with permission from Medzhitov (2009).

triacyl lipopeptides, present within mycobacteria and mycoplasma, are sensed by TLR2 in conjunction with TLR1 and TLR6 (Kumar et al., 2011); the flagellin protein is sensed by TLR5 (Hayashi et al., 2001); and bacterial DNA rich in unmethylated CpG motifs and viral RNA are
recognized by TLRs 9 and 7, respectively (Hemmi et al., 2000; Mancuso et al., 2009). Ligand recognition by the TLRs initiates distinct, yet broadly conserved signaling cascades and outcomes, as will be discussed in more detail in Section 2.2. However, in general, PAMP recognition by TLRs 1, 2, 3, 5 and 6 lead to pro-inflammatory cytokine production, while the endosomal TLRs 7 and 9 induce type I interferons, in line with their function as viral sensors. The function of TLRs as been studied most in macrophages. PAMP recognition by TLRs in tissue-resident macrophages induce production of the cytokines tumour-necrosis factor (TNFα), interleukin-6 (IL-6) and IL-1β, which coordinate the resulting inflammatory response (Medzhitov, 2007). Moreover, TLRs can induce direct antimicrobial responses, for example TLR activation by mouse macrophages induces nitric-oxide synthase expression, which produces nitric oxide that has direct anti-bacterial activity (Thoma-Uszynski et al., 2001).

1.2.2.2 NOD-like receptors (NLRs)

Certain pathogens can invade host cells and exploit the cytosol for growth and replication. Once inside the host cell, these pathogens are not accessible to membrane-bound TLRs and, as such, there exist elegant cytoplasmic surveillance systems to monitor the intracellular compartment for microbial products. PAMPs in the host cytosol are sensed primarily the nod-like receptors (NLRs). There are 23 NLRs in humans, and 34 in mice, of which several are well characterized. The NLRs generally consist of 3 domains: a C terminal leucine rich repeat (LRR) that is thought to mediate ligand recognition in a manner analogous to TLRs; a nucleotide binding and oligomerization (NACHT) domain, which mediates inducible ATP-dependent oligomerization; and an amino-terminal signaling effector domain, consisting of either a death effector domain (DED), Pyrin domain (PYD), a caspase activation and recruitment domain (CARD) or a baculovirus inhibitor repeats (BIR) domain (Kumar et al., 2011). The type of N-terminal domain determines the effector response elicited by NLR, and range from proinflammatory signaling to activation of a massive multiprotein complex, “the inflammasome”, which mediates both post-translational processing of cytokines such as IL-1β and IL-18, and induction of cell death (Martinon et al., 2002).

The NLR family members NOD1 and NOD2 have N-terminal CARD domains and participate in the host response to bacteria by eliciting both proinflammatory and anti-microbial responses (Philpott et al., 2014). These proteins monitor the host cytosol for naturally occurring peptidoglycan degradation products and initiate a nuclear factor-κB (NF-κB) and mitogen-
activated protein kinase – dependent pro-inflammatory transcriptional response. NOD1 recognizes D-glutamyl-
meso-diaminopimelic acid (iE-DAP) found primarily in Gram-negative peptidoglycan, while NOD2 detects muramyl di-peptide (MDP) that is present in most Gram-
positive and -negative bacterial peptidoglycan (Girardin et al., 2003a; 2003b). Ligand binding
initiates nucleotide-dependent self-oligomerization and downstream signaling. Considering their
location within the cytosol, NOD activation is often associated with pathogen invasion. Indeed
the first model describing NOD1 activation was in response to the invasive Gram-negative
bacteria Shigella flexneri (Girardin et al., 2001). However, NOD-dependent signaling can also
occur in the absence of pathogen invasion, for example by delivery of peptidoglycan fragments
via pathogen secretion systems (Viala et al., 2004), bacterial outer membrane vesicles (Irving et
al., 2014) or uptake through endocytosis (Nakamura et al., 2014). Thus, while the NODs can be
considered as sentinels of pathogen invasion, their response to extracellular peptidoglycan
broadens the number of immune responses in which they maybe involved.

1.2.3 Functions of PRRs in immunity

1.2.3.1 Inflammation

PAMP recognition by PRRs has pleotropic effects on host physiology. However, the
most obvious effect may be the induction of an inflammatory response. This response is
designed to be a protective response to remove dangerous stimuli from the body and initiate
mechanisms to repair tissue damage (Medzhitov, 2008). PAMP recognition by the innate
immune system is the major contributor to acute inflammation induced by pathogen infection
(Beutler et al., 2006; Medzhitov, 2008), which is classically associated with redness, swelling,
heat, pain, and loss of tissue function (Takeuchi and Akira, 2010). These symptoms represent an
increase in vascular permeability allowing extravasation of immune cells and leakage of serum
components into tissues to combat the threat. As described above, transcriptional responses
initiated by the TLRs and NOD1 and NOD2 result in production of cytokines, mainly IL-6, IL-8,
TNFα, and IL-β, that coordinate the inflammatory response by modulating endothelial
permeability, acute phase protein production, cell death, and white blood cell (primarily
neutrophils) recruitment (Takeuchi and Akira, 2010). Upon arrival at the damaged site,
neutrophils are themselves activated by the PRR engagement and/or by the local cytokine milieu
and attempt to destroy the foreign invaders through microbial engulfment and/or the exocytosis-
based release of toxic granules (Medzhitov, 2008). While it is clear that inflammation has an
essential role in providing immunity to pathogen infection, rapid termination of the inflammatory response is also necessary since overzealous inflammatory responses are associated with disease caused by numerous bacteria, including *Neisseria gonorrhoeae* (Liu et al., 2011) and *Shigella flexneri* (Sansonetti et al., 1999), which will be discussed in Section 1.5 and are the subject of latter chapters in this thesis. Highlighting the potential dangers of dysregulated innate responses, mutations in PRRs can lead to autoimmune diseases owing to excessive inflammation (Cho, 2008).

1.2.3.2 Innate control of adaptive immunity

An essential function of the innate immune system is to instruct the development of acquired immunity. The antigen receptors expressed on B and T cells have random specificities that have been selected for non-self recognition. These receptors are, therefore, inherently unable to determine the origin of the antigen in question. PRRs, which have evolved to specifically detect microbial-derived products, are required to inform T and B cells if the antigen is ‘foreign’. Thus, in an effort to prevent undesirable immune activation, the adaptive immune system will not be mobilized unless there is a concomitant detection of PAMPs by PRRs; in this case, the innate system “licenses” an appropriate effector immune response (Medzhitov, 2007). This can occur in a cell-autonomous manner using PRRs expressed on the lymphocyte directly, or more commonly, through indirect interactions with signals generated from activated PRRs on antigen presenting cells (APCs). The latter strategy is employed by T cells, as tissue resident dendritic cells possess a broad array of PRRs to monitor the environment for pathogens. Dendritic cells are uniquely adapted to engulf microorganisms through phagocytosis, and process proteins into antigenic peptides that are subsequently displayed on the cell surface by major histocompatibility complex (MHC) class I and class II. The choice of which antigen is selected for presentation is based on whether PRRs are triggered within the phagosome containing the phagocytosed cargo (Blander and Medzhitov, 2006; Nair-Gupta et al., 2014). Thus, dendritic cells preferentially display T cell antigens that have originated from foreign microorganisms. PRR activation also induces dendritic cells to migrate to local lymph nodes where PRR-induced cell surface markers and cytokines promote T cell activation (Banchereau and Steinman, 1998). For B cells, the link between antigen receptor and PRR can either be established through PAMP recognition by PRRs expressed on the B cell itself (when the antigen and PAMP are physically connected) or, when this is not the case, through co-stimulatory signals generated from T-cells that have previously
been activated by PAMP-sensing dendritic cells (Pasare and Medzhitov, 2005). A summary of the host defense systems modulated by PAMP-PRR interactions is summarized in Figure 2A.

Figure 2  PRR-dependent activation of host defense systems. (A) PRR-mediated pathogen recognition influences the choice of innate (hours) and adaptive (days) immune responses launched by the host. (B) Summary of possible effector T-cell subsets generated during immune responses. Adapted with permission from Medzhitov (2009).

Another area in which the innate immune system has an essential role is determining what type of effector immune response to launch against a foreign antigen. When naïve T cells encounter an antigen, they can differentiate into a variety of effector sub-classes (e.g. Th1, Th2 or Th17) that are defined by their respective cytokine profile (Figure 2B). Each differs with respect to the microorganisms the response is designed to combat, as in general, Th1, Th2 or Th17 responses are launched against intracellular pathogens, multicellular parasites and extracellular pathogens, respectively (Reinhardt et al., 2006). The maturation into these T cell subsets is controlled by transcriptional regulators T-bet (Th1), Gata-binding protein 3 (GATA3; Th2) and retinoic-acid-receptor-related orphan receptor γt (RORγt; Th17) (Reiner, 2007). During pathogen recognition by antigen presenting cells, signals produced in response to the PRRs...
induce cytokine production that controls expression of these master regulators and, therefore, determines the type of effector response (Reiner, 2007).

PAMPs differ considerably in their immunostimulatory properties. In fact, the combination of PAMPs expressed by different microbes can be thought of as a PAMP “barcode” that is interpreted by the immune system to tailor the resulting response to specific classes of microbes (Aderem, 2003). For example, Gram-negative bacteria engage TLR4 resulting in interferon-β production that is generally absent from responses to Gram-positive bacteria, which lack LPS (Vance et al., 2009). However, this is not always the case, as cytosolic DNA sensors activate a host type-I interferon response to the Gram-positive bacteria *Listeria monocytogenes* (Stetson and Medzhitov, 2006). In general, TLR activation induces IL-12 production which tends to polarize T\(_\text{H}1\) type responses to intracellular pathogens (Goriely et al., 2008). Alternatively, recognition of fungal β-glucan by dectin-1 induces T\(_\text{H}17\) polarizing IL-23 production (Carvalho et al., 2012). Interestingly, cytosolic detection of peptidoglycan by the NOD proteins drives T\(_\text{H}2\)-type responses that are typically associated with immunity to extracellular pathogens (Fritz et al., 2007; Magalhaes et al., 2011). In contrast, stimulating both TLR and NLRs, as would be the case during infection of APCs, enhances T\(_\text{H}1\) type responses (Fritz et al., 2007). Moreover, NOD1 and NOD2 dependent signaling is also important for T\(_\text{H}17\) responses that provide protection against enteric pathogens in the gut mucosa (Geddes et al., 2011). Clearly, much remains to be discovered before immune responses can be predicted based upon what PAMP-PRR combinations are engaged during infection.

### 1.3 PRR-dependent signaling cascades

#### 1.3.1 Activation of NF-κB

The signaling pathways initiated by the TLRs, RLRs, and the NLRs NOD1 and NOD2 converge upon activation of the transcription factors NF-κB and mitogen-activated protein kinases (MAPK), which function to regulate the inducible transcription of proinflammatory target genes. NF-κB is activated by a vast array of stimuli ranging from cellular stress, PRRs, cytokine receptors and antigenic receptors, all of which converge upon the activation of one of two kinase complexes termed inhibitors of κB (IkB) kinase complex (IkK) (Chen et al., 1996). These complexes are heterotrimeric and are made up of a regulator subunit, NF-κB essential modulator (NEMO/IkKγ) and two kinases IkKa and/or IkKβ (Hayden and Ghosh, 2008). The
identity of the upstream stimuli and what signaling adaptors are engaged determines whether the canonical (IkKβ) or the non-canonical (IkKα) pathway is initiated. Canonical inducing stimuli assemble polybiquitin-based scaffolds that activate IkKβ kinase, which phosphorylates the IkB proteins. IkB proteins are constitutively bound to the NF-κB subunits, masking their nuclear localization signal so as to retain them within the cytosol. Phosphorylation of IkB induces SCFβTrCP-dependent lysine-48 (K48)-linked ubiquitination and targeting of IkB for proteolysis by the proteosome (Chen et al., 1996)(Hayden and Ghosh, 2011). Newly liberated NF-κB subunits migrate into the nucleus where they induce gene expression. There are 5 NF-κB subunits in mammals (RelA/p65, RelB, c-Rel, p50/NF-κB1 and p52) that bind assemble into heterodimers or homodimers to bind consensus oligonucleotide sequences (5´-GGGRNWYYCC-3´) in promoters and enhancers of target genes (Smale, 2010). The differing abilities of homodimers and heterodimers to induce transcriptional transactivation, the effects of post-translational modifications on subunit activities, heterotypic interactions with other transcription factors, and the degenerate nature of the target sequence itself all contribute to the transcriptional specificity of the multitude of target genes regulated by NF-κB (Hayden and Ghosh, 2012; Saccani et al., 2003; Smale, 2011).

1.3.2 Activation of IkK – Role of TRAF6

It is clear that most NF-κB activating signals operate through controlled activation of the IkK complex. The general theme in most NF-κB-activating signaling cascades is that signaling proceeds through successive binding of a series of adaptor proteins that possess shared protein:protein interaction domains. These include the previously mentioned CARD, TIR and death domain (DD). It is believed that signaling adaptor proteins facilitate the assembly of large signaling platforms that function to activate the IkK complex, resulting in either trans autophosphorylation and activation, or phosphorylation by the upstream kinase Transforming Growth Factor-Beta-Activated Kinase (TAK1) (Wang et al., 2001). One family of proteins, called the tumour necrosis factor receptor–associated factor (TRAF) proteins, are the proximal signaling adaptors that activate IkK downstream of a variety of different signals. There are 6 TRAF members in mammals, each defined by the presence of a C-terminal TRAF domain composed of an amino-terminal coiled-coil region and a carboxy-terminal β-sandwich (Xie, 2013). The TRAF domain itself mediates interactions with both upstream and downstream proteins and mediates intermolecular oligomerization and activation (Xie, 2013). Most TRAF
proteins also contain an amino-terminal RING finger domain that has E3 ubiquitin ligase activity, allowing TRAFs to regulate signaling by functioning as both adaptor proteins and ubiquitin ligases (Ferrao et al., 2012). Indeed TRAF-dependent lysine-63 (K63) linked ubiquitination is essential for IκK complex assembly and activation downstream of many signals (Wertz and Dixit, 2010).

Among the TRAF members, TRAF6 is the most well studied, as it is essential for signal propagation downstream of the TLRs and the IL-1 receptor (Cao et al., 1996; Hayden and Ghosh, 2012). Pattern recognition by TLRs triggers a signaling cascade by recruiting TIR containing adaptor proteins via TIR:TIR interactions. The TIR-containing adaptor protein myeloid differentiation protein 88 (MyD88), which mediates signaling for most TLRs, possesses an N-terminal death domain (DD) for signal propagation through inducible recruitment of DD containing IL-1R-associated kinase (IRAK) family members (Medzhitov et al., 1998). The MyD88-IRAK interactions lead to the formation of an oligomeric DD-based signaling scaffold that promotes IRAK activation and recruitment of TRAF6 through TRAF-interacting domains (Watters et al., 2007). Recruitment of TRAF6 to the TLR signalosomes induces TRAF6 oligomerization, which in turn activates its E3 ubiquitin ligase activity (Deng et al., 2000). Together with the E2 enzymes Ubc13 and Uev1A, TRAF6 catalyzes the transfer of K63-linked polyubiquitination onto many proteins, including the IRAKs, IκKγ, and TRAF6 itself (Wu and Arron, 2003). TAK1 binding proteins TAB1, TAB2 and TAB3 then bind to the ubiquitin chains generated by TRAF6 and facilitate TAK1 activation and subsequent phosphorylation and activation of the IκK complex (Kanayama et al., 2004).

As cytosolic PRRs, NOD1 and NOD2 require less complex signaling networks to transmit ligand binding to IκK activation. PAMP recognition by NOD1 or NOD2 induces self-oligomerization, which recruits the CARD-containing serine/threonine kinase RIP2/RICK (RIP-like interacting caspase-like apoptosis regulatory protein kinase) and caspase recruitment domain family member-9 (CARD9) (Hasegawa et al., 2008; Hsu et al., 2007; Park et al., 2007). RIP-2 mediates TAK1 activation via K63-linked ubiquitination, which ultimately drives NF-κB activation (Kobayashi et al., 2002).
1.4 Pattern recognition and evaluation of microbial threat

A complication of pattern recognition theory is that PAMPs are not unique to pathogens, instead they can be found in virtually all microorganisms, including the trillions of commensal microflora that continually inhabit our digestive tract. In fact, many groups now refer to PAMPs as microbe-associated molecular patterns (MAMPs). The PAMP hypothesis by itself is insufficient at explaining how the innate immune system can differentiate between pathogens and non-pathogens, or between colonization and infection. However, there are clearly mechanisms in place for gauging the microbial threat level, as the strength of the resulting immune response varies considerably depending on the type of the microbial encounter (Blander and Sander, 2012). For example, immune responses to bacteria possessing distinct virulence factors, such as secretion systems, tend to be much higher than those that lack them (Freche et al., 2007). It has also been known for years that immunization with live vaccines tends to induce much more vigorous immune responses than their dead counterparts, despite the fact that both may carry the same spectrum of PAMPs (Detmer and Glenting, 2006; Sander et al., 2011). Moreover, invasive pathogens are typically associated with heightened immune responses when compared with microbes that reside on mucosal surfaces (Varol et al., 2010). The elegant mechanisms that permit the evaluation of the threat presented by distinct microbial signatures are dependent upon particular attributes of PRR-signaling (Iwasaki and Medzhitov, 2010), the details of which will be discussed below.

1.4.1 Cell-autonomous immunity

An important theme that has emerged in recent years is that the context in which PAMPs are sensed by host PRRs can have a dramatic effect on the resulting innate immune response. This is best illustrated by the fact that several PAMPs can be sensed independently by two different PRRs, one on the cell surface and one within the host cytosol. The idea is that intracellular PAMPs are an indicator of virulence, and that “violation of the sanctity of the cytosol” is associated with a more vigorous immune response than when the same PAMP is sensed on the cell surface (Lamkanfi and Dixit, 2009). One example is bacterial flagellin, which is sensed on the cell surface by TLR5, and within the cytosol by the NLRC4 inflammasome (Zhao et al., 2011). TLR5 recognition of extracellular flagellin, which can be expressed by commensal or pathogenic microbes, results in a pro-inflammatory transcriptional response, including production of pro-IL-1β (Hayashi et al., 2001). In contrast, NLRC4-dependent
recognition of cytosolic flagellin activates the inflammasome, culminating in activation of the protease caspase-1, which promotes maturation and release of pro-IL-1β into functional IL-1β and initiates a form of proinflammatory cell death termed pyroptosis (Bergsbaken et al., 2009). Another example is bacterial lipopolysaccharide (LPS), which induces a transcriptional response mediated by TLR4-dependent recognition on the cell surface or within the endosomal compartment, and a pyroptotic response following recognition in the cytosol by caspase-4 or -11 in humans and mice, respectively (Shi et al., 2014). Thus, inflammasome activation in response to cytosolic PAMPs can be thought of as a form of cellular altruism, in which the destruction of infected cells acts to limit microbial growth and alert neighboring cells to the presence of an invasive pathogen. Limiting this response to only virulent microbes by virtue of the fact that they enter into spaces that are not accessible to non-pathogenic microbes is essential to ensure vigorous responses are not launched against commensal microflora.

Bacterial PAMPs can reach the cytosol by various means, each of which acts as its own indicator of virulence. One such method is inadvertant translocation of PAMPs by a secretion system, a bacterial virulence factor often associated with pathogenic microorganisms. The extracellular bacteria Helicobacter pylori drives a proinflammatory response by virtue of the fact that its type IV secretion system translocates NOD1-activating peptidoglycan into the host cytosol (Viala et al., 2004). Flagellin can also reach the cytosol via translocation by a type IV secretion system during infection with Legionella pneumophila (Molofsky et al., 2006) and a diverse group of bacteria activate an interferon-β response following translocation of bacterial DNA through type III, IV or VI secretion systems (Vance et al., 2009). Cytosol-invasive bacteria may also translocate PAMPs directly into the cytosol following escape from the vacuole. Salmonella that can escape the vacuole, or invasive strains Burkholderia and Yersinia, can each release LPS into the cytosol, which induces Caspase-11-dependent pyroptosis in mouse macrophages (Aachoui et al., 2013). Shigella flexneri, another invasive Gram-negative bacteria, is detected by NOD1 immediately following vacuolar escape (Girardin et al., 2001). However, not all cytosolic PAMPs require their translocation by bacterial virulence factors. For example, soluble extracellular NOD1 and NOD2 ligands can be taken up by endocytosis and are translocated into the cytoplasm of epithelial cells (Lee et al., 2009). Moreover, bacterial mRNA and MDP reach the cytosol following phagocytosis and degradation of whole bacteria by host macrophages (Herskovits et al., 2007; Sander et al., 2011). Therefore, while sensing
contamination of the host cytosol is a good indicator of the pathogenic potential of a microorganism, other mechanisms are clearly required to ensure proper immune homeostasis is maintained.

1.4.2 Tissue compartmentalization

In addition to sub-cellular compartmentalization of PRRs, the host can detect invasive pathogens via compartmentalization of PRRs on distinct anatomical tissues. In this case, pathogen invasion refers to the breach a mucosal surface into normally sterile tissues. Epithelial cells, through tight junctions and a secreted mucus layer, forms the physical barrier between the host and the external environment, however their apical surface is constantly exposed to a bounty of microbial-derived immune agonists (Peterson and Artis, 2014). This necessitates that specialized systems to ensure the epithelium is relatively refractive to non-pathogenic microbes or the PAMPs that they may liberate. One such mechanism present in the intestinal epithelium is the selective expression of PRRs on the basolateral face of the epithelial surface (Gewirtz et al., 2001). This segregation pattern ensures that only tissue-invasive bacteria that penetrate the epithelial barrier, rather than luminal bacteria, trigger TLR signaling in the gut epithelium. Epithelial cells also express several known negative regulators of PRR-signaling. In support of their importance in maintaining immune homeostasis, disruption of these pathways in mice results in excessive inflammation and in some cases tumorigenesis (Vereecke et al., 2010; Xiao et al., 2007). Important to clarify, while intestinal epithelial cells are hyporesponsive to PAMPs on their apical (luminal) surface, they are not mute to microbial signals since they do express a broad array of cytosolic NLRs that endow them the ability to act as sentinels for cytosol-invasive pathogens (Fritz et al., 2008; Sellin et al., 2015).

While epithelial cells are generally tolerant to commensal microbes, there is evidence that some sampling of the microbiota occurs in the intestinal epithelium. Low-level translocation from the intestinal lumen has been reported (Nolan, 2010), and specialized dendritic cells extend dendrites through epithelial cell monolayers to sample the luminal contents (Rescigno et al., 2001). This basal signaling induced by the commensals has an essential role in lymphogenesis and barrier function (Eberl and Boneca, 2010). Microbes that breach the epithelial cell layer are detected in the lamina propria by specialized dendritic cells, macrophages, and mast cells (Iwasaki and Medzhitov, 2015). Broad expression of PRRs endow these cell types the ability to
rapidly detect microbes and release pro-inflammatory cytokines and chemokines to recruit neutrophils, monocytes, basophiles and eosinophiles to the infection site. If local responses are insufficient at clearing the pathogen and bacteria reach the bloodstream, they are immediately detected by circulating neutrophils and monocytes. PRR engagement on these cell types leads to respiratory burst, degranulation, enhanced phagocytosis and rapid production of inflammatory cytokines (Thomas and Schroder, 2013). Specifically, IL-1β, IL-6 and TNFα have far reaching effects, inducing sickness and fever in the brain (Pecchi et al., 2009) and an acute phase protein response from the liver (Gabay and Kushner, 1999). Therefore, considering that tissue damage during infectious disease is often a direct result of an overzealous immune response, it is especially important for the innate immune system to accurately gauge the level of microbial threat, and to reserve such vigorous immune responses for organisms that pose a serious health risk.

1.4.3 Damage associated molecular patterns (DAMPs)

In addition to sensing PAMPs, host cells can detect endogenous danger signals released from damaged tissues. Originally proposed by Polly Matzinger in 1994 as an alternative to Janeway’s theory of pattern recognition, the model held that sensing of pathogen-inflicted damage would provide a more specific mechanism to distinguish exposure to pathogenic versus commensal microbes (Matzinger, 1994). Evidence has since emerged that support both of these theories, as heat-shock proteins (hsp), uric acid, high mobility group box 1 protein (HMGB1), nucleic acids, heparan sulfate, and adenosine triphosphate (ATP) have all been identified as bona fide DAMPs (Escamilla-Tilch et al., 2013). For a molecule to function as a DAMP, it must be compartmentalized within live cells until damage is inflicted. For example, ATP is present within host cells at a concentration of 1-10 mM, however as it is not membrane permeable and requires energy to produce, the ATP concentration in the extracellular milieu is in the low nM range (Bours et al., 2006). Consequently, an increase in extracellular ATP can be interpreted as a danger signal since a loss of membrane integrity is required for its appearance. Physiological effects from DAMP detection range from dendritic cell maturation, cytokine production, mast cell degranulation and cell death depending upon the cell type involved. The host also employs cell-autonomous mechanisms to detect DAMPs. For example, membrane damage induced by bacterial pore-forming toxins induces a potassium ion flux that activates the NLRP3 inflammasome (Muñoz-Planillo et al., 2013).
Recent evidence has suggested that DAMPs and PAMPs can synergistically activate an immune response. Such a relationship would endow the immune system with the ability to recognize foreign organisms capable of inflicting damage. One such example is the DAMP HMGB1, which can bind to viral ssDNA and bacterial CpG DNA and facilitate their binding to TLR9 (Ivanov et al., 2007). DAMPs and PAMPs can also bind to the same receptor (e.g. mammalian hsp72 and Gram negative bacterial LPS both bind TLR4) and modulate their signaling activity (Drexler and Foxwell, 2010; Hreggvidsdóttir et al., 2012). Finally, some PAMPs can induce the release of immune modulating DAMPs. One example is infection with respiratory syncytial virus, which induces the release of hsp27 that can than activate neutrophils through TLR4 (Wheeler et al., 2009). Therefore, in addition to considering the context in which PAMPs are detected by the host, the DAMPs generated during an infection may also contribute to the resulting immune response.

1.5 Immunopathogenic responses to bacterial pathogens

As described above, the innate immune system employs elegant mechanisms to fine-tune the magnitude and duration of the immune response to maximize protection from infection while minimizing immunopathology. However, these systems do not always function perfectly, as certain bacterial infections are associated with an overzealous immune responses that result in severe tissue damage. Some pathogens may even actively manipulate innate immune sensing mechanisms to cause an immunopathologic response, which benefits the pathogen rather than the host. Two of the bacterial pathogens most often associated with immunopathogenesis belong to the genera of Shigella and Neisseria.

1.5.1 Immune response to Shigella

Shigella is a facultative intracellular Gram-negative foodborne pathogen that invades the colonic epithelium causing shigellosis or dysentry in humans. The global burden of Shigella infections is estimated at 165 million cases/year which results in 1.1 million deaths (Kotloff et al., 1999). Disease is associated with a variety of symptoms, including abdominal cramps, fever, and bloody mucoid stools (Phalipon and Sansonetti, 2007). There are four subroups of Shigella that are classified by the composition of the polysaccharide O-antigen of the LPS: S. flexneri, S. sonnei, S. dysenteriae, and S. boydii. S. flexneri and S. sonnei are endemic to developing countries, while S. dysenteriae is responsible for deadly epidemics attributable to its production.
of Shiga toxin. Despite considerable effort (Phalipon and Sansonetti, 2003), no vaccine currently exits for preventing shigellosis, and treatment relies on second generation antibiotics, of which the emergence of resistant strains has been reported (Talukder et al., 2006).

Following ingestion of as little as 10-100 organisms, *Shigella* crosses the epithelial barrier via M cells and subsequently invades tissue resident macrophages and intestinal epithelial cells (IECs) basolaterally via their type III secretion apparatus (T3S) (Phalipon and Sansonetti, 2007). Bacterial invasion requires the presence of a 213-kB virulence plasmid that encodes upwards of 100 genes, most of which are believed to be virulence effectors (Sansonetti et al., 1982). Contact with host cells is interpreted by *Shigella* as a protein secretion signal, and initiates secretion of effectors from the bacterial cytosol into the membrane and cytosol of the host. Two of these effectors, IpaB and IpaC, are themselves able to insert into the host membrane forming a pore, which elicits cytoskeletal rearrangements that support bacterial entry (High et al., 1992). Once internalized, *Shigella* induces immediate lysis of the entry vacuole, escapes into the cytosol, and uses directed polymerization of host actin by the outer membrane protein IcsA to move within and then spread between cells without exiting the cells (Goldberg and Theriot, 1995). The hallmark of shigellosis is acute inflammation resulting in massive neutrophil recruitment and tissue destruction. Rectal biopsies of humans with shigellosis has identified the cytokines IL-6, IL-8, IL-1β and TNF as upregulated during the disease (Raqib et al., 1995). Among these cytokines, it appears that IL-8 is the main recruiter of neutrophils and is produced primarily by epithelial cells themselves (Sansonetti et al., 1999).

As summarized in section 1.4.2, restricted expression of PRRs in intestinal epithelial cells (IECs) render them refractory to non-invasive bacteria. Indeed, IECs are anergic to *Shigella* that lack the virulence plasmid (Philpott et al., 2000), likely due to low expression the receptors CD14 and TLR4 (Abreu et al., 2001) rendering them non-responsive to LPS. Therefore, the proinflammatory response by IECs to *Shigella* requires intracellular recognition by cytosolic PRRs. The primary PRR thought to mediate intracellular *Shigella* recognition is the NLR family member NOD1. Primary murine epithelial cells display NF-κB activation 30 minutes following injection of supernatants obtained from *S. flexneri*, which is attributable to liberation of a diaminopimelic acid containing fragment of peptidoglycan from bacterial cultures, while NOD1-deficient cells do not (Girardin et al., 2003a). Moreover, invasive *Shigella* induced the formation of a transient signaling complex involving NOD1, RIP2(RICK) and the IkK complex within 20
minutes of infection (Girardin et al., 2001). Signaling by NOD1 is also important for the expression of pro-survival genes like Bcl-2, that counterbalance mitochondria-dependent cell-death mechanisms induced by Shigella in epithelial cells (Carneiro et al., 2009). Indeed, unlike macrophages which rapidly undergo apoptosis following infection due to an IpaB-Caspase-1 dependent mechanism (Hilbi et al., 1997), epithelial cells can survive to late stages of infection despite massive multiplication of intracellular bacteria (Mantis et al., 1996). While NOD1 is clearly activated by invasive Shigella, the contribution of other cytosolic PRR pathways to the innate response has not been resolved. Analysis of human cells infected with Shigella has largely been limited to transfection with dominant negative constructs or silencing with RNA interference (RNAi) (Fukazawa et al., 2007), which makes it difficult to resolve whether the residual responses are attributable to incomplete knockdown or the activity of other effectors. In mice, NOD1 knockout cells display a reduced response to invasive Shigella, however Shigella colonization of the murine colon lacks much of the proinflammatory response apparent during colonization of the human colon (Phalipon and Sansonetti, 2007). While there exist differences in NOD1 specificities between human and mice, Murine NOD1 can efficiently recognize the high tetrapeptide/tripeptide ratio present within Shigella peptidoglycan (Magalhaes et al., 2005), suggesting that the lack of immunopathogenesis in mice does not result from a difference in NOD1 function. Moreover, as described above, many initial studies on the involvement of NOD1 in detecting invasive Shigella or microinjected bacterial supernatants were done at time points within 30 minutes of infection (Girardin et al., 2001; 2003a). Thus, it is possible that there exist additional sensors of Shigella that predominate after NOD1, and that may be more active in human compared with murine IECs.

Inflammation plays a dual role in Shigella pathogenesis. During the initial infection, neutrophil recruitment may facilitate bacterial infection, as cytotoxic granules disrupt the epithelial barrier promoting translocation (Singer and Sansonetti, 2004). At later stages of infection, however, neutrophils play an essential role in bacterial clearance (Phalipon and Sansonetti, 2007). Thus, it is not surprising that Shigella secretes several effectors, including OspG (Kim et al., 2005) and OspI (Sanada et al., 2012), which function to dampen the innate immune response. Finely tuned regulation of Shigella virulence effectors likely permits this bacteria to control the magnitude of the host innate response to a level that promotes its transmission.
1.5.2 Innate response to *Neisseria* sp.

Bacteria belonging to the genus *Neisseria* are Gram-negative non-motile diplococci that inhabit the mucosal membrane of mammals (Virji, 2009). Two pathogenic species are uniquely adapted to humans: *N. gonorrhoeae*, the etiological agent of the sexually transmitted infection (STI) gonorrhea, and *N. meningitidis*, which can exist commensally in the nasopharynx or cause lethal meningitis (Caugant et al., 1994). Gonorrhea, in particular, is a major global health concern, with approximately 106 million new infections occurring each year (World Health Organization, 2012). In men, infection of the urogenital tract induces an intense inflammatory response including urethral discharge of neutrophil-containing pus and painful sensation during urination (Virji, 2009). Infection of the rectum is also associated with intense inflammation, including mucoid discharge and bloody stool (Virji, 2009). In women, gonococci infect the endocervix of the genital tract with symptoms manifesting between 7 – 21 days as cervicitis and purulent exudate (McCormack et al., 1977). Asymptomatic infection is also a serious issue in women, as 80% of infected women have such minor symptoms they do not seek medical treatment (McCormack et al., 1977). In these instances, the infection can ascend into the uterus and fallopian tubes to cause pelvic inflammatory disease (PID) and infertility due to scarring of the reproductive tract (McCormack et al., 1977). No vaccine exists for *N. gonorrhoeae*, and the emergence of multi-drug resistant strains has lead to serious fears concerning untreatable infections (Kirkcaldy et al., 2011).

Similar to *Shigella*, the hallmark of infection with *N. gonorrhoeae* is an overzealous neutrophil response that leads to tissue damage. However, unlike *Shigella*, *N. gonorrhoeae* is an extracellular pathogen and seems to be purposefully immunostimulatory in a variety of ways. Studies in male volunteers have implicated local production of IL-6, IL-8, TNFα and lymphocyte-derived IL-1β as primary drivers of the inflammatory response (Ramsey et al., 1995), however the events that drive this response remain incompletely understood. Historically, it was believed that immunopathogenesis was mediated by LOS, the most immunostimulatory of the neisserial PAMPs. However, that immune recognition and proinflammatory responses can occur in the absence of functional TLR4 in primary cervicovaginal epithelial cells and in vivo (Fichorova et al., 2002; Packiam et al., 2012) suggested that additional PAMPs may exist that facilitate innate recognition of *N. gonorrhoeae*. Subsequent work has now established that infection with *N. gonorrhoeae* generates ligands that engage a variety of PRRs, including: the
major outer membrane porin (PorB) variants expressed by certain strains are TLR2 agonists (Massari et al., 2002); *Neisseria sp.* actively shed outer membrane vesicles that contain TLR4-agonist endotoxin (Packiam et al., 2012) and peptidoglycan (Kaparakis et al., 2010); the pathogenic *Neisseria* have the unique propensity to actively shed peptidoglycan monomers 1,6-anhydro tetrapeptide and 1,6-anhydro tripeptide monomer, which are recognized by both NOD1-dependent and -independent pathways, and which kill ciliated human fallopian tube cultures (Melly et al., 1984); and they actively pump out TLR9-stimulatory DNA through their type IV secretion system (Hamilton et al., 2005). Finally, aside from PRR-mediated immune activation, *Neisseria* is bound by the innate decoy receptor CEACAM3, which is expressed on neutrophils and elicits opsonin-independent phagocytosis and degranulation as well as an inflammatory cytokine response (Sintsova et al., 2014). It is likely that each of these innate receptors contributes at some level to the immunopathogenesis observed during gonorrhea, and it seems clear by the pattern of liberal shedding of immunoactive PAMPs that the overzealous innate response observed during infection must benefit *Neisseria* more than the host. Support for this hypothesis comes from a mouse model of vaginal infection, which demonstrated that infection with *N. gonorrhoeae* elicits an T_{H17} pro-inflammatory immune response while dampening T_{H1} and T_{H2} adaptive immune responses through a mechanism dependent upon TGF-β and regulatory T cells (Liu et al., 2012). Indeed, the persistence of *N. gonorrhoeae* within the population relies on the fact that infection does not generate protective immunological memory responses. Consequently, repeat infections with the same serotype are a common occurrence (Fox et al., 1999; Schmidt et al., 2001).

1.5.2.1 *N. gonorrhoeae* and HIV co-infection

In the context of public health, a major consideration for *N. gonorrhoeae* public health management is its close association with HIV-1. Women with *N. gonorrhoeae* infections are at a significantly higher risk of acquiring HIV-1 during sex (Mlisana et al., 2012). In men, gonorrhea is associated with an increase in viral shedding that can be abolished by treatment with antibiotics (Cohen et al., 1997; Ghys et al., 1997; Moss et al., 1995). Consequently, coinfection is associated with a two- to five- fold increase in male-to-female HIV-1 transmission rates (Fleming and Wasserheit, 1999). Attempts at understanding this synergistic relationship at a molecular level have shown that in different systems, LOS, flagellin and Pam(3)C-Lip, a synthetic Ng-like lipopeptide, can enhance HIV-1 replication in CD4+ T cells in a TLR-
dependent mechanism (Thibault et al., 2009a; 2009b; Zhang et al., 2005). This is not surprising considering that HIV-1 gene expression is controlled by the NF-κB family of transcription factors, the same transcription factors that are activated downstream of the TLRs (Alcamí et al., 1995). However, previous observations in our lab revealed that gonococcal supernatants still induced substantial transcription from the HIV-1 long terminal repeat (LTR) in cell lines lacking functional TLRs. Therefore, a former colleague in our lab undertook a genome-wide mutagenesis approach to identify neisserial genes required for the production of the HIV-1 inducing “factor”. Remarkably, disruption of a single gene abrogated the LTR inducing ability of neisserial supernatants (Malott et al., 2013). Sequencing revealed the gene as NMB0825, annotated as encoding for an ADP-heptose synthase that is RfaE- or HldE-like (Tettelin et al., 2000). The E. coli homologue, HldE, is a bifunctional enzyme that functions as a carbohydrate kinase and an adenylyltransferase involved in the biosynthesis of ADP-heptose, a precursor for LPS biosynthesis (Kneidinger et al., 2001). However, purified LOS preparations from the isolated mutant, and wild type bacteria had similar TLR4 stimulatory ability (Malott et al., 2013). This suggested that the unknown inducer of the HIV-1 LTR was as a metabolite of the LPS biosynthesis pathway. Considering that NF-κB activation is a read out of many PRR signaling cascades, and that LPS is exclusively present in Gram-negative bacteria, it was tempting to speculate that this unknown metabolite may represent a novel PAMP.

1.6 LPS biosynthesis in Gram-negative bacteria

1.6.1 Structure of LPS

Gram-negative bacteria are defined by the presence of a second outer membrane that serves as protection against both the environment and attacks by the host. The external leaflet of the outer membrane is composed of an amphipathic molecule termed lipopolysaccharide (LPS) (Figure 5). The hydrophobic base of LPS is termed lipid A, or endotoxin, a glucosamine-containing phospholipid of which there are estimated $10^6$ molecules in a single E. coli bacterium (Galloway and Raetz, 1990). Lipid A is acylated with a variable number of fatty acid chains that can have a substantial effect on the immunogenicity of LPS (Seydel et al., 2000). While there have been varying reports on the minimal LPS structure that confers immunogenicity, it is generally accepted that Lipid A is the portion of LPS that binds and activates both TLR4 and the caspase-4/11 inflammasomes (Hagar et al., 2013; Raetz and Whitfield, 2002). Attached to the lipid A molecule are hydrophilic carbohydrates that stabilize the lipid A and confer the negative charge.
to LPS. The polysaccharide portion of LPS can be further divided into the core oligosaccharide, consisting of 10 to 12 sugar residues, and a longer chain of repeating polysaccharide units termed the O-specific chain (Caroff et al., 2002). The polysaccharide core is linked to the lipid A via a covalent bond to the acidic sugar 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo). Proximal to the Kdo lies the “inner core” region, which is composed of 1 to 3 residues of the uniquely microbial, seven carbon monosaccharide heptose, which are often decorated with phosphate, pyrophosphate, or diphosphoethanolamine residues (Caroff and Karibian, 2003). The “outer core” region is composed of several units of neutral sugars like glucose, galactose, and glucosamine, which is followed by the O-antigen, a chain of repeating units of sugar residues that confer serotype specificity to Gram-negative bacteria (Caroff et al., 2002). Certain mucosal pathogens, such as the Neisseria species, lack O polysaccharides so instead produce lipoooligosaccharides (LOS) that consist of one or more oligosaccharide chains that branch off the inner core (Raetz and Whitfield, 2002). In general, sugar variation in the polysaccharide region is largest in residues more distal to the lipid A, as the inner core region of most Gram-negative bacteria share close homology while the O-antigen displays considerable heterogeneity, even among members of a single bacterial species. This is not surprising, considering the exposure of the O-antigen to selective pressures from the environment and the host.
Figure 3  Structure and composition of lipopolysaccharide (LPS). Depicted is a schematic of inner and outer membranes of a typical Gram-negative bacteria. The outer leaflet is composed of LPS that can be subdivided into the lipid A and polysaccharide components. The polysaccharide can be further subdivided into the inner and outer core, as well as the O-antigen repeat. The inner core is made up of 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo) sugars, which are proximal to lipid A, and heptose residues. membrane derived oligosaccharides (MDO). Adapted from (Raetz and Whitfield, 2002).

1.6.2 Biological functions of the inner core

The biological importance of the various components of LPS have been established by studying bacterial mutants with LPS truncations. It is generally accepted that minimal LPS structure required for bacterial growth is lipid A linked to two Kdo residues (Raetz and Whitfield, 2002). These heptose-less LPS mutants are referred to as “deep-rough mutants” and while they can survive in the laboratory, they are extremely susceptible to environmental stress (Valvano et al., 2002). The lack of the extended polysaccharide reduces surface hydrophilicity causing an increased susceptibility to hydrophobic detergents, antibiotics, polycyclic hydrocarbons, bile salts and antimicrobial peptides (Valvano et al., 2000). The lack of membrane stability can lead to a decrease in expression of outer membrane proteins, and motility and
attachment factors like pili and flagella (Parker et al., 1992). In terms of virulence, Kdo-lipid A truncations are usually serum sensitive and avirulent in animal models (Valvano et al., 2002). Indeed, in vitro and in vivo studies have revealed that deep-rough mutants of E. coli, Salmonella typhimurium, Shigella flexneri, Burkholderia, Neisseira meningitidis and Neisseria gonorrhoeae are avirulent (Dlabac et al., 1997; Lambotin et al., 2005; Loutet et al., 2006; Plant et al., 2006; Sandlin et al., 1995; Schwan et al., 1995). Considering the dramatic phenotype associated with a lack of the inner core, there have been recent attempts to develop inhibitors of the heptose biosynthetic pathway that could be used to inhibit the virulence of the organism rather then kill it (Desroy et al., 2013).

1.6.3 Synthesis of the inner core

The inner core region of LPS displays considerable homology among distantly related bacteria, reflecting the importance of this region in maintaining membrane integrity (Raetz and Whitfield, 2002). Kdo residues are found in all bacterial LPS structures, and L-glycerol-D-mannoheptose residues are found in the vast majority of LPS structures, only absent in select species such as Moraxella, Rhizobium, Acinetobacter and Legionella (Valvano et al., 2002). Synthesis of LPS begins in the bacterial cytosol, where the Kdo2-lipid A region of LPS is synthesized (Piek and Kahler, 2012). The inner core is then sequentially added onto the growing LPS molecule by glycosyl transferases at the cytoplasmic face of the inner membrane where nucleotide-activated heptose (ADP-heptose), which act as donors for the heptose residues that eventually make up the inner core, are readily available (Raetz and Whitfield, 2002). The core-LPS molecule is then translocated across the inner membrane by ATP-binding cassette (ABC) transporters into the periplasmic space where the O-antigen is added (Zhou et al., 1998). Transfer of LPS from the periplasmic space on the outer membrane is mediated by a series of LPS transport proteins (Lpt) that are still incompletely understood (Piek and Kahler, 2012).

1.6.4 Biosynthesis of ADP-heptose

The precursors for the heptose residues found in the inner core were originally identified as ADP-D,D-heptose and ADP-L,D-heptose in Salmonella enterica serovar Minnesota and Shigella sonnei (Kocsis and Kontrohr, 1984; Kontrohr and Kocsis, 1981). ADP-L,D-heptose is much preferred as the substrate, as ADP-D,D-heptose exhibits much lower efficiency of transfer (Zamyatina et al., 2000; 2003). Transfer of the first ADP-Heptose to Kdo of the conserved Kdo2-
lipid A structure is mediated by the α1,5-heptosyltransferase RfaC (WaaC), forming the Hep1-Kdo2-lipid A. The α1,3-heptosyltransferase RfaF (WaaF), catalyzes the transfer of the second ADP-Hep to Hep1 to form Hep2- Kdo2-lipid A (Gronow et al., 2000; Sirisena et al., 1992).

The donor for all heptose residues found in the inner core, ADP-heptose, is synthesized in a 5-step biosynthetic pathway that is well conserved in Gram-negative bacterial phylogeny (Figure 6). The first substrate in the synthesis of ADP-heptose is sedoheptulose-7-phosphate, an intermediate in the pentose phosphate pathway (Eidels and Osborn, 1974). The phosphoheptose isomerase GmhA, converts sedoheptulose-7-phosphate into D-glycero-D-mannoheptose-7-phosphate (Brooke and Valvano, 1996). The next step in the pathway in most bacteria is catalyzed by the bifunctional enzyme HldE, which phosphorylates heptose-7-phosphate generating glycero-manno-heptose-1,7-bisphosphate (hereafter known as HBP) (Kneidinger et al., 2002). In Neisseria, this step is preformed by the enzyme HldA, which is homologous to the kinase domain of HldE in Enterobacteriaceae and is not bifunctional (Valvano et al., 2000). The C-7 phosphoryl group is then removed from HBP by the phosphatase GmhB generating D,D-heptose-1-phosphate (Kneidinger et al., 2002; Shih et al., 2001). Subsequent transfer of ADP onto the C-1 position of heptose-1-phosphate is catalyzed by the adenyl-transferase domain of HldE in E. coli or by HldC in Neisseria (Valvano et al., 2000). Lastly, the epimerase HldD, converts this compound to the preferred substrate for RfaC (WaaC) ADP-L-glycero-D-manno-heptose (Coleman, 1983). Sequencing of many bacterial genomes has revealed that the genes of the ADP-heptose biosynthetic pathway are present as single copies and are scattered throughout the chromosome (Valvano et al., 2002). One exception is Helicobacter pylori, whose heptose synthesis genes are part of a cluster which also includes flagellin (Valvano et al., 2002).

In general, the broadly conserved nature of the ADP-heptose biosynthetic pathway, the selective pressure for its expression, and the uniquely microbial nature of its metabolic constituents, all contributed to my hypothesis that the uncharacterized heptose-based innate immune agonist shed from Neisseria was a novel PAMP.
Figure 4  Biosynthetic pathway of ADP-heptose in *E. coli*. In *Neisseria*, the bifunctional enzyme HldE is replaced by the enzymes HldA and HldC, which together perform the function HldE. (Adapted with from Raetz and Whitfield, 2002).

1.7 Thesis Objectives

It is clear that pattern recognition by the innate immune system is essential for acute defense against infection and developing long lasting protective immunity. While much progress has been made in the past 20 years in both defining the molecular signals that our innate immune system interprets as “foreign” and on the host sensory apparatus responsible for their detection, models of infection have suggested that additional pathogenic signals may exist. Our observations concerning the stimulatory effect of *N. gonorrhoeae* on HIV transcription (Chen et al., 2003, Malott et al., 2013) led me to hypothesize that an as yet uncharacterized LPS metabolite released from bacteria of the *Neisseria* species was being recognized as an innate
immune agonist. Therefore, the three primary objectives of this work are: 1) Conclusively identify the metabolite released from *Neisseria* species and determine if this molecule represents a novel PAMP; 2) Define the host cell signaling cascades that mediate the detection of this molecule; and 3) Describe how recognition of this molecule can impact host-pathogen interactions.

In Chapter 2, I provide genetic and biochemical evidence that the unknown innate immune agonist shed from *Neisseria* species is heptose-1,7-bisphosphate (HBP), a metabolic intermediate in the LPS biosynthetic pathway common to most Gram-negative bacteria. In Chapter 3, I conduct a genome-wide loss-of-function screen to reveal that host recognition of HBP occurs within the host cytosol, and initiates a signaling cascade of innate immunity that is dependent upon the previously uncharacterized TRAF-Interacting Forkhead-Associated Protein A (TIFA). Finally in Chapter 4, I use the Gram-negative bacteria *Shigella flexneri* as a model for cytosol-invasive bacteria to demonstrate that TIFA-dependent recognition of HBP has an essential role in detecting bacterial pathogens that invade and exploit the cytosol of epithelial cells for growth and replication.
Chapter 2: Heptose 1,7-bisphosphate (HBP) is a novel PAMP that activates the host innate immune response

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**Ryan G Gaudet** designed and performed the experiments, wrote the paper, and analyzed the data.

Dr. Andrew Cox, and Jianjun Li contributed the mass-spectrometry analysis of HBP (Figure 6, and 17)

Cynthia Guo performed the injection into mouse genital tracts, and contributed to Figure 12.

Dr. Anna Sintsova performed experiments with the mouse air-pouch, primary neutrophils, and contributed to Figure 17.

Dr. Nelly Leung, and Dr. Carolyn Buckwalter performed the mouse challenge experiment and contributed to Figure 18.

Dr. Scott Gray-Owen designed experiments, analyzed the data and wrote the paper.

### 2.1 Summary

*Neisseria spp.* release a pro-inflammatory metabolite containing the uniquely microbial sugar heptose (Malott et al., 2013). However, the specific identity of this metabolite, and its prevalence among other bacteria remain unknown. Here I identify heptose 1,7-bisphosphate (HBP), an intermediate in the synthesis of lipopolysaccharide (LPS), as the innate immune agonist shed by *Neisseria spp*. HBP is an essential intermediate in a conserved pathway present in most Gram negative bacteria (Kneidinger et al., 2002), leading us to hypothesize that HBP is a novel PAMP. Indeed, I show that immune-active HBP is retained within the cytoplasm of other Gram-negative bacteria, is released upon loss of cell wall integrity, and activates a potent NF-κB
dependent immune response upon entry into the host cell cytosol. A molecule solely of bacterial origin (Herget et al., 2008), and an intermediate in a highly conserved pathway essential for bacterial virulence (Desroy et al., 2013; Loutet et al., 2006; Plant et al., 2006), HBP satisfies the 3 defining characteristics of a PAMP (Medzhitov, 2007) and contributes to the unique immunological signature of Gram-negative bacteria.

2.2 Overview

The mammalian innate immune system detects microbes by virtue of their expression of a variety of different PAMPs (Medzhitov, 2009). The presence of PAMPs in extracellular, vacuolar, or cytosolic cellular compartments initiates an inflammatory response, largely driven by the transcription factor NF-κB, that is critical for pathogen elimination and engagement of adaptive immunity (Kumar et al., 2011). Only a select group of bacterial molecules have been identified to function as PAMPs. Yet, despite considerable attention, they alone are insufficient at explaining pathogen-specific effector immune responses, suggesting additional PAMPs may exist. Three observations stemming from previous work in our lab suggested that the factor released from \textit{Neisseria} that activates HIV replication in Jurkat CD4 T cells (Chen et al., 2003; Malott et al., 2013) may represent a novel PAMP. First, the factor was uniquely shed by \textit{Neisseria} species, as conditioned media from cultures of other bacteria, such as \textit{E. coli}, \textit{Salmonella}, and \textit{Streptococcus}, had no activity. Second, Jurkat CD4 T cells are refractory to most PAMPs, in fact we tested a variety of TLR, NLR, and RLR agonists and only the TLR5 agonist flagellin was capable of activating NF-κB in this cell line (Malott et al., 2013). \textit{Neisseria} do not express flagellin, and blocking TLR5 with an antagonizing antibody had no affect on the inducing ability of \textit{Neisseria} supernatant, confirming that the factor was not a TLR5 agonist (Malott et al., 2013). Thus, the fact that Jurkat cells respond at all to \textit{Neisseria} culture supernatants suggested that the agonist was an unknown molecule that was being detected by a recognition system independent of known pattern recognition receptors. Third, we conducted a transposon mutagenesis screen and discovered that the \textit{Neisseria} gene \textit{hldA} was required for the NF-κB inducing ability of \textit{Neisseria} supernatant (Malott et al., 2013). HldA is the \textit{Neisseria} homologue to hldE in \textit{E. coli}, which catalyzes the second step in the biosynthesis of adenosine-diphosphate (ADP) activated-heptose, which is used as the precursor to add heptose residues
onto the 3-Deoxy-D-manno-oct-2-ulosonic acid (Kdo) sugar attached to the lipid A during the de novo biosynthesis of LPS (Kneidinger et al., 2002). This suggested that the innate immune agonist may be a metabolite of the ADP-heptose biosynthetic pathway. That heptose is a seven carbon monosaccharide only found Gram-negative bacteria (Herget et al., 2008) suggested that this unknown metabolite may represent a previously unknown PAMP.

2.3 Experimental Procedures

2.3.1 Cell Culture luciferase assay

HEK 293T (293T) cells were maintained in DMEM supplemented with 10% FBS, 1% glutamax, and 1% penicillin streptomycin. Jurkat 1G5 cells contain a stably-integrated LTR-luciferase reporter gene (Aguilar-Cordova et al., 1994), and were maintained in RPMI supplemented with 10% FBS and 1% glutamax. THP-1 cells were maintained in RPMI supplemented with 10% FBS and 1% glutamax and differentiated into macrophages with 50 ng/ml PMA for 48 h, followed by a 48 h rest period prior to stimulation. Immortalized endocervical epithelial cells (END1 E6/E7) have been described previously (Fichorova et al., 1997). To measure LTR-driven luciferase, 1G5 cells were lysed and luminescence determined using the Luciferase Assay kit (Promega) according to manufacturer’s instructions. Results are expressed as fold change compared to untreated cells. 293T cells were transfected in 96 well plates with 90 ng ELAM firefly luciferase reporter plasmid (Chow et al., 1999) and 10 ng pRL-TK Renilla plasmid using TransIT®-LT1 (Mirus). 18 h later, cells were treated for 6 h and luciferase activity determined using the Dual-Glo Luciferase Assay System (Promega). Results are expressed as fold increase relative to transfected, mock-treated cells following normalization to Renilla luciferase. Digitonin permeabilization assays were done as described previously (Girardin et al., 2003a) with the following modifications: 1G5 cells were stimulated with purified HBP-containing supernatants, or 10 µg/ml flagellin (Invivogen) for 15 minutes in the presence or absence of 2 µg/ml digitonin (Sigma). To assess HBP internalization, cells were treated with 80 µM Dynasore (Sigma) or 10 µM cytochalasin D for 1 h prior to stimulation with purified HBP, 10 ng/ml TNFα, or ΔhldA-HBP. M-TriDAP (100 µg/ml), C12-iE-DAP (0.5 µg/ml), c-di-GMP (100 µg/ml), 5’ppp-dsRNA/Lyovec (10 µg/ml), CpG oligonucleotide ODN 2216 (25 µg/ml), Pam3CSK4 (250 ng/ml), heat killed Listeria monocytogenes (HKLM) (10^8/ml), muramyl di-peptide (MDP) (10
µg/ml) were from Invivogen. Peptidoglycan (Pg.) was purified from *N. gonorrhoeae* as previously described (5). TLR4-expressing HEK-Blue™ cells were from Invivogen.

### 2.3.2 Bacteria

Bacterial strains used were the following: *Neisseria gonorrhoeae* MS11 (Opa−, pilus−), Δ*hldA*:Tn5 *N. gonorrhoeae* MS11 (Opa−, pilus−) (Malott et al., 2013), *Neisseria meningitidis* B16B6, *N. meningitidis* B16B6 Δ*hldA*:Tn5 (Malott et al., 2013), *E. coli* DH5α, *E. coli* BL21 (DE3), *Salmonella typhimurium* strain 14028S, *Burkholderia multivorans* pulmonary isolate from CF patient, *Haemophilus influenzae* 1128 middle ear isolate, *Streptococcus pneumoniae* sputum isolate, *Staphylococcus aureus* ATCC 29213 skin wound isolate, and *Listeria monocytogenes* EGD-e. To generate *N. meningitidis* mutants, overnight cultures of *N. meningitidis* B16B6 were spot transformed with 10 µg pUC19 containing a KAN-2 kanamycin cassette (Epicentre) flanked by ~500 bp adjacent regions of *gmhB* or *hldD*. pUC19 Targeting vector: *gmhB* 5ʹ'-acgtggtacctgggggatcctctagagaagttacaatgagcccttttagagg-3' and 5ʹ'-acagtatgaccatagttacgccaagttacatgagcccttttagagg-3'; *hldD* 5ʹ'-acgtggtacctgggggatcctctagagaagttacaatgagcccttttagagg-3' and 5ʹ'-acagtatgaccatagttacgccaagttacatgagcccttttagagg-3'. KAN-2 cassette amplification: *gmhB* 5ʹ'-gaacctgcccaaaccaaaggaaacgcgcaaccatcatcgatgaattgtg-3' and 5ʹ'-tttgcctttggtaatgccggtgatagttacgccaagttacatgagcccttttagagg-3'; *hldD* 5ʹ'-aacatcgtcaaagcacttaatcaacgc-3' and 5ʹ'-cgtgtgtgtccttgatagttacgccaagttacatgagcccttttagagg-3'. Restriction-free cloning was used to replace the *gmhB*, and *hldD* open reading frames in pUC19 with the amplified KAN-2 cassettes (van den Ent and Löwe, 2006). Following transformation and selection using 80 ug/ml kanamycin, genotyping was done with the following primers: *gmhB* 5ʹ'-acctgcccaaaccaaaggaaacgcgcaaccatcatcgatgaattgtg-3' and 5ʹ'-atgttgtttgccttgatagttacgccaagttacatgagcccttttagagg-3'; *hldD* 5ʹ'-aacatcgtcaaagcacttaatcaacgc-3' and 5ʹ'-cgtgtgtgtccttgatagttacgccaagttacatgagcccttttagagg-3'. In *E. coli* DH5α, *gmhA*, *hldE*, and *waaC* genes were deleted using the λ-Red plasmid pTP233 (Poteete and Fenton, 1984). Log phase bacteria were induced for 4 hours with 0.5 mM IPTG in the presence of 25 µg/ml tetracycline, washed 3 times with cold 10% glycerol and transformed via electroporation with the gel-purified Kan cassette. Kanamycin cassettes flanked by homology arms were generated by PCR using the following primers: *gmhA* 5ʹ'-ctgcatattttgtctcattatgtcatgagctggatct-3' and 5ʹ'-
ccgatggcgcgtaacgttttatcggcctacgccagaccatatgaatatcctccttag-3'; hldE 5’-tattatcgegceggaaatttggtgatcgcgcggttgagctgcctc-3’ and 5’-cctgccatgtacgaacgcgatgctgtcggcctacgccagaccatatgaatatcctccttag-3’; waaC 5’-agaaactcaacgcgctatatttgataacagaaagccgctctgctgcttc-3’ and 5’-tcaatgatgaagtttaagagatgtagcatgacatctctctctcctccttag-3’. Following selection with 50 µg/ml kanamycin, genotyping was done by colony PCR using the following primers: gmhA 5’-tagcacctgcccgtacttctcgc-3’ and 5’-agacgcgtcagcgtcgcatcagg-3’; hldE 5’-aggtggtgatccgcagccgctgc-3’ and 5’-acgacactacccagtcgaccgc-3’; waaC 5’-gctgccgttgagcgagttattcctg-3’ and 5’-cttccgccgcatgtcgtttcgcgct-3’. LPS and LOS preparations were prepared from proteinase K-treated cell lysates and visualized by silver staining (Hitchcock and Brown, 1983).

2.3.3 Synthesis and purification of HBP

_N. meningitidis_ gmhA, hldA, and gmhB genes were amplified and cloned into pET28a (Novagen). _E. coli_ BL21(DE3) were transformed, selected with 50 µg/ml kanamycin, and starter cultures grown to an OD$_{600}$ = 0.6. Cultures were induced with 0.5 mM IPTG for 4 hr and harvested by centrifugation. Pellets were re-suspended in lysis buffer: 50 mM TRIS pH 8.0, 300 mM NaCl, 10 mM imidazole, 3 mM 2-Mercaptoethanol. Clarified lysates were prepared by sonication followed by centrifugation at 20,000 x g for 30 min. Proteins were purified with Ni-NTA agarose (Qiagen) using Amicon® Pro purification system with 10 kDa cut-off (Millipore). Proteins were eluted in lysis buffer containing 300 mM imidazole and buffer exchange was done using 50 mM HEPES pH 8.0, 100 mM KCl, 1 mM DTT. Enzymes were stored in 50% glycerol. HBP was enzymatically synthesized in the following reaction: 20 mM HEPES pH 8.0, 20 mM KCl, 10 mM MgCl$_2$, 10 mM sedoheptulose-7-phosphate (Sigma), 20 mM ATP, 5 µg GmhA and 3 µg HldA. Reactions were stopped by incubating at 95°C for 5 min, and then passed through a 0.22 µm filter. Where indicated, filtrate was then incubated with 2 µg GmhB.

2.3.4 Capillary electrophoresis (CE)-MS analysis of HBP

PGC column purified fractions containing NF-κB inducing activity were analyzed by a Prince CE system (Prince Technologies) coupled to a 4000 QTRAP mass spectrometer (AB SCIEX). A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 2.0 uL/min. Separations were obtained on about 90 cm length bare fused-silica capillary using 15 mM
ammonium acetate in deionized water, pH 9.0. The 5.2 kV or −5.2 kV of electrospray ionization voltage were used for positive ion mode and negative ion mode detections, respectively. Tandem mass spectra were obtained using enhance production ion scan mode (EPI) with a scan rate of 4000 Da/s. Nitrogen was used as curtain (at a value of 12) and collision gas (set to scale high).

2.3.5 Bacterial lysate transfection and infections

*Neisseria* strains were grown overnight on GC agar supplemented with IsovitaleX enrichment (BD Biosciences). *E. coli* and *S. typhimurium* were grown overnight on LB agar, *S. pneumoniae* was grown on Columbia blood agar containing 5% sheep blood, and *H. influenzae, B. multivorans, L. monocytogenes*, and *S. aureus* were grown on brain heart infusion (BHI) agar (BD Biosciences). Where culture supernatant was desired, overnight cultures were scraped into PBS, re-suspended in RPMI 1% Isovitalex to an OD$_{600} = 0.2$, grown for 6 hours and the spent medium was filtered through a 0.22 μm filter. For heat-killed bacteria, overnight cultures were scraped into PBS and 1 OD$_{600}$ unit was re-suspended in 100 μl PBS and heated to 65°C for 1 hr, with the exception of *B. multivorans* which was heated to 85°C. Cell pellets were washed, re-suspended in 100 μl PBS, and 2 μl/well used as treatment. To generate lysates, cultures were treated as above and boiled for 15 minutes. Insoluble components were pelleted, and the supernatant treated with RNAse A (10 μg/ml), DNase 1 (10 μg/ml) and Proteinase K (100 μg/ml). Samples were boiled for 10 minutes, insoluble material was pelleted, supernatant passed through a 0.22 μm filter, and 1 μl/well was used as a treatment. To generate transfection complexes, 1 μl lysate was mixed with 1 μl lipofectamine 2000 (Life) in 25 μl Opti-MEM, incubated for 30 minutes, and added dropwise to 293T cells at 70% confluence. For opsonization, overnight cultures of *E. coli* were washed and re-suspended at an OD$_{600} = 0.5$ in 20% heat-inactivated human serum (Chemicon) for 1 hour at 25°C, then washed twice with PBS 10% FBS and added to differentiated THP-1 macrophages in antibiotic free medium, pre-treated with 10 μg/ml cytochalasin D or DMSO for 30 min, at an MOI of 5. After 1 hr, media was removed, washed, and replaced with RMPI complete media containing 50 μg/ml gentamicin.

2.3.6 Purification of HBP from *Neisseria* supernatants

Purified HBP-containing or HBP-deficient supernatants, were isolated from spent *Neisseria* cultures essentially as described previously (Malott et al., 2013). Briefly, *N. gonorrhoeae* or *N.
meningitidis wild-type or ΔhldA were grown from OD₅₅₀ 0.18 to ~0.5 for 6 hours in RPMI containing 1% Isovitalex. Supernatants were digested with DNAse (10 µg/ml), RNAse (10 µg/ml), Proteinase K (100 µg/ml), boiled for 30 minutes, passed through an Amicon 3 kDa MW cutoff filter (Millipore) and then a C18 Sep-Pak® cartridge (Waters). Any residual LOS was removed using endotoxin removal resin (Pierce) according to manufacturer’s instructions.

2.3.7 Measurement of inflammatory cell death

Cell death in THP-1 differentiated macrophages infected with live opsonized E. coli, HBP-containing or deficient supernatants, or transfected with LPS (Sigma) using lipofectamine 2000, were measured using the Cytotox96 cytotoxicity assay (Promega) according to manufacturer’s instructions. LDH release was measured at 24 hr, and quantified as a percentage of total LDH released from lysis 100% of cells. Where indicated, LDH release from untreated cells was used for correction.

2.3.8 Lentivirus production and infection

pLKO.1-based lentiviral particles were produced as previously described (Moffat et al., 2006). For each gene to be targeted, a minimum of 5 shRNAs were first tested for effective titer using Alamarblue viability assays and for gene silencing using real-time qPCR (Blakely et al., 2011). The target cells, 293T epithelial or THP-1 monocytes, were infected in media containing 8 µg/ml polybrene. 24 hours later, cells were selected with 2 µg/ml puromycin. Cells were harvested after 72 hours and knockdown efficiency was again confirmed by qPCR.

2.3.9 Real-time quantitative PCR and ELISAs

RNA was isolated using an RNaseasy kit (Qiagen) per manufacturers’ protocol. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) and treated with TURBO DNase (Life Technologies). cDNA was amplified using SsoAdvanced SYBR Green (Bio-Rad) using a C1000 thermal cycler (Bio-Rad). Target genes were amplified using the following primers:

- **GAPDH** 5ʹ-ttgaggtcataaagggggtc-3ʹ and 5ʹ-gaaggtgaagttcaggtctc-3;
- **NFKBIA** 5ʹ-tcaggtgatggcccaagt-3ʹ and 5ʹ-gtcagggactgctgccagatg-3;
- **CCL1** 5ʹ-aaccaacctcctggagaagg-3ʹ and 5ʹ-atgcagatcatcaccac-3;
- **TNFA** 5ʹ-gcagagggcttggattagaga-3ʹ and 5ʹ-teagctctctctctct-3;
- **CXCL10** 5ʹ-gcaggtcaggtcattcttc-3ʹ and 5ʹ-cagcagagagcatct-3;
- **IL8** 5ʹ-agcactctggcagaactg-3ʹ and 5ʹ-cggaagcaaatctctg-3;
- **LUC** 5ʹ-ctcactgagactcatc-3ʹ and 5ʹ-
tccagatccacaaccttcgc-3' \textit{RIP2K} 5'-ggtgaatggcacttgaaaca-3' and 5'-ggcacaataccagatgaag-3'; 
\textit{MYD88} 5'-aaaggcttctcagcctcctc-3' and 5'-actgtcagactgtcctacca-3'; \textit{MAVS} 5'-
tcagattctggagagggc-3' and 5'-ggtcgcagacttcagg-3'; \textit{TMEM173(STING)} 5'-
atatacagctggtgctc-3' and 5'-gatactggctgctcgtc-3'. Relative expression was calculated using
the $2^{-\Delta\Delta CT}$ method following normalization of target gene abundance to \textit{GAPDH}. Quantitative
measurements of cytokines were performed using ELISA kits form R&D Systems (KC, Il-23, IFN-\(\beta\)) or BD Biosciences (Il-1\(\beta\), Il-8, Il-6, Il-12p70, TNF-\(\alpha\)). Nuclear extracts were prepared
from Jurkat T cells and NF-\(\kappa\)B subunit binding was determined using the TransAM®
Transcription Factor ELISA (Active Motif).

2.3.10 Microarray

Two clonal populations of Jurkat cells were stimulated for 2 hr with purified HBP-containing
supernatants, or supernatants from \textit{M. catarrhalis}. RNA was extracted using RNeasy (Qiagen),
labeled using Illumina TotalPrep RNA Amplification kit (Ambion) and analyzed on a human
HT-12 v4.0 Beadchip (Illumina). Data normalization and analysis were provided as a service by
the Bioinformatics Department of the University Health Network (UHN) Microarray Centre,
Toronto, ON. Data was analyzed using Genespring v11.0.1. Genes with a $\geq 1.5$ fold change (FC)
in gene expression in both clones when treated with HBP-containing supernatants compared to
\textit{M. catarrhalis} supernatants are shown.

2.3.11 Primary cell culture

Whole blood was taken by venipuncture from human volunteers. Peripheral blood mononuclear
cells (PBMCs) were isolated using Ficoll-Paque (GE). PBMCs (2 x 10\(^6\) cells/ml) were incubated
for one hour at 37\(^\circ\)C to allow monocytes to adhere. Following removal of non-adherent cells,
monocytes were re-suspended and incubated for 7 days in RPMI 1640, 10\% FBS, 1\% glutamax,
1\% penicillin streptomycin, containing 100 ng/ml recombinant human Granulocyte macrophage-
coloncy stimulating factor (GM-CSF; BioLegend). For infections, cells were detached using
accutase (Sigma) and seeded at 2 x 10\(^5\) cells/well in 48 well plates without antibiotics. Human
neutrophils were isolated from citrated whole blood taken from healthy volunteers by
venipuncture using Ficoll-Paque (GE) as described previously (McCaw et al., 2003).
2.3.12 Air pouch

FvB mice (6-8 wk) were anesthetized with isoflurane, and dorsal air pouches raised by injecting 3 ml sterile air subcutaneously on day 0 and 2 ml on day 3. On day 5, air pouches were injected with 1 ml RPMI 1% isovitalX or 1 ml HBP purified from spent cultures of \textit{N. gonorrhoeae} wild-type, or \textit{ΔhldA}. Mice were sacrificed 3 hr after the injection and serum samples were collected by cardiac puncture. Air pouches were washed with 2 ml PBS. Neutrophils were quantified using trypan blue exclusion. KC levels in the sera and air pouch were quantified by ELISA as described above.

2.3.13 Mouse challenge

Groups of ten eight week old FVB male mice (Charles River) were challenged on day 0 and day 21 with a non-lethal dose of \textit{N. meningitidis} strain B16B6 ΔgmhB or ΔhldA. To prepare each inoculum, bacteria were grown overnight on GC agar containing 60 µg/ml kanamycin, resuspended in BHI broth, adjusted to an optical density of 0.1 and grown at 37°C with shaking. After two hours, bacteria were diluted in sterile PBS such that each 200 µl aliquot contained 1 x 10^6 CFU. Mice were anesthetized with isofluorane and injected intraperitoneally with each inoculum. Without addition of an exogenous iron source, this dose of bacteria is cleared quickly from the bloodstream (<12 h) and results in no clinical symptoms or lethality. Mice were monitored at least once per day for two days after bacterial challenge for changes in weight or clinical symptoms. No animals showed any signs of clinical illness. Whole blood was collected via facial vein bleed at the indicated time point for analysis of serum antibodies and to ensure bacterial clearance. Animal experiments were conducted in accordance with the Animal Ethics Review Committee of the University of Toronto.

2.3.14 Whole-bacteria ELISAs

Anti-meningococcal antibodies were measured by whole-bacteria ELISA. Each well of a Maxisorp 96 well flat-bottom immuno plate (Nunc) was coated with 50 µl of a standardized suspension of heat-inactivated \textit{N. meningitidis} B16B6 ΔhldA in H_2O and allowed to dry. After washing wells with wash buffer (PBS containing 0.05% Tween-20) and blocking 1 h with PBS containing 5% BSA, 50 µl per well of diluted sera were added to wells and incubated for 2 h. Wells were again washed three times and 50 µl per well of 1:10,000 dilution of AP-goat-anti-
mouse IgG Fc(\(\gamma\)) (total), sub-class specific IgG (Jackson Immunoresearch) or AP-goat-anti-mouse IgM (Jackson Immunoresearch) was added and incubated for 1 h at room temperature. This was followed by an additional three washes and detection with 100 µl per well of BLUEPHOS AP detection substrate (KPL). Immunoglobulin concentrations were determined from a standard curve generated from the detection of sequential dilutions of mouse IgG (ab37355; Abcam), or mouse IgM isotype control (M5909; Sigma) in wells coated with a 1/200 dilution of goat-anti-mouse IgG (Jackson Immunoresearch) or goat anti-mouse IgM (Jackson Immunoresearch).

2.4 Results

2.4.1 Heptose 1,7-bisphosphate is the innate immune agonist shed from *Neisseria*

*Neisseria spp.* secrete a metabolite that activates NF-\(\kappa\)B in 293T epithelial and Jurkat T cell lines; cell types whose ability to respond to previously-described PAMPs is limited to TLR5-dependent detection of flagellin (Malott et al., 2013). While the neisserial gene *hldA* is essential for this process, the identity of the molecule remains unknown. HldA catalyzes the second step in the synthesis of ADP-heptose (ADP-hep), the precursor for the inner core region of LOS the major component of the Gram-negative outer membrane (Kneidinger et al., 2002) (Figure 5A). To identify the molecule, I sought the first step in the ADP-hep biosynthetic pathway downstream of HldA that was dispensable for culture supernatant-mediated NF-\(\kappa\)B activation. Supernatant from the *N. meningitidis Δg mhB* mutant, whose terminal metabolite in the ADP-hep pathway differs from the ΔhldA mutant by a single phosphate group, potently activates NF-\(\kappa\)B (Figure 5B). Thus, by permitting the synthesis of d-glycero-d-manno-heptose-1,7-bisphosphate (HBP), I restored the pro-inflammatory nature of *Neisseria* culture supernatants. Importantly, the Δg mhB and ΔhldA *N. meningitidis* mutants both display the same so-called “deep-rough” LOS truncations after the Kdo sugars (Figure 5C), indicating that NF-\(\kappa\)B activation in the host was dependent upon bacterial production of the metabolite HBP and not on whether heptose was incorporated into the LOS. Next, I enzymatically synthesized HBP from sedoheptulose-7-phosphate (S7P) using GmhA and HldA purified from *N. meningitidis*. The product of the
Figure 5  Heptose-1,7-bisphosphate is the innate immune agonist liberated by Neisseria. (A) Schematic depiction of the ADP-heptose (ADP-hep) biosynthetic pathway in Gram-negative bacteria. Supplied by the pentose phosphate pathway, sedoheptulose-7-phosphate is converted to ADP-D-glycero-D-manno-heptose-1,7-bisphosphate (ADP-hep), the precursor for the synthesis of the inner core of LOS and LPS in five steps (Kneidinger et al., 2002). Neisseria enzymes are indicated in bold. E. coli enzymes, when different than Neisseria, are in parenthesis. (B) NF-κB luciferase activity in 293T cells treated with purified culture supernatants prepared from N. meningitidis of the indicated genotype. (C) Silver stain of LOS extracts from indicated N. meningitidis isogenic strains. (D and E) NF-κB luciferase activity in 293T cells treated with the product of in vitro reactions containing combinations of sedoheptulose-7-phosphate (S7P), His-tag purified GmhA and HldA (D), and then incubated with or without His-tag purified GmhB (E). Results represent the mean of 3 independent experiments ± SEM.

In vitro reaction potently stimulated NF-κB only when the substrate and both enzymes were supplied (Figure 5D). Furthermore, incubation of the product with the downstream phosphatase GmhB decreased NF-κB activation (Figure 5E). Finally, my collaborators performed mass-spectrometry on the enzymatically synthesized HBP following purification to show that the proinflammatory product of the GmhA-HldA reaction was indeed HBP (Figure 6). Thus, HBP is the innate immune agonist shed by Neisseria.
Figure 6  Identification of HBP from a purified fraction from the enzymatic synthesis reaction of GmhA and HldA described in Figure 5. Shown is precursor ion CE-mass spectrum for \( m/z \) 79.0 (phosphate) of the first fraction containing activity from the enzymatic synthesis reaction following purification using a porous graphite column and elution using an acetonitrile gradient. The ion at \( m/z \) 270.9 corresponds to losses of 98 Da (phosphate + water) from the ion \( m/z \) 368.7 corresponding to HBP (M-H). The molecular weight of heptose-1,7-bisphosphate (C7-H12O13P2) is 370.0 Da. The same fractionation pattern was observed when the fraction was spiked with glucose-1,6-bisphosphate. The ion at \( m/z \) 345.9 corresponds to adenosine-monophosphate (AMP), a bi-product of the reaction.

2.4.2 HBP is sensed within the host cell cytosol

I next performed transcriptome analysis on the response of Jurkat T cells to HBP-containing supernatants purified from *N. gonorrhoeae* compared with supernatants derived from *Moraxella catarrhalis*, a Gram-negative bacteria that is closely related to *Neisseria* but, curiously, does not make heptose metabolites for incorporation into its LOS. I found that HBP-containing supernatants up-regulated a variety of NF-κB dependent genes in Jurkat T cells (Figure 7A). Interestingly, the kinetics of HBP-mediated pro-inflammatory transcriptional response was slower, and persisted longer, than stimulation with flagellin or TNFα, two ligands that signal at the cell surface (Figure 7B). This slower kinetic pattern was also reflected in the nuclear translocation of NF-κB subunits upon stimulation with HBP (Figure 7C). Innate immune agonists can be sensed on the cell surface, or within the cytosol. The latter first requires transport of the agonist into the host cytosol, and results in a delayed transcriptional response compared with agonists that signal on the cell surface. Therefore, I hypothesized that HBP may require entry into the host cytosol to signal. Indeed, delivery of HBP-containing supernatants into the cytosol of Jurkat 1G5 cells, which harbor a stable HIV LTR-luciferase construct (Aguilar-Cordova et al., 1994), using reversible digitonin permeabilization (Girardin et al., 2003) resulted
in a dose-dependent increase in luciferase activity, whereas TLR5-mediated activation remained constant (Figure 8A). Moreover, like other cytosolic PAMPs, synthetic HBP cooperatively activated THP-1 macrophages in combination with TLR ligands (Figure 8C). To determine how HBP gains entry to the cytosol, I treated 293T cells with a highly specific inhibitor of the GTPase dynamin (dynasore) (Macia et al., 2006) or cytochalasin D, an inhibitor of actin
polymerization. Dynasore, but not cytochalasin D, attenuated the NF-κB response to HBP (Figure 8B). Thus, in a process similar to endocytosis of the NOD1 and NOD2 activating ligands muramyl di-peptide (MDP)- and diaminopimelic acid (DAP)-containing peptidoglycan fragments (Lee et al. 2009), HBP signals in the host cytosol following internalization via dynamin-dependent endocytosis.

Figure 8 HBP is sensed within the host cytosol. (A) Jurkat 1G5 cells, stably expressing a HIV long terminal repeat (LTR)-driven luciferase, treated with increasing amounts of HBP containing or deficient (ΔhldA) supernatants, or flagellin in the presence or absence of the permeabilizing agent digitonin (Dig) for 15 minutes. Media was replaced and luciferase activity determined after 6 hrs. (B) NF-κB luciferase activity in 293T cells treated with HBP containing or deficient (ΔhldA) supernatants, or TNFα in the presence of vehicle (DMSO), dynasore (Dyn), or cytochalasin D (Cyto D). (C) qRT-PCR analysis of THP-1 macrophages treated with synthetic HBP (sHBP), PAM3CSK4 (PAM3), or flagellin (FLAG) for 4 hr, expressed as fold increase relative to untreated after normalization to GAPDH. Data represent ≥3 independent experiments performed in duplicate. All error bars ± s.e.m. *P < 0.05, **P < 0.01 by ANOVA.

2.4.3 HBP is present within a variety of Gram-negative bacteria

The ADP-heptose biosynthetic pathway is highly conserved among Gram-negative bacteria (Kneidinger et al., 2002) yet is completely absent in eukaryotic cells. Moreover, a complete pathway is essential for virulence of a number of bacterial species (Desroy et al., 2013;
Loutet et al., 2006; Plant et al., 2006). Therefore, I hypothesized that HBP represented a Gram-negative bacterial PAMP. However, being a cytosolic bacterial metabolite, HBP-mediated signaling by other non-*Neisseria* bacteria would only be apparent if HBP was first liberated from within bacterial cytosol. To test this model, I transfected soluble lysates from a variety of bacterial Genera into non-phagocytic 293T cells containing an NF-κB reporter. Consistent with my previous results, only the culture supernatant from *Neisseria* activated NF-κB in the 293T cells (Figure 9A). Strikingly, transfection of Gram-negative lysates, with the notable exception if *M. catarrhalis*, potently activated NF-κB, while Gram-positive lysates had no activity (Figure 9A). Cells were relatively unresponsive to the two known PAMPs unique to Gram-negative bacteria, LPS and the NOD1 ligand m-TriDAP, suggesting that a novel PAMP was responsible for activating NF-κB (Figure 9A). NF-κB activation depended on the release of bacterial cytosolic components, as heat-killed whole bacteria showed no activity. As *M. catarrhalis* is one of the few Gram-negative bacteria that lack the ADP-hep pathway, this supported our proposition that HBP could be responsible for this activity (Caroff and Karibian, 2003). Consistent with this, deletion of genes upstream of HBP in the ADP-hep pathway in either *N. meningitidis* or *E. coli*, completely abrogated lysate-mediated NF-κB activation (Figure 9B). In contrast, mutants lacking genes in the pathway downstream of the HBP intermediate, *waaC* (*rfaC*) in *E. coli*, or *gmhB* in *N. meningitidis*, displayed greater activation of NF-κB than did the respective wild type bacteria (Figure 9B and C). Importantly, these (*ΔwaaC/ΔgmhB*) mutations produced LPS/LOS truncations that phenocopied the HBP-deficient mutants *ΔhldE* and *ΔhldA* (Figure 9C), confirming that it is the metabolite HBP and not the LPS/LOS glycan structure that is detected. Instead, the fact that deletion of either gene significantly increased NF-κB activation implicates an intracellular buildup of HBP (Figure 10A). Importantly, the HBP-effect could be exacerbated in wild type *E. coli*, as over-expression of *Neisseria* HldA, but not other enzymes in the ADP-hep pathway in wild-type *E. coli* (BL21) increased lysate-mediated NF-κB activation by over 100-fold (Figure 10B). Interestingly, HBP did not accumulate in the culture supernatant in the HldA-overexpressing *E. coli*, suggesting a unique mechanism for HBP release exists in *Neisseria* spp.
Figure 9  Immunoactive HBP is prevalent in the cytosol of a variety of Gram-negative bacteria. (A) NF-κB luciferase activity in 293T cells treated for 6 hr with culture supernatant, heat-killed (HK) whole bacteria, soluble lysate, or transfected with soluble lysate prepared from Gram-negative or Gram-positive bacteria. (B) NF-κB luciferase activity in 293T cells transfected with soluble lysates from *N. meningitidis* or *E. coli* lacking the indicated genes in the ADP-heptose biosynthesis pathway. (C) Silver stain of LPS extracts from *E. coli* mutants showing all 3 *E. coli* mutants have the same “deep rough” phenotype. Data from A and B represent ≥3 independent experiments performed in duplicate. C is representative of 2 independent experiments. Error bars ± s.e.m.
Figure 10  A buildup of intracellular HBP in bacteria lacking genes downstream of HBP in the ADP-heptose pathway (A) NF-κB luciferase activity of 293T cells transfected with the indicated volume of soluble lysates form wild-type (Wt) or mutants lacking genes upstream of HBP (ΔhldA or ΔhldE), or downstream of HBP (ΔgmhB, or ΔwaaC) in the ADP-Hep pathway in N. meningitidis or E. coli. (B and C) NF-κB luciferase activity in 293T cells treated with soluble lysates (B) or culture supernatants (C) prepared from E. coli (BL21) cells expressing the indicated N. meningitidis genes from an IPTG-inducible vector. Data represent ≥3 independent experiments performed in duplicate. All error bars ± s.e.m. *P < 0.01, by T test.

2.4.4 Extracellular ATP enhances the host response to HBP

During purification and mass spectrometry analysis of the product of in vitro synthesis reactions described in Figure 6, I noticed that in non-digitonin permeabilized cells, NF-κB inducing activity of each fraction did not necessarily correlate with the intensity of the HBP peak observed in the mass spectrum. As can be seen in Figure 11A, the ion peaks corresponding to HBP is higher in the eluted fraction 12, compared to fraction 15. However, when added extracellularly to non-permeabilized cells, fraction 15 displays much greater NF-κB inducing
Figure 11  Extracellular ATP enhances the host response to to HBP. (A and B) Precursor ion CE-mass spectrum for \( m/z \) 79.0 (phosphate) of fractions 12 and 15 obtained following purification of enzymatically synthesized HBP using a porous graphite column and elution using an acetonitrile gradient and described in (B). (B) depicts the NF-κB inducing activity of each purified fraction in the presence or absence of the permeabilizing agent digitonin. The ion at \( m/z \) 270.9° corresponds to losses of 98 Da (phosphate + water) from the ion \( m/z \) 368.7° corresponding to HBP (M-H). The molecular weight of heptose-1,7-bisphosphate (C\(_7\)H\(_{12}\)O\(_{13}\)P\(_2\)) is 370.0 Da. (C) NF-κB luciferase activity in HEK 293T cells induced by fraction 12 in the presence of increasing amounts of extracellular ATP.
activity than fraction 12 (Figure 11B). Interestingly, this does not hold true for digitonin-permeabilized cells, as both fractions displayed robust activating ability when permitted to enter host cells (Figure 11C). An analysis of the total mass spectrum of each fraction revealed that fraction 15 contained much higher peaks corresponding to adenosine diphosphate (ADP) and adenosine triphosphate (ATP) than fraction 12, which, in contrast, contained a higher peak corresponding to adenosine mono-phosphate (AMP). Co-elution of ATP and its metabolites with HBP was not unexpected, as a high starting concentration of ATP was included in the reaction mixture as a phosphate donor to insure adequate phosphorylation of heptose-7-phosphate into HBP by the enzyme HldA. Therefore, to see if the presence of ATP could explain the difference in the host response to fraction 12 and 15, I added increasing amounts of ATP into fraction 12 and monitored NF-κB activation upon addition to non-permeabilized cells (Figure 11C). Critically, ATP itself had no effect on reporter expression. However, ATP potently increased NF-κB activation of fraction 12 in a dose-dependent manner (Figure 11C), suggesting that this endogenous nucleotide can significantly affect the host response to extracellular HBP.

Well known for its role in intracellular energy metabolism, extracellular ATP is both a danger signal (discussed in section 1.4.3) and a signaling molecule. As a signaling molecule, it can have pleiotropic effects on physiology, including modulating cardiac function, neurotransmission, muscle contraction, vasodilatation, bone metabolism, liver glycogen, metabolism and inflammation (Bours et al., 2006). To further examine the synergy between HBP and ATP, I added ATP to HBP-containing supernatants purified from *N. gonorrhoeae* and observed that 1 mM exogenously added ATP increased NF-κB activation in HEK 293T cells over 100-fold (Figure 12A). I next asked whether this affect could be replicated in vivo. To this end, we injected HBP-containing supernatants purified from *N. gonorrhoeae* in the presence or absence of 1 mM ATP into the urogenital tract of mice and monitored levels of the cytokine KC (CXCL1) in the serum. Remarkably, ATP had a dramatic effect on the response to HBP, increasing the response over 50-fold (Figure 12B). The synergy phenotype was unique to HBP, as 1 mM ATP had no affect on NF-κB activation in 293T cells induced by TNFα, C12-iE-DAP or PAM3CSK4 (Figure 12C).
Figure 12  ATP enhances the host response to extracellular, but not intracellular HBP. (A) NF-κB luciferase activity in T cells following treatment with purified HBP-containing supernatants from *N. gonorrhoeae* (Ngo sup) in the presence of the indicated amount of extracellular ATP. (B) ELISA measurement KC (CXCL1) levels in the serum of mice inoculated intravaginally with HBP-containing supernatants from *N. gonorrhoeae* (N302 sup) in the presence of 1 mM ATP after 3 h. (C) NF-κB luciferase activity in 293T cells transfected with TLR2 or NOD1 expressing plasmid, and stimulated with the indicated ligand in the presence or absence of 1mM ATP for 6 hours. (D) ATP levels in the culture supernatant of *N. gonorrhoeae* cultures at the indicated optical density. (E) NF-κB luciferase activity in 293T cells in the presence or absence of the permeabilizing agent digitonin following treatment with HBP-containing supernatants from *N. gonorrhoeae* treated with the ATP-degrading enzyme Apyrase, or mock treated. Data represent ≥3 independent experiments. All error bars ± s.e.m.

These results raised the question as to whether the presence of extracellular ATP was a requirement for the host response to extracellular HBP. The sensitivity of host cells to HBP-containing supernatants from *N. gonorrhoeae* suggested that for this to be true, *Neisseria* must
release ATP into the culture supernatant during growth. Indeed, I detected significant levels of accumulating ATP in the culture supernatant during the growth curve of \textit{N. meningitidis} (Figure 12D). Treatment of supernatants with the ATP-degrading enzyme Apyrase significantly reduced NF-κB activation in non-permeabilized 293T cells but did not completely abrogate it (Figure 12E). However, in digitin-permeabilized cells Apyrase treatment had no affect on NF-κB activation, confirming that ATP is not required for cytosolic detection of HBP (Figure 12F). Together these results suggest extracellular ATP dramatically enhances the host response to extracellular HBP. Permitting HBP to access the host cytosol removes the requirement for ATP, suggesting that either ATP is inducing host internalization of HBP, or that the signaling cascades induced by both molecules interact in a synergistic manner only apparent when HBP is in limiting quantities, as would be the case if the internalization of HBP is the rate-limiting step in its signaling cascade.

2.4.5 HBP is liberated during intraphagosomal bacteriolysis

In the context of a natural infection, a probable source for HBP release would be during bacterial lysis within the lysosome following phagocytosis. To test this, I treated MyD88 and TRIF deficient THP-1 macrophages (Fig. 13A and B) with serum-opsonized HBP proficient or deficient \textit{E. coli}. MyD88 and TRIF were silenced to reduce signaling by the TLRs. Consistent with our hypothesis, \textit{E. coli ΔwaaC}, capable of synthesizing HBP, induced more IL-6 than bacteria that could not synthesize HBP (ΔgmhA, ΔhldE) (Figure 13C). Inhibiting phagocytosis with cytochalasin D, or lysosomal acidification with chloroquine abrogated the effect, suggesting uptake and degradation of the bacteria within the phagolysosome was required to liberate HBP (Figure 13C). Indeed, while all three \textit{E. coli} mutants were internalized and degraded at a similar rate (Figure 14), HBP-proficient \textit{E. coli ΔwaaC} induced more pro-inflammatory transcription than HBP-deficient \textit{E. coli ΔgmhA} or ΔhldE without affecting IFN-β production or cell death (Figure 15A to C). HBP containing supernatants also did not induce pyroptosis in THP-1 differentiated macrophages upon cytosolic delivery, despite inducing significant IL-6 production (Figure 15D). Given that HBP is only liberated from non-\textit{Neisseria} upon bacterial degradation, the lack of a self-destructive inflammatory cell death response to HBP likely allows the cell to detect degraded bacterial products in the cytosol without undergoing the danger-associated
pyroptosis. Thus, there is immunoeactive HBP in the cytoplasm of many Gram-negative bacteria that is liberated during lysis or phagocytosis, activating NF-κB without triggering cell death.

**Figure 13** HBP is released during phagocytosis. (A) IL-6 levels by ELISA in the supernatants of THP-1 activated macrophages treated with LPS following transduction with non-targeting shRNA (ShCtrl) or targeting either MyD88 or TRIF. To knockdown both MyD88 and TRIF, cells were transduced with a pool of shRNAs targeting both MyD88 and TRIF. (B) mRNA expression of MYD88, TRIF, or β-actin (BACT) by qPCR following normalization to GAPDH and expressed as a percentage of non-transduced cells. THP-1 cells were transduced with shRNAs as per (A). (C) Shown are IL-6 levels by ELISA after infection of THP-1 macrophages expressing the MyD88 and TRIF targeting shRNAs with serum-opsonized E. coli of the indicated genotype with or without pre-treatment with cytochalasin D (CytoD) or chloroquine (Chq). Data represent ≥3 independent experiments performed in duplicate. All error bars ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA.
Figure 14  Opsonized *E. coli* Δ*gmhA*, Δ*hldE*, and Δ*waaC* are internalized and degraded by THP-1 differentiated macrophages. (A) GFP expressing *E. coli* of the indicated genotype opsonized with heat-inactivated human serum were used to infect MyD88/TRIF depleted THP-1 macrophages at an MOI of 20. At the indicated timepoint, cells were fixed, and extracellular bacteria identified with anti-*E. coli* antibody. By this method, bacteria appearing red (or yellow due to colocalization with green) are extracellular whereas bacteria that are green but not red are intracellular. (B) Quantification of *E. coli* internalization and degradation. Macrophage containing intracellular bacteria (green) were quantified from at least 3 fields. A reduction in GFP-mediated fluorescence was considered indicative of bacterial degradation within the phagolysosome. Data represent the mean of 2 independent experiments done in triplicate. Error bars ± s.e.m.
Figure 15  HBP increases pro-inflammatory transcription, but not inflammatory cell death or interferon-β production following bacterial phagocytosis. (A and B) Cell death by lactate dehydrogenase (LDH) release (A) or IFN-β production by ELISA (B) following infection of MyD88/TRIF depleted THP-1 macrophages with serum-opsonized E. coli of the indicated genotype. (C) Gene expression by qRT-PCR at the indicated timepoint following infection as described in (A, B). (D) Cell death by lactate dehydrogenase (LDH) release or IL-6 production by ELISA following treatment with HBP containing or deficient (ΔhldA) supernatants or transfected with LPS (tran LPS). Data represent the mean of 3 independent experiments done in duplicate. Error bars ± s.e.m.
2.4.6 HBP is not detected by any known pattern recognition receptor

I next determined whether any of the known pattern recognition receptor signaling pathways were responsible for sensing HBP. 293T cells have previously been reported to express endogenous levels of NOD1 and NOD2 (Girardin et al., 2003). HBP signaling was independent of NOD1/2, as shRNA knockdown of RIP2, which is essential for NOD1/2 signaling (Kobayashi et al., 2002), had no significant affect on HBP or lysate-mediated NF-κB activation (Figure 16A and B). Moreover, shRNA knockdown of the adaptor proteins MyD88, CARD9, STING and MAVS, which mediate signaling from other known cellular pattern recognition receptors (Hara et al., 2007; Kawai et al., 2005; Medzhitov et al., 1998; Meylan et al., 2005; Parvatiyar et al., 2012; Seth et al., 2005) had no effect on HBP-mediated cytokine production in THP-1 macrophages (Figure 16C and D), suggesting HBP is detected by a previously undescribed pathway.

2.4.7 HBP elicits an innate and adaptive immune response in vivo

To determine the biological significance of HBP detection, I tested the ability of primary human cells to detect HBP. HBP induced IL-8, IL-6 and TNFα production in differentiated primary human macrophages, neutrophils, and immortalized epithelial cells (Figure 17A to C), and infection of macrophages with \( N. meningitidis \Delta gmhB \) induced more IL-6, IL-8 and IL-23, but not IFN-β, than the isogenic \( \Delta hldA \) mutant that differs only in its ability to synthesize HBP (Figure 17D). Notably, HBP induced significant amounts of the Th17 polarizing cytokines IL-6 and IL-23. To assess the activity of HBP in vivo, we used the mouse subcutaneous cytokines IL-6 and IL-23. To assess the activity of HBP in vivo, we used the mouse subcutaneous air pouch as a model to study acute inflammation in a sterile environment (Edwards et al., 1981). Injection of HBP-containing fractions prepared from culture supernatants, which do not contain other microbial PAMPs, into the sterile compartment induced a local and systemic inflammatory response, evidenced by an increase in local and systemic accumulation of the neutrophil-targeting keratinocyte derived chemokine (KC), and culminating in a 3-fold increase in neutrophil recruitment to the air pouch (Figure 17D to F). Therefore, similar to NOD1-mediated recruitment of neutrophils (Masumoto et al., 2006), HBP in the host cytosol is an alarm signal that stimulates innate cytokine production and recruits neutrophils to the site of infection.

Innate recognition of PAMPs provides critical instruction to the onset of adaptive immunity (Iwasaki and Medzhitov, 2010). Moreover, the ability of PAMPs to modulate immune
Figure 16  HBP is not sensed by the classic pattern recognition pathways. (A) NF-κB luciferase activity in 293T cells following RIP2 knockdown and treatment with HBP, the NOD1 ligand mTri-DAP, purified and mutanolysin digested peptidoglycan (Pg. Dig.) from *N. gonorrhoeae*, TNFα, or transfected with lysates from the indicated bacteria. (B) qRT-PCR analysis of the knockdown efficiency of RIP2 in 293T cells after normalization to *Gapdh* and expressed as a percentage of non-transduced cells. (C) IL-6 or IL-8 production by ELISA (top) and knockdown efficiencies (bottom) following transduction of THP-1 cells with MyD88, STING, CARD9, RIP2, or MAVS targeting shRNA and treated with HBP, LPS, c-di-GMP, MDP, or dsRNA. Data represent the mean of 3 independent experiments done in duplicate. Error bars ± s.e.m. *P < 0.05, **P < 0.01, by ANOVA.
Figure 17  HBP elicits an inflammatory response from primary human cells and in vivo. (A to C) Shown is cytokine production following treatment of primary human macrophages (A) primary human neutrophils (B) or immortalized epithelial cells from the human endocervix (C) with purified HBP containing (Wt) or deficient (ΔhldA) supernatants from N. gonorrhoeae or LPS (24hr). (D) ELISA of IL-6, IL-8, IL-23 or IFN-β production in primary human macrophages infected with N. meningitidis ΔgmhB or ΔhldA (6hr). (E and F) KC levels in mouse serum and air pouch washes (AP) (E) or neutrophil counts in the air pouch (F) following injection of HBP-containing or deficient purified culture supernatants from N. gonorrhoeae into previously raised dorsal pouches (n=6) (3hr). Data from A,B, and D are representative of 3 different donors, error bars ± s.t.d . Data from C represents 3 independent experiments, error bars ± s.e.m. *P < 0.05 by t-test.
cell maturation, cytokine production, and antigen presentation offers incredible potential for their use as vaccine adjuvants and cancer immunotherapy (Demento et al., 2011). To test if HBP contributed to the adaptive immunity during an infectious challenge, we analyzed the antibody titers produced following sub-lethal infection of mice with *N. meningitidis* ΔgmhB or ΔhldA, strains that differ only the presence of HBP. The HBP-producing strain (ΔgmhB) induced a transient increase in meningococcal-specific IgM, and significantly more class-switched anti-meningococcal IgG, in particular Th1-associated subclasses IgG2a, b and IgG3, upon rechallenge (Figure 18). This indicated that HBP can prime adaptive immune responses *in vivo*, speaking to its potential as a vaccine adjuvant. Thus, HBP contributes to the unique immunological signature of Gram-negative bacteria, and likely has a role in explaining why Gram negative bacteria are more potent adjuvants than Gram-positive bacteria expressing the same protein (Martner et al., 2013).

**Figure 18**  HBP primes the adaptive immune response *in vivo*. (A and B) Total anti-*N. meningitidis* (*Nm*) IgM or IgG serum titres at the indicated day, or individual IgG subclasses at day 35 (B) by whole bacteria ELISA following immunization and rechallenge of mice with 1 x 10^6 live *N. meningitidis* of the indicated genotype (n=10). Bacteria were cleared within < 12 h of injection. *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA. ns, not statistically significant.
2.5 Discussion

Conserved molecular structures that are essential for fitness and are of uniquely microbial origin are sensed by the host innate immune system to provide a first line of defense against pathogens. These PAMPs are sensed by germ-line encoded PRRs expressed on cellular membranes and within the cytosol, and mediate the production of cytokines via the transcription factor NF-κB. Here in Chapter 2, I showed that HBP is a novel PAMP that elicits an innate immune response upon detection within the cytosol of host cells. HBP is a 7-carbon sugar solely of microbial origin, and is incorporated into the inner core region of LOS/LPS from the ADP-activated heptose precursor. The biosynthetic pathway of ADP-activated heptose is conserved in virtually all Gram-negative bacteria, and disruptions in the pathway result in truncated, heptoseless LPS; a phenotype associated with a severe attenuation of bacterial virulence (Sandlin et al., 1995). Thus, bacterial modifications to this pathway to avoid immune detection are likely difficult. Interestingly, HBP is actively shed from Neisseria, whereas for signaling to be apparent in other Gram-negative bacteria, HBP must first be liberated from within the bacterial cytosol. How HBP is sensed by host cells remains an open question since my RNAi studies show that HBP detection occurs independently of known pattern recognition receptor pathways. Further identification of how HBP elicits a response from host cells is needed.

In considering the effects of HBP, its cytosolic detection does not elicit the inflammasome-activating, aggressive inflammatory characteristics associated with the sensing of cytosolic LPS (Hagar et al., 2013; Kayagaki et al., 2013), flagellin (Franchi et al., 2006) or prokaryotic RNA (Sander et al., 2011) that signify intracellular invasion or host cell membrane ‘piercing’ by bacterial secretion systems. Given that HBP can access the cytosol in the absence of bacterial virulence factors, the detection of HBP likely allows our innate immune system to detect phagosome-degraded bacterial components in the cytosol at lower threat level and with differing kinetics than surface TLRs, alerting the immune response to bacteria without the need to trigger panic-associated inflammatory cell death.
3  Chapter 3: TIFA mediates cytosolic detection of bacterial-derived HBP

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Author Contributions:
Ryan G Gaudet designed and performed the experiments, wrote the paper, and analyzed the data.

Dr. Jason Moffat supplied the RNAi library, and assisted with design and deconvolution of the RNAi screen (Figure 21).

Dr. Alan Cochrane assisted with the design of the HIV-1 reporter cell line (Figure 19).

Dr. Scott Gray-Owen designed experiments, analyzed the data and wrote the paper.

3.1 Summary

Heptose-1,7-bisphosphate, an intermediate in the synthesis of bacterial lipopolysaccharide, is sensed in the host cell cytosol by a mechanism that ultimately activates an NF-κB-dependent inflammatory response. However, how mammalian cells are able to sense cytosolic HBP remains an open question. Here, I describe a novel innate immune signaling pathway, mediated by the protein TRAF-interacting forkhead associated protein A (TIFA), that senses and initiates the cellular response to the presence of cytosolic HBP.

3.2 Overview

Germ-line encoded PRRs on the plasma or endosomal membranes and others in the cytosol sense PAMPs and mediate the production of proinflammatory cytokines via activation of the transcription factor NF-κB. Common bacterial PAMPs, sensed extracellularly or on the
luminal side of the phagosome, include LPS, peptidoglycan, flagellin, and CpG-containing DNA, which are sensed by TLRs -2, -4, -5, and -9, respectively (Blasius and Beutler, 2010; Kumar et al., 2011). In the host cytosol, peptidoglycan degradation products muramyl di-peptide and diaminopimelic acid containing muropeptides activate NOD2 and NOD1 (Girardin et al., 2002), while microbial nucleic acid alerts RLRs (Yoneyama et al., 2004) or the cytoplasmic DNA sensor cGAS (Sun et al., 2013). Following activation, each receptor recruits a defined set of adaptor proteins that mediate transcriptional responses though shared signaling intermediates. Upon stimulation, the TLRs, NLRs, RLRs and CDSs engage the proximal adaptor proteins MyD88, RIP2K, MAVS and STING, respectively, which then converge upon a shared set of signaling mediators, including the TRAF family of proteins. TRAF6 specifically is an E3 ubiquitin ligase essential for signaling downstream of the TLRs, NLRs, and RLRs, and mediates Lys63 (K63)-linked ubiquitination and activation of kinases that control NF-κB and mitogen activated kinase (MAPK) transcription factors (Ferrao et al., 2012).

In Chapter 2, I showed that microbial metabolite HBP represents a new PAMP unique to Gram-negative bacteria. Moreover, I showed that this metabolite is sensed within the cytosol of host cells following endocytosis and occurs independently of the known pattern recognition receptors described to date. Therefore, I undertook a genome-wide loss-of-function screen to identify host proteins that are essential for responding to HBP. Here, I describe this screen and report that it revealed that host sensing of HBP was dependent on the novel innate immune adaptor protein, TIFA, which links HBP sensing to the innate immune signaling hub TRAF6.

3.3 Experimental procedures

3.3.1 Cell culture and luciferase assays

293T were maintained in DMEM supplemented with 10% FBS, 1% glutamax, and 1% penicillin streptomycin. Jurkat 1G5 cells contain a stably-integrated LTR-luciferase reporter gene (Aguilar-Cordova et al., 1994), and were maintained in RPMI supplemented with 10% FBS and 1% glutamax. THP-1 cells were maintained in RPMI supplemented with 10% FBS and 1% glutamax and differentiated to macrophages with 50 ng/ml PMA for 48 hr, followed by a 48 hr rest period prior to stimulation. To measure LTR-driven luciferase, 1G5 cells were lysed and luminescence determined using the Luciferase Assay kit (Promega) according to manufacturers instructions. Results are expressed as fold change compared to untreated. 293T cells were transfected in 96
well plates with 90 ng ELAM firefly luciferase reporter plasmid (Chow et al., 1999) and 10 ng pRL-TK Renilla plasmid using TransIT LTI (Mirus). 18 hours later cells were treated for 6 hours and luciferase activity determined using the Dual-Glo Luciferase Assay System (Promega). Results are expressed as fold increase relative to transfected, mock treated cells following normalization to Renilla luciferase.

3.3.2 Purification of HBP supernatants

Purified HBP-containing (or HBP-deficient) supernatants, were isolated from spent Neisseria cultures essentially as described previously (Malott et al., 2013). Briefly, N. gonorrhoeae or N. meningitidis wild-type or ΔhldA were grown from OD550 0.18 to ~ 0.5 for 6 hours in RPMI containing 1% Isovitalex. Supernatants were digested with DNAse (10 µg/ml), RNAse (10 µg/ml), Proteinase K (100 µg/ml), boiled for 30 minutes, passed through an Amicon 3 kDa MW cutoff filter (Millipore) and a C18 Sep-Pak® cartridge (Waters). Any residual LOS was removed using endotoxin removal resin (Pierce) according to manufacturer’s instructions.

3.3.3 Reporter cell-line generation

The full-length HIV-1 molecular clone pLAI containing the following modifications was used as the reporter backbone: AvrII deletion of the 3’ end of Gag and the entire coding sequence of Pol, and a NdeI/Stul deletion of the 3’ end of Env. The Dsred allele was cloned into the Nef reading frame using BamHI and XhoI. Lentiviral particles were produced by co-transfection into 293T cells with pMDG.2 and psPAX2 to generate mature virus particles. Jurkat cells were transduced with the minimum dose required to see Dsred positive cells following treatment with 10 ng/ml TNFα. 48 hours post transfection, cells were treated with HBP and Dsred positive cells sorted by FACS. These cells were then cultured for 14 days, followed by Dsred negative cell sorting by FACS to remove constitutive expressors. 5 days later, cells were induced with HBP, and Dsred positive cells collected into 96 well plates (0.5 cells/well). Clones were individually tested for low basal Dsred expression, and high Dsred expression following treatment with both HBP and TNFα, with the preferred clone termed RG7.

3.3.4 Pooled genome-wide RNAi screen

A pooled lentiviral shRNA library containing 78,432 shRNAs targeting 16,056 Refseq Human genes (“80K library”) developed by the RNAi consortium (Moffat et al., 2006) and described
previously (Marcotte et al., 2012) was used to transduce $1.2 \times 10^8$ Jurkat-RG7 cells at an MOI of 0.3, resulting in 1500 fold coverage of each hairpin. 24 hours later, cells were re-suspended in complete RPMI containing $4 \mu g/ml$ puromycin. Following 3 days of selection, dead and early apoptotic cells were removed using a dead-cell removal kit (Miltenyi Biotec). 24 hours later, $\sim 2 \times 10^8$ cells were treated with HBP-containing supernatant and incubated for 48 hours in the presence of $2 \mu g/ml$ puromycin. Cells were stained with APC-Annexin V (BD Biosciences), and $5 \times 10^6$ APC-negative cells from both the lowest 5% of the Dsred expressing population, and highest 95% expressing Dsred population were collected. The process was repeated on successive days for a total of 4 replicates. Genomic DNA from each pool was harvested using a Qiagen DNEAsy Kit, precipitated, and re-suspended at 400 ng/ml in H$_2$O. shRNA barcodes were amplified by PCR, subject to Illumina sequencing and analyzed as described previously (Ketela et al., 2011). Data were normalized to reads per million reads, and a threshold was set to 0.1 reads/million reads. The MFC (mean fold change) was determined for each hairpin by dividing the mean number of reads from the ‘Dsred LOW’ fraction by the mean number of reads in the ‘Dsred HIGH’ fraction.

3.3.5 Flow cytometry

Live cells were re-suspended to $1 \times 10^6$ cells/ml in 2% FBS in PBS and analyzed using a FACSCalibur with CellQuest software (Becton Dickinson). Analysis was performed using FlowJo software (TreeStar). Cell sorting was done using an Aria I cell sorter (Becton Dickinson).

3.3.6 Confocal microscopy

293T cells were seeded on collagen-coated glass coverslips, treated for 4 hr with HBP, fixed with 4% paraformaldehyde, and permeabilized with 0.1% saponin. For visualization of FLAG-TIFA and TRAF6, cells were stained overnight at 4°C with Alexa Fluor 488-conjugated rabbit anti-FLAG (Cell Signaling) and mouse anti-TRAF6 (sc-8409; Santa Cruz), followed by 1 hour with Alexa Fluor 594-conjugated anti-mouse Ig (Life Technologies). For visualization of FLAG-TIFA and Lamp2, cells were stained overnight at 4°C with mouse anti-FLAG (M2; Sigma) and rabbit anti-Lamp2 (ab37024; Abcam), followed by 1 hour with Alexa Fluor 488-conjugated anti-mouse (Life Technologies) and Alexa Fluor - 594 conjugated anti- rabbit (Life Technologies). Slides were visualized using an LSM510 (Carl Zeiss) confocal microscope. For analysis, images were processed using ImageJ software.
3.3.7 FLAG-TIFA constructs and cell line generation

The TIFA coding sequence was amplified from cDNA derived from Jurkat cells and cloned into pMSCV-Blast (Clontech) containing one N-terminal FLAG sequence. Point mutations were inserted using QuickChange II mutagenesis kit (Agilent). Infectious virus was produced using the Pantropic Retroviral Expression System (Clontech). Viral titres were determined as above using AlamarBlue viability and target cells were infected at an MOI of 0.5, as described for lentivirus infections. Cells were then selected for 14 days with blasticidin to create polyclonal stable cell lines.

3.3.8 Knockout cell line generation

Human Cas9 and guide RNA expression plasmid hSpCas9-2A-Puro (pX459) (Addgene) was a gift from S.E. Girardin. Tifa targeting RNA sequences 1: 5ʹ-cagatgacggtttattac-3ʹ, or 2: 3ʹ-tgtgatgccatcttcaac-5ʹ were designed using software available at http://crispr.mit.edu. Double stranded templates were generated from single stranded oligonucleotides 5ʹ-caccgcagatgacggtttaccatcc-3ʹ and 5ʹ-aaacggatggtaaaccgtcatctgc-3ʹ (KO-1) or 5ʹ-cacggctgtgatgccatcttcaac-3ʹ and 5ʹ-aaacgttttgaagatgctgacacac-3ʹ (KO-2) and cloned into pX459 as described previously. Cleavage efficiencies were evaluated using SURVEYOR mutation detection kit (Transgenomic). Following transfection into 293T cells and selection with 2 µg/ml puromycin, single cell clones were obtained by limiting dilution. Clones of the desired genotype were selected by cloning and sequencing of PCR-amplified Tifa genomic DNA using PCRTM4Blunt-TOPO® (Life Technologies). KO-1 was homozygous for an in-frame 3 amino acid deletion (23-25), while KO-2 was genotyped as containing 2 heterozygous mutations, each containing a substitution that disrupted the Tifa start codon. When indicated, cell lines were transfected with pUNO1-hNOD1 (Invivogen) or pDUO-hCD12/TLR2 (Invivogen) for 72 hr prior to treatment.

3.3.9 Mass-spectrometry of FLAG-TIFA

Following treatment with HBP and immunoprecipitation and SDS-PAGE described above, bands corresponding to 24 kDa (TIFA-1XFLAG), subject to in-gel trypsin digestion, and incubated overnight with CNBr (Sigma) in 70% trifluoroacetic acid. LC-MS/MS analysis was performed on a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer coupled with
Easy-nLC 1000 LC system (San Jose, CA). The peptide mixtures were separated using a 0 to 90% acetonitrile gradient in 0.1% formic acid over 60 minutes. Tandem mass spectra were extracted, charge state deconvoluted and de-isotoped by Bioworks version 3.3 (Thermo Electron). All MS/MS samples were analyzed using Sequest (XCorr Only) (Thermo Fisher) to search TIFA-Modified-FASTA sequence database assuming digestion with trypsin and CNBr. Fragment ion mass tolerance was 0.030 Da and a parent ion tolerance of 15 PPM. Peptide identifications were accepted if they could be established at 100.0% probability to achieve an FDR (false discovery rate) less than 0.1% using Scaffold (Proteome), and phosphopeptides validated by inspection. The percentage of TIFA-derived MTSFEDAdpTEETVTCLQM peptides, where ‘pT’ indicates phosphothreonine, among the total T9-containing peptides were determined for the HBP treated and untreated samples using Scaffold PTM (Proteome).

### 3.3.10 Immunoprecipitation and Immunobloting

FLAG-TIFA was immunoprecipitated from Jurkat cells using FLAG M2 agarose beads (Sigma) as described previously (Chen and Gingras, 2007). Briefly, cells were lysed in 50 mM Hepes-KOH pH 8.0, 100 mM KCl, 2mM EDTA, 0.1% NP40, 10% glycerol. Soluble cell lysates were pre-cleared for 2 hr with mouse IgG agarose (Sigma) and immunoprecipitated overnight at 4°C. Bound proteins were eluted using 0.5 M NH₄OH pH 11 (for mass-spectrometry) or 3X FLAG-peptide (Sigma) (for immunoblot analysis). TRAF6 was immunoprecipitated from Jurkat cell lysates using rabbit anti-TRAF6 (sc-72201; Santa Cruz) conjugated to protein A/G PLUS-agarose (Santa Cruz) and eluted in sample buffer. Whole cell lysates or immunoprecipitated eluates were immunoblotted with the following antibodies: M2 anti-FLAG (Sigma), rabbit anti-FLAG (Sigma), mouse anti-TRAF6 (sc-8409; Santa Cruz), rabbit anti-TRAF2 (sc-876; Santa Cruz), mouse anti-ubiquitin (sc-8017; Santa Cruz), mouse anti-beta actin (Sigma).

### 3.3.11 Native PAGE

For BlueNative PAGE, cells were lysed in 1% NP-40, 0.1% Triton-X100, 0.1% SDS and soluble lysate separated by gradient PAGE (8-16%) using ExpressPlus system™ (GenScript) as described previously (Kofoed and Vance, 2013). For ClearNative PAGE, cells were lysed in 1% NP-40, 0.1% Triton-X100, 0.1% SDS and soluble lysate separated on 12.5% tris-glycine polyacrylamide gels.
3.4 Results

3.4.1 A genome-wide screen reveals TIFA is essential for the host response to HBP

I conducted a human genome-wide RNAi screen to identify host proteins that mediate the response to HBP. To maximize the RNAi phenotype, I created novel latent HIV-1 reporter Jurkat T cell lines to take advantage of a unique characteristic of LTR-driven transcription: a TAT-mediated positive feedback loop that drives phenotypic bifurcation (Weinberger et al., 2005) (Figure 19A and B). HBP was able to potently induce latent HIV gene expression (Figure 19C). I then optimized screening conditions by titrating an NFKB1-specific small hairpin RNA (shRNA) into a lentiviral pool containing 78,000 (78K) unique sequences, and monitored HBP-mediated LTR-Dsred expression following transduction (Figure 20). Conditions were selected that maximized the number of Dsred negative cells in the highest titer of the NFKB1-specific shRNA in the total pool. I transduced the reporter-containing cell line with a pooled genome- Hits were defined as genes with at least 2 targeting hairpins found at a 4-fold greater frequency in the LOW vs. HIGH fractions following sequencing of hairpin barcodes amplified from the genomic DNA. NFKB1 and RELA, known to be required for HBP-mediated LTR activation, were both identified as hits, validating the screen (Figure 21A). To identify HBP-specific signaling regulators, I chose the top 10 scoring genes after filtering out general cellular machinery and conducted a secondary screen assessing the respective proteins’ involvement in other NF-κB signaling pathways, specifically TLR5 and TNFR. Of these 10, TRAF-interacting forkhead associated protein A (TIFA), a ubiquitously expressed cytoplasmic protein known to activate NF-κB in 293T cells (Kanamori et al., 2002; Takatsuna et al., 2003) seemed uniquely required for HBP signaling, as 2 non-overlapping sequence-targeting hairpins abrogated HBP-mediated Dsred expression, while showing little effect on flagellin- and TNFα-driven expression (Figure 21B). Furthermore, TIFA knockdown specifically attenuated the HBP-induced pro-inflammatory transcriptional response of a variety of immunologically relevant genes 2 hours post treatment with HBP (Figure 21C). I then rescued the RNAi phenotype by creating Jurkat cell lines expressing a recombinant 1XFLAG epitope-tagged TIFA and then treating these with an shRNA targeting the 3’ UTR absent in the recombinant construct (Figure 21D). Importantly, TIFA knockdown abrogated Gram-negative lysate-mediated NF-κB activation without affecting the cellular responses to TNFα in 293T cells (Figure 22A), and the defective phenotype could be rescued by stable
Figure 19  Construction of a HBP-sensitive latent HIV-1 Jurkat T cell line. (A and B) A depiction of the construct termed RG5 - the genome of HIV-1 modified to include the DsRed open reading frame in the Nef position. Depicted in black are the expressed viral proteins. The construct was engineered into Jurkat T cells to generate a stable latent HIV-1 reporter cell line following successive FACS sorting of DsRed positive and DsRed negative cell populations following treatment with or without HBP shown in (B). (C) Flow cytometry analysis of DsRed expression in a single selected clone termed “RG7” following treatment with an increasing amount of HBP-containing supernatants. (C) is representative of 2 independent experiments.
Figure 20  Optimization of the RNAi screen. Flow cytometry analysis of DsRedd expression expressed as a percentage of max following titration of an *NFKB1* targeting shRNA into the 78 000 shRNA library at the indicated percentage, transducing Jurkat RG7 cells at an MOI = 0.3, and monitoring the change in resulting DsRed negative cells following treatment with HBP. Depicted on the right is the quantification of the percentage of cells gated as DsRed negative.

expression of FLAG-TIFA (Figure 22A). To further confirm my RNAi results, I created 293T cell lines that were knocked out for TIFA using CRISPR/Cas9 technology. Strikingly, knockout of TIFA in 293T cells completely abolished HBP and *E. coli* mediated NF-κB activation (Figure 22B). Conversely, ectopic expression of TLR2 or NOD1 rendered TIFA knockout cells responsive to Pam3CSK4 or C12-iE-DAP, respectively, distinguishing the TIFA-dependent pathway from the TLR and NLR pathways. These results implicate TIFA as an essential component of the HBP signaling pathway.
Figure 21  Identification of TIFA as an essential gene for the cellular response to HBP. (A) is a schematic of the RNAi screen used to identify HBP signaling mediators; The 78K lentiviral library was used to transduce Jurkat reporter (RG7) cells harboring a latently integrated HIV-LTR-DsRed construct. Following selection and treatment with HBP, live cells were sorted into LOW and HIGH DsRed fractions and the abundance of each hairpin in each fraction determined using Illumina sequencing. The mean fold change (MFC) of each hairpin was calculated from the normalized number of reads in the LOW and HIGH fractions from 4 replicates performed on separate days. Genes were classified as hits if >2 unique targeting shRNAs had an MFC of >4. Shown in empty circles are the position of the two shRNAs targeting the indicated genes: NFKB1, RELA, and TIFA. (B) Knockdown of TIFA abrogates HBP-mediated DsRed expression. Jurkat RG7 reporter cells were transduced with one of two TIFA targeting shRNAs (red histograms), or a scrambled shRNA (black histograms) and either left untreated (grey filled histogram), or treated with HBP, TNFα, or flagellin. DsRed expression was determined 48 hr later using FACS. (C) Knockdown of TIFA abrogates the HBP induced pro-inflammatory transcriptional response. qRT-PCR analysis of previously identified HBP-upregulated genes in Jurkat cells transduced with shRNAs targeting TIFA, RelA, or scrambled, then treated with HBP, TNFα, or flagellin for 2 hours. (D) Luciferase activity of Jurkat 1G5 cells transduced with lentiviral MSCV-driven FLAG-TIFA, or empty vector, and treated with shRNAs targeting the TIFA-untranslated region (UTR), or the coding sequence (CDS) then treated with HBP (6hr). Results from B to D are representative of 3 individual experiments, error bars ± s.e.m.
Figure 22  TIFA is required for the host response to Gram-negative bacterial lysates. (A) NF-κB luciferase activity following TIFA knockdown in 293T cells and treated with HBP containing supernatants, TNFα, or transfected with the indicated Gram-negative lysate. White-bars represent stable expression of FLAG-TIFA (eTIFA) and knockdown with the TIFA-untranslated region (UTR) targeting shRNA. (B) NF-κB luciferase activity in wildtype or TIFA knockout 293T cells (KO-1 and KO-1 are two individual clones). Cells were transfected with E. coli lysate, or treated with HBP, PAM3CSK4, C12-iE-DAP or TNFα. Where indicated cells were transfected with TLR2 or NOD1 expression vectors prior to treatment. Data represent 3 independent experiments, errors bars s.e.m.

3.4.2 TIFA is uniquely essential for the response to HBP

TIFA is not essential for the NF-κB response to bacterial flagellin through TLR5, or to the cytokine TNFα. However, to prove that TIFA is uniquely required for HBP sensing, I tested the effect of silencing TIFA in THP-1 macrophages, a cell-line capable of responding to a broad array of microbial signals. TIFA knockdown prevented the HBP-dependent increase in IL-6 following infection of THP-1 macrophages with N. meningitidis (Figure 23A) or live opsonized E. coli ΔwaaC (Figure 23B). TIFA knockdown also abrogated IL-6 and IL-8 production in THP-1 macrophages otherwise seen upon HBP treatment (Figure 24). However, this effect was remarkably specific for HBP, as TIFA was completely dispensable for macrophages to respond to a variety of other PAMPs of bacterial and viral origin (Figure 24). Thus, TIFA is an essential component of the HBP signaling pathway specifically.
Figure 23  TIFA is required for HBP sensing by THP-1 macrophages. (A and B) TIFA knockdown and IL-6 production in THP-1 macrophages infected with live *N. meningitidis* (6hr) or live-opsonized *E. coli* (24 hr) (B) of the indicated genotype by ELISA. Data represent 3 independent experiments, error bars s.e.m. *P < 0.001 by ANOVA.
TIFA is not essential in THP-1 macrophages for responding other PAMPs. ELISA measurement of IL-6 (top two rows) or IL-8 (bottom row) from supernatants from THP-1 macrophages expressing TIFA or scrambled shRNA and treated with the indicated PAMP ligand for 6 hr; Pam3SK4 (Pam3), *N. gonorrhoeae* derived peptidoglycan (Pg.), flagellin (Flag), zymosan (Zym), muramyl dipeptide (MDP). Data are from 3 independent experiments (error bars s.e.m). **P < 0.01 by t-test.

3.4.3 TIFA links HBP detection with TRAF6 activation

TIFA overexpression studies suggest that TIFA-mediated NF-κB activation occurs via its constitutive association with and activation of the ubiquitin ligase TRAF6 (Ea et al., 2004). However, since these studies were done prior to my identification of any agonist of TIFA, it remained uncertain whether this was a physiologically relevant cellular response. To determine if HBP has an effect on the TIFA-TRAF6 interaction, I created stable Jurkat and 293T cell lines expressing FLAG-TIFA from an MSCV promoter and knocked down endogenous TIFA using a TIFA-UTR targeting shRNA. In this system, there is not a constitutive association between TIFA and TRAF6.
Figure 25  HBP induces a TIFA-TRAF6 interaction. (A) immunoprecipitation (IP) of FLAG-TIFA in Jurkat cells expressing a TIFA UTR targeting shRNA and treatment with HBP-containing or deficient supernatant, and immunoblot for TRAF6, TRAF2, or FLAG-TIFA (2 hr). (B) Immunofluorescence microscopy in 293T cells of the formation of a TIFA - TRAF6 complex with or without HBP (3 hr), scale bars, 10 µm. Data are representative of ≥2 independent experiments.

and TRAF6, presumably because TIFA is expressed at more physiologically relevant levels. However, HBP did associated with TRAF6 upon exposure to HBP, and this was apparent as early as 30 min after treatment (Figure 25A). No interaction was observed with TRAF2, another proposed TIFA binding partner (Kanamori et al., 2002). Immunostaining indicated that TIFA co-localized with TRAF6 in distinct foci upon HBP treatment (Figure 25B). The large TIFA and TRAF6-containing structures are reminiscent of the large oligomeric complexes seen following recruitment of TRAF6 to TLR- or IL-1R-induced signalosomes, which activate the TRAF6 E3 ubiquitin ligase activity (Ferrao et al., 2012). Correspondingly, ubiquitin chains could be detected in both the N. gonorrhoeae-infected or HBP-induced TIFA-TRAF6 complexes following TIFA immunoprecipitation, indicating the ubiquitin ligase activity of TRAF6 was also being activated in response to HBP (Figure 26A). This was TIFA-dependent, as TIFA depletion abrogated HBP-mediated TRAF6 ubiquitination, whereas TLR5-mediated ubiquitination of TRAF6 was unaffected (Figure 26B). TRAF6 is recruited to the TLR and IL-1R signalosomes by the IRAK family of serine and threonine kinases. Yet, RNAi depletion indicated HBP signals independently of IRAK1, -2, and -4 (Figure 27). Therefore, TIFA is a novel entry point into the TRAF6-mediated NF-κB activation pathway downstream of HBP.
Figure 26 TIFA mediates HBP-inducible TRAF6 activation and ubiquitination. (A) Immunoprecipitation (IP) of FLAG-TIFA and immunoblot of TRAF6, FLAG-TIFA, or ubiquitin, in Jurkat cells treated with HBP-containing or deficient supernatant (sup), or infected with *N. gonorrhoeae* of the indicated genotype and MOI (2hr). (B) TIFA knockdown and TRAF6 IP analysis of the TIFA-TRAF6-ubiquitin complex in Jurkat cells treated with HBP or flagellin. Data are representative of ≥2 independent experiments.

Figure 27 HBP signaling is independent of IRAK-1,-2 and -4. (A) shRNA knockdown of IRAK1, IRAK2, or IRAK4 and IL-8 production in THP-1 macrophages treated with HBP-containing supernatants, or flagellin (24 hr). (B) qRT-PCR assessment of the knockdown efficiency of IRAK-1, IRAK-2, or IRAK-4 shRNA. Data were normalized to *GAPDH*, and expressed as a percentage of the mRNA observed in cells not expressing an shRNA. Data are from 3 independent experiments. *P < 0.05 by ANOVA (error bars s.e.m).
3.4.4 HBP triggers TIFA phosphorylation-dependent oligomerization

It has been previously shown that TIFA overexpression results in self-association via constitutive phosphorylation at threonine-9 (pT9) leading to oligomerization via intermolecular pT9 binding with the central forkhead-associated domain (FHA) (Huang et al., 2012). I hypothesized that constitutive TIFA phosphorylation and oligomerization previously observed were a result of overexpression, and is in fact, a HBP-specific signaling mechanism. To test this, FLAG-TIFA was immunoprecipitated and analyzed by LC-MS/MS from HBP- or mock-treated Jurkat cells stably expressing FLAG-TIFA. Indeed, Thr9 was phosphorylated only after treatment with HBP (Figure 28A). Furthermore, cells treated with the TIFA-UTR-specific hairpin and re-constituted with recombinant non-phosphorylatable TIFA T9A, TIFA containing conserved mutations in the FHA domain (G50E/S66A), or TIFA containing a mutation in the TRAF6 binding site (E178A), were unresponsive to HBP treatment (Figure 28B), nor could they bind TRAF6 in a HBP-dependent manner (Figure 28C). T9 phosphorylation is only essential for HBP-dependent signaling, since upon over-expression in 293T cells, the T9A mutant can still activate the HIV LTR when co-transfected, whereas the FHA (G50E/S66A), and TRAF6 (E178A) mutants cannot (Figure 28D). HBP also triggered TIFA oligomerization when analyzed by clear native-PAGE (Figure 29A and B) or blue native PAGE (Figure 29C), in a process independent of TRAF6 binding, but completely dependent on Thr9 phosphorylation, as TIFA T9A failed to oligomerize upon HBP treatment. Moreover, treatment of lysates with protein phosphatase destroyed the high molecular weight TIFA complexes (Figure 29B). Thus, HBP induces phosphorylation of TIFA Thr9, triggering intermolecular binding between pT9 and the FHA domain, leading to TIFA oligomerization and subsequent TRAF6 recruitment and activation.

3.4.5 HBP induces TIFA oligomerization at the lysosomal compartment

Given that soluble HBP gains access to the cytosol via endocytosis, and that phagocytosis of E. coli liberated HBP in macrophages, I hypothesized that lysosomes may play a role in mediating TIFA signaling. Treatment of 293T cells with soluble HBP induced the formation of the large TIFA aggregates, or “TIFAsomes”, that co-localized with the late endosomal/lysosomal marker Lamp 2 (Figure 30). This was independent of TRAF6 binding, but was dependent on Thr9 phosphorylation and a functional FHA domain, as HBP-induced TIFAsomes were evident.
in cells re-constituted with TIFA E178A but not TIFA T9A or G50E/S66A. Thus, HBP treatment induces TIFA phosphorylation-dependent complex formation at the lysosome.

Figure 28  TIFA phosphorylation at Threonine 9 is essential for HBP-inducible signaling. (A) is a depiction of the primary structure of TIFA and quantification of a phospho-threonine 9 (pT9) peptide of FLAG-TIFA immunoprecipitated from stable 293T cells with or without HBP treatment. (B) LTR-driven luciferase activity in Jurkat 1G5 cells stably expressing FLAG-TIFA wild-type (WT), T9A, G50E S66A, or E178A, then treated with scrambled shRNA, or shRNA specific for the TIFA 3’ UTR, (UTR) or coding sequence (CDS) and treated with HBP (6hr). (C) Immunoprecipitation (IP) analysis of the HBP-induced TIFA-TRAF6 interaction in Jurkat cells stably expressing FLAG-TIFA wildtype (wt), T9A, G50E S66A, or E178A, then treated with TIFA 3’ UTR specific shRNA. (D) Co-transfection of 293T cells with a HIV-1 LTR-DsRed construct, and pMSCV-FLAG-TIFA of the indicated genotype and FACS analysis of the number DsRed positive cells after 36 hours. Data from B and D represent 2 independent experiments (error bars ± s.e.m.), C is representative of ≥3 independent experiments.
Figure 29 HBP induces TIFA phosphorylation-dependent oligomerization. (A) Clear native PAGE (top) or SDS-PAGE (bottom) and immunoblot analysis of Jurkat cells stably expressing the indicated FLAG-TIFA construct, transduced with a TIFA 3’ UTR specific shRNA, and treated with HBP. (B) Clear native PAGE and Immunoblot analysis of FLAG-TIFA oligomerization in HBP treated Jurkat cells. Lysates were treated with or without λ protein phosphatase (λPPase) before running the gel. (C) Blue native PAGE analysis of Jurkat cells stably expressing FLAG-TIFA, a TIFA UTR-targeting shRNA, and treated with HBP for the indicated time. Estimated molecular weight markers based on the NativeMARK™ protein standards are indicated on the right. Data are representative of of ≥2 independent experiments.
Figure 30  HBP induces TIFA oligomerization at lysosomal compartments. Shown is confocal microscopy of FLAG-TIFA (green) and Lamp2 (red) in 293T cells stably expressing the indicated FLAG-TIFA construct, transduced with TIFA 3’ UTR specific shRNA, and treated with HBP (4hr). Scale bars, 10 µm. Data are representative of at least 3 independent experiments.

3.5 Discussion

Together, these results indicate that HBP is sensed in the host cytosol by a novel cytosolic surveillance pathway that requires the protein TIFA. Whether TIFA is the receptor for HBP is currently not known, however, consistent with TIFA being a proximal signaling molecule in the sensing of HBP, HBP-induced TIFA aggregates were found at the lysosome, where HBP initially gains access to the cytosol. HBP-dependent activation stimulates TIFA
phosphorylation, oligomerization and activation of the ubiquitin ligase TRAF6, which leads to the activation of NF-κB-dependent transcription of proinflammatory genes. Considering that other innate immune adaptor proteins are not essential for HBP-detection, TIFA is the key molecule in linking HBP detection with the common PAMP signaling hub TRAF6. An unanswered question is whether TIFA polymorphisms exist that predispose individuals to either inflammatory diseases, or infection by Gram-negative bacteria. As the TIFA activating signal was unknown prior to this work, very little is known about the role of TIFA in the development of human disease. In depth analysis of TIFA using genome-wide association scanning (GWAS) may shed light on this issue.

It is intriguing why HBP detection occurs in the cytosol. Indeed, HBP had to be liberated (through shedding or lysis) and subsequently pass through a host membrane (plasma or endosomal) to access the cytosol in each of the settings described in Chapter 2. If these were the only contexts in which HBP could be sensed, then evolution would seem to dictate that the receptor for HBP be located in the endosomal membrane where HBP concentrations would be highest. However, all the data presented thus far has pointed to the receptor for HBP being strictly cytosolic. Moreover, every cytosolic PRR pathway described to date primarily detects pathogens that either access the cytosol via secretion systems or directly through vacuolar escape. Therefore, I hypothesized that a primary physiological role of TIFA-mediated HBP detection would be in sensing intracellular bacteria.
4 Chapter 4: Innate immune sensing of intracellular bacterial replication by the TIFA-dependent cytosolic surveillance pathway

The contents of this chapter have been submitted to Immunity.

Author Contributions:

Ryan G Gaudet designed and performed the experiments, wrote the paper, and analyzed the data.

Dr. Raphael Molinaro and Dr. Stephen Girardin constructed the NOD1, NOD2 and NOD1/2 double knockout HCT 116 cell lines.

Halia Kottwitz and Dr. John R. Rohde constructed the PTA and ACKA Shigella flexneri mutants.

Dr. Scott Gray-Owen designed experiments, analyzed the data and wrote the paper.

4.1 Summary

In Chapters 2 and 3, I showed that the bacterial metabolite HBP represents a PAMP unique to Gram-negative bacteria. HBP is sensed within the host cytosol by a novel pattern recognition pathway that depends upon the conserved vertebrate protein TIFA. Despite the fact that the TIFA signaling axis operates within the cytosol, whether this pathway is important for sensing intracellular Gram-negative bacteria remains unknown. Indeed, in the context of an infection, the previous 2 chapters demonstrate that TIFA-dependent detection of HBP contributes to innate immune recognition of the extracellular bacteria Neisseria meningitidis, N. gonorrhoeae and E. coli. I reasoned that if the TIFA pathway was only important for sensing extracellular bacteria, than sensing HBP would occur on the cell surface or within the endosomal pathway, rather than within the host cytosol. Therefore, I hypothesized that TIFA-dependent detection of HBP has a role in innate recognition of invasive Gram-negative bacteria.

Intestinal epithelial cells (IECs) act as frontline sentinels for cytosol-invasive bacteria through the intracellular receptors NOD1 and NOD2. Here I report that a TIFA-dependent cytosolic surveillance pathway senses invasive bacteria in a manner complementary to, yet
functionally independent from, NOD1 and NOD2. Invasive *Shigella flexneri*, or a vacuole-escaping *Salmonella* mutant, released the bacterial metabolite heptose-1,7-bisphosphate (HBP) during cytosolic growth, triggering a sustained wave of TIFA activation that followed transient activation of the NOD1 pathway. Bacterial growth within the cytosol was essential for HBP release and concomitant TIFA activation, since prolonged infection with wild-type *Shigella*, but not metabolically attenuated invasive mutants, triggered TIFA-oligomerization and signaling to drive a massive NOD-independent NF-κB response from human IECs. My findings identify the TIFA pathway as an immunosurveillance system that temporally complements the NODs, and one that is essential for escalating the immune response to invasive Gram-negative bacteria that exploit the host cytosol for growth and replication.

### 4.2 Overview

Pattern recognition by the innate immune system provides the first line of defense against infection (Janeway, 1989). Sensing of PAMPs, invariant molecules broadly present in microbes yet different from self, alert the host to a microbial presence and, depending on the context in which they are recognized, elicit an immune response that is normally commensurate with the microbial threat presented. The cellular compartment in which the microbial product is sensed is of particular importance as contamination of the cytosol serves as an indicator for virulence, often eliciting a more robust response than the same PAMP sensed on the cell surface (Vance et al., 2009). The mechanisms for scaling microbial threats are especially important in the epithelium, the physical barrier between the host and external environment. Intestinal epithelial cells (IECs), through mechanisms that remain poorly defined, maintain immune homeostasis with trillions of commensal microbiota, yet mount robust inflammatory responses in settings of pathogen infection (Peterson and Artis, 2014). Indeed, the relative tolerance of IECs to non-invasive bacteria, coupled with their broadly expressed repertoire of cytosolic NOD-like receptors (NLRs), allow IECs to act as sentinels for bacterial invasion of the cytosol (Philpott et al., 2014; Sellin et al., 2015).

A classic model for invasive intestinal pathogens is *Shigella flexneri*, a foodborne bacteria that invades the colonic epithelium causing shigellosis in humans. *Shigella* crosses the epithelial barrier via M cells, then invades IECs basolaterally through the activity of their type III secretion apparatus (T3S) (Phalipon and Sansonetti, 2007). Following internalization, *Shigella* immediately escapes the entry vacuole and multiplies rapidly in the cytoplasm, using actin-based
motility to spread to adjacent cells. The innate response to *Shigella* is characterized by massive production of the chemokine IL-8, which recruits neutrophils to control the infection at the intestinal level but can also contribute to immunopathogenesis characteristic of shigellosis (Sansonetti et al., 1999). Most of the IL-8 induced by *Shigella* is produced by IECs, and is thought to occur primarily following intracellular recognition by the NLR family member NOD1 (Girardin et al., 2001; 2003a). NOD1 recognizes a unique D-Glu-meso-diaminopimelic acid (DAP) component of Gram-negative peptidoglycan, and drives a pro-inflammatory transcriptional response via activation of the transcription factor NF-κB. While evidence that invasive *Shigella* activates NOD1 is abundant, whether additional pathways are also required to drive NF-κB activation remains unclear. However, the lingering *Shigella*-induced cytokine responses by murine embryonic fibroblasts (MEFs) deleted for RIP2 (Sorbara et al., 2013; Travassos et al., 2010), the adaptor essential for NOD1 and NOD2 signalling, hints at the existence of additional NF-kB-activating sensors for cytosol-invasive bacteria.

In Chapter 2, I demonstrated that a metabolic intermediate in LPS biosynthesis, heptose-1,7-bisphosphate (HBP), represents a new PAMP specific to Gram-negative bacteria (Gaudet et al., 2015). Host recognition of HBP requires its liberation from within the bacterial cytosol, which can occur through active shedding by *Neisseria* species (Malott et al., 2013) or, in the case of enteric bacteria, through extracellular or intraphagosomal bacteriolysis (Gaudet et al., 2015). In Chapter 3, I show that sensing of liberated HBP occurs within the host cytosol following endocytosis, and requires the TRAF-interacting forkhead associated protein A (TIFA), the central mediator of a cytoplasmic surveillance pathway specific for HBP. Contamination of the host cytosol with HBP induces TIFA phosphorylation-dependent oligomerization, which activates the E3 ubiquitin ligase TRAF6 to drive an NF-κB-dependent pro-inflammatory transcriptional program. Although epithelial cells can internalize HBP from the surrounding milieu, the requirement for cytosolic sensing suggested that an essential physiological function of the TIFA pathway might be in detecting intracellular bacteria. However, bacteria other than *Neisseria sp.* tend not to liberate abundant HBP and the initiating event in TIFA-mediated HBP detection has not been described, so it remained unknown whether intracellular pathogens would trigger TIFA activation in the context of an infection.

Here, I report that the TIFA-TRAF6 signaling axis constitutes a NOD-independent, cytoplasmic surveillance pathway specific for invasive Gram-negative bacteria. Invasive *Shigella*
*flexneri* and *Salmonella* Typhimurium that can escape the vacuole both triggered the TIFA signaling cascade. In contrast to NOD1, which was activated within minutes of infection, TIFA activation occurred later and required shedding of HBP during bacterial proliferation within the host cytosol. Despite the functional independence of NOD1 and TIFA pathways, they are also intimately linked, as NOD1 activation during the initial bacterial invasion further induced *TIFA* transcription. Our results suggest that TIFA-dependent detection of HBP represents a previously unappreciated mechanism whereby gut IECs act as dynamic sensors for cytosol-invading microbes.

### 4.3 Experimental Procedures

#### 4.3.1 Cell Culture and Reagents

All cells were cultured continuously in antibiotic free medium. HCT 116 cells were maintained in McCoy’s 5A medium, Caco-2 cells and 293T cells were maintained DMEM, HeLa cells were maintained in RPMI, and T84 cells maintained in DMEM/F12. All media was supplemented with 10% FBS and 1% glutamax. Immortalized mouse intestinal epithelial cells (m-ICc12) were maintained in DMEM/F12 supplemented with 100 nM dexamethasone, 2 nM tri-iodinated L-Thyronine, 10 nM epidermal growth factor, 1% insulin-transferrin-selenium A (Gibco), and 2% FBS as described previously (Bens et al., 1996). Flagellin (10 µg/ml), TNFα (50 ng/ml), C12-iE-DAP (20 µg/ml), LPS (100 ng/ml) and muramyl di-peptide (MDP; 10 µg/ml) were each from Invivogen.

#### 4.3.2 Luciferase assays

293T cells were transfected in 96 well plates with 90 ng ELAM firefly luciferase reporter plasmid and 10 ng pRL-TK *Renilla* plasmid using TransIT®-LT1 (Mirus). 18 h later, cells were treated for 6 h and luciferase activity determined using the Dual-Glo Luciferase Assay System (Promega). Results are expressed as fold increase relative to transfected, mock-treated cells following normalization to *Renilla* luciferase.

#### 4.3.3 Bacterial strains and infections

The following bacterial strains were used: *Shigella flexneri* M90T, and its non-invasive variant BS176, *N. gonorrhoeae* MS11, *E. coli* DH5α *ΔhldE* and Δwaac have been described previously (Gaudet et al., 2015), S. Typhimurium strain 14028S and ΔsifA were a generous gift from W.W.
Navarre, *L. monocytogenes* EGDe. *Shigella* M90T Δ*pta* and ΔackA were constructed as described below. For *Shigella* infections, single colonies from tryptic soy agar with 0.1% congo-red were grown overnight in tryptic soy broth (TSB; BD), then diluted 1/100 and grown to an OD$_{600} = 0.5$, washed twice and infected by spinoculation for 15 min at 2000 x g. Following incubation for 30 min at 37°C, samples were washed three times and replaced with fresh growth medium containing 80 µg/ml gentamicin. Where indicated, 100 mM acetyl-phosphate (Sigma) was included in the infection media and during all subsequent washes and incubations.

### 4.3.4 *Shigella* mutant construction

Knock-out cassettes were generated using PCR with the indicated primers to amplify linear fragments of DNA containing the kanamycin resistance gene flanked by 150-460 bp of homology sequence to the gene of interest using the *pta::kan* or *ackA::kan* deletion mutant strains from the Keio collection as template (Baba et al., 2006). The following primers were used: *pta* 5ʹ-ggtacctgctctggtggtaatccc-3ʹ and 5ʹ-caccaacgtatcgggcattgccc-3ʹ; *ackA* 5ʹ-cecgattttcgacaagacggggcaaagcg-3ʹ and 5ʹ-cagcggttcagcggccgtcgtggtggaagttcgc-3ʹ. To prepare template, a colony from the appropriate Keio mutant strain was suspended in 100 µL of distilled water and 1 µL was added to the 25 µL PCR reaction. Knock-out cassettes were purified using a PCR purification kit (Qiagen) and used for λ red-mediated recombination to create the mutants as described previously (Sidik et al., 2014) but with kanamycin used in the recovery media. Integration of the knock-out cassette at the desired location was confirmed by PCR with the reverse primer used to create the knock-out cassette and the indicated forward check primers *pta* 5ʹ-cggtggtttccgtctgctatccgcaacggt-3ʹ; and *ackA* 5ʹ-gcaggcgacggtaacgttcagcatttgccgg-3ʹ upstream of the region deleted for each mutant.

### 4.3.5 Heptose-1,7-bisphosphate (HBP) synthesis

HBP was synthesized enzymatically from sedoheptulose 7-phosphate (Sigma) using recombinant GmhA and HldA purified from *N. meningitidis* as previously described (Gaudet et al., 2015).

### 4.3.6 Lentivirus production and infection

pLKO.1-based lentiviral particles were produced as previously described (Moffat et al., 2006). For each gene targeted, a minimum of 5 shRNAs were first tested for effective titer using alamar blue viability assays and for gene silencing using real-time qPCR. Target cells were infected at
an equal titer in media containing 8 µg/ml polybrene. 24 hours later, cells were selected with 4 µg/ml puromycin. Cells were harvested after 72 hours and knockdown efficiency was again confirmed by qPCR.

4.3.7 Real-time quantitative PCR and ELISAs

RNA was isolated using an RNeasy kit (Qiagen) per manufacturer’s protocol and treated with TURBO DNase (Life Technologies). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) and amplified using SsoAdvanced SYBR Green (Bio-Rad) using a C1000 thermal cycler (Bio-Rad). Human target genes were amplified using the following primers: GAPDH 5’-ttgaggtcaatgaaggggtc-3’ and 5’-gaaggtgaaggtcggagtca-3’; IL8 5’-agcaactcttgccaaactg-3’ and 5’-cgggaaggaacatctcactg-3’; TIFA 5’-caacaggttcccaggttca-3’ and 5’-tgccacgatcagttgtcc-3’. NOD1 5’-gcgaaaccaaggaattg-3’ and 5’-ggctgcaagctgttaaccc-3’; NOD2 5’-gcctagtagtgttac-3’ and 5’-atcctcaccagtcaccagacgagac-3’ IFNB1 5’-tggaagaacacaacaggaga-3’ and 5’-aacccctgagctgtttgtc-3’. Murine targets were amplified with the following primers: Tifa 5’-gccactggaagactctcagg-3’ and 5’-aacgtatctggcactggttg-3’, Gapdh 5’-tgaccaagagggcctcattc -3’ and 5’-agggcctctgtattag -3’; Cxcl1 5’-actgtcaccaccagaaagcgcctggtgtgc-3’ and 5’-gaggccctccctggtgtattg -3’; Cxcl2 5’-cccaaccaccaggtcacc-3’ and 5’-cggtcaccacggtctggcctg -3’. Amplification efficiencies were calculated for each primer pair. Relative expression was calculated using the 2-ΔΔCT method following normalization of target gene abundance to Gapdh. Quantitative measurements of cytokines were performed using ELISA kits from R&D Systems (KC, MIP-2) or BD Biosciences (IL-8). Nuclear extracts were prepared and NF-κB p65 binding was determined using the TransAM® Transcription Factor ELISA as per manufacturer’s protocol (Active Motif).

4.3.8 Immunofluorescence Microscopy

HCT 116 or 293T cells were seeded on collagen-coated glass coverslips, infected, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton-X100. Staining was done overnight at 4°C with mouse anti-FLAG (M2; Sigma) or p65 (sc-109; SantaCruz). Slides were visualized using an Axio Observer 7.1 inverted microscope (Zeiss) using ZEN 2 (Blue edition) pro-imaging software microscope. For analysis, images were processed using ImageJ software.
4.3.9 FLAG-TIFA constructs and cell line generation

HCT 116, and 293T stably expressing FLAG-TIFA were constructed by transducing with MSCV-1XFLAG-TIFA retrovirus (Clontech) and selecting stable colonies using 10 µg/ml blasticidin.

4.3.10 Knockout cell line generation

Construction of 293T TIFA knockout cells have been described previously (Gaudet et al., 2015). To generate HCT 116 (HCT) knockout cell lines, TIFA targeting RNA sequences 1: 5’-cagatgacgttacattc-3’, or 2: 3’-tgtgtcagacctttaaac-5’, NOD2 targeting 5’-ctctcctggagactagca-3’ and NOD1 is 5’-tgtggttgagactctgat-3’ were designed using software available at http://crispr.mit.edu. Double stranded templates were generated from single stranded oligonucleotides and cloned into hSpCas9-2A-Puro (pX459) or LentiCas9-Blast (Addgene) as described previously (Ann Ran et al., 2013). Polyclonal cleavage efficiencies were evaluated using SURVEYOR mutation detection kit following transfection or transduction (Transgenomic). To generate the TIFA knockout, pX459-guide1 and px459-guide2 were co-transfected into HCT cells. To generate NOD1 and NOD2 double knockouts, NOD2 knockout cells were transduced with the NOD1 construct and isolated by blasticidin selection. Following transfection or transduction of each guide RNA into HCT cells and selection with 2 µg/ml puromycin, or 10 µg/ml blasticidin single cell clones were obtained by limiting dilution. Expanded clones of the desired genotype were selected by cloning and sequencing of the TIFA, NOD1 or NOD2 genomic DNA. To assess CRISPR/Cas9 indel mutation, PCR products from target locus were sequenced by Sanger sequencing. The TIFA knockout contains a homozygous 62 base pair deletion (7-69) causing a frame-shift and lack of protein expression as determined by western blot. To assess functional assay, each cell line were tested by NF-κB luciferase activity loss.

4.3.11 qPCR Assessment for TIFA expression in primary tissues

Pre-normalized cDNAs derived from poly(A)-selected DNase-treated RNAs purified from pools of healthy human digestive system tissues were obtained from Clontech (lot# 1408485A). 12 week old FVB mice (Charles River) were euthanized and solid organs were removed and
immediately frozen at -80°C. Total RNA was isolated following Trizol extraction using RNA purification kit (Ambion). cDNA was synthesized and qPCR performed as described above.

4.3.12 Immunoprecipitation and immunobloting

FLAG-TIFA was immunoprecipitated using FLAG M2 agarose (Sigma). Cells were lysed in 50 mM HEPES-KOH pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% NP40, 10% glycerol. Soluble cell lysates were pre-cleared for 2 h with mouse IgG agarose (Sigma) and immunoprecipitated overnight at 4°C. Proteins were eluted using 3X FLAG-peptide (Sigma). TRAF6 was immunoprecipitated using rabbit anti-TRAF6 (sc-72201; Santa Cruz) or mouse anti-TRAF6 (sc-8409; Santa Cruz) bound to protein A/G PLUS-agarose (Santa Cruz) and eluted in sample buffer. Antibodies used for immunoblotting were the following: TRAF6 (sc-8409; Santa Cruz), P-SAPK/JNK (9251; Cell Signaling), phospho-IκBα (14D34; Cell Signaling), NF-κB p65 (sc-372; Santa Cruz), β-Actin (A1978; Sigma), phospho-TAK1 (4508; Cell Signaling), K63-linked polyubiquitin (5621; Cell Signaling) and MyD88 (sc-11356; Santa Cruz). Pooled-serum from mice immunized with His-tagged purified recombinant human TIFA was used to visualize endogenous TIFA in whole cell lysates.

4.3.13 Native PAGE and cell fractionation

Cells were lysed for 30 min on ice in 1% NP-40, 0.1% Triton-X100 and soluble lysates separated on 12.5% tris-glycine polyacrylamide gels, or denatured in 1X Laemmli sample buffer and separated on SDS-containing gels. The insoluble pellet was solubilized in sample buffer and separated on SDS containing gels.

4.3.14 Cytoplasm extractions and digitonin assays

Mammalian cytoplasm was extracted essentially as previously described (Beuzón et al., 2002). Briefly, 1 x 10⁹ HCT 116 cells were scraped into PBS, washed and re-suspended in 0.5 ml ice-cold PMEE (35 mM PIPES pH 7.4, 5 mM MgSO4, 1 mM EGTA, 0.5 mM EDTA, 250 mM sucrose). Cells were lysed by passaging through a 21G needle, subject to freeze-thaw, and clarified by centrifugation. Cleared lysates were then centrifuged at 150 000 x g for 1 h at 4°C, with the supernatant used directly for growth assays. Overnight cultures of Shigella were subcultured and grown to a final OD₆₀₀ of 0.1 (lag), 0.5 (log) or 2 (stationary), washed, and used to inoculate cytoplasm extracts at the desired bacterial density. Following 2 h shaking at 12°C or
37°C, a 10 µl aliquot was removed for CFU enumeration, bacteria were pelleted, and the supernatant (CFS) filtered through a 0.22 µm SpinX filter. Where indicated, the CFS were normalized to final CFU/ml before treatment. Overnight cultures of *E. coli ΔhldE* and *ΔwaaC* were washed and diluted 1/80 in LB media, grown for 6 h until each reached an OD600 of 0.6 and then re-suspended in an equal volume of fresh LB and grown for an additional 2 h at 37°C before the CFS was processed as per *Shigella*. Reversible digitonin assays were performed as previously described (Girardin et al., 2003a). Briefly, cells were stimulated for 20 minutes in permeabilization buffer (50 mM HEPES, pH 7, 100 mM KCl, 3 mM MgCl2, 0.1 mM DTT, 85 mM sucrose, 0.2% BSA, 1 mM ATP and 0.1 mM GTP) in the presence or absence of 5 µg/ml digitonin, washed 3X, then incubated for 4 to 6 h in complete growth medium before supernatants were harvested for ELISA measurements or luciferase assay.

### 4.4 Results

#### 4.4.1 TIFA is essential for NOD1- and NOD2- independent detection of invasive *Shigella flexneri*

I hypothesized that TIFA had a role in sensing cytosol-invasive bacteria in IECs and chose *Shigella* as a model pathogen to study TIFA activation. Considering the abundance of literature on NOD-dependent detection of *Shigella*, we began by definitively assessing whether NOD1 and NOD2 could account for the entirety of the IL-8 response to invasive *Shigella*. We generated human HCT 116 (HCT) colonic epithelial cells in which either NOD1, NOD2 or both were knocked out via CRISPR/Cas9 technology. While I did observe a statistically significant decrease in IL-8 produced by cells knocked out for NOD1, most of the IL-8 produced 6 hours after infection with invasive *Shigella* was independent of both NOD1 and NOD2 (Figure 31A). Knockdown of MyD88 with RNAi confirmed that the NOD-independent IL-8 was not derived from endogenous TLRs (Figure 31B). To see if the NOD-independent IL-8 response was mediated by TIFA, I created TIFA knockout HCT 116 cells using CRISPR/Cas9. Strikingly, there was a dramatic decrease in IL-8 production upon infection with invasive *Shigella* in the TIFA knockout cells (Figure 31C). In contrast, TIFA knockout cells were not compromised in their response to the NOD1 ligand C12-iE-DAP or the cytokine TNFα. Interestingly, *IL8* mRNA induced by *Shigella* in TIFA knockout cells was unaltered within 60 minutes, yet abolished by
240 minutes of infection (Figure 31D), suggesting a kinetic requirement to TIFA activation. TIFA was also essential for the response to invasive Shigella in HeLa cells and T84 colonic epithelial cells (Figure 32) confirming that the effect was not limited to a single cell line. To confirm that TIFA signaling was independent of NOD1, I conducted shRNA knockdown of TIFA or NOD1 in NOD1 or TIFA knockout cells, respectively (Figure 33A and B). In each case, IL-8 production was further reduced to almost undetectable levels in the double deficient cell lines (Figure 33C and D), indicating that TIFA signaling is independent of NOD1.

**Figure 31** TIFA mediates NOD1- and NOD2-independent detection of invasive Shigella. (A) ELISA measurement of IL-8 production in the supernatants of NOD1, NOD2 knockout (KO), or NOD1 and NOD2 double knockout (dKO) HCT 116 cells infected with invasive S. flexneri (S.f) M90T, the non-invasive strain BS176, or stimulated with C12-iE-DAP (20 µg/ml), muramyl dipeptide (MDP; 10 µg/ml), or TNFα (50 ng/ml) for 6 hours. (B) ELISA of IL-8 production in HCT 116 cells expressing a MyD88 targeting or scrambled shRNA and infected at the indicated MOI (multiplicity of infection) with S. flexneri. (C and D) ELISA measurement of IL-8 production (C) or IL8 mRNA expression by qPCR (D) in TIFA or NOD1 knockout cells transduced with NOD1 or TIFA targeting shRNAs, and infected with S. flexneri (moi=50) or treated as indicated. Results are mean ± SEM, and represent 3 independent experiments. (ns) not significant (P > 0.05). *P < 0.05, ***P < 0.001 by ANOVA.
**Figure 32** TIFA is essential for the IL-8 response to invasive *Shigella* in HeLa and T84 cell lines. HeLa or T84 colonic epithelial cells were transduced with the indicated shRNA, then infected at an MOI of 80 with *S. flexneri* M90T. 6 hours later, IL-8 levels in supernatants were measured by ELISA. Results are mean ±SEM, and represent 3 independent experiments. **P < 0.01 by ANOVA.

**Figure 33** HCT 116 cells deficient in both TIFA and NOD1 are refractory to invasive *Shigella*. (A and B) qPCR measurement of mRNA levels of *GAPDH*, *TIFA* or *NOD1* expressed as a percentage of untransduced following shRNA knockdown of TIFA or NOD1 in wildtype, NOD1 or TIFA knockout HCT 116 cells respectively. (C and D) ELISA measurement of IL-8 production in supernatants of wild-type, NOD1 knockout, or TIFA knockout HCT 116 cells expressing a TIFA, NOD1 or scrambled targeting shRNA and infected with *S. flexneri* (6h) or stimulated with the indicated microbial product. Results are mean ±STD, and are representative of 3 independent experiments. **P < 0.01, ***P < 0.001 by ANOVA.
I then assessed whether the defect in IL-8 production was caused by a decrease in TIFA-dependent activation of NF-κB. Indeed, phosphorylation of and transforming growth factor β-activated kinase 1 (TAK1) and IκBα (Figure 34A), and nuclear translocation of NF-κB p65 (Figure 34B) induced by Shigella invasion was markedly reduced in TIFA knockout cells. Invasive Shigella induced dramatically more TIFA-dependent NF-κB activation (Figure 34C) and IL-8 production (Figure 34D) compared with extracellular stimulation with soluble bacterial lysate or cell-free supernatant (CFS) derived from Shigella, confirming that TIFA was not activated by HBP released from extracellular bacteria. In line with this, all cell lines were refractory to non-invasive Shigella BS176. Together, these results demonstrate that TIFA is essential for NOD1- and NOD2-independent recognition of invasive Shigella in human epithelial cells.

**Figure 34**  TIFA is required for Shigella-induced NF-κB activation.  (A) Immunoblot analysis of phosphorlylated IκBα (top) or TAK1 (bottom) in wildtype or TIFA knockout cells infected with S. flexneri. Blots for p-TAK1 and β-actin were exposed for the same amount of time.  (B) Nuclear localization of NF-κB p65 during S. flexneri infection of wildtype or TIFA knockout HCT116 cells visualized by fluorescence microscopy.  (C and D) NF-κB luciferase activity (C) or IL-8 production by ELISA (D) in wildtype or TIFA knockout 293T cells infected with S. flexneri (moi 10), or stimulated with soluble lysate, or cell-free supernatant (CFS) derived from 100 fold the amount of bacteria used in the infection. all data represent mean ± SEM. Results are representative of 3 independent experiments.
4.4.2 Escape from the entry vacuole drives TIFA activation

To further explore the pathogenic signature required to activate the TIFA-signaling axis, I infected wild type or TIFA knockout cells with bacteria that establish their niche in differing cellular compartments. While *Shigella* immediately escapes into the cellular cytoplasm, *Salmonella enterica* Typhimurium remains in the phagosome, using its type 3 secretion systems to translocate effector proteins into the host and maintain a stable bacteria-containing vacuole (Ray et al., 2009). Indeed, the potent TIFA-dependent NF-κB activation apparent during *Shigella* infection was absent during infection with vacuole-confined *Salmonella* (Figure 35A), despite the fact that soluble lysates from both species had comparable levels of TIFA-stimulating activity when introduced into digitonin-permeabilized cells (Fig. 35B). I next tested whether *Salmonella* that escape the vacuole could trigger TIFA signaling. Loss of its effector SifA causes rupture of vacuole and eventual release of *Salmonella* into the cytosol (Beuzón et al., 2000). Consistent with TIFA as a sensor of cytosolic bacteria, *Salmonella ΔsifA* induced significantly more TIFA-dependent NF-κB activation (Figure 35A) and TIFA oligomerization (Figure 35C) than wild type bacteria. IECs express endogenous levels of TLR5, confounding analysis of NF-κB activation during infections with flagellated bacteria such as *Salmonella*. To overcome this, I knocked down MyD88 to suppress TLR5 signaling, and observed a markedly amplified TIFA-dependent difference in NF-κB induced by *Salmonella ΔsifA* (Figure 35D). Moreover, allowing the infection to proceed for 20 h, when some wild type *Salmonella* can enter the cytoplasm of epithelial cells (Knodler et al., 2010), further uncoupled TIFA-dependent *Salmonella* recognition from that which occurs through TLR5 (Figure 35E). To confirm that bacterial entry to the cytoplasm did not, in itself, cause TIFA activation, we considered whether invasive Gram-positive bacteria, which do not make heptose metabolites, initiated a similar response. Consistent with our expectations, *Listeria monocytogenes* (*L.m*) did not elicit a TIFA-dependent response (Figure 35F). However, HBP was able to recapitulate the response to invasive Gram-negative bacteria, since repeating the *L.m* infections in the presence of HBP caused a dramatic increase in TIFA-dependent NF-κB activation (Figure 35F) without affecting interferon-beta (*IFNB1*) induction (Figure 35G). Together, these observations suggest that TIFA is activated by cytosolic Gram-negative bacteria that escape the vacuole.
Figure 35 Gram-negative bacteria that escape the vacuole activate TIFA. (A and B) NF-κB luciferase activity in wildtype or TIFA knockout 293T cells infected with the indicated bacteria (A), or stimulated with soluble lysates derived from *S. flexneri* (*S.f*) or *Salmonella* Typhimurium in the presence or absence of the permeabilizing agent digitonin (Dig). (C) TIFA aggregation measured by clear-native PAGE (CN-PAGE) in 1X-FLAGTIFA expressing 293T cells infected with *Salmonella* of the indicated genotype for 6 h. (D) NF-κB luciferase activity in wildtype or TIFA knockout 293T cells expressing scrambled (shCtrl.) or MyD88 targeting shRNA and infected for 8 h with *Salmonella* of the indicated genotype. Inset immunoblot indicates protein expression. (E) ELISA measurement of IL-8 production by wild-type or TIFA knockout HCT 116 cells expressing scrambled (shCtrl.) or MyD88 targeting shRNA and infected for 20 h with *Salmonella* of the indicated genotype. (F) NF-κB luciferase activity in wildtype or TIFA knockout 293T cells treated with increasing amounts of HBP during infection with *Listeria monocytogenes* (*L.m*). (G) *IFNB1* mRNA induction by qPCR in HCT 116 cells infected with *L.m* in the presence or absence of HBP. Data represent ≥ 3 independent experiments. (ns) not significant (*p > 0.05*). *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA.
4.4.3 TIFA senses cytosol-invasive bacteria after initial detection by NOD1

NOD1 and NOD2 are localized to host cell membranes, meaning they can rapidly respond to invading microbes at the site of bacterial entry (Philpott and Girardin, 2010). Indeed, NOD1 is recruited to *S. flexneri* entry sites (Kufer et al., 2008; Travassos et al., 2010) and interacts with the effector RIP2 (RICK/CARDIAK) within minutes of bacterial invasion (Girardin et al., 2001). NOD2 also forms a sensor complex on the endosomal membrane within minutes of treatment with muramyl dipeptide or *Salmonella* infection (Nakamura et al., 2014). Therefore, in light of the TIFA-independent p-IκBα and *IL8* induction within 60 minutes of *Shigella* infection, we hypothesized that the NOD1 and TIFA pathways are activated at different stages of the infectious process: NOD1 responding to the initial bacterial escape from the vacuole, and TIFA responding later to freely cytosolic bacteria. Consistent with our hypothesis, nuclear p65 was entirely NOD1-dependent and TIFA-independent within 60 minutes of infection (Figure 36A). Strikingly, the phenotype was reversed at later time points, with nuclear p65 being TIFA-dependent, and NOD1 independent 2 and 4 hours after infection (Figure 36A). Nuclear p65 was absent in cells deficient in both NOD1 and TIFA, confirming that both pathways are independent and are cumulatively responsible for bacterial sensing. This temporal pattern was also reflected in phospho-Iκbα levels during infection (Figure 36B). In addition to NF-κB, invasive *Shigella* activates the stress response kinase SAPK/JNK (Girardin et al., 2001). Phosphorylated SAPK/JNK were reduced in the TIFA and NOD1/2 knockout cells respectively, yet in a different pattern of kinetics than NF-κB, as inactivation of both pathways was required to eliminate activation (Figure 36C). Given that both NF-κB and JNK control pro-inflammatory gene expression in IECs, I next analyzed the kinetics of *Shigella*-induced NOD1 and TIFA-dependent *IL8* transcription. Whereas NOD1 mediated the initial wave of *IL8* induction, the robust and sustained increase in *IL8* beginning 90 minutes after infection was almost entirely driven by TIFA (Figure 36D), and is reflected by the total IL-8 protein level being essentially TIFA-dependent by 4 hours of infection (Figure 36E). If the NOD1 pathway is sensing the initial breach of the cytoplasm, then using a higher multiplicity of infection (MOI) would shift IL-8 production to be more NOD1-dependent, as a higher dose of NOD1-agonist would enter cells. Indeed, only at high doses of bacteria (MOI = 80) was there was a significant defect in NOD1-dependent IL-8 production (Figure 36F). In contrast, a reduction in TIFA-dependent IL-8 was
observed at all MOIs examined, with the phenotype being more apparent at lower MOIs. Together, these results suggest that the NOD1 and TIFA pathways are not redundant, and instead represent separate defense mechanisms that are activated temporally during infection.

**Figure 36**  TIFA is activated after NOD1 during infection with invasive *Shigella*. (A) Timecourse of nuclear p65 translocation by transcription factor ELISA at in HCT116 cells of the indicated genotype during infection with *S. flexneri* (*S.f*). (B and C) Time course immunoblot analysis of phosphorylated IκBα (B) or SAPK/JNK (C) levels in HCT 116 cells of the indicated genotype during infection with *Shigella*. (D to F) Timecourse of IL8 mRNA levels by qPCR (D), or IL-8 protein production by ELISA (E and F) in HCT 116 cells of the indicated genotype during infection with *S. flexneri*. Results (A,D,E,F) represent ≥ 3 independent experiments or are representative of ≥ 2 independent experiments (B,C). **P < 0.01 by ANOVA.**
4.4.4 Endogenous TIFA expression determines the cellular responsiveness to HBP and sensitivity to invasive Shigella

To confirm that TIFA can function as an important regulator of immunity in the intestinal tract, I next analyzed TIFA expression levels in primary human tissues. TIFA was constitutively expressed at high levels in the gastrointestinal epithelium when considered relative to NOD1 (Fig. 37A). TIFA has been identified as a microbial-inducible gene in other vertebrate species (Chang et al., 2005; Sommer et al., 2015), and the TIFA mRNA sequence contains A+U rich elements in the 3’UTR common to short lived cytokines and proto-oncogenes (Kanamori et al., 2002). Therefore, considering the temporal cascade of Shigella-induced NF-κB activation mediated by NOD1 and TIFA, I asked whether the initial bacterial detection by NOD1 may further stimulate TIFA expression. Stimulation of NOD1, NOD2, TLR5 or TIFA itself with their cognate PAMPs lead to upregulation of TIFA mRNA in HCT 116 cells (Figure 37B). Moreover, overexpression of NOD1 alone or together with c12-iE-DAP increased TIFA expression in a dose-dependent manner in 293T cells (Figure 37C). TIFA inducibility was dependent on NF-κB activation as depleting the subunit RelA abrogated TIFA upregulation (Figure 37D). I then measured TIFA mRNA levels during infection with invasive Shigella and found that TIFA expression was significantly induced at high multiplicities of infection in a NOD1/2 dependent mechanism (Figure 37E). To see if this could affect the amplitude of Shigella-induced NF-κB activation, I first stimulated TIFA induction with C12-iE-DAP for 6 h, or TNFα or flagellin for 2 h, rested the cells for 2 h, then infected with Shigella. Priming through stimulation of NOD1, TLR5 or TNFR increased S. flexneri induced NF-kB activation in a TIFA-dependent manner (Figure 37F). Importantly, the priming effect was absent in TIFA knockout cells complemented with a non-inducible TIFA construct (Figure 37F), confirming that the increase in NF-κB activation was a direct result of endogenous TIFA upregulation. Moreover, preventing translation with cyclohexamide (CHX) reduced IL8 induced by S. flexneri in dose-dependent manner that required both NOD1/2, and endogenously controlled TIFA expression (Figure 37G). Importantly, kinetic analysis of IL8 induction revealed that this effect was only apparent at later time points, when NF-κB activation in vehicle-treated cells would be primarily TIFA-dependent (Figure 37H).
Figure 37  NOD1-dependnet TIFA expression induced by invasive Shigella.  (A) TIFA and NOD1 mRNA expression by qPCR in primary human tissues pooled from healthy donors. All data was normalized and expressed as a ratio relative to GAPDH. (B to E) TIFA mRNA expression by qPCR in HCT 116 cells stimulated with C12-iE-DAP (20 µg/ml), HBP (1/100), flagellin (10 µg/ml) or TNFα (20 ng/ml) (B) 293T cells transfected with 5, 10, or 25 ng pNOD1 with or without C12-iE-DAP (C), or in wildtype HCT 116 cells expressing a scrambled or RelA targeting shRNA and stimulated as indicated (D), or in wildtype and NOD1 and NOD2 double knockout cells infected with S. flexneri (E). (F) NF-κB luciferase activity in wildtype, TIFA knockout, or TIFA knockout 293T cells complemented with pMSCV-TIFA. Cells were pre-stimulated for 2 h (flagellin, TNFα) or 6 h (C12-iE-DAP), rested for 2 h, then infected with S. flexneri. (G and H) Induction of IL8 mRNA by qPCR in HCT 116 cells of the indicated genotype treated for 1 h with cyclohexamide (CHX, 10 µg/ml) then infected for 3 h with S. flexneri at the indicated MOI (F) or at an MOI of 50 for the indicated time in the continued presence of CHX (G). Results in A are mean ± S.T.D. Results (B-H) represent ≥ 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA.
Finally, to obtain more direct evidence that TIFA expression levels dictate the cellular responsiveness to both HBP and invasive Shigella, I screened microarray meta-data for cell lines that exhibit naturally low TIFA expression levels (Hruz et al., 2008). Among the greatest perturbations across human and murine data sets were human Caco-2 colonic epithelial cells and a trans-immortalized mouse intestinal epithelial cell-line (mIC-Cl2) that maintains a crypt-like phenotype in culture. I first analyzed TIFA expression in Caco-2 cells, which have been used in the past to model Shigella interactions with the colonic epithelium (Pédron et al., 2003), and observed dramatically lower TIFA expression than primary human intestinal tissues despite NOD1 expression being similar (Figure 38A). Strikingly, while Caco-2 cells were naturally unresponsive to HBP, transduction with a TIFA-expressing retrovirus was sufficient to confer the ability to detect HBP, and significantly increased IL-8 production upon infection with Shigella (Figure 38B). I observed similar results with mIC-Cl2 cells, as low Tifa levels compared with murine spleen or intestinal tissues prompted an insensitivity to HBP that could be restored by expression of TIFA in trans (Figure 38C and D). TIFA overexpression had no affect on LPS-induced cytokine production, but significantly increased Shigella-induced keratinocyte-derived chemokine (KC, or CXCL1) and macrophage inflammatory protein 2 (MIP-2), the murine functional homologues of human IL-8 (Figure 38D). TIFA-mediated inflammation was more apparent at later time points, consistent with the temporal pattern of TIFA activation observed previously (Figure 38E). These data indicate that cellular sensitivities to both HBP and invasive Shigella are determined by endogenous TIFA expression levels. Moreover, activation of the NOD pathways during bacterial invasion induces a feedback mechanism that ensures sufficient TIFA expression to robustly respond to the continued presence of freely cytosolic bacteria.

### 4.4.5 Invasive Shigella triggers the TIFA signaling cascade

I previously identified phosphorylation-dependent oligomerization as the molecular mechanism whereby TIFA is activated by soluble HBP (Gaudet et al., 2015). To characterize the molecular mechanism of Shigella-driven TIFA activation, I examined TIFA oligomerization during infection by clear-native PAGE (CN-PAGE). Similarly to what is induced by soluble HBP, TIFA aggregates of higher molecular weight are induced by invasive Shigella (Figure 39A). However, I was surprised to notice that the TIFA aggregates disappear from the detergent soluble fraction during the infection, as TIFA became completely insoluble by 4 h after infection (Figure 39A). This is in contrast to TIFA-aggregates remaining soluble during stimulation with
Figure 38  Endogenous TIFA expression levels determine cellular sensitivities to HBP and invasive Shigella. (A) TIFA and NOD1 expression by qPCR in primary human colon or intestinal tissue compared with HCT116 or Caco-2 cell lines. (B) IL8 mRNA or protein levels induced in Caco-2 cells transduced with an empty, or TIFA encoding retrovirus and stimulated with HBP, TNFα or infected with S. flexneri. (C) Tifa expression by qPCR in primary murine tissues compared with the mIC-Cl2 cell line. (D and E) ELISA measurements of protein (D) or mRNA levels by qPCR (E) of KC and MIP-2 produced by mIC-CL2 cells transduced with an empty, or TIFA encoding retrovirus and stimulated with HBP, LPS (100 ng/ml), or infected with S. flexneri for 6 h (D) or for the indicated time (E). Data represent ≥ 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA.
*Figure 39*  *Shigella* induces TIFA aggregation and TRAF6 activation. (A to D) Cell-fractionation and immunoblot for: FLAG-TIFA in 293T cells during infection with *S. flexneri* (*S.f*) (A) or treated with soluble HBP (B); FLAG-TIFA in TIFA knockout cells complemented with 1XFLAG-TIFA of the indicated genotype and infected with *S.f* (C); TRAF6 and lysine 63 (K63)-linked polyubiquitin in wildtype or TIFA-knockout 293T cells infected with *S.f*. Soluble or insoluble (pellet) fractions were separated under native or denaturing (denat.) conditions. (E and F) Immunoprecipitation (IP) and immunoblot analysis (E) or ELISA measurement of IL-8 in the supernatants (F) of TIFA knockout 293T cells complemented with 1XFLAG-TIFA of the indicated genotype and infected with *S.f*. Data are representative of ≥ 3 independent experiments.

extracellular HBP (Figure 39B). TIFA aggregation was dependent on phosphorylation of TIFA on Threonine 9 (Thr9), as TIFA mutated to prevent phosphorylation (T9A) did not migrate to the
insoluble pellet during infection (Figure 39C). This is consistent with our report that HBP triggers phosphorylation of TIFA on Thr9 (Gaudet et al., 2015), and the recently solved structure of TIFA, which confirmed that TIFA oligomerization is mediated by intermolecular binding of the central forkhead-associated domain (FHA) with phospho-Thr9 (Weng et al., 2015). As TIFA oligomerization activates the E3 ubiquitin ligase TRAF6 (Ea et al., 2004), I next asked whether TRAF6 was also becoming detergent-insoluble. Indeed, invasive *Shigella* triggered the migration of TRAF6 from the soluble to insoluble fraction in a TIFA-dependent manner (Figure 39D). Notably, the detergent insoluble pellet from infected wild type but not TIFA knockout cells was enriched in lysine 63 (K63)–linked polyubiquitin, suggesting *Shigella* induced TIFA-dependent TRAF6 activation (Figure 39D). Moreover, immunoprecipitation analysis revealed that invasive *Shigella* triggered the formation of a complex involving TIFA, TRAF6 and phosphorylated TAK1 (Figure 39E). The presence of TIFA-dependent K63 linked ubiquitin chains in the *Shigella*-triggered TIFA-TRAF6 complex confirmed TRAF6 was being activated by TIFA (Figure 39E). Moreover, complementation of TIFA knockout cells with TIFA variants unable to oligomerize (T9A or G50E/S66A) or interact with TRAF6 (E178A) (Gaudet et al., 2015) prevented the *Shigella*-induced formation of the TIFA-TRAF6-TAK1-Ub complex (Figure 39E), and largely abolished the massive IL-8 response to *Shigella* infection (Figure 39F). Considering the insoluble nature of the *Shigella*-induced TIFA complexes, I next examined the cellular localization of TIFA in *Shigella* infected cells by immunofluorescence. Large TIFA aggregates were readily apparent in infected IECs 3 h after infection, an effect that was independent of NOD1 (Figure 40A). Kinetic analysis revealed that while TIFA aggregates were occasionally visible by 45 minutes after infection, foci became larger and more apparent at later time points and seemed to coincide with intracellular bacterial proliferation (Figure 40B). In contrast to what is observed for NOD1 and NOD2 which surround the bacterial entry foci (Travassos et al., 2010), TIFA did not colocalize with bacteria, as TIFA aggregates were readily apparent at sites distal to *Shigella* (Figure 40B). These data suggest that freely replicating bacteria in the cytosol may liberate HBP, which triggers TIFA phosphorylation-dependent oligomerization and activation of TRAF6.
**Figure 40**  *Shigella* induced TIFA aggregation. (A) TIFA aggregation in HCT 116 WT or NOD1 knockout cells stably expressing 1XFLAG-TIFA infected with *Shigella* (*S.f*) and analyzed by immunofluorescence microscopy. (B) TIFA aggregation in 293T TIFA knockout cells complemented with 1XFLAG-TIFA during infection with mCherry expressing *Shigella* and analyzed by immunofluorescence microscopy. Results are representative of ≥ 3 independent experiments. Quantification indicates the percentage of cells with TIFA aggregates after counting ≥ 20 fields in each of 3 independent experiments (mean ± s.e.m).

### 4.4.6 TIFA is activated by HBP released from growing Gram-negative bacteria

Conditioned media from *Shigella* grown in broth does not contain sufficient HBP to activate IECs when supplied extracellularly (Figure 31C). Therefore, I investigated the context through which intracellular *Shigella* presents HBP to the TIFA signaling axis. I hypothesized that either HBP is liberated during infrequent bacteriolysis in the cytosol, or it is shed from bacteria,
but in quantities so low that accumulation and concentration within the host cell cytoplasm is required for its detection. Provoking intracellular bacteriolysis in infected 293T cells using the cell-permeable antibiotic imipenem (Tattoli et al., 2008) had no appreciable effect on NF-κB activation (Figure 41).

**Figure 41** Provoking intracellular bacteriolysis of *Shigella* does not increase NF-κB activation. (A and B) NF-κB luciferase activity in 293T cells (A), or recovered bacterial counts (B), following infection with *S. flexneri* (MOI 50). Following spinnoculation and incubation for 30 min, cells were incubated in media containing the cell-impermeable antibiotic (gentamicin 80 µg/ml) for 1 hour. Infected cells were then subject to 3 hours in media containing either gentamicin, or cell-permeable antibiotic imipenem (100 µg/ml) to induce intracellular bacteriolysis. Cells were then harvested for NF-κB luciferase measurements or bacteiral counts. Results represent 2 independent experiments.

Therefore, to determine if HBP is shed during growth, we cultured *Shigella* in undiluted cytoplasmic extracts prepared from IECs to simulate cytosolic conditions (Figure 42A). Following 2 hours of growth, we collected the cell-free supernatant (CFS) and presented it into the cytosol of HCT cells using reversible digitonin permeabilization. Growing *Shigella* for 2 hours in cytoplasm extracts or tryptic soy broth (TSB) resulted in abundant proteinase K- and DNASE-resistant TIFA stimulating activity in the CFS (Figure 42B). Interestingly, there was almost as much TIFA-stimulating activity in the CFS than in total soluble lysate prepared from the end culture, suggesting infrequent bacteriolysis was not the principal source of HBP. Moreover, there was more NOD1 stimulating activity in cultures grown in TSB broth when compared to cultures grown in cytoplasm extracts (Figure 42B).
Figure 42. Intracellular growth and IL-8 stimulating activity released from growing S. flexneri. (A) Overnight cultures of S. flexneri were diluted 1/100 and grown to the indicated bacterial density. Cultures were then inoculated into PMEE buffer, cytoplasm extract prepared from HCT 116 cells (Cyto), or tryptic soy broth (TSB). Samples were taken at 1 and 2 hours post inoculation and plated to determine growth rate. (B) ELISA measurement of IL-8 released from digitonin permeabilized wildtype, NOD1/2 double knockout, TIFA knockout, or NOD1/2 double knockout cells expressing a TIFA targeting shRNA stimulated with the indicated fraction from S. flexneri. Cell-free supernatants (CFS), or soluble lysate (Sol. lysate) were harvested from log phase bacteria cultured in cytoplasm extracts (Cyto) or tryptic soy broth (TSB) for 2 hours at 37°C. Where indicated, fractions were treated with proteinase K and nuclease (Benzonase ®) for 1 hour before treatment. Results are mean ±STD, and are representative of 3 independent experiments.

This was not limited to invasive bacteria, as intracellular presentation of the CFS from nonpathogenic E. coli also stimulated TIFA (Fig. 43A and B). Previous studies have attributed the NF-κB inducing activity of Gram-negative supernatants to LPS (Philpott et al., 2000) or peptidoglycan (Girardin et al., 2003a). Therefore, to prove that the TIFA agonist was HBP, we collected the CFS from E. coli deleted for hldE, the gene that synthesizes HBP, or waaC (rfaC),
a gene downstream of HBP in the ADP-heptose biosynthesis pathway. The ∆waaC mutant is a control for the LPS phenotype of the ∆hldE mutant, since both have the same truncated “deep-rough” LPS structure (Gaudet et al., 2015), and thus differ solely by the ability to synthesize heptose metabolites downstream of HldE. Whereas the CFS from the ∆waaC mutant stimulated robust IL-8 production from permeabilized cells, the CFS from E. coli ∆hldE, grown to the same optical density, failed to induce IL-8 (Figure 43C). This provides genetic proof that HBP is the TIFA activating agonist in the CFS. Importantly, there was no difference in NOD1 activation induced by the CFS from ∆hldE and ∆waaC E. coli (Figure 43D), confirming the specificity of the TIFA pathway for HBP.

Considering the activation kinetics of the TIFA pathway during infection, we next asked whether HBP release required bacterial replication. We inoculated Shigella into cytoplasmic extracts at different bacterial densities and cultured at different temperatures to allow a variety of bacterial growth rates. After 2 hours, we collected the CFS and presented it into the cytosol of digitonin-permeabilized cells. Strikingly, incubation at 12°C abrogated the TIFA-stimulating activity of the CFS even at high bacterial density (Figure 43E). Moreover, when the CFS extracts from 37°C cultures were normalized to account for final bacterial densities, TIFA-, but not NOD1-, dependent IL-8 production correlated with the bacterial growth rate from which the CFS was derived (Figure 43F); this indicates TIFA, but not NOD1, can act as a dynamic sensor of bacterial proliferation. Intracellular presentation of CFS from bacteria grown at 37°C but not 12°C triggered TIFA oligomerization, with activity accumulating during growth (Figure 43G). Moreover, starting cultures at log phase resulted in more normalized TIFA-stimulating activity in the CFS than cultures started at lag or stationary phase (Figure 43H). Together, these data suggest HBP is generated and released solely by actively replicating Gram-negative bacteria. Importantly, the failure of non-permeabilized cells to respond to HBP-containing supernatants, implies that entry into the cytosol is the limiting factor that determines whether TIFA is engaged by HBP, providing a mechanism whereby IECs can discriminate between extracellularly and intracellularly growing bacteria.
Figure 43  HBP is shed by Gram-negative bacteria during growth. (A) Illustration of the assay used to assess TIFA stimulating activity released from cytosolic Shigella. (B) ELISA measurement of IL-8 production by HCT 116 cells of the indicated genotype in the presence or absence of the permabilizing agent digitonin (Dig), stimulated with the cell-free supernatant (CFS) from S. flexneri cultured for 2 h in cytoplasmic extracts, or E. coli in LB broth. (C) ELISA measurement of IL-8 production by digitonin permeabilized HCT 116 cells stimulated with the CFS from E. coli of the indicated genotype. (D) NF-κB luciferase activity from digitonin permeabilized wildtype or TIFA knockout 293T cells transfected with pNOD1 and stimulated with the CFS from E. coli of the indicated genotype. (E) ELISA measurement of IL-8 production (left y axis) by digitonin permeabilized HCT 116 cells of the indicated genotype stimulated with equal volumes of CFS collected from S. flexneri. Cultures were inoculated into cytoplasm extracts at the indicated density, incubated at 37°C or 12°C for 2 h and bacterial counts pre- and post- incubation determined (right y axis). (F) IL-8 production with by digitonin permeabilized TIFA-, or NOD1- knockout HCT 116 cells stimulated with the CFS from Shigella cultures that were inoculated at varying densities to stimulate different growth rates then normalized to final CFU/ml. Linear regression indicates the correlation between IL-8 and fold increase in bacteria. (G and H) TIFA oligomerization by clear-native (CN) PAGE in 293T cells stimulated with the CFS from Shigella cultures inoculated from cultures at different growth stages (G) or incubated at different temperatures (H) in the presence or absence of digitonin (Dig). CFS was normalized to CFU/ml prior to stimulating cells. (m = mock). Results represent 3 independent experiments, or are representative of 2 independent experiments (G and H).
4.4.7 Metabolically attenuated invasive *Shigella* fail to activate the TIFA pathway

To confirm that TIFA was responding to the proliferation of cytosolic bacteria in the context of an infection, we generated invasive *Shigella* mutants that display a reduced intracellular growth rate. Host-derived pyruvate is an important energy source for intracellular *Shigella* (Kentner et al., 2014). Consequently, mutants lacking either phospho-transacetylase (PTA) or acetate kinase (ACKA), which are required for the metabolism of pyruvate, are less metabolically active and display longer intracellular generation times than wild-type (Figure 44A). Consistent with our hypothesis, the $\Delta pta$ and $\Delta ackA$ mutants induced markedly less TIFA-driven IL-8 6 hours after infection compared with wild-type *Shigella* (Figure 44B). In contrast, NOD1-driven IL-8 produced within 1 hour of infection was intact (Figure 44C), which is consistent with each strain being equally invasive (Figure 44D). Notably, the robust and sustained TIFA-driven wave of IL8 transcription was absent in cells infected with the $\Delta ackA$ mutant (Figure 44E). Complementation of the $\Delta pta$ mutant with acetyl-phosphate (Acetyl-P), the product of PTA, restored both the intracellular growth rate (Figure 44F) and the TIFA-driven IL-8 response (Figure 44G). Strikingly, in TIFA-knockout cells overexpressing NOD1, the NF-κB response induced by the $\Delta pta$ mutant was unaffected by complementation with Acetyl-P (Figure 44H). This confirms that TIFA, not NOD1, discriminates between proliferating and stagnant intracellular *Shigella*. Together with the temporal signature of TIFA activation, these data lead us to suggest that TIFA is stimulated by trace amounts of HBP released from actively growing intracellular bacteria, and represents an important means to escalate the immune response to invasive pathogens that exploit the host cytosol for growth and replication.
Figure 44 Metabolically attenuated invasive *Shigella* fail to activate TIFA. (A to D) Quantification of *S. flexneri* (*S.f*) of the indicated genotype recovered after infecting HCT cells (A, D), and ELISA measurement of IL-8 in the supernatants 6 h (B) or 1 h (C) after infection. (E) *IL8* mRNA by qPCR at the indicated time in HCT WT or TIFA KO cells infected with WT or ΔackA *S.f*. (F and G) Recovered *S.f* of the indicated genotype (F) or ELISA measurement of IL-8 produced 6 h after infection (G) in the presence or absence of 100 mM acetyl-phosphate. (H) NF-κB luciferase activity in 293T cells of the indicated genotype, transfected with pTIFA or pNOD1 and infected with WT or Δpta *S.f* in the presence or absence of acetyl-phosphate. Results represent 3 independent experiments. ns = not significant. **P < 0.01, ***P < 0.001 by ANOVA.

4.5 Discussion

In this chapter, I provide evidence that the TIFA-dependent surveillance pathway constitutes a NOD1 and NOD2-independent detection mechanism for invasive bacterial pathogens in the intestinal epithelium. TIFA was essential for cytokine production in response to invasive *Shigella flexneri*, and *Salmonella* Typhimurium that could escape the vacuole. While NOD1 was required for early detection of *Shigella*, TIFA was activated at later time points and at sites distal to the bacterial entry vacuole, with freely replicating cytosolic bacteria inducing TIFA phosphorylation, oligomerization and activation of the ubiquitin ligase TRAF6 (Figure 45).
Indeed, bacterial growth within the cytosolic compartment was an important requisite for TIFA activation, and therefore represents a novel mechanism for scaling microbial threats.

**Figure 45**  NOD1 and TIFA sequentially respond to invasive *Shigella*. Schematic depiction of our model describing the events that lead to optimal innate recognition of *Shigella*. Upon vacuolar escape, initial recognition of *Shigella* is dependent upon NOD1. Freely replicating bacteria later release trace amounts of HBP during growth that is detected by the TIFA-dependent surveillance pathway.

NOD1 has long been attributed as the sole sentinel for Gram-negative bacteria inside epithelial cells (Girardin et al., 2003). We speculate there are several reasons why the TIFA response has previously escaped attention. First, we show that cellular reactivity to HBP and invasive *Shigella* is sensitive to endogenous TIFA expression and that cell lines differ considerably in TIFA mRNA levels. For example, a common cell line used to model the human colonic epithelia, Caco-2, owe their insensitivity to HBP to low endogenous TIFA expression. We also noticed relatively low Tifa mRNA levels in the murine intestinal tract compared with human. This could, in part, explain why the response to invasive *Shigella* in cells from NOD1 knockout mice tend to display a greater defect than we observe here using human NOD1.
knockout cells (Carneiro et al., 2009). Interestingly, *Shigella* colonization of the murine colon does not display the intense pro-inflammatory response that typifies its colonization of the human colon. Further examination of the function of the TIFA pathway in murine versus human colonic tissues is needed, and could help resolve why mice are naturally resistant to shigellosis. Moreover, that microbial products induced TIFA expression is consistent with a report that showed complementation of the commensal microflora into germ free mice induced TIFA expression in the murine ileum crypt (Sommer et al., 2015). In this respect, TIFA may resemble NOD2 in that it’s expression may depend upon signaling pathways induced by the commensal microbiota (Petnicki-Ocwieja et al., 2009). Second, a prior lack of human NOD1 and NOD2 knockout cells required studies to be performed in systems in which the NLRs were depleted with RNAi (Fukazawa et al., 2008) or with dominant negative constructs (Girardin et al., 2001). In each case, the NOD-independent response to *Shigella* could be attributed to either lingering expression, or signaling by the non-targeted NLR. Finally, it is likely that a combination of time points examined, and MOIs used in various infection protocols shifts the balance between NOD1- and TIFA-dependent responses. Our results points to a NOD1 requirement at early time points and high MOIs, with the TIFA requirement evident at later time points and lower MOIs. Indeed the pioneering studies on the NOD1 response were largely done within 30 minutes of infection (Girardin et al., 2001; 2003). Thus, our use of NOD1, NOD2 and NOD1/2 double knockout human cells and careful examination of the response kinetics combined to expose TIFA as a central player in the innate recognition of cytosolic *Shigella*.

When characterizing the context in which HBP is presented to the cytosol during *Shigella* infection, I noticed that TIFA activation correlated with the intracellular bacterial growth rate. Moreover, infection with metabolically-deficient *Shigella* induced markedly less TIFA-dependent IL-8 production than wild-type bacteria. In this respect, HBP may serve as an indicator of microbial metabolism and replication. Several microbial components have been proposed to fulfill this role, such as bacterial pyrophosphates (Hintz et al., 2001), quorum sensing molecules (Zimmermann et al., 2006), tracheal cytotoxin (TCT) (Luker et al., 1995), isoprenoid metabolites (Tanaka et al., 1995), and cyclic-dinucleotides (McWhirter et al., 2009; Woodward et al., 2010). HBP is a unique member of this class for several reasons. First, it can withstand bacteriolysis, differentiating it from viability-associated PAMPs such as TCT and bacterial mRNA (Sander et al., 2011). Second, unlike isoprenoid and quorum sensing
metabolites, HBP is sensed within the host cytosol and can be recognized efficiently by non-immune cells. Third, in contrast to cyclic dinucleotides, which induce a host type I interferon response, HBP activates the NF-κB and SAPK/JNK pathways, driving production of pro-inflammatory cytokines like IL-8 (human) and KC (mice) that serve to recruit neutrophils. Thus, TIFA-dependent detection of HBP may allow the epithelia to serve as a dynamic sensor for especially virulent Gram-negative bacteria that can escape the entry vacuole, avoid cell-autonomous defense mechanisms, and exploit the cytosol for growth and replication, calibrating the amplitude of inflammatory response based upon the rate of intracellular bacterial growth.

Another mechanism whereby the inflammatory response to Shigella is amplified is through cell-cell propagation of NF-κB activation from infected to uninfected bystander cells (Kasper et al., 2010). In line with this, we occasionally noticed TIFA aggregates in cells that did not appear to be infected. While HBP would seem to satisfy the characteristics of the diffusing small molecule, additional studies are required to establish this link. Moreover, our results provide an explanation for the role of the Shigella effector OspI, a bacterial deamidase that interferes with TRAF6 function (Sanada et al., 2012). Thus, it is tempting to speculate that cell-cell propagation of TIFA activation could circumvent OspI function.

Finally, an essential role of the epithelium is to distinguish between signals from commensal and pathogenic microorganisms and direct an appropriate inflammatory response. While the mechanisms whereby the host evaluates the level of the microbial threat have been extensively studied in the context of immune cells (Blander and Sander, 2012; Franchi et al., 2012), the relative contribution of these mechanisms in IECs is less understood (Peterson and Artis, 2014). As invasion of the host cytosol is a defining characteristic of virulence, activation of the inflammasome likely plays an important role in gauging the microbial threat level (Sellin et al., 2015). However, they fail to account for the robust transcriptional response to bacterial invasion evident in IECs. Our results identify TIFA as the mediator of a cytosolic sensory system that functions non-redundantly with NOD1/NOD2 pathway to provide comprehensive immunosurveillance of the cytoplasmic compartment within IECs. Whereas NOD activation is indicative of cytosol-invasion, stimulation of the TIFA pathway informs the host of both invasion and intracellular proliferation, providing the contextual signal to dramatically amplify the inflammatory response.
Chapter 5: Conclusions and future directions

Host defense against infection relies upon accurate and immediate identification of potentially pathogenic microorganisms. The innate immune system employs evolutionarily ancient detection mechanisms for providing broad surveillance against pathogens displaying divergent virulence strategies. Despite over 20 years of intensive investigation on the host sensory apparatus responsible for initiating innate immune responses, novel innate sensing systems continue to be identified. Several recent examples include: cyclic GMP-AMP synthase, which mediates sensing of cytosolic DNA introduced into the host cell by viruses or bacterial secretion systems (Sun et al., 2013; Wassermann et al., 2015); aryl hydrocarbon receptor-mediated sensing of bacterial pigments (Moura-Alves et al., 2014); inflammatory caspases which sense intracellular LPS (Shi et al., 2014); and an innate antiviral pathway that senses O-linked glycans at epithelial surfaces (Iversen et al., 2016). A common theme among these discoveries is that for each sensory system is associated with detecting microorganisms of distinct virulence strategies; meaning that triggering of each pathway provides the host with specific information about the pathogen involved. Intriguingly, TIFA-mediated HBP detection is activated in a number of different settings by a diverse group of bacteria (summarized in Figure 46), the implications of which will have significant impact on our understanding of how the innate immune system responds to, and interprets, microbial threats.

5.1 Release of HBP from Neisseria

In Chapter 2, I describe the first setting in which HBP-induced inflammatory signaling was discovered: Active liberation of HBP from cultures of Neisseria spp. In this context, intracellular detection of HBP is indicative of extracellular bacteria. In this respect, HBP detection resembles NOD1 and NOD2 sensing of peptidoglycan fragments shed from extracellular bacteria, which is thought to play a key role in maintaining immune homeostasis in
Summary of ways which HBP may engage the TIFA-signalling axis. For HBP to activate TIFA-dependent signaling, it must be first liberated from within the bacterial cytosol. This can be accomplished through 1) release of HBP from Neisseria cultures; 2) inducing extracellular lysis of non-Neisseria Gram-negative bacteria; 3) intraphagosomal lysis of bacteria by activated macrophages; 4) release of HBP from replicating invasive Gram-negative bacteria in the host cytosol.

Figure 46    Summary of ways which HBP may engage the TIFA-signalling axis. For HBP to activate TIFA-dependent signaling, it must be first liberated from within the bacterial cytosol. This can be accomplished through 1) release of HBP from Neisseria cultures; 2) inducing extracellular lysis of non-Neisseria Gram-negative bacteria; 3) intraphagosomal lysis of bacteria by activated macrophages; 4) release of HBP from replicating invasive Gram-negative bacteria in the host cytosol.

the intestine (Clarke and Weiser, 2011). I showed that cultures of N. gonorrhoeae and N. meningitidis release HBP into the extracellular space during growth, while non-Neisseria bacteria do not. HBP is subsequently internalized into host cells by endocytosis and drives a potent NF-κB-dependent proinflammatory response upon entry into the cytoplasm. Indeed, that supernatants from Neisseria activated NF-κB in mammalian cell lines that were unresponsive to most, if not all, other PAMPs was the driving factor behind my initial hypothesis. In fact, it is likely that without its active liberation from Neisseria, the immunostimulatory properties of HBP would still be unrecognized. It is unclear how HBP is released from Neisseria; however, since this genus of bacteria is prone to autolysis, it remains possible that no active mechanism for HBP
export exists. Regardless, the release of HBP is in line with the pro-inflammatory strategy of the pathogenic *Neisseria*. Indeed, HBP joins the likes of endotoxin-containing outer membrane vesicles, peptidoglycan monomers, and immunostimulatory DNA that are all released from *N. gonorrhoeae* during growth. Clearly, there is no attempt by these bacteria to avoid the innate immune system and limit the massive inflammatory response that characterizes symptomatic gonorrhea. In fact, it appears that ongoing stimulation of the innate response is a specific strategy employed by the bacteria and, therefore, must benefit the pathogen and not the host. The excessive inflammation induced by *Neisseria*, in part due to HBP release, may contribute to damage of the mucosal lining, allowing access to the nutrient rich sub-mucosal space. In addition, recent work has suggested that infection with *N. gonorrhoeae* stimulates a robust pathogenic T\(_{h}\)17 response and hampers the development of T\(_{h}\)1/ T\(_{h}\)2 adaptive immune responses that are associated with immunological memory (Liu et al., 2012). This would be in line with historical observations that protective memory responses are not induced to *N. gonorrhoeae*, a characteristic that is crucial for the persistence of this pathogen in the general population (Fox et al., 1999; Schmidt et al., 2001). Work designed at dissecting the contribution of HBP to this phenomena is underway in the laboratory. However, complications to these studies are numerous, including the fact that *Neisseria* are strictly human pathogens and are thus difficult to study in mouse models of infection. Moreover, mutants of *N. gonorrhoeae* that are unable to synthesize HBP inherently possess the truncated “deep-rough” LOS structure. While control strains that can synthesize HBP but possess the same LOS exist, the defects associated with the truncated LOS structure (described in chapter 1.6.2) may themselves render the bacteria non-pathogenic. These issues aside, considering the potent response to HBP exhibited by immortalized human endocervical cells, it is likely that HBP liberation plays a role in the overzealous inflammatory response induced by *N. gonorrhoeae*. Deciphering the consequences of HBP detection in the context of an infection may yield novel immunomodulatory strategies designed at decreasing immunopathogenic responses and promoting protective immunity. In fact, inhibitors of the Neisserial HldA homologue in *Burkholderia* exist (Lee et al., 2013). Provided they have similar efficacy against *N. gonorrhoeae*, their use in combination with antibiotics for treatment of gonorrhea would reduce gonococcal virulence, render the bacteria more susceptible to antibiotics, and inhibit HBP production, thereby decreasing the bacterial burden and immunopathogenesis.
5.2 Release of HBP from extracellular- or phagosomally-lysed enteric bacteria

Supernatants derived from non-*Neisseria* Gram-negative bacteria showed no NF-κB-activating activity when supplied extracellularly to my reporter cell lines. Therefore, considering that HBP is an essential intermediate in the ADP-heptose biosynthesis pathway of every heptose-containing Gram-negative bacteria, the identification of HBP as the immunactive molecule released from *Neisseria* was initially surprising. This lead to my discovery that for HBP-mediated signaling by non-*Neisseria* Gram-negative bacteria to be apparent, HBP had to be first liberated from within bacterial cytosol. In Chapter 2, I described two examples by which this can occur: First by artificially inducing extracellular bacterial lysis; and second, during lysis of opsonized bacteria within the phagosome of activated macrophages. The generation of specific immune ligands during bacterial degradation within the phagosome of activated macrophages has been described previously. In the case of both peptidoglycan fragments (Herskovits et al., 2007) and bacterial mRNA (Sander et al., 2011), degradation of intact bacteria in the phagosome leads to transport (either active or leakage) of previously unavailable agonists into the macrophage cytosol where innate cytosolic surveillance pathways are triggered. These responses are associated with distinct transcriptional profiles, and in some cases, inflammasome activation. Phagosomal degradation also generates bacterial peptide ligands for antigen presentation via major histocompatibility complex class II (Ziegler and Unanue, 1982), and antigens are preferentially selected for their display based upon whether they trigger TLR signaling (Blander and Medzhitov, 2006). Currently, the role of HBP release during phagocytosis is unknown; however, I speculate that the TIFA/HBP-dependent-sustained increase in NF-κB activation observed following *E. coli* degradation in the phagosome could have an affect on the T-cell activating ability of the antigen presenting cell. This could occur through transcriptional upregulation of co-stimulatory molecules, increased cytokine expression, or by some process yet to be discovered. Experiments designed to dissect these relationships will be described in Section 5.4.

The immunological information gleaned from host sensing of HBP originating from extracellularly lysed bacteria is less obvious. Whether, in the context of an infection, extracellular lysis occurs at a frequency high enough to liberate sufficient HBP to activate host cells is unclear. In Chapter 2, I showed that an exceptionally small dose of soluble lysate derived
from Gram-negative bacteria can activate epithelial cells if introduced into the host cytosol through digitonin-permeabilization. This process was dependent on the ability of the bacteria to synthesize HBP. In contrast, for HBP to be detected in lysates by non-permeabilized cells, soluble lysates had to be derived from concentrated bacterial cultures far exceeding the densities seen in a physiological context. This indicated that access to host cytosol was the limiting factor in HBP detection, and that only high doses of extracellular HBP gain sufficient access to the cytosol and induce immune signaling. It is possible that from the perspective of the host, HBP could be used as signal for the presence of a massively expanding population of extracellular bacteria, in which frequent bacteriolysis would occur. A similar hypothesis has been proposed for NOD1/2 function in the intestine (Clarke and Weiser, 2011). However, I speculated that rather than function as an indicator of large numbers of extracellular bacteria, HBP was primarily used by the host as a signal for cytosol-invasive intracellular bacteria.

5.3 Release of HBP from cytosol-invasive bacteria during intracellular growth

In Chapter 4, I describe how TIFA-mediated detection of bacterial-derived HBP in the host cytosol is responsible for much of the innate immune response to prolonged infection with invasive *Shigella flexneri*. Considering that the central dogma in the field was that the innate response to *Shigella* in epithelial cells was entirely driven by NOD1, the importance of the TIFA pathway in this response was unexpected. However, upon discovering that the TIFA pathway was triggered after transient activation of the NOD1 pathway and at sites distal to bacteria, this finding added a crucial piece to understanding how cytosol-invasive bacteria are detected by the host. Although NOD1 and NOD2 are cytosolic sensors, activation of each pathway is tightly linked with their association with host membranes. This allows the NODs to alert the host to an immediate breach in the cytosol, and positions these sensors within areas of the cell where activation signals are likely to be of highest concentration (Philpott and Girardin, 2010). In fact, evidence that the NODs can be activated by intracellular bacteria not associated with host membranes, for instance bacteria freely moving in the host cytosol, is distinctly lacking (Philpott and Girardin, 2010). In addition, a large body of evidence suggests that NOD signaling is initiated immediately after infection with *Shigella*, and occurs independently from intracellular bacterial replication (Girardin et al., 2001; Sorbara et al., 2013; Travassos et al., 2010). This would seem to be at odds with how other systems of innate immunity function to evaluate
microbial threats, as rapidly multiplying intracellular bacteria would present a greater threat than stagnant intracellular bacteria, or bacteria that has invaded the cell but have targeted by cell-autonomous defense pathways. Therefore, TIFA-mediated detection of HBP allows the host to detect invasive bacteria freely replicating in the cytosol after the initial alert provided by NOD1. Moreover, TIFA amplifies the innate response to invasion based upon the intracellular bacterial growth rate. Thus, TIFA is a mediator of the cytosolic sensory system that functions non-redundantly with NOD1/NOD2 pathway to provide comprehensive immunosurveillance of the cytoplasmic compartment within IECs. Whereas NOD activation is indicative of cytosol-invasion, stimulation of the TIFA pathway informs the host of both invasion and intracellular proliferation, providing the contextual signal to dramatically amplify the inflammatory response to especially virulent pathogens.

Unlike culture supernatants from *Neisseria* species, supernatants derived from other Gram-negative bacteria such as *E. coli* and *S. flexneri* require digitonin-mediated access to the host cytosol to stimulate TIFA. My evidence suggests that, whereas growing *Neisseria* cultures release HBP in such a large amount (either by autolysis or active transport) that some may be internalized by dynamin-dependent endocytosis, cultures of *E. coli* and *Shigella* release HBP in such small quantities that accumulation and concentration within the host cell cytoplasm is required for its detection. The failure of non-permeabilized cells to respond to HBP-containing supernatants from enteric bacteria, implies that entry into the cytosol is the limiting factor that determines whether TIFA is engaged by HBP, providing a mechanism whereby IECs can discriminate between extracellularly and intracellularly growing bacteria. Although the process by which HBP is released from bacteria is currently unknown, the polar nature of the molecule implies that it cannot simply diffuse across the bacterial membrane. I speculate that HBP release occurs via leakage during replication, or accompanies the export of other bacterial small molecules.

It is also unclear whether the release of HBP by *Shigella* is beneficial or detrimental to the pathogen. In epithelial cells, *Shigella* induces mitochondrial dysfunction and cell death. This process, in part, is counterbalanced by NOD1-mediated induction of NF-κB-dependent anti-apoptotic gene products (Carneiro et al., 2009). Therefore, it is possible that HBP-induced expression of anti-apoptotic genes regulated by NF-κB promotes the continued survival of infected epithelial cells, providing the bacteria with a nutrient rich niche for replication.
Moreover, in a manner similar to *Neisseria*, *Shigella* induces a massive IL-8 response that mediates neutrophil recruitment. This results in epithelial destruction, which facilitates bacterial transepithelial translocation (Sansonetti et al., 1999). Thus, the HBP-dependent IL-8 response may promote bacterial invasion. An alternative view would be that the IL-8 response and concomitant neutrophil recruitment mediated by the HBP-TIFA pathway is essential for the eventual clearance of the bacteria, ensuring that the infection is limited at the intestinal level. The presence of multiple *Shigella* effectors that function to limit the host innate response seems to support this view. It is likely that the innate response to *Shigella*, while essential for bacterial clearance, can be manipulated by the bacteria to promote invasion and dissemination.

5.4 Future Directions

5.4.1 Further characterize the TIFA signalling cascade

This work opens up several avenues of further investigation. The principal unanswered question in this thesis is whether TIFA is the receptor for HBP. This could be directly tested by enzymatically synthesizing HBP to incorporate radioactive phosphorous-32 (P32) into the position of one of the phosphates. Binding assays and pull-downs could then be conducted with recombinant TIFA to see if there is an interaction between HBP and TIFA in a cell-free system. In addition, once sufficiently pure HBP and recombinant TIFA are available, isothermal titration calorimetry (ITC) could be used to assay whether HBP affects the thermodynamics of soluble TIFA. Indeed, recombinant TIFA is inherently insoluble in solution and is stabilized by the anion citrate. Thus, the similarly negative charged HBP may also function to stabilize TIFA in solution, which would be consistent with TIFA being receptor for HBP.

If TIFA is not the receptor for HBP, two different approaches may be taken to identify proteins upstream of TIFA in the HBP-induced signaling cascade. The first strategy would be to identify proteins that physically associate with TIFA in the presence of HBP. Immunoprecipitation of TIFA in the presence and absence of HBP coupled with LC/MS/MS could identify potential HBP-inducible TIFA-interacting partners. In fact, I have conducted a version of this experiment (Table 1). The fact that TRAF6 was pulled down by TIFA only in the
### Table 1

Co-IP coupled to LC/MS/MS identification of TIFA-binding partners in the presence or absence of HBP. 293T cells expressing 1XFLAG-TIFA (or empty vector) were treated for 1 or 2 hr with soluble HBP. TIFA was pulled down using anti-FLAG agarose and the resulting eluant was analyzed by LC/MS/MS for protein identification. Values indicated number of times a peptide corresponding to the indicated protein was identified in each fraction. Shown are the top 19 scoring gene products.

<table>
<thead>
<tr>
<th>Identified Proteins</th>
<th>Treatment:</th>
<th>Untreated</th>
<th>Untreated</th>
<th>HBP 1hr</th>
<th>HBP 2hr</th>
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</table>
presence of HBP confirms the validity of this approach. While no obvious candidates for the HBP receptor were pulled down by TIFA in the presence of HBP, proteins corresponding to the lysosomal compartment and endoplasmic reticulum were identified in the eluant, and warrant further investigation. The low spectral counts of eluted proteins in this experiment may have hampered efforts at identification of candidate genes. I suspect that several changes to the experimental protocol could improve the results. These include: 1) The creation of cell-lines expressing 3X-FLAG TIFA rather then 1X-FLAG TIFA would improve the immunoprecipitation; 2) Perform the IP after cross-linking TIFA with a reversible crosslinker such as DSP (dithiobis(succinimidyl) propionate), this would be especially important if the protein upstream of TIFA is a kinase that interacts transiently with its target; 3) Use the E178A TIFA mutant that does not interact with TRAF6, this would remove downstream TIFA binding partners from the IP eluant and perhaps enrich the sample with upstream proteins; 4) Due to the ability of HBP-induced TIFA aggregates to become insoluble, the cell-lysis buffer used needs to be optimized to promote solubilization of the TIFA complexes, while maintaining transient binding partners.

To complement this approach, a second loss-of-function screen could be undertaken with the goal of identifying all proteins involved in the HBP-induced TIFA signaling cascade. Improvements in screening technology, including CRISPR/Cas9-based screens (Hart et al., 2015), may yield novel protein candidates for future investigations. I suspect that in addition to obvious question marks such as the kinase that phosphorylates TIFA, potential transporters that facilitate entry of HBP into the cytoplasm, and any possible proteins upstream of TIFA, there remain to be identified many accessory proteins that participate in the signalling cascade including scaffolding proteins, members of the ubiquitin pathway, and positive and negative regulators. In fact, several negative regulators of TIFA function have been identified (Matsumura et al., 2009; 2004; Minoda et al., 2006); however, as the function of TIFA was unknown at the time, it is unclear how each of these regulators affects the HBP-detection pathway.

5.4.2 What are the immunological consequences of HBP recognition

In Chapter 2, I showed that infectious challenge of mice with *N. meningitidis* that are capable of synthesizing HBP induced more class-switched meningococcal specific IgG than bacteria that are deleted for HBP. This suggests that HBP is capable of priming the onset of
adaptive immunity, however the mechanism that promotes this response is unknown. Possible explanations include recognition of HBP by macrophages or dendritic cells and a consequent HBP-dependent increase in the expression of co-stimulatory molecules, cell-intrinsic recognition of HBP by T-cells or B-cells promoting activation and/or proliferation, or HBP-mediated proinflammatory and immunomodulatory cytokine production by immune or non-immune cells. Alternatively, tissue resident innate lymphoid cells may be specialized to detect HBP and promote immune maturation via cytokine production. The *in vivo* studies required to decipher this mechanism largely await the construction of a conditional TIFA-knockout mouse model. However, HBP is unique among bacterial PAMPs in that bacteria lacking HBP are viable, albeit likely to have reduced fitness *in vivo*. Therefore, HBP- proficient or -deficient *E. coli* engineered to express the model antigen ovalbumin (OVA) could be used in *in vivo* and *ex vivo* studies to examine the effect of HBP on T-cell and B-cell antigen specific responses. Engineering HBP+/− *E. coli* to invade the cytosol of host cells by expressing both *Yersinia* invasin protein and the Listeriolysin O (Castagliuolo et al., 2005) may amplify the HBP effect.

An unanswered question raised by my work in Chapter 4 is whether TIFA is required *in vivo* for protection against cytosol invasive bacteria. Since a robust TIFA response is induced by invasive *Shigella* in human colonic cell lines, this is the obvious pathogen to test first. However, even if TIFA knockout mice were available, *Shigella* is a challenging pathogen to study, as mice are naturally resistant to developing shigellosis. One way to circumvent these issues is to use human primary epithelial organoid culture models. Organoids are 3D models of the human intestinal epithelium derived from induced pluripotent stem cells, and which recapitulate the cell types present in the intestinal epithelium *in vivo*. Moreover, the technology for shRNA-targeted knockdown in organoids exists (Onuma et al., 2013), as do protocols for infection with invasive bacteria (Forbester et al., 2015). Therefore, the effect of TIFA depletion in human organoids infected with *Shigella* would both validate my findings in Chapter 4 and provide greater understanding of the TIFA-dependent phenotype. Another approach would be to take advantage of the fact that mice do not develop the massive inflammatory response to *Shigella* that typifies its colonization of the human colon. As discussed in Chapter 4, I speculated that differences in either the expression or function of TIFA in the murine vs. human colon may contribute to the lack of shigellosis in mice. Comparing the responses of murine and human organoid tissues to HBP would shed light on this issue. If the response to HBP is less robust in mice, overexpression
of human TIFA in the murine colon via lentiviral delivery and subsequent infection with *Shigella* would establish if TIFA promotes the clinical features of shigellosis in mice. The ensuing development of TIFA knock-in mice would provide a possible animal model for *Shigella* infection, the lack of which has continually hampered efforts at developing a vaccine to *Shigella* (Phalipon and Sansonetti, 2007).

Alternatively, other cytosol-invasive Gram-negative bacteria that are better suited for animal models may be used, provided they display similar TIFA-dependent responses in model cell lines. Examples of invasive pathogens that synthesize HBP include *Burkholderia thailandensis*, *Yersinia pseudotuberculosis*, or adherent-invasive *Escherichia coli* (AIEC). The effect of TIFA deletion on mouse models of infection would speak directly to the essentiality of the TIFA-dependent cytosolic surveillance pathway in protection against infection.

### 5.5 Summary

The work presented in this thesis describes the discovery of a novel pathogen-associated molecular pattern (PAMP), the host signalling cascade that detects it, and the immunological consequences of its detection in the context of a pathogenic infection *in vitro*. Future work must focus on understanding the implications of TIFA-mediated HBP detection on host-pathogen interactions *in vivo*. As discussed in Chapters 2 and 4, the TIFA response to HBP in some settings, for example to *Neisseria* and *Shigella*, may promote immunopathogenic responses that damage the host. However, the presence of the TIFA-pathway in many human cell types (myeloid and nonmyeloid), and across vertebrate phylogeny, suggests this sensory system plays an essential role in host immunity. Future studies will decipher the contexts in which the TIFA signalling axis contributes to the protective versus immunopathogenic responses of commensal and pathogenic Gram-negative bacteria.


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