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THE ACTIVATION, RECEPTOR COMPLEXING AND ENDOGENOUS REGULATION OF THE TYPE-I INTERFERON RESPONSE AS IT PERTAINS TO INNATE IMMUNITY

James D. Marion Jr.
Virginia Commonwealth University

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THE ACTIVATION, RECEPTOR COMPLEXING AND ENDOGENOUS REGULATION OF THE TYPE-I INTERFERON RESPONSE AS IT PERTAINS TO INNATE IMMUNITY

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctorate of Philosophy at Virginia Commonwealth University.

by

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May 2013
First, I dedicate this thesis to my mother and father who have been there for me every step of the way! Even though I’ve been difficult to deal with at times, you’ve both stood by me! Your love and support has gotten me through many difficult times and I thank you both for that! I love you both very much!

I also want to dedicate this thesis to Jessica and Ellis Bell who have been like a second set of parents to me! Without the support and guidance from both of you, I don’t think I would still be in science! You both have pushed me to achieve things I never thought were possible and I thank you both very much for that!
Acknowledgements

This thesis is the culmination of five years of work in Dr. Jessica Bell’s lab and the help of numerous colleagues, mentors, family members and friends.

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I would next like to thank my other mentor, Dr. Ellis Bell. He had no reason to accept me into his lab during my college years as my focus was on golf, not on academics. He saw something in me that nobody else did and pushed me to work hard. After college, it was his suggestion, working for him for two years as a lab manager and research assistant, that kept me in science and gave me a purpose in life. Again, my grades were not amazing and I had done nothing that made me stand out from the rest of the crowd but he took a chance on me and shaped what I have accomplished today. I don’t think I’ll ever be able to re-pay him for everything he has done for me but I want to thank him for pushing me to be the person I am today!

I would also like to thank my parents and my sister. Their love and support has pushed me through some of the best and some of the worst times in my life! Words cannot begin to describe how grateful I am that they stand by me in every situation and in every decision I have
made in my life. Whether I listen to their suggestions, or ignore them because I obviously think I
know better (when I really don’t), I owe them for every bit of guidance and support they have
given me in my life. Without them, I would not be where I am today!

I would like to thank the faculty and staff at Virginia Common wealth University. Their
support and advice has made this thesis possible and without their suggestions, the completion
of this thesis would not have been possible.

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friends. Their advice, support and ability to make me laugh has made these five years fly by with
relative ease!

I never thought this day would come, but it has and I am indebted to all of those that
made this possible. Thank you all!
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<td>AB</td>
<td>Adaptor-binding</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AQP</td>
<td>Aquaporin</td>
</tr>
<tr>
<td>AUC</td>
<td>Analytical ultracentrifugation</td>
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<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<td>CARD</td>
<td>Caspase recruitment domain</td>
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<td>CCR</td>
<td>Chemokine (C-C motif) receptor</td>
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<td>CBP</td>
<td>CREB-binding protein</td>
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<td>CD#</td>
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<td>CD</td>
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<td>C-type lectin receptor</td>
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<td>CMR</td>
<td>C-type mannose receptor</td>
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<td>CREB</td>
<td>cAMP response element-binding protein</td>
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<td>DUF</td>
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<td>dsRNA</td>
<td>Double-stranded ribonucleic acid</td>
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<td>Acronym</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>ECD</td>
<td>Ectodomain</td>
</tr>
<tr>
<td>eEF</td>
<td>Eukaryotic elongation factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
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<td>Fab</td>
<td>Fragment antigen binding</td>
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<td>FGFR1OP2</td>
<td>FGF receptor 1 oncogene partner 2</td>
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<tr>
<td>FITC</td>
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<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
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<td>IFN</td>
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<td>IFNAR</td>
<td>IFN-α/β receptor</td>
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<td>Immunoglobulin G</td>
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<td>IKK</td>
<td>Inhibitor of κB kinase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IPS</td>
<td>Interferon promoter-stimulating factor</td>
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<td>IkBα</td>
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<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<td>Major histocompatibility complex</td>
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<td>Myeloid differentiation primary response gene 88</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
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<td>NF-κB activating kinase</td>
</tr>
<tr>
<td>NAP1</td>
<td>NAK-associated protein 1</td>
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NEMO - NF-κB essential modulator
NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells
NK - Natural killer
NLR - NOD-like receptor
NOD - Nucleotide-binding oligomerization domain
NPM - Nucleophosmin
OPTN - Optineurin
OZR - Obese Zucker rat
PAMP - Pathogen associated molecular pattern
PCV - Pathogen containing vacuoles
PDB - Protein Data Bank
PE - Phycoerythrin
pI:pC - polyinosinic:polycytidylic acid
PKR - Protein kinase R
PRR - Pattern recognition receptor
PYR - Pyrin domain
RERO - Rapid equilibrium random order
RGA - Reporter gene assay
RIG-I - Retinoic acid-inducible gene I
RIP - Receptor-interacting protein
RLR - RIG-I-like receptor
SDD - Scaffold dimerization domain
SDS - Sodium dodecyl sulfate
SEC - Size exclusion chromatography
SIKE - Suppressor of IKKε
SINTBAD - Similar to NAP1 TBK1 adaptor
SPR - Surface plasmon resonance
STAT - Signal transducer and activator of transcription
T2K - TRAF2-associated kinase
TAK - Transforming-growth-factor-β-activated kinase
TANK - TRAF-associated NF-κB inhibitor
TBK1 - TANK-binding kinase 1
TBS - Tris-buffered saline
TEV - Tobacco Etch Virus
TIR - Toll/interleukin 1 receptor
TLR - Toll-like receptor
TRAF - Tumor-necrosis-factor-receptor-associated factor
TRAM - TRIF-related adaptor molecule
TRIF - TIR-domain containing adaptor protein inducing interferon β
UBC - Ubiquitin-conjugating enzyme
UEV1A - Ubiquitin-conjugating enzyme E2 variant 1
ULD - Ubiquitin-like domain
VACV - Vaccinia virus
V\text{\textsubscript{MAX}} - Maximum velocity
VPS - Vacuolar protein sorting
VSV - Vesicular stomatitis virus
WNV - West Nile Virus
WT - Wild-type
Abstract

THE ACTIVATION, RECEPTOR CLUSTERING AND ENDOGENOUS REGULATION OF THE TYPE-I INTERFERON RESPONSE AS IT PERTAINS TO INNATE IMMUNITY

By James D. Marion, Jr.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctorate of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2013

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To defend against pathogen challenge, multi-cellular organisms mount an immune response that recognizes, sequesters and eradicates invading infectious agents. Critical to this safeguard is the receptor-mediated detection of pathogens. Pathogen recognition then initiates a variety of signaling cascades that lead to the modulation of genes orchestrating an immune response. Toll-like receptor 3 (TLR3), a transmembrane receptor found in endosomes, is vital to the innate immune response against viruses. Double-stranded RNA (dsRNA) stimulation of TLR3 initiates a signaling cascade that leads to the production of type-I interferons and pro-inflammatory cytokines necessary to trigger the protective defenses of the immune system. Critical to this pathway is the activation of a kinase, TANK binding kinase 1 (TBK1), which
phosphorylates the downstream transcription factors, IRF3 and IRF7, and leads to the production of IFNβ. Interestingly, TBK1 function has been implicated in a number of other signaling cascades ranging from the insulin response and vesicle transport to xenophagy and anti-viral immunity. Increasingly, however, TBK1 dysregulation has been linked to autoimmune disorders and cancers, heightening the need to understand regulatory controls of TBK1. As a result, this dissertation investigates three components of the TLR3 signaling cascade in an attempt to further advance our understanding of the innate immune response. First, investigations into the adjuvant potential of dsRNA reveal that a 139bp dsRNA molecule is a viable candidate for vaccine adjuvant studies. Next, structural and functional studies of TLR3 with neutralizing antibodies provide evidence for a new TLR-signaling model in which dsRNA:TLR3 signaling units laterally cluster to achieve efficient signaling. Finally, cell-based assays, biophysical experiments and kinetic investigations into the mechanism by which an endogenous regulator of the TLR3 response, SIKE, functions, reveal that SIKE not only inhibits TBK1-mediated IRF3 phosphorylation, but is also a high affinity substrate. Findings from this study further suggest that SIKE regulates a critical catalytic hub not by direct repression of activity, but by redirection of catalysis through substrate affinity. Taken together, the results presented in this dissertation establish a foundation for building long-term studies on the function, regulation and viral subversion of the innate immune response.
CHAPTER I
INTRODUCTION

1.1 Immunology and the Immune Response

Immunology is widely known as a branch of biomedical science that investigates the ability of an organism to respond to antigenic challenge while also differentiating between “self” and “non-self [1].” Its origin is attributed to Edward Jenner who, in 1796, made the observation that his patients, who worked with cattle and had come in contact with a relatively mild disease called cowpox, never became infected with the deadly smallpox disease that had reached epidemic proportions at the time [1, 2]. By taking pus from a cowpox pustule and inserting it into an incision on the arm of an 8-year old boy, Jenner proved that having been inoculated with cowpox, the boy, who was subsequently inoculated with “variolous matter (Small-pox matter),” was immune to smallpox [1-3].

Interestingly, however, the prevailing theory of Jenner’s era was that chemical toxins were carried from an ill patient to others, causing them to contract the same disease [3]. The bacteria that were present were considered to be a symptom of the disease and not its cause. It was not
until the late 19\textsuperscript{th} century, when Louis Pasteur, who was working on a wine souring problem in which wine fermentations were producing a sour taste and very little alcohol, realized that the solution to the sour wine problem, a contaminating microbe that was generating lactic acid instead of alcohol from sugar, may be related to infectious diseases [4, 5]. In his work entitled, “Germ Theory of Disease,” Pasteur postulated that infections and disease were caused by invading microorganisms [5-7]. In 1876, Robert Koch proved Pasteur’s “Germ Theory of Disease,” when he successfully isolated infectious bacillus, a bacteria which he showed was the cause of both anthrax and cholera [8, 9]. Koch then defined a set of postulates which are still used today to demonstrate microbial pathogenicity amongst the four categories of disease-causing microorganisms; viruses, bacteria, pathogenic fungi, and parasites [1, 8, 9].

After it was established that microbes were the cause of disease, researchers went on to investigate how a host was able to defend against these pathogens. In 1890, using a non-lethal form of the diphtheria toxin (remnants of diphtheria with the active bacilli filtered out), Emil von Behring and Kitasato Shibasaburo injected guinea pigs and discovered that their tissues were able to acclimate to the toxin, producing a substance capable of neutralizing the diphtheria toxin itself [1, 10]. Using this antitoxin in humans, the mortality rate from diphtheria was reduced to negligible levels. This was the first demonstration of antibody activity and the first signs of the humoral theory of immunity.

While Behring and Shibasaburo were reporting their instrumental work on antibodies, another theory on the cellular basis of immunity began to take shape. Elie Metchnikoff, a Russian born biologist and zoologist, was making interesting observations in starfish larvae. By inserting thorns from garden plants into these transparent larvae and observing what he called “wandering cells,” he noted that these cells no longer moved around aimlessly but instead
aggregated around the foreign bodies as if to drive them out [1, 11]. Further experiments by Metchnikoff involved the insertion of spores from an infectious fungus into Daphnia, a type of water flea [1, 11, 12]. He observed mobile cells in the fleas surrounding and engulfing the infectious spores [1, 11]. From these findings, Metchnikoff laid a foundation for the theory of phagocytosis as he theorized that, under appropriate conditions, protector cells within an organism mount an immune response by attacking and ingesting foreign matter [1, 11].

At the time, an intense debate erupted as to which theory on the cellular basis of immunity was correct; the antibody theory of Behring and Shibasaburo or the theory of phagocytosis by Metchnikoff [1, 10, 11]. As we now know today, both theories were correct and are fundamental to the body’s ability to oppose microbial infection.

A specific immune response, such as the production of antibodies against a particular pathogen seen by Behring and Shibasaburo, is now known as an acquired or adaptive immune response. This response is highly adaptable due to V(D)J recombination of antibody heavy and light chains to create a unique immunoglobulin gene per B-cell [1, 13]. This is followed by somatic hyper-mutations that introduce variability into a clonal B-cell population [1, 13]. The end result is a small number of genes that are able to generate a vast number of different, specific antigen receptors. Of great significance to the host is the fact that this adaptability requires several (three to five) days to develop after the initial infection, but can confer lifelong resistance to infection by a pathogen exhibiting the same antigen [1].

On the other hand, a non-specific, immediate (minutes to hours), but short-lived response also exists. It is known as the innate immune response and is limited to germ-line encoded sensors that recognize a danger signal [1, 14, 15]. This response can be facilitated by phagocytic cells, such as those proposed by Metchnikoff, which engulf and destroy microorganisms, but is
also characterized by its many other roles including recognition of pathogens, recruitment of immune cells to sites of infection and communication with the adaptive immune response [1, 16, 17]. Because our lab focuses on the role of the innate immune response as part of the host defense system, the remainder of this document will focus on innate immunity.

1.2 Innate Immunity

The innate immune system is a host’s first line of defense against invading infectious agents. It is found, in some form, in all multicellular organisms and is thought to constitute an evolutionarily older defense strategy [18]. While it does not recognize pathogen specific antigens, it employs a number of other strategies in an attempt to protect the host from invading microorganisms.

Anatomical barriers, inherent to organisomal development, provide the first, and arguably, most important defense system of innate immunity [1, 19, 20]. In humans, epithelial cells are held together by tight junctions, forming a physical barrier that is impermeable to many infectious agents [1, 20, 21]. These epithelia comprise the skin and linings of the body’s tubular structures including the gastrointestinal, respiratory and urogenital tracts [1,20,21]. On the skin, desquamation, or skin peeling, helps remove bacteria and other infectious agents that may have adhered to the surface. Commensal microbes, known as normal flora, also live on the skin and compete with pathogenic microorganisms for nutrients and attachment sites on epithelial cells [1,20,21]. These nonpathogenic bacteria produce antimicrobial substances such as bacteriocidins, cationic proteins and lactoferrin, which work to destroy pathogenic organisms [21]. Internally, pathogens are expelled from the gastrointestinal and respiratory tracts by the peristalsis or ciliary action of tiny hairs [1,20,21]. Mucosal epithelia in the respiratory and gastrointestinal tracts also
secrete a viscous liquid called mucus which traps microorganisms and expels them in the flow of mucus driven by the beating of epithelial cilia [20]. Other epithelia in the respiratory and urogenital tracts produce antimicrobial peptides, such as β-defensins, that kill bacteria by damaging the bacterial cell membranes [20,21]. Saliva, tears, nasal secretions and perspiration also contain enzymes such as phospholipase A and lysozyme, which destroy gram positive bacteria [21]. Taken together, these barriers produce a strong defense against invading pathogens.

However, infections can occur when pathogens colonize or cross these anatomical barriers through cuts or scratches. These microorganisms can also invade by binding to molecules on the epithelial planes of internal organs while adhering to or colonizing the surfaces [21]. In most cases, when this happens, the pathogen is immediately recognized by mononuclear phagocytes, or macrophages, which are located in sub-mucosal tissues. Macrophages mature continuously from monocytes that leave the blood circulation and migrate into tissues throughout the body in a process called diapedesis [1,20,21]. These macrophages are soon reinforced by neutrophils, which are short-lived phagocytic cells that are abundant in the blood [21]. Phagocytic macrophages and neutrophils recognize, ingest and destroy many invading organisms. Their ability to recognize pathogens is mediated by cell-surface receptors, known as PRRs (pattern recognition receptors), which are found on each of the cells that mediate the innate immune response [1, 14]. These receptors have evolved to recognize evolutionarily conserved characteristics in broad classes of infectious microorganisms [14]. As these receptors are critical to the innate immune response, they will be discussed at great length in Chapter 1.3.

Upon recognition of a pathogen, cells of the innate immune response, through activation of a multitude of signaling cascades, are able to up-regulate co-stimulatory molecules on their surface
to communicate with the adaptive immune system and are able to produce proteins, such as
cytokines, chemokines, and cell-adhesion molecules, that guide the migration of effector cells to
the site of an infection [1,14,20,21]. Activation of these elements has several ramifications for
the host. Up-regulation of co-stimulatory molecules invokes the adaptive immune system’s
defenses, including the subsequent activation of T-cells and B-cells for antigenic specific
immune responses such as production of cytotoxic killer T-cells or antibodies [14,21].
Production of cytokines and chemokines mediates an immediate inflammatory response which
provides a physical barrier, in the form of microvascular coagulation, to prevent the spread of
infection in the blood stream [14,21]. Subsequently, this response mediates the delivery of
phagocytic macrophages and neutrophils to the site of infection effectively aiding in the
destruction of invading pathogen [1,20]. As the invading pathogen is contained, the innate
immune system signals to the adaptive immune system to help eradicate the remaining pathogen
and program an inflammatory response for future invasions by the same microorganism (Figure
1.1). While the innate immune response may be short-lived and non-specific, its initial reaction
to a pathogen is critical to host survival.

1.3 Pattern Recognition Receptors: Evolution, Redundancy and Cross-talk

As mentioned in Chapter 1.2, the innate immune system does not recognize pathogen-
specific antigens. Instead, it identifies evolutionarily conserved repeating patterns of molecular
structure found on the surfaces of or in the nucleic acid sequences of related pathogenic microbes
(PAMPs (pathogen-associated molecular patterns)), as well as endogenous molecules released
from stressed, injured, infected or transformed human cells (DAMPs (damage-associated
molecular patterns)) [1,14,21]. Recognition of these molecules is mediated through PRRs. These
Figure 1.1 Progression of the Immune Response
receptors reside on various immune cells (macrophages, neutrophils, dendritic cells, etc.), cells lining surfaces that may be exposed to pathogen (epithelial, endothelial, keratinocytes, etc.) or are secreted to act as sentinels in the bloodstream and lymph nodes [14]. Currently, three functionally distinct classes of PRRs have been identified; endocytic, secreted, and signaling PRRs (Table 1.1) [1,14].

Endocytic PRRs are found on the surface of phagocytes and promote the attachment, engulfment and destruction of microorganisms without relaying an intracellular signal [14, 22]. These PRRs recognize carbohydrates and range from the mannose receptors, which bind terminal mannose and fucose groups on microbial glycoproteins and glycolipids, to the scavenger receptors, which bind to bacterial cell wall components such as LPS, peptidoglycan and teichoic acid [1,20,21].

Secreted PRRs, on the other hand, bind to microbial cell walls and facilitate their recognition by phagocytes or the complement pathways [1,20]. These pathways, once activated, then mediate the regulation of antibody responses and promote the clearance of immune complexes and apoptotic cells. A primary example of these secreted PRRs is the mannose-binding protein [23], a lectin that is synthesized by the liver and released into the bloodstream. Once there, it can bind to carbohydrates on bacteria, yeast, viruses and parasites leading to the activation of the lectin-complement pathway [21,23]. This triggering effect results in the activation of C3b, a molecule that promotes opsonization [23].

Finally, signaling PRRs bind a number of microbial molecules which consequently promote the synthesis and secretion of intracellular regulatory molecules such as pro-
Table 1.1 Representative members of the three functionally distinct classes of PRRs.

<table>
<thead>
<tr>
<th>ENDOCYTIC PRRs</th>
<th>Receptor</th>
<th>Recognition</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-type mannose receptor 1</td>
<td>Glycoprotein/glycolipid</td>
<td>Endocytosis by phagocytic cells</td>
<td></td>
</tr>
<tr>
<td>C-type mannose receptor 2</td>
<td>Glycoprotein/glycolipid</td>
<td>Endocytosis by phagocytic cells</td>
<td></td>
</tr>
<tr>
<td>SCARA1</td>
<td>LDL, Bacterial cell walls</td>
<td>Endocytosis, platelets</td>
<td></td>
</tr>
<tr>
<td>SCARA2</td>
<td>acLDL, oxLDL</td>
<td>Endocytosis, platelets</td>
<td></td>
</tr>
<tr>
<td>SCARB1</td>
<td>HDL, LDL, oxLDL</td>
<td>Endocytosis, eNOS</td>
<td></td>
</tr>
<tr>
<td>SCARB2</td>
<td>HDL</td>
<td>Endocytosis, eNOS</td>
<td></td>
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</tbody>
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<thead>
<tr>
<th>SECRETED PRRs</th>
<th>Receptor</th>
<th>Recognition</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein</td>
<td>Phosphocholine</td>
<td>C1Q complex</td>
<td></td>
</tr>
<tr>
<td>Mannose-binding lectin</td>
<td>Glycoconjugates</td>
<td>Lectin complement pathway</td>
<td></td>
</tr>
<tr>
<td>Serum amyloid protein</td>
<td>HDL, oxLDL, acLDL</td>
<td>C1Q complex</td>
<td></td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>SIGNALING PRRs</th>
<th>Receptor</th>
<th>Recognition</th>
<th>Activation</th>
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</thead>
<tbody>
<tr>
<td>TLRs</td>
<td>Triacylated lipopeptide</td>
<td>NF-κB (MyD88-dependent)</td>
<td></td>
</tr>
<tr>
<td>TLR1-TLR2</td>
<td>Diacylated lipopeptide</td>
<td>NF-κB (MyD88)</td>
<td></td>
</tr>
<tr>
<td>TLR2-TLR6</td>
<td>dsRNA</td>
<td>NF-κB and IRF3 (TRIF)</td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>LPS</td>
<td>NF-κB and IRF3 (TRIF and MyD88)</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>Bacterial flagellin</td>
<td>NF-κB (MyD88)</td>
<td></td>
</tr>
<tr>
<td>TLR5</td>
<td>ssRNA</td>
<td>NF-κB and IRF3 (MyD88)</td>
<td></td>
</tr>
<tr>
<td>TLR7</td>
<td>ssRNA</td>
<td>NF-κB and IRF7 (MyD88)</td>
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<td>TLR8</td>
<td>CpG-DNA</td>
<td>NF-κB and IRF3 (MyD88)</td>
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<td>TLR9</td>
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<td>TLR10</td>
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</tr>
<tr>
<td>RLRs</td>
<td>Short, blunt dsRNA</td>
<td>NF-κB and IRF3 (IPS-1), Inflammasome</td>
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<tr>
<td>RIG-I</td>
<td>Long dsRNA</td>
<td>NF-κB and IRF3 (IPS-1)</td>
<td></td>
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<tr>
<td>MDA5</td>
<td>dsRNA</td>
<td>Positively regulates RLR signaling</td>
<td></td>
</tr>
<tr>
<td>LGP2</td>
<td>dsRNA</td>
<td>NF-κB and IRF3 (TRIF)</td>
<td></td>
</tr>
<tr>
<td>DDX1-DDX21-DHX36</td>
<td>dsRNA</td>
<td>NF-κB (RIP2)</td>
<td></td>
</tr>
<tr>
<td>NLRs</td>
<td>Muramyl dipeptide</td>
<td>NF-κB (RIP2)</td>
<td></td>
</tr>
<tr>
<td>NOD2</td>
<td>Uric acid crystal, silica</td>
<td>Inflammasome</td>
<td></td>
</tr>
<tr>
<td>NALP3</td>
<td>Flagellin</td>
<td>Inflammasome</td>
<td></td>
</tr>
<tr>
<td>IPAF</td>
<td>dsDNA</td>
<td>Inflammasome</td>
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</tr>
<tr>
<td>AIM2</td>
<td>dsDNA</td>
<td>Inflammasome</td>
<td></td>
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<tr>
<td>CLRs</td>
<td>SAP130 nuclear protein</td>
<td>Syk-dependent signaling</td>
<td></td>
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<tr>
<td>Mince</td>
<td>Necrotic cells</td>
<td>Syk-dependent signaling</td>
<td></td>
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<tr>
<td>Clec9a/DNGR-1</td>
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inflammatory cytokines, type I interferons, chemokines, antimicrobial proteins and other co-stimulatory factors [1,14,20,21]. Currently, signaling PRRs have been organized into four distinct families; the RLRs (RIG-I (Retinoic acid-inducible gene)-like receptors) [1, 24], the NLRs (NOD-like receptors) [1, 25], the CLRs (C-type lectin receptors) [1, 26] and the TLRs (Toll-like receptors) [1, 16]. While the RLRs and NLRs are cytoplasmic protein receptors, the CLRs and TLRs are transmembrane protein receptors [16].

RLRs, consisting of RIG-I, MDA5 (melanoma differentiation associated gene 1), LGP2 (laboratory of genetics and physiology 2), and a complex of three RNA helicases, DDX1-DDX21-DHX36, are intracellular PRRs that act as viral sensors through direct interaction with dsRNA, the replicative intermediate of RNA viruses [24]. RIG-I and MDA5 are each comprised of two N-terminal CARD domains (caspase recruitment domains), which induce a cellular response through interactions with the adaptor protein, IPS-1 (interferon promoter-stimulating factor 1), and a DExD/H-box helicase domain, which mediates the recognition and binding of dsRNA to the central cavity of the protein [24]. The complex of RNA helicases, DDX1-DDX21-DHX36, also contains a DExD/H-box helicase domain but differs from RIG-I and MDA5 in that a Helicase A domain in DDX1 recognizes and binds dsRNA while an HA2-DUF and a PRK domain in DDX21-DHX36 recruit an adaptor protein, TRIF (Toll/interleukin 1 receptor (TIR)-domain-containing adaptor protein inducing interferon-β), the consequences of which will be discussed in more detail in Chapter 1.5 [27]. The final member of the group, LGP2, lacks the ability to induce signaling alone due to the absence of a CARD domain, but it can recognize dsRNA through interactions with its DExD/H-box helicase domain [28]. Upon recognition of viral dsRNA, all RLRs activate downstream transcription factors (NF-κB and IRF3/7) that mediate production of pro-inflammatory cytokines and type-I interferons [16].
To recognize cytoplasmic PAMPs as well as endogenous danger signals, NLRs use a tripartite-domain organization, consisting of a carboxy-terminal leucine-rich-repeat motif, which senses the presence of invading microorganisms (ligand sensing is the focus of these receptors as there is only weak evidence supporting a direct interaction of the NLRs and their cognate ligands), a central NACHT domain (nucleotide binding domain) with ATPase activity that promotes ATP-dependent self-oligomerization, and a variable N-terminal domain that is responsible for homotypic protein-protein interactions [16, 25]. NLRs are subdivided into four families based on the type of N-terminal domain they use to mediate protein-protein interactions; NLRA members have an acidic N-terminal transactivating domain, NLRB members have a BIR domain (baculovirus inhibitor of apoptosis protein repeat N-terminal domain), NLRC members have an N-terminal CARD domain, and NLRP members have a PYR domain (pyrin domain) [1,16,25]. While recognition of pathogen-derived ligands by the NLRC family leads to activation of NF-κB and subsequent production of pro-inflammatory cytokines, NLRA, NLRB, and NLRP recognition of pathogen-derived ligands leads to the formation of an inflammasome that mediates the activation of Caspase-1, an enzyme important in the proteolytic processing and activation of the pro-inflammatory cytokines IL-1β and IL-8 [25].

CLRs are transmembrane proteins broadly expressed on a myriad of innate immune cells ranging from those as complex as immature dendritic cells to those as small as platelets [16, 26]. When expressed, they interact with pathogens through the recognition of carbohydrate moieties [26]. CLRs have at least one carbohydrate recognition domain, which determines carbohydrate specificity, and are broadly classified into two distinct groups [16,26]. Group I CLRs are members of the mannose receptor family with an N-terminal cysteine rich domain, a single fibronectin type II domain and eight to ten C-type lectin-like domains [16,26]. These receptors,
such as CMR1 and CMR2 (C-type mannose receptor 1 and 2), are primarily present on the surface of macrophages and dendritic cells where they recognize and bind to repeated carbohydrate moieties on the surface of infectious agents [26]. Group II CLRś belong to the asialoglycoprotein receptor family and contain one C-type lectin-like domain [26]. These receptors, such as the Dectin-1 receptor (DC-associated C-type lectin 1), are expressed on a variety of immune cells and recognize pathogens ranging from M. tuberculosis to HIV-1 [16,26]. Together, CLRś are able to recognize most classes of human pathogens [16]. This recognition, in turn, leads to pathogen internalization, degradation and antigen presentation [16]. CLR activation can also lead to the induction of specific cytokines that determine T-cell polarization fate [16].

Finally, TLRś, which will be discussed at length in Chapter 1.5, are membrane-spanning, non-catalytic receptors found in sentinel cells [1, 16, 29]. They are comprised of an ECD (ectodomain), which contains LRRś (leucine-rich repeats) that mediate the recognition of PAMPś, a single transmembrane region, and a TIR (cytosolic Toll-IL-1 receptor) domain that activates downstream signaling pathways [29, 30]. TLRś recognize a wide variety of PAMPś spanning bacterial cell-surface LPS (lipopolysaccharides) and bacterial flagella to viral dsRNA and the unmethylated CpG islands of bacterial/viral DNA [16]. TLR-mediated recognition of a pathogen leads to the activation of intracellular signaling cascades that induce the production of inflammatory cytokines as well as type-I interferons [16].

While a multitude of defense systems exist to recognize the ever expanding list of invading pathogens, questions have been raised as to the redundant nature of signaling PRRś and their activation of similar signaling cascades (Figure 1.2). Microbes harbor a variety of PAMPś that induce signaling pathways leading to an amplified host response. These responses, as was detailed in Chapter 1.2, are essential for eradicating the invading microorganism. However, over-
activated responses can be dangerous to the host. In the case of sepsis, also known as systemic inflammatory response syndrome, effector proteins induced by an immune response are over produced leading to a state of sustained inflammation, a drop in blood pressure and a lack of perfusion of vital tissues and organs [31]. The deadly nature of these over-activated responses has led to research focused on the cross-talk between PRRs and their signaling pathways [16, 32]. The redundant nature of both entities seems to induce cooperative activation that is critical to an effective immune response, but may contribute to detrimental effects on the host if not tightly regulated [32].

Examples of cross-talk have been shown between similar PRRs such as the TLRs [16,32]. Experiments indicate that stimulation of TLR3 and TLR4 can up-regulate TLR2, TLR7 and TLR9 expression based on the adaptor proteins that are recruited to the cytoplasmic domain of each TLR [32, 33]. Other investigations have shown that TLR3 and TLR4 can also interact, synergistically, with TLR7, TLR8 and TLR9 to increase the production of a Th1-polarizing cytokine known as IL-12p70 [33]. This TLR synergy can be further boosted by IFNγ and CD40L, products of activated NK- and T-cells, suggesting that cross-talk between innate and adaptive immune responses can also occur to control optimal immune cell activation [32,33].

In addition to cross-talk within one class of PRRs, researchers have shown that cross-talk also exists amongst different PRRs [32]. While the RLRs, RIG-I and MDA5, are IFN inducible, specific TLRs, TLR3, TLR4, TLR7, TLR8 and TLR9 are known to induce IFN production. Experiments done on human melanoma cells were able to show that the stimulation of TLRs led to a RIG-I/MDA5 response that caused these cells to terminally differentiate and irreversibly lose proliferative capacity suggesting that the activation of the TLRs can potentiate an RLR response [34]. Further experiments done on the NLRs and TLRs have shown that NOD1 and
**Figure 1.2 The redundant nature of PRRs.** Depicted is a small representation of PRRs that recognize pathogenic dsRNA. Upon recognition, each PRR activates a distinct signaling cascade. Detrimental effects will occur if a coordinated response is not regulated by the host.
NOD2 act synergistically with TLR3, TLR4 and TLR9 in human dendritic cells to induce IL-12p70 production and promote Th1 cell differentiation [35]. Additional experiments done on the TLRs and the CLRs have shown that Dectin-1, a CLR, and TLR2 collaborate synergistically to produce pro-inflammatory cytokines such as TNFα in bone-marrow derived dendritic cells and IL-10, an anti-inflammatory cytokine, in splenic dendritic cells [36].

While numerous other PRR cross-talk examples exist, researchers are just beginning to understand how the immune response is so tightly regulated with the number of seemingly redundant structures and pathways found in many hosts. Recent studies, attempting to investigate the coordinated activity of large networks of intracellular signaling pathways in one specific cell, used RAW 264.7 macrophages to look at 22 receptor-specific ligands and their ability to induce calcium mobilization, cAMP synthesis, phosphorylation of numerous signaling proteins and cytokine production [37]. Using a combination of computational clustering methods and experimental cAMP, calcium and cytokine assays, the investigators found a large network of complex non-linear signaling cascade interactions that generated both known and unexpected novel interactions amongst different pathways [37]. The authors were able to conclude that while many PRRs and signaling pathways do exist, only a small number of interaction mechanisms and signaling cascades coordinate a response towards many stimuli to provide for context-dependent signaling and prevent a chronic or sustained immune response [37].

The cross-talk examples provided here focused on the TLRs as their ability to recognize a diverse set of pathogens has led to mechanistic studies both on their structures and signaling cascades. As will be discussed in Chapter 1.4, the TLRs use their diverse set of recognition capabilities and cross-talk mechanisms to not only initiate immediate inflammatory responses, but also to shape the adaptive immune response.
1.4 Toll-like Receptors: Evolution and Structure

The first Toll family member, Drosophila Toll, was discovered in 1985 by the Nusslein-Volhard group [38]. Identified as a maternal effect gene, mutations caused a fruit-fly phenotype that surprised German researchers to the point of exclaiming, “Das ist ja toll,” which translates to “That’s great,” and subsequently gave the Toll-like receptor family its name [1,38]. Further research has since shown that, in the fly, the Toll receptor recognizes a cleaved protein product known as Spatzle [39]. In the fly embryo, this recognition induces formation of the dorso-ventral axis, while in the adult fly, recognition triggers an intracellular cascade that leads to the activation of a transcription factor termed Dorsal, a fly homologue of the transcription factor NF-κB [39]. In 1996, Jules A. Hoffman and coworkers made the ground-breaking discovery that Toll was critical to a fly’s antifungal innate immune response [40]. They showed that mutations to the Toll receptor dramatically reduced survival of the host after fungal infection [40].

It was not until 1997 that Charles Janeway, the scientist who, a decade earlier, had hypothesized that a set of factors, which he termed PRRs, were capable of detecting PAMPs in the immune response [41], discovered the first human Toll receptor [42]. Through cloning experiments in which Janeway’s group transfected into human cell lines a constitutively active construct of human Toll, they were able to show an induced activation of NF-κB and the expression of NF-κB controlled genes for the inflammatory cytokines IL-1, IL-6 and IL-8 [42]. Further studies with the constitutively active human Toll showed an induced expression of the co-stimulatory molecule B7.1, required for activation of naïve T-cells, and suggested an essential role for this receptor in the human immune response [42]. A year later, building off of Janeway’s results, Bruce Beutler, investigating what he called the most powerful microbial stimulant of the innate immune response, LPS, discovered TLR4 to be the sole LPS receptor and the gateway to
the endotoxin response [43]. Through missense mutations in the TLR4 gene of C3H/HeJ LPS response locus mice, Beutler was able to show that the mammalian TLR4 protein had been adapted to primarily recognize LPS and, as he postulated, transduce the LPS signal across the plasma membrane [43]. Knockout mutations of the TLR4 gene further corroborated Beutler’s work as these mice were resistant to the development of Gram-negative sepsis but susceptible to infection [44]. Subsequent studies by several groups [45] found additional Toll-like receptors that could similarly activate a transcriptional based response.

To date, 10 human Toll-like receptors (13 Toll-like receptors in mice) have been characterized (Figure 1.3), each recognizing a variety of evolutionarily conserved PAMPs including lipopeptides (TLR2 associated with TLR1 or TLR6) [46, 47], viral dsRNA (TLR3) [48], LPS (TLR4) [43], bacterial flagellin (TLR5) [49], viral or bacterial ssRNA (TLR7 and TLR8) [50, 51] and CpG-rich unmethylated DNA (TLR9) [52], among others [16](Table 1.2). While these receptors seem to diverge in the ligands they recognize, clustering studies have found that human TLR1, TLR2, TLR6 and TLR10 converge into a TLR subgroup (TLR1/2/6/10) based on sequence homology as do TLR7, TLR8 and TLR9 (TLR7/8/9) [53, 54].

Further, structural studies have determined that a high degree of homology also exists in the framework of each of these receptors. Each TLR is now known to be an evolutionarily conserved type-I integral membrane glycoprotein that consists of an N-terminal ligand recognition domain (TLR-ECD), a single transmembrane helix that contains approximately 20 uncharged, mostly hydrophobic residues, and a C-terminal cytoplasmic signaling domain, known as the TIR domain [29]. So named for its homology with the signaling domains of IL-1R family members, TIR domains are also found in many adaptor proteins that interact with the TIR domain of TLRs and in plant proteins that confer resistance to pathogens [55]. This has suggested to numerous
Figure 1.3 The 10 human Toll-like receptors and the pathogens they recognize.
<table>
<thead>
<tr>
<th>TLRs</th>
<th>Localization</th>
<th>Exogenous Ligands</th>
<th>Source of Exogeneous Ligands</th>
<th>Endogenous Ligands</th>
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<tbody>
<tr>
<td>TLR1</td>
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<td>Lipopeptides</td>
<td>Bacteria and Mycobacteria</td>
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<td>Plasma Membrane</td>
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<td>Gram-positive bacteria, Mycoplasma, Mycobacteria, Spirochetes</td>
<td>HSP60, HSP70, HSP96, HMGB1, Hyaluronic acid</td>
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<td>Treponema pallidum</td>
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<td>Lipoteichoic Acid</td>
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<td>Treponema pallidum</td>
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<td>Glycoinositolphospholipids</td>
<td>Trypanosoma cruzi</td>
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<td>Phospholipomannan</td>
<td>Candida albicans</td>
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<td>Hemagglutinin</td>
<td>Mycobacteria</td>
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<td>Lipoparabinomannan</td>
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<td>Saccharomyces</td>
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<td>Double-stranded RNA (dsRNA)</td>
<td>RNA Viruses</td>
<td>mRNA</td>
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<tr>
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<td>Flagellin</td>
<td>Gram-positive or Gram-negative bacteria</td>
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<tr>
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<td>RNA Viruses</td>
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<td>Bacteria and viruses</td>
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<td>Unknown, may interact with TLR1 and TLR2</td>
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</table>
researchers that the TIR domain actually represents an evolutionarily conserved motif that may have had an immune function prior to the divergence of plants and animals.

In observing the extracellular domain of each crystallized TLR, it has been noted that all are constructed of 19-25 tandem copies of a motif known as the LRR (leucine-rich repeat) motif (Figure 1.4) [53, 56]. A single LRR motif is typically 22-29 residues in length and contains hydrophobic residues spaced at distinct intervals [57]. In three dimensions, all LRRs adopt a loop structure that begins with three residues in a β-strand configuration [58]. When assembled consecutively in the TLR-ECDs, the LRR motifs form a solenoid structure with all consensus hydrophobic residues pointing to the interior, to form a stable core, and all β-strands aligning to form a hydrogen-bonded parallel β-sheet [57,58]. Because the β-strands in the LRR motif are packed more closely than the rest of the structure, the solenoid is forced into a curved configuration in which a concave surface is formed by the β-sheets [57,58]. This effectively creates a structure with a concave surface, a convex surface, an ascending lateral surface, and a descending lateral surface on the opposite side [57,58]. To date, the structures of the ECDs of TLR1[59], TLR2[59], TLR3[30], TLR4[60], TLR5[61], TLR6[62], and TLR8[63] have been reported. In common with all of these structures is the fact that the solenoid motif is the basis for ligand recognition as ligand binding occurs on the ascending lateral face, the only portion of the molecule that completely lacks N-linked glycan and is free to interact with ligand (Figure 1.4). Interestingly, however, while all of the TLR-ECDs assume a typical horseshoe-shape, attempts to predict these structures in modeling studies have been unsuccessful due to variations in the curvature of the structure.
Figure 1.4 hTLR3-ECD consisting of 23 leucine-rich repeats. hTLR3 contains 23 leucine-rich repeats, each of which follows the typical 24-residue repeat pattern of the LRR motif [57,58]; $xL^2xxL^5xxN^{10}xxL^{12}xxL^{15}xxxxF^{20}xxL^{23}x$ where L represents conserved hydrophobic residues, N represents a conserved asparagine and F represents a conserved phenylalanine. Also depicted is the glycan-free side (ascending side) of the TLR3-ECD, involved in ligand binding in all TLRs [58].
As specific ligands of each receptor were identified, focus shifted to the mechanistic understanding of the resulting activated signaling pathways. As will be discussed at great length in Chapter 1.5, the identification of players in the activated TLR signaling cascades and the elucidation of products from each of these pathways led to the scientific community’s re-evaluation of the importance for these receptors in immunity. The culmination of this recognition came in 2011 with the awarding of the Nobel Prize in Physiology or Medicine to Bruce Beutler, Jules Hoffman and Ralph Steinman for revolutionizing our understanding of the immune system by discovering key principles for its activation.

1.5 Toll-like Receptors: Pathogen Recognition and Signaling

Classifying specific TLRs with the pathogens they recognize was a critical first step in understanding how TLRs aid in the innate immune response. In addition, detailing the mechanism by which TLRs recognized specific pathogens and understanding how subsequent signal transduction events translated pathogen recognition into a host response were essential in understanding the important role TLRs have in protecting a host from microorganism invasion. This is because each TLR and TLR: pathogenic-ligand complex elicits distinct downstream gene expression patterns that lead to innate immune responses tailored towards specific pathogens and to the development of antigen-specific adaptive immune responses.

Long established was the fact that signal transduction by type I transmembrane receptors required ECD dimerization events [64]. Originally observed with the full-length Drosophila Toll ECD, unstable dimers were able to form in solution and thought to exist in pre-assembled, low affinity complexes before ligand binding [65]. However, it was not until 2003, when Nicholas Gay’s group investigated the mechanism by which a protein, Spatzle, activated the Toll-receptor
in adult Drosophila flies, that researchers were able to propose a mechanism by which microbes induced TLR activation. Using analytical ultracentrifugation in combination with chemical cross-linking and isothermal titration calorimetry experiments, Gay’s group was able to show that the Toll-receptor-activating ligand, Spatzle, formed a heterotrimeric complex containing two molecules of Toll-receptor ECD for every one molecule of ligand [66]. From these results, Gay’s group proposed that Toll-receptor activation required ligand-induced dimerization events which caused secondary receptor-receptor interactions effectively stabilizing and producing an active signaling complex [66]. Subsequent structural studies have found that in common with most TLR subfamilies, PAMP binding occurs when two TLR extracellular domains form an “m-shaped” dimer and effectively trap the ligand molecule (Figure 1.5) [29]. This “sandwiching” effect brings the TLR transmembrane and cytoplasmic domains in close proximity and triggers a downstream signaling cascade. However, while the ligand-induced dimerization events of all TLRs have many common features, the recognition events leading to TLR-ECD dimerization are markedly different between TLR paralogs as individual TLRs use unique subsets of LRRs for ligand recognition. This effectively suggests a means by which a limited number of TLRs are able to recognize a diverse array of ligands.

Best understood due to the elucidation of crystal structures for three of the four family members (TLR10 is the only member without a known structure), the TLR1/2/6/10 subfamily differentiates between different PAMPs based on specific dimerization events. Structural studies have shown that TLR2 is able to dimerize with either TLR1 or TLR6 (Figure 1.5A and 1.5B, respectively) [59, 62] and that the specific heterodimeric complex that is formed determines whether these TLRs bind PAMPs containing di-acylated cysteine-rich lipopeptides (TLR2/6) or tri-acylated cysteine-rich lipopeptides (TLR1/2). Further experiments have revealed the reason
Figure 1.5 Representative examples of the ligand-induced “M-shaped” dimer complex formed by the TLR-ECDs. A. TLR1 (green)-TLR2 (blue) heterodimer induced by the binding of tri-acylated lipopeptide (purple) (PDB ID: 2Z7X)[59]. B. TLR2 (blue)-TLR6 (red) heterodimer induced by the binding of di-acylated lipopeptide mimetic (black) (PDB ID: 3A79)[62]. C. TLR4 (raspberry) – MD-2 (yellow) complex induced by the binding of LPS (PDB ID: 3FXI)[67].
for this difference as crystallographic analysis has shown that three distinct subdomains exist in each ECD of the subfamily; N-terminal, central, and C-terminal [59,62]. The border between the central subdomain and C-terminal subdomain (LRRs 9 to 12) contains a group of hydrophobic residues which form a ligand binding pocket on the convex side of the ECD [59,62]. This pocket can increase or decrease in size depending upon the formation of distinct TLR complexes. Complexes between TLR1 and TLR2 form a pocket that is large enough to accommodate tri-acylated cysteine containing lipoproteins [59] while complexes between TLR2 and TLR6 form a pocket that is much smaller, due to constraints placed on the structure by phenylalanine residues, effectively binding only di-acylated lipopeptides [62]. While the fourth member of the family, TLR10, has not been structurally characterized, homology models refined through molecular dynamic simulations suggest that TLR2/10 heterodimeric complexes form a ligand binding pocket similar to the TLR1/2 complexes, effectively recognizing tri-acylated cysteine-containing lipopeptides, while TLR1/10 heterodimeric complexes and TLR10 homodimeric complexes form ligand binding pockets similar to those of the TLR2/6 complex, effectively recognizing di-acylated cysteine-containing lipopeptides [68].

In the TLR3 subfamily, ligand recognition and binding are dependent upon two interactions between dsRNA and the TLR3-ECD, one near the N-terminus, encompassing LRR-NT and LRRs1-3, and one near the C-terminus, involving LRRs 19-23 [30]. These interactions correctly position four ligand binding sites in a TLR3 homodimeric complex with dsRNA [30]. As TLR3 is the focus of much of the work presented in this thesis, these associations and subsequent signaling events will be discussed in more detail in Chapter 1.6.

For the TLR4 subfamily, advances on Beutler’s work [43], discussed earlier, have led to a crystallographic analysis of the receptor structure which depicts similarities to the TLR2
heterodimeric structures previously elucidated [67]. The TLR4-ECD is composed of three subdomains located in similar regions to that of the TLR2-ECD (LRR-NT to LRR5 and LRR8-10); an N-terminal, central, and C-terminal subdomain [67]. However, the β-sheet present in the central subdomain of the receptor contains unusually small radii and large twist angles that prevent a ligand binding pocket, seen in TLR2 heterodimeric complexes, from forming [67]. Instead, it has been found that a co-receptor, MD-2, binds to TLR4 on the concave surface of the N-terminal and central subdomains [67]. LPS is then extracted from the bacterial membrane and transferred to TLR4-MD-2 heterodimers by two accessory proteins, LPS-binding protein and CD14 [67]. LPS interacts with a large hydrophobic pocket in MD-2 and induces the formation of a receptor multimer, composed of two copies of the TLR4-MD-2-LPS complex, by forming hydrophobic interactions with conserved residues on TLR4 [67]. While it has been determined that PAMPs are not directly recognized by TLR4, LPS binding to MD-2 and subsequent interaction with TLR4 induces the formation of the classical “m-shaped” dimeric receptor complex (Figure 1.5C) which is then able to transduce a signal through membranes to the cytosol.

The TLR5 subfamily is the only protein-binding (Flagellin) TLR that is conserved in vertebrates from fish to mammals. However, even with its vast evolutionary background, the human TLR5 structure has not been determined due to technical challenges in expression of the protein in a functionally active, soluble form. Recent crystallographic studies, however, have elucidated a TLR5 structure in zebrafish using a baculovirus system and C-terminal deletion variants that, along with computer based prediction algorithms, have led to an understanding of how TLR5 interacts with bacterial flagellin [61]. This PAMP is composed of three specific domains, D1/D2/D3, which are responsible for the ability of flagellin to polymerize into a
filament in bacterial flagellum, and effectively provide motility to the pathogen [69]. From crystallographic analysis of the zebrafish structure, TLR5 is shown to primarily interact on its lateral side (LRR-CT and LRRs12-13) with three helices that make up the D1 domain of flagellin [61]. This interaction forms an extensive primary binding interface that defines a 1:1 heterodimer [61]. Upon formation of this complex, further ligand-induced oligomerization events lead to the formation of a symmetric 2:2 complex where TLR5 from the first heterodimer creates additional, weaker interactions with flagellin and TLR5 from the second heterodimer [61]. Ligand-induced assembly of two TLR5 receptors juxtaposes the C-terminal tail regions of TLR5-ECD for signaling in a similar manner to all other agonist-bound TLRs [61].

Similar to TLR3, members of the TLR7/8/9 subfamily are all located in the endosome. However, distinct amino-acid sequence differences and the recent elucidation of the TLR8-ECD structure [63] indicate that the ligand binding mechanisms of TLR7/8/9 are different from their endosomal dsRNA receptor counterpart. The recent elucidation of the TLR8-ECD has revealed that similar to all other TLRs, ligand recognition is mediated by a dimerization interface formed by two protomers [63]. However, three-dimensional structures, based on homology modeling studies of the LRR motifs in the TLR7/8/9 subfamily [70], and the recently reported TLR8-ECD structure [63], have shown that all members contain large insertions in LRR2, LRR5 and LRR8 which give rise to distinct structures that loop out from the dimerization surface of the ECDs. These extensions were postulated, and have been confirmed for TLR8, to provide additional support to the receptors upon ligand binding [63,70]. These studies also report that all members of this subfamily contain a 40-amino-acid stretch of residues between LRR14 and LRR15 that have a high degree of species variability amongst the paralogs and are implicated in signal transduction events which will be discussed in the next paragraph [63,70]. Finally, studies have
also suggested that residues in the insertion face of the ECD (LRR17) for TLR8 (Asp543) and TLR9 (Asp535 and Tyr537) are essential for ligand binding [70]. While recent structural studies of TLR8 have confirmed the importance of Asp543 in ligand binding, a more detailed structural analysis of the entire subfamily is still required for a complete understanding of the ligand recognition mechanism used by these compartmentalized TLRs.

Interestingly, investigations have revealed that specific proteolytic cleavage events are required for effective downstream signaling in the TLR7/8/9 subfamily [63]. Studies report that lysosomal proteolysis is required for TLR7/8/9 signaling and that this processing occurs in the undefined regions between LRR14 and LRR15 of the ECDs (proteolytic processing events have also been implicated in TLR3 signaling [71, 72] but these will be discussed in more detail in Chapter 1.6) [63]. Experimental data shows that TLR7/8/9 are escorted from the endoplasmic reticulum, by a chaperone molecule, Unc93b1, to the endolysosome where they are cleaved in a multistep process that has created much controversy in the scientific community [63, 73, 74]. Researchers agree that primary cleavage events, performed by asparagine endopeptidase and other undefined cathepsin family members [75], remove the majority of the ECD domain from the TLRs. However, following this initial proteolytic processing event of TLR7/8/9, researchers diverge on the reasons for these events as experimental data leads to two different conclusions. Further studies done on the cleaved fragments of TLR7/8/9 have suggested that subsequent proteolytic trimming events process the C-terminal fragments of the TLR-ECDs so that only the processed forms are capable of recruiting adaptor molecules, effectively activating a signaling cascade and inducing an immune response [76]. However, other studies mutated various residues in the N-terminal fragment of the cleaved TLR-ECDs and showed that these TLRs were inactive, suggesting that the N-terminal fragments were just as important as the C-terminal fragments for
signal transduction [77]. To this point, no conclusions have been made regarding the nature of these cleaved fragments, but researchers agree that the initial proteolytic processing event is required for effective TLR7/8/9 signaling and the induction of an immune response.

Taken together, the events above suggest that TLR ligand recognition and activation is a specific, concerted and sequential process that is critical for transmission of signal to the cytoplasm. Upon ligand-induced dimerization of the TLR-ECDs, TLRs signal a response to pathogen by dimerization of their cytoplasmic TIR domains. This event creates a new platform on which signaling complexes can be built as TIR-domain containing adaptor proteins (MyD88, MAL, TRIF and TRAM) recognize the dimerization event [16]. Subsequent activation of a MyD88 (myeloid differentiation primary-response protein 88)-dependent or TRIF-dependent pathway is dependent upon which receptors are involved in the recognition process and which TIR-domain containing adaptor molecules are recruited to the TIR domain platform.

The first pathway, the MyD88-dependent pathway, is so named for the MyD88 protein which functions as an adaptor molecule linking the TIR domains of every TLR (except for TLR3 and, at times, TLR4) with downstream signaling molecules so as to induce the activation of the transcription factor NF-κB [78]. MyD88 is directly recruited to the TIR domain of TLR5, TLR7, TLR8 and TLR9, or recruited by an adaptor, the MyD88-adapter-like (MAL) protein, and functions to recruit IL-1R-associated kinase 4 (IRAK4), a kinase which is essential in signaling for NF-κB [78]. This recruitment then leads to a pathway which involves the activation of IRAK1, TRAF6 (tumor-necrosis-factor-receptor-associated factor 6) and TAK1 (transforming-growth-factor-β-activated kinase) through their modification by ubiquitylating factors UEV1A (ubiquitin-conjugating enzyme E2 variant 1) and UBC13 (ubiquitin-conjugating enzyme 13) [79]. Subsequent activation of the IκB kinase complex (IKKα/β) and phosphorylation of an
inhibitor protein, IκBα (Inhibitor of kappa light chain gene enhancer in B-cells alpha) leads to the activation of the transcription factor, NF-κB [80]. Release from IκBα allows NF-κB to translocate to the nucleus where it is able to induce the expression of inflammatory cytokines, various anti-viral and anti-microbial proteins and initiate the adaptive immune response (Figure 1.6) [81].

A second signaling pathway, defined by the recruitment of TRIF to the TIR domain of TLR3 or to the bridging adaptor, TRAM (TRIF-related adaptor molecule), and subsequent recruitment to the TIR domain of TLR4, leads to the activation of the transcription factors NF-κB or IRF3/7 [82]. These transcription factors then translocate to the nucleus where they induce the production of immune response genes, or the induction of apoptosis through the activation of the FAS-Associated with Death Domain protein (FADD)/Caspase 8 pathway [82]. As much of my research and part of this thesis focuses on the induction and regulation of the interferon response through the TLR3 signaling cascade, the TRIF dependent pathway, which is used partially by TLR4 and entirely by TLR3, will be discussed in greater detail in Chapter 1.6.

1.6 Toll-like Receptor 3: Structure and Function

As was mentioned in Chapter 1.4 and 1.5, the TLRs are a family of innate-immune recognition receptors that are able to recognize a variety of PAMPs and induce the activation of a number of host defenses. Similar to the rest of the TLR family, TLR3 is a type I integral membrane glycoprotein with an N-terminal ligand recognition domain, a single transmembrane domain and a C-terminal cytoplasmic signaling domain [30, 83]. Shown to recognize dsRNA, a molecular pattern associated with viral infection, TLR3 is known to induce the activation of NF-
Figure 1.6 Representation of the NF-κB response following ligand-induced pathway activation.
κB and the production of type I interferons [84-86]. Structurally, while all three domains of TLR3 have not been crystallized together, the TLR3-ECD has been characterized, independently, by two groups [30, 83]. Both groups showed that the TLR3-ECD displayed a heavily glycosylated horseshoe-shaped solenoid structure that consisted of 23 leucine-rich repeat motifs. The structure was further shown to be capped on the N- and C-terminals by leucine-rich repeat domains (LRR-NT and LRR-CT) (Figure 1.7). Interestingly, while both groups had elucidated similar ECD structures, they disagreed on the predicted location of the ligand binding site as well as the mechanism by which TLR3 was able to recognize viral dsRNA and initiate signaling. Choe et al. postulated that dsRNA would bind to the convex, glycan-free surface of the TLR3-ECD [83]. This, they hypothesized, would enable dsRNA to bind to the positively charged residues of the TLR3-ECD surface. Bell et al., however, proposed three dsRNA binding sites [30]. In their crystal structure, two sulfate molecules from the crystallization medium had bound to the concave surface of the TLR3-ECD. Since the sulfate ions shared a similar atomic arrangement with phosphate groups, the researchers reasoned that the sulfate sites could represent two potential dsRNA binding sites. The third predicted site was proposed to be a binding site on the glycan free face of the receptor. These discrepancies were not resolved until 2008 when the crystal structure of mouse TLR3-ECD (mTLR3), bound to a 46 base-pair dsRNA molecule, was published (Figure 1.8) [87]. As was detailed in Chapter 1.5, the TLR3-ECD exists as a monomer in solution and upon ligand binding, dimerization occurs. The mTLR3-dsRNA structure showed that dsRNA interacted with basic residues at both the N- and C-terminal sites on the lateral, glycan-free side of the TLR3-ECD [87]. When bound to dsRNA, TLR3-TLR3 direct interactions at the C-terminal were also observed effectively defining three points of
Figure 1.7 Structures of the TLR3-ECD solved independently by two groups. A. TLR3-ECD solved by Choe et al. in 2005 (PDB ID: 1ZIW) [83]. B. TLR3-ECD solved by Bell et al. in 2005 (PDB ID: 2A0Z) [30]. The two sulfate groups, which were found to have crystallized with the ECD, are depicted in red spheres.
interaction in the dsRNA:TLR3 complex; N-terminal with dsRNA, C-terminal with dsRNA, and C-terminal receptor interactions (this terminus could contribute to the formation of a TLR3 signaling unit) [87]. Interestingly, while the N and C-terminal sites were separated by 55-60 Angstroms in each ECD, the two N-terminal sites in the complex were separated by 110 Angstroms. The latter distance was equivalent to that of the length of a 45 base pair dsRNA molecule, effectively preventing shorter molecules of dsRNA from binding and suggesting to investigators that this was a mechanism for preventing auto-reactive responses against self dsRNA (endogenous siRNA consists of 25 base-pairs or less) [87].

Further analysis of the mTLR3:dsRNA complex revealed that dsRNA retains a typical A-DNA like structure in which the ribose phosphate backbone and the position of the grooves are the determinants in binding [87]. The TLR3-ECD interacts not with the individual bases of dsRNA, but rather through electrostatic interactions between the phosphate groups of the dsRNA backbone and the imidazole rings of four histidine residues (three in the N-terminal site and one in the C-terminal site) in the TLR3-ECD. These features have been postulated to effectively prevent viruses from escaping detection by dsRNA [29].

Further experiments done in 2008 focused on dsRNA, its requirements for binding to the TLR3-ECD and the minimal signaling unit required for effective TLR3 signal transduction [88]. Using a multitude of biophysical techniques, Leonard et al. were able to show that TLR3 ligand recognition was specific for dsRNA and that this event was dependent upon pH and dsRNA length [88]. Using SPR (surface plasmon resonance), SEC (size exclusion chromatography) and AUC (analytical ultracentrifugation) experiments, Leonard et al. defined the length of dsRNA that would accommodate a TLR3 dimer, the proposed TLR3 signaling unit, as 48 base-pairs.
Figure 1.8 mTLR3 bound to 46bp dsRNA. Crystal structure solved in 2008 by Liu, et al. (PDB ID: 3CIY) [87].
[88]. However, further experiments showed that multiple TLR3-ECD dimers bound to long dsRNA strands (dsRNA that was 90 base-pairs in length bound two TLR3-ECD dimers while dsRNA that was 139 base-pairs in length bound to three TLR3-ECD dimers) suggesting a more detailed analysis was necessary [88]. Using a GFP-reporter gene assay, Leonard et al. were able to show that when TLR3 was located in an environment where the pH was approximately 6.0-6.5, such as the early endosome, dsRNA ligands had to be greater than 90-basepairs in length to activate TLR3 complexes (2-3 TLR3-ECD dimers) [88]. However, TLR3 proteins that were located in environments where the pH was below 5.5, such as that of the late endosomes, could bind dsRNA ligands of greater than 48-basepairs to activate TLR3 complexes (1-2 TLR3-ECD dimers) [88]. The mouse TLR3-dsRNA crystal structure confirmed these findings as the ligand interaction sites on the TLR3-ECD (two TLR3-ECD N-terminal regions) were shown to be separated by 120 angstroms, the perfect length for stable binding of a TLR3-ECD dimer to 40-50 base-pairs of dsRNA [87]. However, given the observation that dsRNA length, location and pH could influence the nature of TLR3:dsRNA complexes, the minimum signaling unit for TLR3 remained ambiguous.

While previous reports suggested that ligand-induced dimerization was the only necessary event for transmission of signal to the cytoplasm and activation of an immune response, recent reports have suggested that proteolytic processing of TLR3 may also be required prior to recognition of viral ligand and TLR3’s subsequent competent signaling to downstream effector molecules [71, 72]. Newly synthesized TLR3 in the ER (endoplasmic reticulum) is associated with the chaperone protein Unc93b1 [73, 74]. This association has been shown to be required and retained through TLR3’s trafficking from the ER, to the Golgi, and then to the endosomal compartments [73,74]. Once in the endosome, several groups have now shown that the TLR3-
ECD can be cleaved by Cathepsins B and H [73,74]. This cleavage event was localized to amino acids 323 to 343, encompassing LRR12 and its unique loop structure [73,74]. Yet, just as with the proteolytic processing events in TLR7/8/9, researchers have yet to agree on the importance of this event. Some groups suggest that cleavage does not, in fact, dissociate the receptor into two fragments. Rather, they postulate that this event acts to induce stability and create a required, “cleaved/associated” TLR3 that results in a functional receptor which is able to detect and react to viral dsRNA [73]. However, other groups have shown that un-cleaved TLR3 can still induce signal transduction events and postulate that this cleavage event has other, yet undefined, roles in the cell [74].

Whether proteolytic processing is required for ligand recognition of TLR3 or is involved in other facets of TLR3-ECD function is not yet known. However, what researchers do agree upon is the fact that ligand-induced dimerization of TLR3-ECDs is required for TLR3 signal transduction [89]. The dimeric orientation of receptor ECDs is proposed to bring the cytoplasmic TIR domains of the TLR3 homodimer complex into close proximity with one another [90]. This non-enzymatic event is then thought to create a signaling platform upon which the organization of downstream signaling cascades can occur [90, 91]. At this point, TLR3 diverges from classical TLR signaling as it does not induce a MyD88-dependent signaling cascade. Instead, the TLR3 TIR-based signaling platform acts to recruit an adaptor protein known as TRIF. This 712 amino acid adaptor protein has distinct protein-interaction motifs that allow it to recruit effector proteins, which result in the activation of, at least, three possible signaling cascades (Figure 1.9) [82].

The first two cascades, mediated by the recruitment of TRIF to the TIR domain of TLR3, involve the subsequent recruitment of RIP1 kinase (receptor-interacting protein 1), a kinase that
associates with the RIP homotypic interaction motif in the TRIF domain [82]. From this point, one of two downstream signaling cascades is activated depending upon the ubiquitination state of RIP1. Lysine residue 377 of RIP1 has been shown to be an acceptor site for K63-linked polyubiquitination [92]. Ubiquitination of this site in RIP1 results in the activation of the IκB kinase complex (IKK) composed of a heterodimer of the catalytic IKKα and IKKβ subunits and a master regulatory protein termed NEMO (NF-κB essential modulator) [92]. Once activated, this complex phosphorylates two serine residues on the downstream protein IκBα (inhibitor of κBα). IκBα maintains the NF-κB protein complex, consisting of two subunits, RelA (p65) and NF-κB1, from a five subunit family (RelB, c-Rel, NF-κB2 (p52 and its precursor p100) are the other members), in a dormant state until this phosphorylation event occurs [91, 92]. Upon phosphorylation, IκBα is further modified through ubiquitination events that lead to its degradation by the proteasome [91]. Upon dissociation from IκBα, NF-κB is then able to translocate to the nucleus where it can mediate the induction of genes involved in the immune response, the cell survival response or cellular proliferation, as well as up-regulate the expression of its own repressor, IκBα, which forms an inhibitory feedback loop and results in oscillating levels of NF-κB activity [82].

On the other hand, if ubiquitination does not occur on lysine 377 of the RIP1 kinase, RIP1 complexes with the cell death-associated protein FADD (FAS-Associated with Death Domain protein) and Caspase 8 in a complex which initiates downstream Caspase 8 activation [93]. Caspase 8 activation subsequently promotes cell death by triggering the receptor-mediated extrinsic apoptotic pathway [93].
Figure 1.9 Three possible TRIF-dependent signaling cascades
A third signaling cascade, mediated by the recruitment of TRIF to the TIR domain of TLR3, requires the recruitment of effector molecules that have been shown to include molecular bridge proteins such as TRAF1 (tumour necrosis factor receptor-associated factor), TRAF2, TRAF3 and TRAF6 [94]. These proteins mediate signal transduction pathways by interacting with downstream protein kinases, acting as components of ubiquitin ligase machinery and other adaptor proteins. In the TLR3 signaling cascade, once recruited to the cell surface receptor domain, these bridging proteins are able to mediate the activation of TBK1 (TANK-binding kinase 1), a kinase that will be discussed at great length in Chapter 1.7 for its critical roles in phosphorylating substrates involved in cell proliferation, vesicle transport, xenophagy and the anti-viral response [94]. Currently, two competing theories exist regarding the molecular interactions of TBK1 and its surrounding proteins. Originally, a proposed kinase complex was believed to exist that consisted of three adaptor proteins, TANK (TRAF-associated NF-κB inhibitor), NAP1 (NF-κB activating kinase (NAK)-associated protein 1) and SINTBAD (Similar to NAP1 TBK1 adaptor) and two kinases, TBK1 and IKKε (IκB kinase epsilon) [94-97]. Recent systematic affinity purification-mass spectrometry experiments have shown a mutually exclusive interaction may exist between the adaptors and the kinases, suggesting distinct alternative complexes rather than one large signalosome (Figure 1.10) [98]. Yet, with either a large signalosome or distinct mutually exclusive complexes, activation of TBK1 leads to the phosphorylation of an important downstream transcription factor, IRF3 (interferon regulatory factor 3) [94-97]. IRF3, a 427 amino acid protein, is an important transcriptional regulator of the antiviral immune response [94-97]. Upon phosphorylation (phosphorylated by TBK1 on residues 385 and 386 for dimerization and 396-405 to alleviate autoinhibition and allow for interaction with co-activators), IRF3 dimerizes and translocates to the nucleus where it regulates the
expression of numerous host defense genes including type I interferons [94-97]. These interferons, including IFN-α and IFN-β, are able to stimulate both macrophages and NK cells to elicit an anti-viral response or bind to the IFN-α/β receptor (IFNAR1/2) in either an autocrine or paracrine manner to initiate a positive feedback loop that results in the activation of the JAK/STAT pathway and the production of further anti-viral genes [99, 100].

These three defined pathways initiate pro-inflammatory responses that recruit additional immune cells to the site of infection, inhibit bacterial and viral replication, communicate danger signals to the surrounding cells, and induce apoptosis in cells with overwhelming infection. Because of this critical response, our work has focused on the activation of type I interferons, the critical effector molecules which were discussed for their production by the pro-inflammatory response of the TLR3 signaling cascade. In particular, we have examined the activation and regulation of the key kinase in this pathway, TBK1. As will be detailed in Chapter 1.7, TBK1 has become demarcated as a centralized catalytic hub and an essential modulator of the host responses mentioned above. As such, understanding the mechanisms by which the function of TBK1 is both activated and regulated provides insight into how this kinase dictates the shape of a multitude of downstream innate immune responses.

1.7 TANK-Binding Kinase 1 (TBK1): Structure, Function and Regulation

Several signaling pathways converge to activate TBK1 (Figure 1.11) [94, 95, 101]. Originally discovered as a kinase that interacted with the effector proteins, TANK and TRAF2, in a ternary complex that could activate NF-κB [102], TBK1 has since been characterized as a key regulator of substrates ranging from cell proliferation [103] and vesicle transport [104] to the xenophagic elimination of bacteria [105, 106] and the antiviral immune response [94-97].
Figure 1.10 Possible configurations of the proposed TBK1 kinase complex.
TBK1 [102], also known as NAK (NF-κB-activating kinase) [107] or T2K (TRAF2-associated kinase) [108], is a 729 amino-acid serine/threonine kinase that is a ubiquitously expressed non-canonical IκB kinase family member. The canonical IκB kinases, IKKα and IKKβ, phosphorylate IκBα in the NF-κB signaling pathway [109], while TBK1 targets the transcription factors IRF3 and IRF7 in the type I interferon response [94]. TBK1 is composed of an N-terminal KD (kinase domain), which contains an activation loop between subdomains VII and VIII that controls its catalytic activity, and three C-terminal regulatory domains; a ULD (ubiquitin-like domain), which does not interact with known ubiquitin-binding proteins, but rather with the KD and appears to be necessary for the full activation of the kinase along with substrate presentation, a leucine-zipper containing DD (dimerization domain), and finally a small helix-loop-helix protein interaction module that has been termed the AB (adaptor-binding) motif (Figure 1.12) [110-112]. From TBK1 structural studies, the kinase has been shown to be a dimeric entity in which the KD, ULD and DD form a three-way interface that positions the kinase active sites, consisting of the critical activation loops, away from one another (Figure 1.13) [112]. This configuration, as is postulated by numerous researchers, prevents productive autophosphorylation events, which, as will be discussed in the next paragraph, are required for TBK1 activation.

Upon recognition of an upstream signal, TBK1 is recruited to signaling complexes via its AB motif [111]. Once there, TBK1 requires phosphorylation of serine residue 172 within its kinase domain activation loop to form a productive active site center (Figure 1.14) [113]. This event has been shown to be achieved through the kinase activity of IKKβ [114]. Recent crystal structures of the KD and ULD of TBK1 have also shown that this post-translational modification

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Figure 1.11 Multiple signaling pathways converge to activate TBK1.
Figure 1.12 Structure of TBK1. A. Structure of the kinase domain of dimeric TBK1 with a potent small molecule inhibitor, BX795 (depicted in red spheres), bound to the active site (PDB ID: 4EUT). B. Structure of the asymmetric unit of TBK1. The KD is depicted in red spheres, the ULD in yellow spheres and the DD in green spheres (PDB ID: 4IM0). C. Ribbon diagram of the domain structure of TBK1 highlighting the KD, ULD and SDD of the kinase (PDB ID: 4IM0).
Figure 1.13 Structural organization of the inactive TBK1 molecule. A. Structural configuration of the KD and ULD of inactive TBK1. The activation loop of each monomer is depicted in sphere representation with the critical, S172 residue, highlighted in red (PDB ID: 4EUT). B. Detailed view of a single chain of TBK1 in the inactive state. The activation loop is again represented in green spheres with the critical, S172 residue, in red (PDB ID: 4EUT).
can occur through a trans-phosphorylation event mediated by the local clustering of TBK1 molecules upon recognition of a signal [112]. In this case, neighboring TBK1 molecules interact via an activation loop-swapped conformation. These interactions help to supply critical structural contacts required to achieve an active kinase conformation, as well as to place the activation loop within the catalytic cleft of the adjacent TBK1 KD for phosphotransfer to S172. While kinetic analysis suggests that the initial loop-swapped phosphorylation mechanism is slow, once activated, TBK1 is able to readily phosphorylate activation loop sequences of the remaining unphosphorylated TBK1 molecules leading to activation of any dormant TBK1 constructs [112].

Upon autophosphorylation of S172, the TBK1 activation loop folds back onto the C-terminal lobe of its KD to complete an apparent binding site for polypeptide substrates [111]. Investigations into the composition of this site along with TBK1 substrate sequence alignments have suggested that a consensus sequence for TBK1 phosphorylation exists [112]. This sequence reveals that the kinase favors a hydrophobic residue immediately proceeding the modified serine (P0) which is then mirrored by several hydrophobic residues lining the P+1 position. Indeed, the TBK1 activation loop sequence which is phosphorylated by activated TBK1, contains a leucine at the P+1 position. Similarly, a more detailed sequence analysis found that hydrophobic residues comprise the P+1 position in approximately 70% of TBK1 substrates [112, 115]. Other attempts to derive a TBK1 phosphorylation consensus sequence have suggested that polar amino acids are also a requirement at the P+3, P+5 and P+8 positions, or that specific amino acids, defined in each study, are a requirement at the P+1, P+3 and P-2 positions, but analysis of a large panel of natural TBK1 substrates suggests that the most prominent requirement is that of the hydrophobic residue at the P+1 position (Figure 1.15) [112,115].
Figure 1.14 Active conformation of TBK1 kinase domain. A. Structural configuration of the KD for active TBK1 with a phosphorylated S172 residue (PDB ID: 4EUU). The activation loop of each monomer is depicted in sphere representation with the critical, S172 residue, highlighted in red (PDB ID: 4EUU). B. Detailed view of a single chain of TBK1 in an active confirmation with phosphorylated S172. The activation loop is again represented in green spheres with the critical, S172 residue, in red (PDB ID: 4EUU).
While the composition of the kinase active site appears to be critical to substrate specificity, researchers were also interested in elucidating the enzymatic mechanism by which TBK1 functions. Using an IκBα peptide (amino acids 19-41), that is known to be phosphorylated by TBK1 at serine residue 36, in conjunction with ATP as a varied substrate, researchers observed almost parallel lines in double reciprocal plots, suggesting that the kinase acted through a ping-pong mechanism [116]. However, additional kinetic experiments soon revealed that TBK1 could not function through a ping-pong mechanism as product inhibition studies showed that ADP was a non-competitive inhibitor of TBK1 with respect to the IκBα peptide [116]. If the enzyme functioned through a ping-pong mechanism, ADP would have functioned as a competitive inhibitor with respect to IκBα as both substrates would have to access the same form of TBK1 during the reaction. Excluding a ping-pong mechanism, sequential versus ordered mechanisms were evaluated [116]. The non-competitive inhibition by ADP with respect to the IκBα peptide also excluded an ordered sequential mechanism with IκBα peptide binding first as ADP would then have functioned as an uncompetitive inhibitor [116]. To determine if the TBK1 mechanism was therefore random sequential or ordered sequential with ATP binding first, the pattern of inhibition with an IκBα peptide inhibitor was examined [116]. With respect to ATP as the varied substrate, the double reciprocal plots intersected at the 1/ATP axis and to the left of the 1/rate axis, indicating the presence of a non-competitive inhibitor [116]. This inhibition pattern, as with all IKK family members, was consistent with a random sequential mechanism for TBK1 [116-118]. Interestingly, further kinetic experiments found that ATP decreased the affinity of IκBα for TBK1 and that the dissociation constant of IκBα for TBK1 was significantly higher than that of the IKKα/IKKβ heterodimer (also known to phosphorylate IκBα subsequently leading to the
Figure 1.15 Postulated optimal TBK1 phosphorylation motif. Highlighted in red is the modified serine residue (S172) of TBK1 which is required for kinase activation.
activation of NF-κB) suggesting to researchers, at the time, that other substrates for TBK1 may exist [116-118].

Since that initial characterization, activated TBK1 has now been shown to phosphorylate several substrates involved in a multitude of molecular events. Best understood today for its role in the induction of type I interferons [101], activated TBK1 has been shown to target two sites (seven serine/threonine residues) near the C-terminus of the transcription factor IRF3 [94, 109, 119]. Phosphorylation of residues at the first site (Ser385 and Ser386) promotes the dimerization of IRF3 [119], required for its translocation to the nucleus, while phosphorylation of residues at the second site (Ser396-Ser405) permits IRF3 interaction with its co-activators p300/CBP (CREB-binding-protein) [120]. These events, in turn, mediate the formation of a nuclear IRF3 nucleoprotein complex, at the promoter region of the IFN-β gene, which induces the production of type I interferons and facilitates an immediate inflammatory response.

While primarily studied in the antiviral response, TBK1 has also been shown to play a role in endosomal sorting and trafficking [104]. VPS37C, another protein defined as a TBK1 substrate, is a subunit of ESCRT-1 (endosomal sorting complex required for transport 1), a complex in the class E-VPS (vacuolar protein sorting) pathway required for sorting ubiquitinated transmembrane proteins into internal vesicles of multivesicular bodies [114]. Unfortunately, this pathway is subverted by retroviruses to mediate budding from host cells and facilitate the spread of infection [22]. TBK1’s phosphorylation of VPS37C negatively regulates PTAP-dependent (an HIV-1 motif) viral budding as this post-translational modification prevents the competent assembly of viral budding complexes [114].

In addition to the antiviral response and vesicle budding, TBK1 has also been shown to mediate the sequestration of intracellular bacteria through a number of roles in xenophagy.
Infection or invasion by a bacterial pathogen tends to result in compartmentalization of the invading organism within PCVs (pathogen containing vacuoles) [105]. This restriction event limits nutrient intake by the invading microorganism and prevents collateral host damage. In 2007, using TBK1-/- cells, a group reported that TBK1 kinase activity was required for the restriction of bacterial infection as TBK1 was able to regulate the integrity of PCVs [106]. Further experiments, done by the same group, showed that AQP-1 (Aquaporin-1), a water channel that regulates the swelling of secretory vesicles, associated with PCVs [104]. In TBK1 deficient mice, AQP-1 levels were elevated causing PCV destabilization. This suggested to researchers that TBK1 may control AQP-1 expression, a critical regulatory point in host cell protection [104].

Further investigations into TBK1’s role in xenophagy focused on ubiquitination and the tendency of polyubiquitinated proteins to accumulate around cytosolic invaders. While it is not known whether invading bacteria are ubiquitinated while in the cell or if polyubiquitinated proteins surround the invading microorganisms, these studies revealed two roles for TBK1 in mediating the selective autophagy of bacteria displaying a polyubiquitinated-coat [105, 106]. First, TBK1 has been shown to mediate the activity of a ubiquitin binding protein, NDP52, through recruitment of adaptor molecules that recognize bacterial ubiquitin coats [105]. This event signals cells to activate autophagy against the bacteria attempting to colonize the host cytosol [105]. Second, TBK1 has also been shown to enhance the affinity of an autophagy receptor, Optineurin, for ubiquitinated proteins, through its phosphorylation of a residue (serine-177) known to enhance the autophagic clearance of cytosolic Salmonella [106]. This, in turn, is known to promote the selective autophagy of invading intracellular bacteria through interactions with xenophagosomes.
Interestingly, however, for all of its positive attributes, aberrant activation of TBK1 has been shown to contribute to human disease. For example, in oncogenic transformation studies, TBK1 activation has been shown to support the suppression of a programmed cell-death response to oncogene activation [103]. In these investigations, biophysical studies revealed that RalB, a key component of the oncogenic Ras signaling network, once activated, promoted a direct interaction between Sec5, a critical Ral effector protein, and TBK1 which resulted in kinase activation [103]. Using TBK1−/− cells, researchers then showed that TBK1 directly interacted with and activated AKT, which, in turn, is associated with tumor cell survival, oncogenic proliferation and invasiveness [103].

Similarly, in the insulin response, TBK1 has been implicated in genetic models of diabetes [103, 121]. In co-immunoprecipitation assays with the insulin receptor, researchers have found that TBK1 and the insulin receptor interact in obese Zucker rat (OZR) models [121]. Through further investigations, researchers showed that TBK1 was able to phosphorylate serine residue 994 in the insulin receptor, a post-translational modification that has been shown to cause reduced insulin sensitivity in genetic models of diabetes [121].

As is evident from the experiments above, while TBK1 activation can promote host survival during infection, an unchecked TBK1 response can lead to detrimental effects ranging from cellular proliferation in transformed cells and insulin resistance, as were detailed above, to numerous autoimmune disorders, obesity and glaucoma. For this reason, effector molecules have been sought and identified that control TBK1 activation and prevent its rampant activation from negating its beneficial effects.

In searching for regulators of the TBK1 response, two independent groups discovered K63-linked ubiquitination of TBK1 (one group showed ubiquitination on residues K69, K154, and
K372 [122] while the other group showed ubiquitination on residues K30 and K401 [110]) in response to RNA virus infection (Figure 1.16). While the E3 ligase responsible for the addition of modifications on residues K30 and K401 has not been elucidated, the ubiquitination modifications on residues K69, K154 and K372 have been shown to be induced by the E3 ligases Mind Bomb 1 and Mind Bomb 2 [122], in response to RNA virus infection, or NRDP1, in response to LPS [123]. From this post translational modification, subsequent recruitment of the downstream adaptor NEMO, through ubiquitin binding domains, has been shown to lead to the assembly of a NEMO/TBK1 complex [122]. This complex, in turn, is known to be able to activate TBK1 kinase activity, leading to the phosphorylation of the downstream transcription factor, IRF3. While ubiquitination events have not been shown to be required for the activation of TBK1, the presence of this post-translational modification has been shown to upregulate the type I interferon response and promote host cell defenses.

Further investigations into the regulation of TBK1 activity have identified endogenous inhibitors that have been shown to prevent the TBK1-mediated downstream phosphorylation of numerous substrates. One such inhibitor, A20, a ubiquitin-editing enzyme, has been shown to disrupt the K63-linked ubiquitination events discussed previously [124]. Another such inhibitor, SHP-2, a tyrosine phosphatase has been shown to negatively regulate TBK1-mediated signal transduction [125]. Since tyrosine phosphorylation of recombinant active TBK1 has not been detected, researchers postulate that this protein binds to the KD of the kinase and prevents the autophosphorylation of S172 in the active loop of the KD [125]. In addition, a yeast two-hybrid screen for interaction partners of the closely related, non-canonical, IκB kinase, IKKε, identified the protein SIKE (Suppressor of IKKε) as a physiological inhibitor of TBK1 that acts through an undefined mechanism [126]. As investigations into the mechanism by which SIKE inhibits
Figure 1.16 K63-linked ubiquitination of TBK1. A. Ribbon diagram of a single TBK1 molecule. Residues in TBK1 found to be ubiquitinlated by Wang et al. (K69, K154, and K372) are highlighted in red spheres. Residues found to be ubiquitinlated by Tu et al. (K30 and K401) are highlighted in blue spheres (PDB ID: 4IM3). B. Sphere representation of dimeric TBK1 with ubiquitination residues discovered by Wang et al. displayed in red spheres and ubiquitination residues discovered by Tu et al. displayed in blue spheres.
TBK1 function are a major component of this work, characteristic SIKE structure and function details will be explained at length in Chapter 1.8.

1.8 SIKE: Discovery, Structure, and Function

As was discussed in Chapter 1.7, over-activation of TBK1 can negate the positive attributes associated with its induction of critical substrates and downstream signaling cascades. To prevent aberrant kinase activity, endogenous inhibitors have been discovered that regulate TBK1 function. Of interest to our lab was a 207 amino acid protein, SIKE, which was first discovered in 2005 by Huang et al. and characterized as a physiological suppressor of the kinases, IKKε and TBK1 [126].

With hopes of identifying IKKε interacting proteins, Huang et al. used a yeast two-hybrid system that screened a human B-cell cDNA library, consisting of approximately $8 \times 10^6$ independent clones, with full-length IKKε as bait [126]. The group was able to obtain 123 positive hits, of which, four belonged to a previously uncharacterized protein which they termed SIKE [126]. Studies into this ubiquitously expressed protein revealed that SIKE was encoded by a gene that mapped to human chromosome 1p13.2 [126]. Interestingly, human chromosome 1 spans 260 million base pairs, contains over 3000 genes, comprises approximately 8% of the human genome, and encodes a large number of disease-associated genes, including those that are involved in Parkinson’s disease, Usher syndrome and schizophrenia [127]. Blast searches of the GenBank databases revealed that SIKE was an evolutionarily conserved protein (from Xenopus to human) that belonged to an uncharacterized family consisting of proteins such as FGFR1OP2. While SIKE and FGFR1OP2 differed by 40 amino acids and were shown to be approximately 50% homologous (Figure 1.17), studies
revealed that FGFR1OP2 was encoded by a gene that mapped to chromosome 12p12.1 [128]. Further investigations revealed that FGFR1OP2 was a protein found in tissues ranging from the spleen and thymus to bone marrow [129]. Additionally, studies indicated that FGFR1OP2 had the ability to dimerize, associate with coiled-coil structures, and interact with cytoskeleton networks of oral wound fibroblasts to enhance wound healing [129].

Analysis of the SIKE sequence revealed that this protein had three predicted coiled-coil domains; a low probability predicted domain from amino acids 5-42, and two high probability predicted domains from amino acids 72-102 and 163-197 [126] (Figure 1.18). Since coiled-coil motifs were known to mediate protein-protein interactions and multimerization events, researchers questioned whether SIKE had the ability to dimerize or oligomerize. Using co-immunoprecipitation experiments, investigators were able to show that a FLAG-tagged SIKE construct was able to interact with an HA-tagged SIKE construct indicating that SIKE could form homodimers or even oligomers [126].

Taking interaction studies one step further, researchers knew that, while SIKE interacted with IKKe, this kinase was undetectable under physiological conditions (inducible in lymphoid and other cell types). Scientists wondered whether SIKE, therefore, could interact with TBK1, the kinase which was constitutively expressed in most cell types. In co-immunoprecipitation experiments, endogenous SIKE did, in fact, interact with endogenous TBK1 under physiological conditions [126]. In an attempt to map the interaction sites between TBK1 and SIKE, researchers postulated that the coiled-coil motifs of each protein would be the basis for association events. Using further co-immunoprecipitation experiments, investigators were able to reveal that the
Figure 1.17 Sequence similarity of SIKE and its homologue, FGFR1OP2. Basic Local Alignment Search Tool (BLAST) sequence alignment of the two isoforms of FGFR1OP2 and the two isoforms of SIKE. Conserved residues are highlighted in yellow. Amino acid inserts in the FGFR1OP2 sequence are highlighted in blue. Amino acid inserts in the SIKE sequence are highlighted in red.
Figure 1.18 Predicted domain organization of SIKE and the kinase TBK1.
high probability predicted coiled-coil domains of SIKE, amino acids 72-207, interacted with the two coiled-coil domains of TBK1, amino acids 601-729 [126] (Figure 1.18), suggesting that coiled-coil motifs did, in fact, mediate the interactions between these two proteins and that the first 72 amino acids of SIKE were not necessary for interactions with TBK1. Interestingly, however, this study did not detect an interaction between the TBK1-KD and SIKE.

As TBK1 had been shown to play a significant role in the TLR3 signaling cascade, researchers next questioned whether SIKE also associated with the kinase upon pathway activation. Upon stimulation with either poly(I:C) (polyinosinic:polycytidylic acid), a dsRNA mimicetic, or VSV (vesicular stomatitis virus), SIKE dissociated from TBK1 effectively terminating the interaction [126]. Huang et al. questioned the functional basis of this dissociation event and were able to show that SIKE acted in a more influential manner in the TLR3 signaling cascade. Using transient transfections and co-immunoprecipitation experiments, Huang et al. showed that SIKE was able to disrupt TBK1 interactions with the upstream adaptor molecule, TRIF, and the downstream substrate, IRF3, effectively sequestering TBK1 in an inactive complex in the TLR3 signaling cascade [126]. Understanding that TLR3 was able to activate both NF-κB and IRF3, Huang and colleagues examined whether SIKE disrupted both pathways. Interestingly, the use of a SIKE RNAi plasmid in reporter gene assays potentiated poly(I:C)- and VSV-triggered activation of the IFNβ promoter, but had no effect on NF-κB [126]. Further studies showed that SIKE did not disrupt interactions between TRIF and TRAF6 or RIP1, associations required for TLR3-mediated NF-κB activation [126]. SIKE also did not inhibit NF-κB activation triggered by TNF, poly(I:C), and VSV suggesting that SIKE specifically inhibited the IKKe- and TBK1-mediated IFNβ activation pathways [126]. Subsequent studies by Andrei Medvedev’s group showed that LPS-induced pathway activation led to an increase in SIKE
mRNA levels [130]. This suggested to researchers that TLR3-pathway activation induced the expression of pro-inflammatory cytokines and chemokines as well as the expression of SIKE which could return to the TLR3 pathway to negatively regulate TBK1 activity. The production of SIKE would, therefore, effectively form a negative feedback inhibition loop in the TLR3 signaling cascade (Figure 1.19).

SIKE based studies seemingly terminated following its characterization as a physiological inhibitor of IKKe- and TBK1-mediated antiviral responses. Intriguing to our lab, however, was the unknown mechanism by which SIKE inhibited TBK1 prior to pathway stimulation and the unknown role SIKE had in the cell following its dissociation from TBK1. Further confounding our thoughts was the fact that SIKE and its homologue, FGFR1OP2, were 50% similar in sequence identity yet their defined physiological roles, one as an inhibitor of the innate immune response and one as a cytoskeleton protein mediator in wound healing, varied greatly. As will be conveyed in Chapter 4, our investigations have defined SIKE as much more than an endogenous inhibitor of the TLR3 signaling cascade.

1.9 Current Study

To defend against pathogens, decades of research have shown that multi-cellular organisms mount an immune response that recognizes, sequesters and eradicates invading infectious agents. Essential to this safeguard is the innate anti-viral response mediated by PRRs such as TLR3. Given the important nature of the TLR3 signaling cascade in antiviral immunity, the current study examines three aspects of a TLR3-mediated immune response. First, this study investigates the potential for TLR3 ligands to act as adjuvants, pharmacological agents that are included in
Figure 1.19 Postulated mechanism of SIKE inhibition.
vaccines to enhance an antigenic response. This study continues by exploring the role of receptor clustering, a key spatial feature that influences effective and proper physical and biochemical cellular responses, in the TLR3:dsRNA signaling complex. Finally, the current study concludes by exploring the role of endogenous regulation, a critical control to aberrant immune activity, on downstream TLR3 signaling pathways. While these studies cover both extracellular and intracellular events in innate immunity, the findings as a whole establish a previously undefined clustering mechanism as well as a novel substrate that will enhance the scientific community’s understanding of TLR3 and add to its already established function in the immune response.
2.1 Introduction

Investigators have established that mature, immunologically competent dendritic cells (DCs) are one of the most potent antigen presenting cells (APCs) uniquely able to initiate primary immune responses [1, 21]. DC precursors, immature DCs (iDCs), derived from hematopoietic bone marrow progenitor cells, migrate from the bone marrow to the blood performing sentinel-like functions, internalizing and processing antigens from surrounding microenvironments in the cell [1,21]. These iDCs can also be recruited to sites of inflammation in peripheral tissues following pathogen invasion through receptor expression on their cell surfaces (iDCs may express chemokine receptors, such as CCR1, CCR2, CCR5, CCR6 and CXCR1, or receptors from the CD1 family of molecules such as CD1a, CD1b and CD1c) [1, 21, 131]. Upon recognition of inflammatory mediators, microbial products and other pathogens, iDCs phagocytose these danger signals and degrade their proteins into small peptides [1,21].
iDCs then migrate to the draining lymph nodes simultaneously undergoing maturation into mature DCs which effectively induces the expression of co-stimulatory molecules and cytokines on their cell surface (up-regulation of chemokine receptors CCR6 and CCR7 is observed along with the enhanced expression of cell-surface receptors that act as co-receptors in T-cell activation such as CD80 (B7.1), CD86 (B7.2) and CD40) [1, 21]. Once migration into these T-cell rich areas has occurred, DCs act as APCs, using MHC molecules on their cell surface to present danger signals and effectively induce cognate T-cells to differentiate into antigen specific CTL and Th cells [1, 21]. As these cells are a requirement for long-term protection against infection and the generation of acquired immunity, researchers have long investigated the adaptability of this process as it pertains to vaccination.

A major goal of vaccination is the generation of a strong immune response to an administered antigen that can provide long-term protection against infection. To achieve this objective in the past, medical professionals used either inactivated vaccines [132], which consisted of virus particles that cannot replicate but are stable enough to be recognized by the immune system and evoke a response, or attenuated vaccines [133], which consisted of live virus particles with low virulence. However, with the ever expanding list of pathogens and the growing infection rate from these invading microorganisms, vaccines today tend to be developed through pure recombinant or synthetic antigens and are less immunogenic than their predecessors [134]. Therefore, in addition to the initial vaccination, doctors also administer adjuvants which are pharmacological or immunological agents that modify effects of the vaccine. Adjuvants are used for various purposes; to enhance the immunogenicity of highly purified or recombinant antigens; to reduce the amount of antigen necessary to elicit a response or the number of immunizations needed for protective immunity; to improve the efficacy of vaccines in newborns,
the elderly or other immune-compromised persons; or as antigen delivery systems for the uptake of antigens by the mucosa. While these pharmacological agents are co-administered with a multitude of vaccines today, adjuvants are not a recent concept.

In the 1920s, Ramon et al. noted that horses that developed an abscess at the inoculation site of diphtheria toxoid generated higher specific antibody titres [135]. They subsequently found that an abscess generated by the injection of unrelated substances along with the diphtheria toxoid increased the immune response against the toxoid. In 1926, further research was able to elucidate the adjuvant activity of aluminum compounds when Glenny et al. demonstrated that the diphtheria toxoid absorbed to alum, a specific chemical compound defined as hydrated potassium aluminum sulfate [136]. Interestingly, to this day, aluminum-based compounds (specifically aluminum phosphate or hydroxide) remain the predominant adjuvants with alum being the sole adjuvant approved for human use in the majority of countries worldwide.

While alum is able to induce a good antibody (Th2) response, it does not have the capacity to stimulate cellular (Th1) immune responses which are important for protection against many pathogens [137]. In addition, alum has been shown to cause severe local and systemic side-effects including sterile abscesses, eosinophilia and myofascitis [138]. Concerns have also been raised regarding the possible role of aluminum in neurodegenerative diseases such as Alzheimer’s disease [137, 138]. For these reasons, a demand for safer and more effective adjuvants, suitable for human use, has been brought to the attention of the scientific community and researchers world-wide.

To develop an alternative to alum that effectively enhances both an antibody and cellular immune response, research has begun to focus on pathogen and microbially-derived ligands.
These ligands, recognized by PRRs, are known to strongly activate an inflammatory response and corresponding release of cytokines. This barrage of immune activators signals immune cells to sequester and eradicate invading infectious agents. At the forefront of PRR-ligand adjuvant studies are the TLRs, the receptors that, as discussed in Chapters 1.4 and 1.5, recognize a multitude of PAMPs. Initial research has shown that synthetic TLR2 ligands, MALP-2 and Pam3, were effective in the induction of CTL [139] while synthetic TLR7, TLR8 and TLR9 ligands, R-848 and CpG ODN, were able to augment both T-cell mediated immune responses [140]. Of interest to our lab was the potential adjuvancy of dsRNA with respect to its activation of multiple PRRs, including TLR3. Once activated, TLR3 triggers signaling cascades, discussed in Chapter 1.6, that lead to both an immediate inflammatory response and a subsequent adaptive immune response. Unfortunately, the widely used, synthetic dsRNA analogue, pI:pC, the obvious choice for dsRNA adjuvant development, has toxic side effects that require doses for systemic therapeutic applications to be below effective concentrations [141]. The chemical structure and biological function of pI:pC has also been shown to vary leading to the promiscuous activation of non-TLR3 dependent pathways with unpredictable consequences [141]. Recent studies have investigated different delivery methods for this potential adjuvant, but results have been inconclusive [142]. Therefore, attention has turned to defined dsRNA oligonucleotides.

Previous studies had shown that TLR3 activation is dependent upon dsRNA length [88]. The minimal length for efficient in vitro binding was shown to be ~48bp, while in vivo, a robust response was not observed until dsRNA of ≥90bp was used for stimulation [88]. The objective of this study was to determine the adjuvant potential of a 139bp dsRNA molecule as assessed
through the maturation of iDCs to DCs. Our goal from this work was to develop a specific PRR ligand that would act as a safe agonist of TLR3.

2.2 Materials and Methods

2.2.1 Reagents

Chemicals were purchased from Sigma-Aldrich unless otherwise specified. The T7 RiboMAX Express Large Scale RNA Production System kit was purchased from Promega. GM-CSF (granulocyte macrophage-colony stimulating factor) and IL-4 (interleukin-4) were purchased from R&D Systems. Hank’s Buffered Saline Solution was purchased from GIBCO. Anti-hIgG antibody was purchased from Sigma-Aldrich. FITC-mouse and human CD86 was purchased from BD Biosciences (SSS687). PE-mouse-Anti-hCD80 (305207), PE-mouse-Anti-hCD1a (300105), PE-mouse-Anti-hCD54 (322707), FITC-mouse-hCD14 (301803), and FITC-mouse-Anti-hHLA-DR (307603) antibodies were purchased from BioLegend.

2.2.2 Synthesis of dsRNA

The enzymatic synthesis of dsRNA requires a dsDNA template. As TLR3 recognizes dsRNA molecules of any sequences, any PCR template is suitable, but dsRNA length (>48bp) is required to accommodate recognition by a TLR3 dimer. Templates in this study consisted of 48bp, 139bp and 540bp sections of a WNV (West Nile Virus) sequence. The sequences were PCR amplified and included a 5’SacII restriction enzyme site and a 3’SpeI restriction enzyme site. PCR products were ligated into a pGEM-T Easy vector containing a T7 RNA polymerase promoter upstream. DNA templates in the pGEM-T Easy vector were completed for the forward (sense) and reverse (antisense) orientation of the WNV sequence. Prior to the RNA polymerase reaction, sense and antisense DNA templates (100 µg) were treated with Proteinase K.
(100μg/mL) in Tris-HCl (50mM, pH 7.5) and CaCl₂ (5mM) containing 0.5% SDS for 30 minutes at 37°C to digest any protein present, especially RNases. Following the 30 minute incubation period, a Phenol:Chloroform extraction was performed to separate digested proteins and Proteinase K from the DNA templates. This was followed by an ethanol precipitation (Sambrook/Russell Molecular Cloning: A Laboratory Manual 3rd edition (Appendix A8.9)) to purify and concentrate the DNA templates. Sense and antisense DNA templates (50μg each) were then linearized by digestion with NdeI restriction enzyme (75units) (the NdeI site in pGEM-T Easy is 26bp downstream of the target DNA template allowing RNA polymerase to “run off” after completing synthesis of the target sequence) through incubation overnight at 37°C. Two micrograms of each solution were then run on a 2% agarose gel to confirm linearized product (Figure 2.1B). Sense and antisense ssRNA were then synthesized simultaneously by combining, in a single tube, sense (6μg) and antisense (6μg) DNA templates with the remaining reagents at levels detailed in the manufacturer’s protocol for “Synthesizing Large Quantities of RNA” reaction. Following synthesis, RNA strands were annealed by heating the reaction tube to 70°C for 10 minutes and allowing it to cool slowly to room temperature (~1 hour) to anneal RNA complimentary sense/antisense strands. To remove the DNA template and ssRNA (overhangs of annealed strands and unpaired RNA strands), RNase A (80pg/μL) and RQ1 DNase (4μL, RiboMax kit) were added to the reaction tube and incubated at 37°C for 30 minutes. The target dsRNA was isolated via a second Phenol:Chloroform extraction and concentrated using an ethanol precipitation reaction. Purity and quality of the resulting dsRNA products was assessed by separation on a 6% TBE-Urea gel and visualized by silver stain.
Figure 2.1 Synthesis of 48bp, 139bp and 540bp dsRNA ligands. A. Protocol for the synthesis of dsRNA ligands from DNA. B. 2% agarose gels containing sense (S) and antisense (AS) DNA templates either exposed (NdeI) or unexposed (blank) to the restriction enzyme NdeI. C. 6% TBE-Urea gels depicting purified 48bp, 139bp and 540bp dsRNA ligands.
2.2.3 Cell Culture

Peripheral blood mononuclear cells (PBMCs) were prepared in the Segal Lab, NIH. Briefly, Buffy coats (~20mL) were received from the NIH Blood Bank. To ~10mL Buffy coat, PBS was added to a total volume of 30mL in a 50mL tube. Buffy coat was dispersed by slow pipetting (1mL). Ficoll reagent (13mL) was added to the bottom of the tube. Lymphocytes were separated by Ficoll gradient (400xg, 20 minutes, RT, no brake) and transferred to a new tube. Lymphocytes were washed three times with PBS (~40mL) (1500rpm, 10 minutes, RT). The final pellet was resuspended in complete RPMI (10% FBS, 2mM L-glutamine, 1mM NaPyruvate, 1X non-essential amino acids, 50μg/mL Pen/Strep) and counted by trypan blue exclusion. Purified PBMCs were stored at 1x10^7 cells/mL in 90% serum and 10% DMSO in liquid phase N_2.

Monocytes were selected from PBMC stocks and differentiated into iDCs following the method described by Sallusto et al. Briefly, PBMCs were plated in complete RPMI (sans FBS) and 5-8x10^6 cells/mL and cultured at 37°C and 5% CO_2 for two hours. Non-adherent cells were removed by gently washing wells with complete RPMI. The remaining adherent population was almost exclusively composed of monocytes. To differentiate iDCs from the monocyte population, cells were grown in complete RPMI (with 50μM βMe) supplemented with GM-CSF (50ng/mL) and IL-4 (10ng/mL). Cells received 50% fresh complete RPMI (with 50μM βMe) supplemented with GM-CSF and IL-4 on days 1, 3, 5 and 7 following observation (Figure 2.2). On day 7, following visual confirmation of monocyte differentiation to iDCs, cells were stimulated with LPS (200ng/mL) or pI:pC (20μg/mL) or 139bp dsRNA (20μg/mL). Following 24-hours of stimulation, cells were collected and analyzed for cell surface markers by flow cytometry.

2.2.4 Flow Cytometry
Following 24-hour stimulation, cells were harvested by pipetting, counted by trypan blue stain and placed in 50mL falcon tubes. Cells (2.5x10^5) were then aliquoted into 10mL flow cytometry tubes and pelleted at 1000rpm for 6 minutes. The supernatant was removed and cells were washed with FACS buffer (1X Hank’s buffered saline solution, 0.1% bovine serum albumin, and 0.1% sodium azide). Cells were pelleted and then resuspended in blocking solution (100μLFACS buffer containing 0.1mg Human IgG antibody). Cells were incubated in blocking solution for 15 minutes at 4°C and then pelleted and washed three times with FACS buffer (1000xg, 3 minutes, RT). Cells were resuspended in 200μL FACS buffer and tubes were labeled as follows; unlabeled, PE-Ctl (PE-mouse-Anti-hCD54), FITC-Ctl (FITC-mouse-Anti-hHLA-DR), CD80/CD86 (PE-mouse-Anti-hCD80 / FITC-mouse-Anti-hCD86), CD1a/CD14 (PE-mouse-Anti-hCD1a / FITC-mouse-Anti-hCD14). Antibodies (5μL/2.5x10^5 cells) as indicated by tube, were added (manufacturer’s suggested dilutions) and incubated for 45 minutes at 4°C in the dark. Following antibody incubation, cells were pelleted and washed three times with FACS buffer (1mL) (1000xg, 3 minutes, RT). Cells were resuspended in FACS buffer (100μL) and analyzed via flow cytometry on an FC500 using CXP v.1 acquisition/analysis software (Beckman Coulter). Gates and voltages were optimized using PE and FITC control labeled cells prior to analysis. For each sample, a minimum of 10,000 events was recorded. A representative figure of 4 separate experiments is recorded in this document.

2.3 Results and Conclusions

As a result of work done by Leonard et al. and their findings elucidating the robust activation of a TLR3 response following stimulation by a 139bp dsRNA ligand [88], our group
set out to synthesize three dsRNA ligands, 48bp, 139bp and 540bp, in an attempt to test a range of dsRNA ligands in adjuvant potential studies. Successful synthesis of all three dsRNA constructs provided approximately 1.4mg of pure product for each ligand (Figure 2.1C).

After confirming the successful synthesis of dsRNA ligands, monocytes were differentiated into iDCs to use in the measurement of dsRNA adjuvant potential studies. iDC phenotype was confirmed visually through the transformation of cells from adherent to loosely adherent clusters (isolated floating cells or clusters) (Figure 2.2). Successful loss of receptor CD14 expression and gain of receptor CD1a expression, evaluated through flow cytometry analysis, also confirmed the successful differentiation of monocytes to iDCs (Figure 2.3).

To evaluate the adjuvant potential of synthesized dsRNA, we examined iDCs for increased expression of CD80/CD86, cell surface markers of DC maturation. Candidates for future development as vaccine adjuvants must successfully mature iDCs to DCs. Untreated iDCs, serving as negative controls, showed no surface expression of CD80 or CD86 receptors (Figure 2.4A). This negative control confirmation was critical as iDCs can undergo some level of maturation if roughly handled (i.e. jarring of culture dishes, pelleting of cells during harvest, or pipetting to resuspend). LPS treated iDCs, as a positive control, showed that all cells now expressed CD80 and CD86 receptors (Figure 2.4B) confirming that our iDC preparation could undergo maturation. iDCs, treated with pI:pC, the dsRNA mimetic known to induce TLR3 activity but shown to have toxic side effects in humans, exhibited an 82.9% gain in expression of the CD80 receptor while only a 24.2% gain in the CD86 receptor (Figure 2.4C). Similarly, 139bp dsRNA treated iDCs displayed an 82.2% gain in CD80 receptor expression but only a 29% gain in CD86 receptor expression (Figure 2.4D). These studies showed that 139bp dsRNA could
Figure 2.2 Visual confirmation of monocyte differentiation. Appearance of cells stimulated with GM-CSF (50ng/mL) and IL-4 (10ng/mL) over a 7 day period. Notice increased clustering and cells losing adherent phenotype. Microscope magnification; Day 1 = 40X, Day 3 = 10X, Day 5 = 10X and Day 7 = 10X.
Figure 2.3 Flow cytometry analysis of monocyte differentiation. Flow cytometry results of cells stained with CD14-FITC and CD1a-PE following 7 days of co-culturing with GM-CSF (50ng/mL) and IL-4 (10ng/mL).
match the maturation of DCs by pI:pC, but neither pI:pC or 139bp dsRNA could increase CD86 expression to levels observed in LPS treated iDCs.

Prior to completing our studies with the 48bp and 540bp dsRNA ligands, a paper was published investigating the capacity of dsRNA oligonucleotides of defined sizes to activate DC subsets from WT and TLR3-/- mice, to induce antigen specific CTL responses and to serve as adjuvants for an experimental influenza vaccine [143]. The authors were able to show that dendritic cell activation and adjuvant effectiveness increased with increasing dsRNA length. This study confirmed that dsRNA oligonucleotides could effectively serve as vaccine adjuvants.

While progress towards the completion of the study proposed in this document included the successful synthesis of three dsRNA ligands, the successful differentiation of monocytes to iDCs and the successful maturation of iDCs to DCs through stimulation with a 139bp dsRNA ligand, the results of the paper published by Jelinek et al. [143] were too similar to the remaining objectives for our lab to continue to pursue this project. Therefore, this study was terminated.
Figure 2.4 Assessment of the maturation of iDCs to DCs following stimulation. Flow cytometry analysis of cells stained with CD86-FITC or CD80-PE antibodies following A. no treatment, B. 24-hour treatment with LPS (200ng/mL), C. 24-hour treatment with pI:pC (20μg/mL), or D. 24-hour treatment with synthesized 139bp dsRNA (20μg/mL).
Chapter III
Elucidating the Role of Receptor Clustering in TLR3 Signal Transduction

3.1 Introduction

The events leading to the induction of TLR signaling occur in a sequential process in which pathogen recognition induces subsequent ligand binding and simultaneous receptor dimerization. TLR3, as was discussed in Chapter 1.6, recognizes viral dsRNA and associates with its ligand to form a 2:1 TLR3 to dsRNA complex. This 2TLR3:1dsRNA complex has been termed the signaling unit [88]. Previous cell-based studies of the TLR3:dsRNA complex had shown that a minimum of two signaling units were required to initiate endosomal TLR3
signaling and suggested that competent signaling may also occur through the clustering of complexes upon recognition of different length dsRNA ligands [88].

A multimerization event in which TLR complexes clustered together would not be unusual as researchers had shown previously that receptor clustering was required for TLRs that signal through the MyD88 adaptor molecule [144]. Upon activation of the MyD88–dependent pathway, signaling components interact to form a 14-subunit left-handed helical oligomer [144]. Formation of this Myddosome requires multiple, clustered receptor:adaptor complexes that have yet to be investigated.

Further evidence for a TLR3 multimerization event was discovered in cross-linking experiments done by the Caux group [145] who, at the time, were investigating both TLR3 and the FC-gamma receptor, CD32a. CD32a, a cell surface receptor, was known to have a well-defined mode of action in which multimeric IgG induced receptor multimerization [145]. This led to the activation of a cytosolic ITAM (immunoreceptor tyrosine-based activation motif) domain and a subsequent rapid rise in intracellular calcium levels. By generating a TLR3-CD32a chimeric molecule in which the TLR3-ECD was fused to the cytosolic ITAM domain of CD32a, the investigators were able to show that an intracellular Ca$^{2+}$ flux was only observed upon pI:pC stimulation or Anti-IgG crosslinking of Anti-TLR3 antibody suggesting that, in the same manner as endogenously expressed CD32a, the TLR3-CD32a chimera required receptor multimerization for signaling [145].

Although multiple 2TLR3:1dsRNA signaling units appear required for signaling, the parameters dictating clustering are unknown. In this study, three TLR3-ECD specific monoclonal antibodies (mAbs) were generated. Monoclonal antibody 1068 down-regulated dsRNA-induced pro-inflammatory mediators (mAb1068), whereas mAb12 and mAb15 ablated
dsRNA-induced TLR3 activity. Characterization of these antibodies through structural, cell-based and biophysical methods uncovered a role for lateral receptor clustering in TLR3 signal transduction.

3.2 Materials and Methods

3.2.1 Reagents

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. mAb1068, Fab1068, mAb12, Fab12, mAb15 and Fab15 were supplied by Janssen Biotech Inc. through a Materials Transfer Agreement. Structural determination of the quaternary complex and molecular modeling studies were completed by Janssen Biotech Inc.

3.2.2 TLR3-ECD purification and dsRNA production

The coding sequence for the TLR3-ECD (residues 22-702) fused to an N-terminal GP67 secretion signal sequence and a C-terminal TEV cleavage site followed by FLAG and 6XHis tags was inserted into a baculovirus expression system and expressed in High Five cells (Invitrogen) by using a multiplicity of infection of 3. Supernatant was harvested at 48 hours post-infection and passed over Anti-FLAG M2 agarose resin (Sigma) three times. Resin was washed with 20-column volumes of 1X TBS. Target protein was eluted with five 10mL volumes of 1X TBS plus FLAG peptide (100µg/mL). Resin was regenerated and stored as per manufacturer’s protocol. Samples from each step were analyzed by SDS-PAGE. Elution fractions containing target protein were pooled and stored at 4°C. To remove FLAG peptide from target protein, samples were separated on a Superdex 200 column (GE Healthcare).
dsRNA production was as described in Chapter 2.2.2

3.2.3 SEC and gel analysis of TLR3-ECD:dsRNA:Fab complexes

TLR3-ECD and/or Fab were mixed with dsRNA in a 2:1 molar ratio. Component or complexes were incubated for 1 hour at 4°C before separation. Individual components or complexes were separated on an Akta FPLC System (GE Healthcare) using a Superose 6 Tricorn column (GE Healthcare) equilibrated in 0.02M Pipes, pH 6.0, 150mM NaCl, and 1mM beta-mercaptoethanol prepared with diethylpyrocarbonate-treated water. Separation was monitored by absorbance at 280nm. Samples from each peak fraction were analyzed by SDS-PAGE. Fraction (18μL) plus loading dye (6μL – 4X reducing SDS-loading dye (Life Technologies)) were boiled for 10 minutes, briefly spun to sediment condensate and 20μL separated onto a 10% Tris-glycine SDS-PAGE gel (run at 200V for 35 minutes). Novex Sharp pre-stained molecular weight marker (Invitrogen) and/or RNA century marker (Applied Biosystems) were used as molecular weight standards. Gels were stained with SimplyBlue SafeStain (Invitrogen) for protein followed by silver stain for nucleotides. Gel documentation was completed using an Alpha Innotech gel imaging system. Contrast adjustment and cropping of gel images was completed in Adobe Photoshop.

3.2.4 Stimulation Assays

Reporter gene assays with luciferase readout were completed as reported by Janssen Biotech Inc. TLR3hi cells were a gift from the Segal Lab and stably expressed TLR3 in endosomal and cell surface locations along with a GFP-based NF-κB reporter. This system provides a sensitive readout of TLR3 activation. TLR3hi cells were plated at 1x10^6 cells/2mL in a 6-well plate. Cells were stimulated with dsRNA (25μg/mL), or Fab (100μg/mL), or control
mAb (100µg/mL), or dsRNA + Fab/control mAb for 48 hours. Cells were harvested by washing wells with PBS + 20mM ethylenediaminetetraacetic acid and counted by trypan blue exclusion method. Cells were pelleted, washed three times with FACS buffer (1X Hank’s buffered salt solution, 0.1% bovine serum albumin, and 0.1% sodium azide), and resuspended in 200µL FACS buffer. GFP fluorescence was analyzed via flow cytometry on an FC500 using CXP v.1 acquisition/analysis software (Beckman Coulter). For each sample, 10,000 events were recorded. The mean of three separate experiments ± SEM is reported. The two strands for the 49bp ligand were purchased from Dharmacon, mixed 1:1 and annealed. The 540bp dsRNA ligand was prepared as previously described in Chapter 2.2.2.

3.3 Results and Conclusions

3.3.1 A 49bp dsRNA ligand does not induce TLR3 signaling in RGA

Initial efforts to investigate receptor multimerization events, as they pertained to the induction of TLR3 signaling, utilized an NF-κB-driven reporter gene assay (RGA) [88]. In this assay, successful ligand-mediated activation of the TLR3 signaling cascade results in luciferase production under NF-κB promoter control. This luciferase response can be quantified by measuring emitted photons in a standard luciferase assay. Similar to the results obtained by Leonard et al. [88], pI:pC and long dsRNA (139bp and 540bp) ligands induced robust signaling in this assay, mediated through TLR3 in HEK293 cells transiently expressing human TLR3 (Figure 3.1). In contrast, the 49bp dsRNA ligand, that supports formation of a single signaling unit (2TLR3:1dsRNA), did not induce detectable activation in this assay (Figure 3.1). These results are consistent with another study that showed increasing pI:pC length proportionally
increased the receptor signaling response [146]. Taken together, this data suggests that individual signaling units are not sufficient to induce downstream TLR3 signaling, but rather multiple, linked signaling units are required for competent transduction of signal.

### 3.3.2 Three high-affinity antibodies generated to probe TLR3 function

In order to probe the clustering events of multiple signaling units as they pertain to TLR3 function, three high-affinity anti-TLR3-ECD mAbs (mAb1068, mAb12, and mAb15) were developed and their mechanistic inhibitory modes of action were characterized (Janssen Biotech Inc.). The affinities of the Fab fragments of each antibody for TLR3-ECD were shown to be in the low picomolar range (Table 3.1). All three mAbs dose-dependently inhibited the TLR3-mediated pI:pC-induced signals in the luciferase RGA (Figure 3.2). MAb1068 only exhibited partial neutralization of pI:pC-induced TLR3 signaling as opposed to complete inhibition by mAb12 and mAb15 (Figure 3.2). For the latter two antibodies, the potency of inhibition suggested the antibodies may block TLR3-ECD binding to dsRNA. Size-exclusion chromatography (SEC) experiments were performed to answer this question using the TLR3-ECD, a 139bp-dsRNA ligand, and the Fab fragments of mAb12 (Fab12) and mAb15 (Fab15) to avoid potential complications of the bivalency of mAbs (Figure 3.3). In these studies, the TLR3-ECD and a 139bp-dsRNA formed an apparent binary complex as shown by the absence of free RNA and TLR3-ECD peaks (T+R, Figure 3.3). Fab15 and Fab12 each formed an apparent binary complex with the TLR3-ECD (T+F15, T+F12, respectively) while Fab15, Fab12 and the TLR3-ECD were able to form a ternary complex (T+F12+F15). When Fab15 was combined with the TLR3-ECD and the 139bp dsRNA ligand (T+R+F15), a peak corresponding to T+F15 was apparent suggesting that the dsRNA ligand had been excluded. However, in contrast, Fab12 did not form a T+F12 peak when combined with the TLR3-ECD and dsRNA (T+R+F12) suggesting
Figure 3.1 Length-dependent induction of NF-κB-driven TLR3 signaling RGA. HEK293T, cells transiently expressing human TLR3, stimulated with varying concentrations of pI:pC, 49bp dsRNA, 139bp dsRNA, or 540bp dsRNA.
that Fab12 and Fab15 used different mechanisms to neutralize pI:pC-induced TLR3 signaling. When all four components were mixed (T+R+F12+F15), a peak corresponding to T+F12+F15 was apparent. Taken together, these data indicated that Fab15 prevented the TLR3-ECD from binding to dsRNA, while Fab12 could bind the TLR3-ECD that was bound to dsRNA. From this, we concluded that Fab15 inhibited TLR3 signaling by blocking TLR3-ECD binding to dsRNA. However, Fab12 neutralized TLR3 function even though a TLR3:dsRNA complex formed.

3.3.3 Structure of TLR3-ECD + Fab1068 + Fab12 + Fab15

To further elucidate the mechanisms by which each mAb neutralized ligand-induced TLR3 signaling, a structure of the quaternary complex of the TLR3-ECD, Fab1068, Fab12 and Fab15 was determined through a collaboration with Janssen Biotech, Inc. (Figure 3.4). The asymmetric unit contained one TLR3-ECD and one molecule of each Fab, all retaining their respective unbound structures. Interestingly, in the quaternary structure, each Fab bound a unique TLR3-ECD surface (Figure 3.4). While Fab12 was found to bind near the N-terminus of the TLR3-ECD on the convex surface of the solenoid from LRRs 3-7, Fab1068 was found to target the convex surface of the solenoid from LRRs 16-20 and Fab15 was found to bind the non-overlapping epitopes spanning LRRs 15-23 near the C-terminus of the TLR3-ECD.

3.3.4 Fab15 neutralizes by blocking TLR3:dsRNA binding

From the structural characterization of the quaternary complex, it was found that the Fab15 binding epitope contained amino acid residues N517, H539 and N541 which are part of the C-terminal dsRNA binding site of the TLR3-ECD and have been shown to be critical for ligand binding [87, 147]. TLR3-ECD binding of Fab15 would therefore prevent the binding of dsRNA which was consistent with SEC data (Figure 3.3) showing that Fab15 forms a dsRNA-free TLR3-ECD:Fab15 complex in the TLR3-ECD:dsRNA:Fab15 mixture. Thus, Fab15
Table 3.1 Fab binding affinity and buried surface area (SA) upon TLR3 binding.

<table>
<thead>
<tr>
<th>Fab</th>
<th>$k_a \times 10^9$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_d \times 10^{-4}$ (s$^{-1}$)</th>
<th>$K_d$ (pM)</th>
<th>$SA_{Ag}$ (Å$^2$)</th>
<th>$SA_{Ab}$ (Å$^2$)</th>
</tr>
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<tbody>
<tr>
<td>Fab12</td>
<td>4.11 (1)</td>
<td>2.83 (0)</td>
<td>68.8 (4)</td>
<td>928</td>
<td>896</td>
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<tr>
<td>Fab1068</td>
<td>3.54 (1)</td>
<td>1.03 (1)</td>
<td>29.1 (4)</td>
<td>914</td>
<td>963</td>
</tr>
<tr>
<td>Fab15</td>
<td>3.33 (1)</td>
<td>1.12 (5)</td>
<td>3.1 (1)</td>
<td>1084</td>
<td>1080</td>
</tr>
</tbody>
</table>
Figure 3.2 mAbs inhibit NF-kB driven TLR3 activity induced by pI:pC. The ratios of luciferase activity induced by pI:pC between HEK293T cells transfected with a TLR3 expressing construct and those transfected with a control Renilla vector in the presence of antibodies are shown for (A.) mAb1068, (B.) mAb12, (C.) mAb15 and (D.) a control antibody CNTO3161, respectively. PIC: pI:pC.
Figure 3.3 Fab15 inhibits TLR3-ECD:dsRNA binding. SEC chromatograms of various combinations of TLR3-ECD (T), Fab15 (F15), Fab12 (F12), and 139bp dsRNA (R). Fab15 forms a dsRNA-free TLR3+Fab15 complex (T+R+F15) indicating that it blocks the TLR3-ECD from binding to dsRNA. The excess TLR3-ECD was loaded onto dsRNA. Fab12 molecules were completely bound by the TLR3-ECD:dsRNA complex (T+R+F12). In the presence of Fab15, Fab12 can form a dsRNA-free TLR3-ECD+Fab15+Fab12 ternary complex (T+F12+F15). Components in peak fractions of SEC experiments were confirmed by SDS-PAGE separation and Coomassie / silver staining as described in Chapter 3.2.2. Individual chromatographs with corresponding SDS-PAGE analysis can be found in Appendix 1.
competes with dsRNA for TLR3 binding thereby preventing ligand-induced receptor
dimerization, which is required for the formation of TLR3 signaling units. This finding further
corroborates the luciferase RGA results in which mAb15 is able to completely neutralize pI:pC-
induced TLR3 activation (Figure 3.2).

3.3.5 Fab12 and Fab1068 are compatible with the TLR3-ECD:dsRNA signaling unit

In contrast to Fab15, the Fab1068 and Fab12 binding sites did not overlap any of the amino acid
residues involved in dsRNA binding to the TLR3-ECD (Figure 3.4). Based upon the structures of
the TLR3-ECD:3Fabs and the TLR3-ECD:dsRNA, composite molecular models of Fab12 and/or
Fab1068 bound to the TLR3-ECD:dsRNA dimer (Figure 3.5A) were generated. The bound
Fab1068 and Fab12 did not sterically clash with the dsRNA ligand or the TLR3-ECD dimer
partner in the signaling unit. Additionally, both bound Fabs were nearly parallel to the proposed
cell membrane surface and would therefore be unlikely to disrupt the orientation of the
TLR3:dsRNA complex with respect to the membrane surface and subsequent TIR domain
dimerization. Thus, we predicted that Fab1068 and Fab12 would each be able to bind to a
signaling unit without disrupting its function. SEC data confirmed this hypothesis as both
Fab1068 (Figure 3.5B) and Fab12 (Figure 3.5C) were able to bind TLR3:dsRNA complexes.
Taken together, these data indicate that Fab1068, and similarly Fab12, can bind simultaneously
to two TLR3 molecules of a signaling unit without preventing dsRNA binding or TLR3
dimerization. This further indicates that Fab12 and Fab1068 are unlikely to neutralize TLR3
function by direct inhibition of signaling unit formation.
Figure 3.4 Molecular structure of the quaternary complex of TLR3-ECD:3Fabs. Overall structure of the complex in ribbon (A.) and surface (B.) representations. TLR3 colored in rainbow scheme (blue-to-red for N-to-C), Fab12 in yellow-green, Fab15 in light blue, and Fab1068 in blue. Carbohydrates are omitted for clarity. (B.) Distinct binding epitopes are highlighted on TLR3. The epitopes are colored on the TLR3-ECD as for the Fabs in (A.). The C-terminal dsRNA binding site is indicated. The overlap of the Fab15 epitope and dsRNA binding site is indicated in cyan.
3.3.6 Lateral clustering model of TLR3 signaling

From SEC (Figure 3.3) and structural data (Figure 3.4), in collaboration with Janssen Biotech Inc., we propose a modified model of TLR3 signaling following ligand induced dimerization (Figure 3.6). As discussed in the introduction and alluded to by Leonard et al., longer dsRNA ligands are not only important for the induction of TLR3 activity, but they also support TLR3 multimerization events on a single dsRNA molecule. The apparent affinities of the TLR3-ECD for longer dsRNA ligands has also been shown to be much higher than for 49bp dsRNA ligands [88], potentially indicating the occurrence of lateral signaling unit interactions and avidity effects of multiple binding sites on the longer dsRNA ligands. To interpret our results, we will use a model of two adjacent signaling units assembled on dsRNA (Figure 3.6). Binding of Fab12 and Fab1068 laterally separates, but does not disrupt, individual signaling units along the dsRNA molecules. The Fab-induced signaling unit separation likely provides the neutralizing activity of these antibodies and indicates that maintaining a short distance between the signaling units, termed “lateral clustering,” is required for TLR3 signaling. Fab12 and Fab1068 neutralized lateral clustering by separating the signaling units to avoid steric clashes between bound antibody and a neighboring signaling unit. This would suggest that increasing neutralization effects of antibodies would be dependent upon the distance between signaling units resulting from antibody binding. This separation, however, would not inhibit the basal activity of a single signaling unit. As shown in Figure 3.6, Fab12 causes a larger signaling unit separation than Fab1068. Fab12’s larger signaling unit separation correlates with mAb12’s ability to consistently exhibit greater neutralizing activity than mAb1068 (Figure 3.2) even though Fab12 has a slightly lower binding affinity (Table 3.1).
Figure 3.5 Fab12 and Fab1068 bind the TLR3-ECD in the TLR3-ECD:dsRNA signaling unit. A. Model of Fab1068 and Fab12 binding to a single signaling unit. Fab binding does not inhibit dsRNA binding, TLR3 dimerization, or membrane association of TLR3. B. SEC of various combinations of TLR3-ECD (T), Fab1068 (F), and 49bp dsRNA (R) indicate the presence of the R+T+F ternary complex. C. SEC of various combinations of TLR3-ECD (T), Fab12 (F12), and 49bp dsRNA (R) indicate formation of R+T+F12 complex. Components in peak fractions of SEC experiments were confirmed by SDS-PAGE separation and Coomassie / silver staining as described in Chapter 3.2.2. Chromatograms with corresponding SDS-PAGE analysis can be found in Appendix 1.
Figure 3.6 Lateral signaling unit clustering mediated TLR3 signaling. Model of two signaling units clustered on a 76bp dsRNA ligand (top panel) and separation of signaling units (bottom panel) on long dsRNA by Fab1068 and Fab12 due to steric clashes between the antibodies and neighboring signaling units. The separation distances were calculated using the center of gravity of a group of TLR3-ECD C-terminal atoms.
3.3.7 Experimental support for lateral clustering model

To test the validity of a lateral clustering model which depends upon signaling unit distance for competent TLR3 signaling, we carried out neutralization assays in TLR3hi cells that stably express TLR3 and the GFP reporter gene driven by NF-kB (Figure 3.7). As opposed to the transient TLR3 RGA, short dsRNA ligands were able to generate robust TLR3 activation in this cell line (Figure 3.7A). Cells were stimulated with a 49bp (single signaling unit) (Figure 3.7A) or 540bp (multiple signaling unit binding) (Figure 3.7B) dsRNA ligand in the presence of Fab. Fab15 completely inhibited GFP fluorescence induced by both ligands consistent with Fab15 ability to directly block dsRNA binding to TLR3. In contrast, Fab12 did not significantly inhibit 49bp dsRNA (single signaling unit)-induced fluorescence, as predicted by our structural analysis that shows Fab12 does not affect formation of individual signaling units. In contrast, Fab12 did partially inhibit 540bp dsRNA-induced fluorescence. This, we postulate, is due to Fab12 altering the distance between individual signaling units. These results were further corroborated in an NF-κB luciferase RGA in HEK293 cells transiently expressing human TLR3, where both Fab15 and Fab12 strongly inhibited the RGA luciferase activity induced by pI:pC in a dose dependent manner (Figure 3.7C). Taken together, these results are consistent with the model in Figure 3.6 in which Fab12 (and Fab1068 by inference) reduces TLR3 signaling via disruption of signaling unit-signaling unit interactions but has little impact on signaling from the single signaling unit.

3.3.8 Lateral clustering study concluded

Prior to this study, the functional differences observed between short (~48bp) dsRNA ligands that form a single signaling unit and longer (>~90bp) dsRNA ligands with multiple
Figure 3.7 Antibody inhibition of TLR3 activation induced by dsRNA and pI:pC. Fab15 completely inhibits fluorescence of TLR3hi cells (A. and B.). Fab12 partially inhibits signal induced by 540bp dsRNA (B.) and has no significant impact on that of 49bp dsRNA (A.). Percentage of GFP-positive cells after 48h treatment with dsRNA (49bp or 540bp) ± antibody treatment as indicated. Error bars indicate mean and SEM from three independent experiments. Tests for significant differences were performed by Bonferroni posttests of one-way ANOVA. NS, not significant. C. Fab15 and Fab12 nearly completely inhibit pI:pC-induced TLR3 signaling in NF-kB RGA in HEK293 cells with transient TLR3 expression.
signaling units docked to the ligand could not be addressed by a single signaling unit structure. Through structural and functional studies of TLR3, utilizing neutralizing antibodies, we provide evidence for a new TLR3 signaling model in which dsRNA:TLR3 signaling units laterally cluster to achieve efficient signaling. While multimerization events do not contribute to single signaling unit signal transduction, we show here that the requirement for multiple signaling units on longer (>~90bp) dsRNA ligands is mediated by our novel lateral clustering mechanism. This mechanism is critical for competent TLR3 signaling in response to longer dsRNA ligands and may be a mechanism by which other TLR proteins mediate downstream signaling cascades.
Chapter IV

SIKE: A Novel TBK1 Substrate and Endogenous Regulator of the Type-I Interferon Response

4.1 Introduction

Inhibitor of κB kinases (IKKs) are essential regulators of innate immunity, inflammation, cell proliferation and apoptosis [109]. Canonical IKK signaling is mediated by the IKK complex consisting of two kinases, IKKα and IKKβ, and the regulatory scaffold, NEMO [148]. As discussed in Chapter 1.7, the canonical IKKs activate the transcription factor NF-κB by phosphorylating the inhibitor of κB proteins. This post-translational modification releases NF-κB from an inhibitory complex and permits its translocation to the nucleus where it mediates gene transcription.
In addition to the IKKα and IKKβ kinases, the IKK kinase family contains two non-canonical family members, a constitutively expressed serine/threonine kinase, TBK1, and an expression inducible serine/threonine kinase, IKKε (largely immune-cell specific) [101]. In response to receptor-mediated pathogen detection, these kinases play distinct roles in inducing type I interferon production and modulating NF-κB signaling. In addition, several substrates have been identified that implicate these kinases in functions ranging from insulin response and cell growth to xenophagy.

Increasingly, however, dysregulation of the non-canonical members of the IKK family has been linked to autoimmune disorders and cancers. IKKε has been shown to be up-regulated in breast, ovarian and prostate cancer cell lines as well as in patient-derived tumors [149]. TBK1 has been implicated in KRAS-signaling dependent cancers, such as colorectal and lung cancer, as RALB-mediated activation of TBK1 has been shown to promote cancer cell survival [150]. Furthermore, studies have found that viruses, such as Hepatitis C (HCV), the Vaccinia virus (VACV) and certain Hantaviruses have evolved to target these kinases in order to evade immune pathway activation and subsequent anti-viral responses [22]. These findings have elevated the need to understand regulatory controls of kinase activity in an effort to therapeutically manipulate the pathways involved in aberrant kinase activity.

To control the activity of TBK1, the study presented herein focuses on the 207 amino-acid protein termed SIKE, which, as discussed in Chapter 1.8, was previously characterized as an endogenous inhibitor of the TLR3 signaling cascade. Using a multitude of biophysical techniques and cell-based assays, this document explores the undefined mechanism by which SIKE inhibits the type-I interferon response and, the regulatory release mechanism of SIKE from
TBK1 mediated by viral activation of the TLR3 signaling cascade. Additional experiments attempt to elucidate the potential role of SIKE following its release from TBK1. Taken together, this study not only highlights potential therapeutic targets in the type-I interferon response, but also establishes a novel role for a critical member of the TLR3 signaling cascade.

4.2 Materials and Methods

4.2.1 Reagents

Chemicals were purchased from Sigma-Aldrich unless otherwise specified. $\gamma^{32}$P-ATP was purchased from Perkin Elmer. Recombinant TBK1 was purchased from Life Technologies. Anti-phospho-serine antibody was purchased from Qiagen. Anti-FLAG-HRP was purchased from Sigma-Aldrich. Anti-HA antibody was purchased from Covance. Anti-full-length IRF3 and anti-actin-HRP antibodies were purchased from Santa Cruz. Anti-phospho-S396 IRF3 antibody was purchased from Cell Signaling. Anti-rabbit-IgG-HRP and anti-mouse-IgG-HRP antibodies were purchased from Southern Biotechnology Associates, Inc. pFLAG-CMV5a-IRF3 was a gift from Dr. T. Kordula.

4.2.2 Cell Culture

HEK293 cells were purchased from American Type Culture Collection. Cells were cultured in complete medium (RPMI 1640 supplemented with 10% low-endotoxin FBS, 20mM L-glutamine, 100mM HEPES, 10mM Na-pyruvate, and 1X nonessential amino acid solution) at 37°C in 5% CO$_2$. 

4.2.3 Constructs
The human SIKE sequence was cloned from total RNA isolated from the 786O cell line using the OneStep RT-PCR kit according to the manufacturer’s protocol (Qiagen). The primers incorporated a 5’ NdeI restriction enzyme site (5’-ATTATCATATGAGCTGCACCATCGAGAAGATC-3’) and a 3’ BamHI restriction enzyme site (5’-TAATAGGATCCTTATTTGATGGCTTGGGAAGC-3’) compatible with insertion into the pET15b vector (Novagen). The SIKE72 construct (residues 72-207) was amplified using 5’-TATACATATGCTGCTGCCAAGAAGAC-3’ and inserted into the pET15b vector using the same strategy. Full-length SIKE was amplified from FL-SIKE pET15b using standard molecular biology techniques with primers including a 5’ EcoRI restriction enzyme site (5’-TATAGAATTCAGAGCTGCACCATCGAGAAG-3’) and 3’ SalI restriction enzyme site (5’-TATAGTCGACCTTATTTGATGGCTTGG-3’) compatible with insertion into the pCMV HA vector (Clontech). SIKE113-207 was amplified from FL-SIKE pET15b using standard molecular biology techniques and primers with a 5’ EcoRI restriction enzyme site (5’-TATAGAATTCAGAGCTGCACCATCGAGAAG-3’) or 5’ NdeI restriction enzyme site (5’-TATACATATGCTGCTGCCAAGAAGAC-3’) and the 3’ primer used for FL-SIKE pCMV HA or pET15b compatible with insertion into the pCMV HA or pET15b vector, respectively. SIKE point mutations (S133A/E, S185A/E, S187A/E, S188A/E, S190A/E, S198A/E) and insertions (stop codons after 112 (SIKE 1-112), 184 (SIKE72-184) and N-terminal FLAG sequence into pUNO-TBK1 vector (Invivogen) were made using the Quikchange site-directed mutagenesis kit following the manufacturer’s protocol (Agilent Technologies). All constructs and mutations were confirmed by DNA sequencing. GST-IRF3 173-427 pGEX4T1 vector was a kind gift from Dr. Katherine Fitzgerald.

4.2.4 Expression and purification of proteins
For pET15b SIKE72 construct expression, vector was transformed into chemically competent BL21-CodonPlus (DE3)-RIPL following the manufacturer’s protocol (Agilent Technologies). A single colony was used to inoculate an overnight culture of Luria broth (LB) plus 100 μg/ml ampicillin. The overnight culture was sub-cultured, 1:100, into 2-1L LB/amp flasks grown at 37°C until the cell density reached an A600 ≈ 0.6. Cultures were induced with 1 mM isopropyl-β-D-galactopyranoside (IPTG) and allowed to grow for an additional 4 hours at 37°C. Cells were harvested by centrifugation at 7,000 xg and the pellet solubilized in guanidine hydrochloride (GudHCl) buffer (6 M guanidine hydrochloride, 100 mM sodium phosphate (pH 8.0), 500 mM NaCl and 1 mM 2-mercaptoethanol) (5ml buffer/g of cell pellet). Lysate was clarified by centrifugation at 14,000 xg. The supernatant was mixed with 5ml Ni-NTA agarose resin (Qiagen), pre-equilibrated in GudHCl buffer. The lysate-resin mixture was loaded into a column (BioRad) and washed by gravity with 50 column volumes (CV) of GudHCl buffer. Bound protein was refolded on the column using a 40-CV reverse gradient of GudHCl buffer to 100 mM sodium phosphate (pH 8.0), 500 mM NaCl and 1 mM 2-mercaptoethanol (Buffer 1) and eluted with 5-CV of Buffer 1 plus 500 mM imidazole. Elution fractions were separated on a HiLoad 16/60 Superdex 200 column (GE Healthcare) and peak fractions screened for target protein by SDS-PAGE analysis. CD experiments of WT and mutant SIKE72 proteins indicated primarily helical secondary structure (Figure 4.1A), consistent with a predicted coiled coil domain structure, while a thermal melt denaturation experiment of WT SIKE72 revealed a ≥ two-state unfolding curve typical of globular proteins (Figure 4.1B).

For GST-IRF3 173-427 expression, pGEX4T1-IRF3 173-427 was transformed into chemically competent BL21-CodonPlus (DE3)-RIPL cells following the manufacturer’s protocol (Agilent Technologies). Cell culture was identical to pET15b expression prior to induction.
When cell density reached $A_{600} \approx 0.6$, cultures were incubated on ice for 30 minutes, induced with 1 mM IPTG, and allowed to grow at 16°C for an additional 14 hours. Cells were harvested by centrifugation at 7,000 $xg$ and the cell pellet was resuspended in Buffer A (2X PBS, 10mM DTT, 1mM EDTA), 5ml buffer/g of cell pellet. Cells were emulsified to lyse (Emulsiflex C3, Avestin) and clarified by centrifugation at 12,000 $xg$. The supernatant was mixed with glutathione Sepharose 4b resin pre-equilibrated in Buffer A (5 mL)(GE Healthcare). The resin-supernatant solution was loaded into a column (Biorad) and washed by gravity with 100 CV Buffer A, 100 CV 20 mM Tris, pH 7.5, 500 mM NaCl, 1 mM EDTA, 10 mM DTT, 10% glycerol (Buffer B) and eluted with 2-5CV fractions of Buffer B plus 10 mM glutathione (pH 8.0). The elution fractions were concentrated in an Amicon Ultra-15 centrifugal filter (Millipore) to 6 mg/ml. GST-IRF173-427 was incubated with 20U/ml thrombin (GE Healthcare) for 24 h at 4°C. IRF3 173-427 was separated from GST using a HiLoad 16/60 Superdex 75 column (GE Healthcare) equilibrated in 20 mM HEPES, pH 7.5, 500 mM NaCl, 1 mM 2-mercaptoethanol. Peak fractions were screened for cleaved, pure IRF3 173-427 by SDS-PAGE analysis. Fractions were pooled and stored at 4°C.

Protein concentrations were determined using the Bradford method (BioRad).

### 4.2.5 In vitro kinase assays: TBK1 activation and linear range controls

TBK1 (Life Technologies) was pre-activated (S172 phosphorylated) through auto/transphosphorylation prior to kinetic assays. To determine the optimal pre-incubation time, a time course following $^{32}$P incorporation into TBK1 was completed. TBK1 (4.93 nM) in 50 mM Tris, pH 7.5, 10 mM MgCl$_2$, 2 mM DTT and 0.025% Triton-X 100 (assay buffer) was incubated.
Figure 4.1 Characterization of WT and mutant SIKE72. Recombinant WT SIKE72, S6A SIKE72 and S6E SIKE72 were dialyzed into 5mM NaH/H$_2$PO$_4$, pH 8.0 and 0.15M NaCl and diluted to ~1 mg/mL. A. CD spectra were collected on a Jasco 720 Circular Dichroism spectrophotometer. Five scans were accumulated, buffer subtracted and smoothed using the Jasco spectra manage software. B. Ellipticity at 222 nm was monitored over the temperature range of 4-90$^\circ$C. The thermal melt was completed on the sample used in A. Increases in temperature were controlled by a Peltier cell holder set to 1$^\circ$ increase/minute.
with 100 μM ATP plus 0.1 mCi γ-32P-ATP at 30°C for 240 minutes (200μL total volume). 20μL aliquots were removed at indicated times and the reaction was terminated by the addition of SDS loading dye (Invitrogen) and boiling for 10 minutes at 98°C. Duplicate samples (10μL) were separated by SDS-PAGE on a 4-12% Bis-Tris polyacrylamide gel (Life Technologies). Gels were mounted on filter paper and exposed to a storage phosphor screen (Imaging Screen K, BioRad). The screen was read by a Molecular Imager FX system (BioRad). Band densitometry was measured and converted to 32P μM via a γ-32P-ATP standard curve. Incorporation of 32P into TBK1 stabilized at 25 minutes (Figure 4.2).

Initial rate parameters for TBK1 phosphorylation of IRF3 were also established. 20μL aliquots from a reaction (250μL total volume) containing 20 μM IRF3 173-427, 100 μM ATP, 0.1 mCi γ-32P-ATP and 4.93 nM TBK1 (preactivated for 30 minutes with 100 mM “cold” ATP) in assay buffer were taken at the indicated times and reaction terminated as described above. Duplicate samples (10μL) were analyzed for 32P-IRF3 as described above. The initial rate remained linear between 5-200 minutes (Figure 4.3).

4.2.6 In vitro kinase assays: Experimental

Michaelis-Menten kinetic assay reactions (50 μl volume) contained 0.1 mCi γ-32P-ATP, 100 μM ATP and 0.042-20.8 μM IRF3 173-427 for IRF3 varied assays; 0.1 mCi γ-32P-ATP, 20 μM IRF3 173-427 and 0.48-83 μM ATP for ATP varied assays; and 0.1 mCi γ-32P-ATP, 100 μM ATP and 0.043-8.4 μM SIKE72 for SIKE varied assays. Reactions were initiated by addition of 29.6 nM TBK1 (10 μL, 4.93 nM final concentration, pre-activated for 30 minutes with 100 μM ATP in assay buffer, Figure 4.2). For ATP varied reactions, excess cold ATP from pre-
Figure 4.2 Control experiment investigating the activation of TBK1. Activation of TBK1 via autophosphorylation at S172 is required for \textit{in vitro} experiments. TBK1 (4.93 nM) was incubated with ATP (100 \textmu M) for 240 minutes at 30\textdegree C. At indicated times, 20 \textmu L aliquots were collected and the reaction stopped by addition of 4X Loading Dye sample buffer and boiling at 98\textdegree C for 10 minutes. Reactions were analyzed as described in Chapter 4.2.5.
**Figure 4.3 Control experiment investigating initial rate parameters for the TBK1-mediated phosphorylation of IRF3.** Initial rate kinetic parameters were determined from a reaction containing IRF3 (20 μM), ATP (100 μM), and TBK1 (4.93 nM) and incubated for 720 minutes at 30°C. At indicated times, 10 μL aliquots were collected and the reaction stopped by the addition of 4X Loading Dye sample buffer and boiling at 98°C for 10 minutes. Reactions were analyzed as described in Chapter 4.2.5.
activation of TBK1 was removed by desalting column (Zeba spin, 7 MWCO, 2 ml -Pierce) prior to initiating reactions. Reactions were incubated for 120 minutes at 30°C (determined in Figure 4.3) and terminated by boiling in reducing SDS sample buffer (20 μL, Life Technologies).

Duplicate samples (10 μL ea.) were separated by SDS-PAGE on a 4-12% Tris-Bis-Tris polyacrylamide gel (Life Technologies). Gels were mounted on filter paper and exposed to a storage phosphor screen (Imaging Screen K, BioRad). The screen was read by a Molecular Imager FX system (BioRad). Band densitometry was measured and converted to $^{32}$P μM via a $\gamma$-$^{32}$P-ATP standard curve. Inhibition experiments were carried out using the assay methodology described for IRF3 varied and ATP varied assay with the addition of SIKE72 at 20.8 or 83.3 nM final concentration. All reactions were analyzed by SDS-PAGE/autoradiography as described above. A minimum of quadruplicate repeats was completed for each reaction. Data were plotted as a Michaelis-Menten plot or double reciprocal plots and fit to a single rectangular hyperbola or linear polynomial equations, respectively, in SigmaPlot (Systat Software, Inc.). Errors were reported as standard deviation. From the intercepts and slopes derived from the double reciprocal plot fit to a linear polynomial, inhibition constants were calculated for the SIKE + ATP or IRF3 varied data using:

\[
\text{Intercept}_{\text{inh}} = \text{Intercept}_{\text{none}}(1 + \left(\frac{[I]}{K_i}\right)) \quad (1)
\]

\[
\text{Slope}_{\text{inh}} = \text{Slope}_{\text{none}}(1 + \left(\frac{[I]}{K_i}\right)) \quad (2)
\]
To examine inhibition of IRF3 phosphorylation by SIKE72, 4.17 μM IRF3 173-427, 100 μM ATP, 0.1 mCi γ-32P-ATP and SIKE72 or SIKE72 mutant, at indicated concentrations, were prepared in assay buffer (50 μL volume). TBK1 (10 μL of 29.6 nM, 4.93 nM final concentration) pre-activated as above, was added to initiate the reaction. Reactions were incubated at 30°C for 120 minutes and analyzed as described above to derive initial rates for IRF3 173-427 phosphorylation. Data were plotted as the percentage of uninhibited reaction rate versus SIKE72 concentration. Errors were reported as standard deviation. To derive the $K_{i,app}$ parameter, inhibited reaction rate versus SIKE72 or SIKE72 mutant concentration was fit to a 2-parameter rectangular hyperbola.

4.2.7 DNA transfection, immuneprecipitation and immunoblot analysis

Approximately $0.5 \times 10^6$ cells were plated into 10 cm² wells and transfected with 2.5 μg total DNA of the different expression plasmids (1:0.9:0.6 ratio of (pUNO-FLAG-TBK1 or mTurq-TBK1 or FLAG-IRF3 or HA-SIKE):pCDNA3.1:pCMV-HA-SIKE) using Lipofectamine 2000 following the standard procedure (Life Technologies). After 24 h, cells were stimulated with 50 μg/ml polyinosinic:polycytidylic acid (pI:pC) for 3 hours, harvested and lysed in a lysis buffer (200 μL, 0.02 M HEPES, pH 7.4, 0.15 M NaCl, 10 mM NaF, 2 mM DTT, 2 mM EGTA, 1.5 mM MgCl₂, 1 mM Na₃VO₄, 2.7 mg/ml β-glycerophosphate, 1 mg/ml N-ethylmaleimide, 0.5% Triton-X100, 1X Complete, EDTA-free protease inhibitor cocktail (Roche)). Lysates were cleared by centrifugation (14,000 xg for 30 minutes at 4°C). Protein concentration was quantified by Bradford method (BioRad). For whole cell lysates, 50 μg of total protein per sample were boiled in sample buffer (Life Technologies), and separated by SDS-PAGE (10% Tris-Glycine) and transferred to nitrocellulose membrane. The membrane was blocked in 5% non-fat dry milk diluted in Tris-buffered saline (TBS) containing 0.1% Tween20 and probed
with indicated antibodies. Blots were developed with chemiluminescent reagents, ECL Plus (GE Healthcare). For immunoprecipitations, cell lysates (500 μg) were incubated with 40 μL of anti-FLAG M2 antibody affinity gel (Sigma Aldrich) for 24 hours in TBS at 4°C. Resin was washed with TBS (3x1mL) and bound proteins eluted with 100 μL of 125ng/μL FLAG peptide (Sigma Aldrich). Eluted proteins were analyzed by immune-blot as described above. Each experiment was repeated in triplicate.

4.2.8 Characterization of phosphorylation by MALDI-TOF

Protein (500 nM SIKE72, 5000 nM IRF3 173-427, 714 nM gMDH) was incubated with 4.93 nM TBK1 and 100 μM ATP for indicated times at 30°C. Reactions were terminated by addition of an equal volume of 6 M guanidine hydrochloride and target protein recovered by absorption onto Ni-NTA resin. Proteins were eluted from Ni-NTA as described above, desalted and concentrated using a ZipTipC4 (Millipore). ZipTipC4 eluent was mixed 1:1 with α-cyano-4-hydroxycinnamic-acid and spotted onto a Scout49 target. Data were collected on a Bruker OmniFlex MALDI-TOF mass spectrometer. Spectra for time points 0, 24 and 96 hours were recorded.

4.2.9 Phospho-peptide mapping

Recombinantly expressed SIKE72 (500 ng) was incubated with TBK1 (4.93 nM) and ATP (100 μM) in assay buffer (100 μL volume) for 24 hours at 30°C. The reaction was terminated by diluting the reaction 1:1 with 6M GudHCl buffer. SIKE72 was isolated from the reaction by incubation with 40 μL of Ni-NTA resin, washed with GudHCl buffer and bound SIKE eluted with 50 μL of GudHCl buffer plus 0.5 M imidazole. Eluent was separated by SDS-
PAGE (10% Tris-Glycine) and target band excised for analysis. The band was submitted for analysis to the Chemical and Proteomic Mass Spectrometry VCU Core (Dr. K.T. Nelson). The method for analysis was as follows: The gel slice was washed and de-stained in 200µL 50% methanol overnight. The gel pieces were dehydrated in acetonitrile, rehydrated in 30µL of 10mM dithiothreitol in 0.1M ammonium bicarbonate and reduced at room temperature for 30 minutes. The DTT solution was removed and the samples were alkylated in 30µL 50mM iodoacetamide in 0.1M ammonium bicarbonate at room temperature for 30 minutes. The reagent was removed and the gel pieces were dehydrated in 100µL acetonitrile. The acetonitrile was removed and the gel pieces were rehydrated in 100µL 0.1M ammonium bicarbonate. The pieces were dehydrated in 100µL acetonitrile, the acetonitrile removed and the pieces completely dried by vacuum centrifugation. The gel pieces were rehydrated in 1µg trypsin in 50 mM ammonium bicarbonate on ice for 10 min. Any excess trypsin solution was removed and 20µL 50mM ammonium bicarbonate added. The samples were digested overnight at 37°C and the peptides that were formed were extracted from the polyacrylamide in two 30µL aliquots of 50% acetonitrile/5% formic acid. Extracts, evaporated to 15 µL, were separated by C18 reversed-phase capillary column (Waters NanoAcquity) coupled with nanospray tandem mass spectrometry (LTQ-Orbitrap hybrid, Thermo Electron). The digest was analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. The data were analyzed by database searching using the Sequest search algorithm against custom databases generated from the SIKE72 sequence. All potential phosphor-peptides were manually examined for correct identification of the modified site.

4.2.10 Tandem mass spectrometry for identification of SIKE interaction network
Approximately $0.5 \times 10^6$ cells were plated into 10 cm$^2$ wells and transfected with 4 μg pCMV-HA-SIKE using Lipofectamine 2000 following the standard procedure (Life Technologies). After 24 h, cells were stimulated with 50 μg/ml polynosinic:polycytidylic acid (pI:pC) for 3 hours, harvested and lysed in a lysis buffer (200 μl, 0.02 M HEPES, pH 7.4, 0.15 M NaCl, 10 mM NaF, 2 mM DTT, 2 mM EGTA, 1.5 mM MgCl$_2$, 1 mM Na$_3$VO$_4$, 2.7 mg/ml β-glycerophosphate, 1 mg/ml N-ethylmaleimide, 0.5% Triton-X100, 1X Complete, EDTA-free protease inhibitor cocktail (Roche)). Lysates were cleared by centrifugation (14,000 xg for 30 minutes at 4°C). Protein concentration was quantified by Bradford method (BioRad). For immunoprecipitations, cell lysates (500 μg) were incubated with 50 μL of monoclonal-Anti-HA agarose for 24 hours in PBS at 4°C. Resin was washed with PBS (3x1mL) and bound proteins incubated with 120 μL of 0.1M glycine-HCl, pH 2.5 for 8 hours (elution). Following incubation, resin was pelleted at 12000xg for 2 minutes and the supernatant was added to 40 μL of 1M Tris buffer, pH 8.0. Quality of IP was analyzed via SDS-PAGE gel / silver-stain. Remaining solutions (~500 μL) were submitted for analysis to the Chemical and Proteomic Mass Spectrometry VCU core (Dr. K.T. Nelson). The method for analysis was as follows: Solutions were diluted to 150 μL with 100mM ammonium bicarbonate to reduce the concentration of salts prior to digestion. Samples were reduced with 5 μL of 10mM dithiothreitol in 0.1M ammonium bicarbonate at room temperature for 30 minutes. Samples were then alkylated with 5 μL 50mM iodoacetamide in 0.1M ammonium bicarbonate at room temperature for 30 minutes. Samples were then digested with 1 μg trypsin overnight and then quenched with 5% (v:v) glacial acetic acid. Sample analysis was completed on an LC-MS system consisting of a Thermo Electron LTQ-Orbitrap hybrid mass spectrometer with a nanospray ion source interfaced to a Waters SCX trap column and a Waters NanoAcquity C18 reversed-phase capillary column. Seven μL of the
final solution was injected onto the trap column, and the peptides were eluted from the column by an acetonitrile/0.1% formic acid gradient at a flow rate of 0.4 μL/min over 60 minutes. The nanospray ion source was operated at 3.5kV. The digests were analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine amino acid sequence in sequential scans. This mode of analysis produces approximately 10000 CAD spectra of ions ranging in abundance over several orders of magnitude. Final data analysis was done by database searching using the Sequest search algorithm against the IPI Human database.

**4.2.11 Cell lysate separation into nuclear and cytoplasmic fractions**

HEK293 cells, transiently transfected with S6A SIKE72 or S6E SIKE72, were stimulated with 50 μg/mL pI:pC. Cells were harvested by washing with ice cold 1X PBS and placed in a microcentrifuge tube. Cells were then pelleted via centrifugation at 2000 xg for 3 minutes and the supernatant removed. Cells were then gently resuspended in 400 μL of cold Solution A (0.01M Hepes, pH 7.8, 0.01M KCl, 0.1mM EDTA, 1mM NaVO₄, 1mM DTT, and 1X complete protease inhibitor). Cells were incubated on ice for 15 minutes. Following the incubation period, Solution B (10% IGEPAL) was added to create a final IGEPAL percentage of 0.7% (30 μL Solution B to 400 μL Solution A). Cells were immediately vortexed for 10 seconds and pelleted at 14000 xg for 30 seconds at 4°C. Following this centrifugation step, the supernatant was transferred to a new tube as it was the soluble cytosolic fraction (leaving 15μL to avoid contamination with pelleted nuclear fraction) . The remaining pelleted material was then resuspended in Solution A and washed three times (pelleted at 14000xg for 30 seconds at 4°C between washes and supernatant discarded). Following the final wash, 50 μL of ice cold Solution C (0.02M HEPES, pH7.8, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM NaVO4, 1mM DTT, and 1X complete
protease inhibitor) was added to the pelleted material and the pellet gently resuspended. The solution was placed on ice for 15 minutes with gentle agitation every 2-3 minutes. The solution was then spun at 14000 xg for 5 minutes and the supernatant transferred to a new tube as it was the soluble nuclear extract fraction. Both cytosolic and nuclear fractions were stored at -80°C.

4.3 Results and Conclusions

4.3.1. SIKE acts as a mixed-type inhibitor in the TBK1-mediated phosphorylation of IRF3

SIKE was originally classified as a physiological suppressor of TBK1 [126]. In response to viral or dsRNA stimulation, overexpression of SIKE disrupted TBK1 interactions within the dsRNA receptor pathways, including the upstream adaptor TRIF and the kinase’s downstream substrate IRF3 [126]. Although IRF3 phosphorylation is essential for the anti-viral response, the mechanism by which SIKE selectively represses the type-I interferon response remains undefined.

Our studies into the mechanism of SIKE’s inhibitory activity focused on the TBK1-mediated phosphorylation of IRF3. The IRF3 construct used in these studies included residues 173-427, encompassing the IRF3 activation domain and TBK1 phosphorylation sites (residues 385, 386, 396, 398, 402, 404, and 405). The Michaelis-Menten plot of IRF3 as the varied substrate gave initial kinetic parameters for $K_M$ (Michaelis constant) and $V_{MAX}$ (maximum velocity) as 2.69 μM and 10.5 nM/min reaction, respectively (Figure 4.4A, Table 4.1). Similarly, using ATP as the varied substrate, initial kinetic parameters for the $K_M$ and $V_{MAX}$ values of the reaction were 35.3 μM and 12.3 nM/min reaction respectively (Figure 4.4B, Table4.1). To examine the effect of SIKE72 on IRF3 phosphorylation, SIKE72 was added at ~20 nM and ~80
nM to the IRF3 varied or ATP varied (Figure 4.4C,D) assays. The primary effect of SIKE72 for IRF3 varied reactions occurred on $V_{\text{MAX}}$ (~1.8 fold decrease), indicative of a non-competitive inhibitor (Table 4.1). Interestingly, the $K_M$ and $V_{\text{MAX}}$ values calculated from double reciprocal plots of the ATP varied reactions indicated a 2.2-3.0 fold change in both $K_M$ and $V_{\text{MAX}}$ consistent with a mixed-type inhibitor (Table 4.1). The $K_{i,\text{app}}$ values were calculated from the double reciprocal plot slope and intercept parameters using equations 1 and 2, described in Chapter 4.2.6 (Table 4.2).

4.3.2 TBK1 directly phosphorylates SIKE in vitro

TBK1-mediated $^{32}$P incorporation was assessed by phosphor K screen of SDS-PAGE separated reactions with saturating ATP (100 μM), IRF3 held constant at 5 μM (~$K_M$ value), and increasing SIKE72 concentrations (5-5000 nM). Analysis of the $\gamma$-$^{32}$P-ATP kinase assays revealed two $^{32}$P-labeled species corresponding to IRF3 and SIKE72 (Figure 4.5A). Inhibition of TBK1-mediated phosphorylation of IRF3 was apparent at 500nM SIKE72, 10 fold less than the substrate concentration. Moreover, as IRF3 phosphorylation diminished, SIKE72 phosphorylation increased (Figure 4.5A) suggesting that SIKE had a competitive nature associated with its inhibitory mode of action.
Figure 4.4 SIKE is a non-competitive inhibitor of TBK1. A. Michaelis-Menten plot of TBK1-mediated phosphorylation of IRF3 with saturating ATP (100 μM), pre-activated TBK1 (5.6 nM) and IRF3 varied from 0.042-20.8 μM. Data were fit to a 2-parameter rectangular hyperbola (SigmaPlot). B. Michaelis-Menten plots of TBK1 phosphorylation of IRF3 with increasing SIKE (0-83nM) fit to a single rectangular 2-parameter hyperbola curve (SigmaPlot). C. Michaelis-Menten plot of TBK1 mediated-phosphorylation of IRF3 with saturating IRF3 (20.8 μM), pre-activated TBK1 (5.6 nM) and ATP varied from 0-150 μM. Data were fit to a 2-parameter rectangular hyperbola (SigmaPlot). D. Michaelis-Menten plots of TBK1 phosphorylation of IRF3 (20.8 μM) with ATP varied (0.08-150 μM) and increasing SIKE (0-83 nM) fit to a single rectangular, 2-parameter hyperbola curve (SigmaPlot). Circle, no SIKE; Triangle, 20.8 nM SIKE; Square, 83 nM SIKE. E. Lineweaver-Burk plots of TBK1-IRF3 assays with ATP IRF3 varied from 0.042-20.8μM, 100μM ATP. Data for 0.042-0.42μM and 1.7-20.8μM IRF3 were fit to a linear polynomial equation (Sigma Plot). F. Lineweaver-Burk plots of TBK1-IRF3 assays with ATP varied from 0.08-150μM, 20.8μM IRF3. Data were fit to a linear polynomial equation (Sigma Plot).
Table 4.1 Michaelis-Menten and LineWeaver-Burk derived kinetic parameters

| Substrate      | $^{a}K_m\text{, app}$ & $^{b}V_{\text{max, app}}$ & $k_{\text{cat}}$ |
|----------------|----------------------|-----------------|-----------------|
| 1RF3, (173-427)| 2.69 ± 0.18          | 10.5 ± 0.18     | 2.13 ± 0.04     | This study |
| ATP            | 35.3 ± 3.6           | 12.3 ± 0.43     | 2.5 ± 0.087     | This study |
| SIKE (72-207)  | 0.41 ± 0.0083        | 7.4 ± 0.36      | 1.5 ± 0.073     | This study |
| 1RF3 (2-427)   | 71 ± 19              | ND               | ND              | Ma et al. 2012 PNAS |
| 1κBα (19-41)   | 69.9                 | ND               | ND              | Huynh et al. 2002 JBC |
| ATP            | 1.57                 | ND               | ND              | Huynh et al. 2002 JBC |
| ATP            | 7.5                  | ND               | ND              | Huttí et al. 2012 PLoS ONE |

| SIKE, nM       | Intercept & Slope   | $K_m\text{, app}$ & $V_{\text{max, app}}$ |
|----------------|----------------------|-----------------|-----------------|
| 0              | 95.6 ± 12.5          | 2495 ± 25.9     | 26.1±3.4        | 10.5±1.4 |
| 20.8           | 123.4 ± 9.7          | 2582 ± 20       | 20.9±1.6        | 8.1±0.6 |
| 83.3           | 289 ± 35             | 3349 ± 72.7     | 11.6±1.4        | 3.5±0.4 |

$^{a}K_m\text{, app}$ refers to parameter describing $K_m$ for all 7 1RF3 or 6 SIKE phosphorylation sites. Values derived from fitting single rectangular, 2-parameter hyperbola to the $V_o$ versus [S] plots.

$^{b}V_{\text{max, app}}$ refers to parameter describing $V_{\text{max}}$ for phosphorylation of 1RF3 or SIKE at multiple sites. Values derived from fitting single rectangular, 2-parameter hyperbola to the $V_o$ versus S plots.

$^{c}$Substrates are listed with inclusive residues in parentheses.

$^{d}$SIKE construct included residues 72-207.

$^{e}$Intercept and slope values were derived from fitting the 1/$V_o$ versus 1/[S] plots (Figs. S1C,D) to a linear polynomial.

$^{f}$1RF3 construct included residues 173-427 and parameters derived from data at listed concentrations.
4.3.3 DsRNA stimulates SIKE phosphorylation in vivo and dissociates a TBK1-IRF3-SIKE complex

With the observation that SIKE is phosphorylated in vitro, but kinetic characterizations suggesting that SIKE functioned as a non-competitive inhibitor, we examined whether this post translational modification occurred in vivo. In HEK293 cells transiently transfected with HA-epitope-tagged WT-FL SIKE, serine phosphorylation of WT-FL SIKE was observed following stimulation with pI:pC (Figure 4.5B).

From these results, taken together with the in vitro data discussed previously (Figure 4.5A), we next examined the effect of SIKE on the TBK1:IRF3 interaction required for competent TLR3 signaling. In HEK293 cells transiently transfected with mTurq (GFP variant)-tagged TBK1, FLAG-tagged IRF3 and HA-tagged SIKE72, co-IP/immunoblot analysis revealed that prior to pathway stimulation, a TBK1-IRF3-SIKE complex existed (Figure 4.6). Upon dsRNA stimulation, however, SIKE was shown to be released from this complex (Figure 4.6) meaning that in the absence of over-expressed TBK1, SIKE is excluded from a TBK1:IRF3 complex. Interestingly, IRF3 remained bound to TBK1 following dsRNA stimulation suggesting that a hetero-substrate may be bound to the two active sites of TBK1. While these results cannot rule out SIKE as a truly competitive inhibitor, this data raises the possibility that the release of SIKE from TBK1, upon pathway stimulation, may be mediated by the post-translation modification observed both in vitro (Figure 4.5A) and in vivo (Figure 4.5B).
Table 4.2 Parameters for SIKE inhibition of TBK1 mediated IRF3 phosphorylation

<table>
<thead>
<tr>
<th>bSIKE, nM</th>
<th>(^a\text{K}_{\text{app}}) nM</th>
<th>intercept</th>
<th>slope</th>
</tr>
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<tr>
<td>ATP</td>
<td>20.8</td>
<td>83.3</td>
<td>20.8</td>
</tr>
<tr>
<td>(^c\text{IRF3} (1.7-20.8 \mu M))</td>
<td>72 ± 9.3</td>
<td>41 ± 5</td>
<td>597 ± 6.2</td>
</tr>
<tr>
<td>(^c\text{IRF3} (0.042-0.42 \mu M))</td>
<td>66 ± 3.2</td>
<td>100 ± 4.8</td>
<td>168 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>92.9</td>
<td>133.1</td>
<td>79.3</td>
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</tbody>
</table>

\(^a\text{K}_{\text{app}}\) refers to parameter describing \(K_i\) for all IRF3 phosphorylation sites assayed. \(K_i\) derived from \(\text{Intercept}_{\text{lab}} = \text{Intercept}_{\text{nom}} (1 + [\text{Inh}]/K_i)\) where Inh=SIKE values/ concentrations and intercept derived from linear regression of LWB plots.

bSIKE construct included residues 72-207.

cIRF3 construct included residues 173-427 and parameters derived from data at listed concentrations.
Figure 4.5 SIKE is directly phosphorylated by TBK1. A. SIKE (5-5000 nM) was added to the TBK1 assay containing 5 μM IRF3 (~K_m concentration), 100 μM ATP and 4.93 nM TBK1. Reactions were completed and analyzed as described in Chapters 4.2.6 and 4.2.7. O = p-IRF3; ▽ = p-SIKE. B. HEK293 cells were transiently transfected with HA-SIKE. Cells were stimulated for 3 hours with pI:pC (50 μg/ml). HA-SIKE was immunoprecipitated from lysates with α-HA resin. Total and phospho-Serine SIKE was assessed via Western blot using α-HA or α-pSer antibody, respectively. One representative experiment is shown.
4.3.4 TBK1 phosphorylates SIKE on six serine residues that mimic the IRF3 phosphorylation motif

TBK1 prefers a hydrophobic residue following the targeted serine/threonine (S/T) residue as noted in Chapter 1.7. In SIKE, 23 S/T residues occur in the protein sequence of which 11 conform to the preferred S/T-hydrophobic residue motif for TBK1 phosphorylation. To determine the number of TBK1-mediated SIKE phosphorylation sites, we assessed TBK1-mediated phosphorylation by MALDI-TOF mass spectrometry. Over a 96 hour time course, we observed a mixture of singly to triply phosphorylated SIKE72 (Figure 4.7A). Under the same conditions, single to 7 out of 7 known IRF3 phosphorylation sites were modified, whereas glyoxosomal malate dehydrogenase, not known to be phosphorylated by TBK1 but containing 16 preferred S/T motif sites out of 36 total S/T residues, remained unmodified (Figure 4.7B,C). To identify the positions of TBK1-mediated phosphorylation in SIKE72, we completed phosphopeptide mapping by tandem mass spectrometry. Six SIKE72 phospho-serine residues (S133, S185, S187, S188, S190, S198) were identified (Figure 4.8.1 and Figure 4.8.2). When compared to the multiple phosphorylation sites of IRF3, SIKE72 and IRF3 shared a remarkable, conserved phosphorylation motif (Figure 4.9).

4.3.5 SIKE phosphorylation status alters its inhibitory activity

To determine the relationship between the multiple SIKE phosphorylation sites and SIKE-mediated inhibition of IRF3 phosphorylation by TBK1, we probed these sites via truncated mutants or site-directed mutagenesis and assessed the effect of these SIKE mutations on TBK1-IRF3 assays. The broad effect of SIKE phosphorylation was analyzed by phosphomimetic mutant, Ser to Glu mutation at the six identified sites (S6E), and phosphoknockout mutant, Ser to
Figure 4.6 TBK1-IRF3-SIKE complex exists prior to TLR3 pathway stimulation. HEK293 cells were transfected with mTurquoise-TBK1, FLAG-IRF3 and HA-SIKE. Cells were (un)stimulated with 50μg/mL pI:pC and harvested 3 hours later. Immunoblots were performed using α-GFP, α-FLAG and α-HA antibodies from α-FLAG co-immunoprecipitation experiments.
Figure 4.7 MALDI-TOF mass spectra of SIKE, IRF3 and gMDH exposed to TBK1. Protein (500nM SIKE72, 500nM IRF3 173-427, 714nM gMDH) was incubated with TBK1 (4.93 nM) and ATP (100 μM) for indicated times at 30°C. Reactions were terminated by the addition of an equal volume of 6M guanidine hydrochloride and target protein recovered by absorption onto Ni-NTA resin (SIKE, gMDH). Proteins eluted from Ni-NTA and IRF3 were desalted and concentrated using a ZipTipC4 (Millipore). ZipTipC4 eluent was mixed 1:1 with α-cyano-hydroxycinnamic-acid and spotted onto a Scout49 target. Data were collected and analyzed as described in Chapter 4.2.8. Spectra for 0, 24 hours and 96 hours are shown. Addition of a phosphate moiety (~80 Daltons) is indicated along the top y-axis and vertical dotted lines. A. WT SIKE72, B. IRF3 173-427, and C. gMDH.
Ala mutations at the six identified sites (S6A). Subsets of phosphoknockout mutants were created to further probe sites essential for the inhibitory activity of SIKE: S4A (S185, 187, 188, 190A), S2A (S133, 198A), and S185A. SIKE’s phosphorylation sites cluster in the C-terminal portion of SIKE. To examine the role of the N-terminal sequence in SIKE inhibition, SIKE truncation mutants containing one (SIKE72-184) or retaining all phosphorylation sites (SIKE 113-207) were constructed. The panel of constructs is summarized in Figure 4.10A.

Inhibition curves for the phosphomimetic and phosphoknockout mutants showed that the negative charge introduced at phosphorylation sites of SIKE72 reduced the ability of SIKE to inhibit TBK1-mediated IRF3 phosphorylation, whereas unmodified SIKE72 had greatly enhanced inhibitory activity (Figure 4.11). Using four concentrations that defined SIKE’s inhibitory effect, kinase assays were completed with the full panel of mutants (Figure 4.10B). Apparent inhibition constants (Ki,app) for each mutant were derived (Table 4.3). The Ki for the phosphomimetic mutant increased ~3 fold over WT SIKE72, suggesting that the negative charge introduced by multiple glutamic acid residues may reduce the interaction between TBK1-SIKE and/or enhance release of SIKE from TBK1. The Ki,app for the C-terminal 113-207 construct, retaining all of the phosphorylation sites, was similar to WT SIKE72. Surprisingly, the Ki,app for the N-terminal 72-184 construct was 196 nM, even though maximal inhibition was only ~25% of the reaction. Loss of the two peripheral phosphorylation sites, S133 and S198, in the S2A construct also did not alter the Ki,app value, whereas phosphoknockout of the four clustered serines, S185, S187, S188, and S190, reduced the Ki,app parameter by 2.5 fold. Within this cluster, the Ki,app for the point mutation S185A was similar to the S6A mutant, implying that this position regulates the TBK1-SIKE interaction (Table 4.3).
**Figure 4.8.1 TBK1 phosphorylates SIKE on six serine residues.** A. Mass spectrum resulting from analysis of indicated phosphopeptide sequence. Two separate ion series were recorded simultaneously; b- and y-ion series, which represent sequencing inward from the N- and C-termini, respectively. Lower case “s” indicates phosphorylated residue. B. Summary of phosphopeptide sequences identified using a Sequest search algorithm against custom databases generated from the 6xHis-SIKE72 sequence. “?” indicates that a phosphorylation site exists in peptide but cannot be uniquely assigned to a single serine residue.
**Figure 4.8.2 Spectra of SIKE phosphopeptides.** Mass spectra resulting from analysis of indicated phosphopeptide sequence showing mixture of uniquely phosphorylated peptides. Two separate ion series were recorded simultaneously; b- and y- ion series, which represent sequencing inward from the N- and C- termini, respectively. Lower case “s” indicates phosphorylated residue.
Figure 4.9 TBK1-mediated SIKE phosphorylation sites mimic phosphorylated IRF3 sequence. Sequence alignment of known phosphorylation sites on IRF3, required for dimerization and activation, with TBK1 phosphorylation sites on SIKE found through phosphopeptide mapping. Gray letters = phosphorylated residues; *, conserved site; :, functionally conserved site.
Figure 4.10 Phosphorylation status modulates SIKE inhibitory activity. A. Cartoon diagram of SIKE constructs highlighting the six potential sites of serine phosphorylation, mutations used to probe phosphorylation sites and truncation mutants. Serines listed from left to right; 133, 185, 187, 188, 190, 198. CC, coiled coil. B) WT or mutant SIKE (4-1658 nM) was added to the TBK1 assay containing 5 μM IRF3 (~K_m concentration), 100 μM ATP and 4.93 nM TBK1. Reactions were completed and analyzed as described in Chapter 4.2.7.
Figure 4.11 Effects of SIKE mutants on IRF3 phosphorylation to define inhibitor concentration parameters. WT or S6A or S6E SIKE (5-5000nM) was added to the TBK1 assay containing 5 μM IRF3 (~K_M concentration), 100 μM ATP and 4.93 nM TBK1. Reactions were completed and analyzed as described in Chapter 4.2.6.
4.3.6 SIKE phosphorylation status controls TBK1-SIKE interaction in vivo

To investigate the effect of SIKE phosphorylation on the TBK1-SIKE interaction, we utilized co-immunoprecipitation assays of epitope-tagged TBK1 and WT or mutant FL SIKE. Prior to stimulation with pI:pC, all HA-tagged SIKE constructs co-immunoprecipitated with FLAG-tagged TBK1 (Figure 4.12A). The S6E, S185E and 1-112 constructs showed limited interaction with TBK1 relative to input protein as observed in whole cell lysates. Following pI:pC stimulation, WT-FL, 1-112 and 113-207 SIKE constructs were released from TBK1, whereas the S6A, S185A, S6E, and S185E SIKE interactions with TBK1 were unchanged from unstimulated conditions (Figure 4.12A). These findings suggest that SIKE 1-112, absent TBK1 phosphorylation sites, has reduced affinity for activated TBK1 and that the phosphorylation of SIKE does, in fact, regulate the interaction between SIKE and TBK1.

Further investigations into the role of phosphorylated SIKE on downstream cascade activation revealed that the release of WT-FL SIKE from TBK1 correlates with increased TBK1-mediated phosphorylation of IRF3 (Figure 4.12B). The reduced interaction of S6E or S185E and TBK1 had no effect on dsRNA stimulated IRF3 phosphorylation, but the stable interaction of S6A SIKE or S185A SIKE with TBK1, irrespective of dsRNA stimulation, negated dsRNA stimulated IRF3 phosphorylation.

4.3.7 SIKE is a TBK1 substrate

SIKE is directly phosphorylated by TBK1 (Figure 4.8). To establish kinetic parameters for SIKE as a TBK1 substrate, TBK1 assays with saturating ATP (100 μM) and SIKE72 varied from 0.043-8.3 μM were completed. The K_M and V_MAX parameters were 0.41 μM and 7.4 nM/min reaction, respectively (Figure 4.13A and Table 4.1). Strikingly, the double reciprocal
Table 4.3 $^{a}K_d$ values for SIKE inhibition of TBK1-mediated IRF3 phosphorylation

<table>
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<tr>
<th>SIKE construct</th>
<th>$^{a}K_{\text{app}}$ (nM)</th>
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<tbody>
<tr>
<td>WT</td>
<td>350 ± 16</td>
</tr>
<tr>
<td>72-184</td>
<td>196 ± 7.9</td>
</tr>
<tr>
<td>113-207</td>
<td>363 ± 52</td>
</tr>
<tr>
<td>S6E</td>
<td>900 ± 56</td>
</tr>
<tr>
<td>S6A</td>
<td>77 ± 6</td>
</tr>
<tr>
<td>S4A</td>
<td>140 ± 7</td>
</tr>
<tr>
<td>S2A</td>
<td>368 ± 14</td>
</tr>
<tr>
<td>S185A</td>
<td>41 ± 8</td>
</tr>
</tbody>
</table>

$^{a}K_{\text{app}}$ derived from fit of 2-parameter rectangular hyperbola to % inhibited $V_o$-pIRF3 μM/min versus SIKE concentration.

$^{b}$SIKE construct included residues 72-207 except where explicitly stated.
Figure 4.12 Phosphorylation status modulates TBK1-SIKE interaction and p-IRF3 in vivo.

A. HEK293 cells were transiently transfected with FLAG-TBK1 and HA-tagged WT or mutant SIKE. Cells were (un)stimulated with pI:pC (50 μg/ml) for 3 hours. FLAG-TBK1 was immunoprecipitated from lysates with α-FLAG resin, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with α-HA or α-FLAG antibody. Immunoblots of whole cell lysates show expression of each construct. Blots are representative of three independent experiments. B. HEK293 cells were transiently transfected with empty vector (none), or TLR3 and WT or mutant SIKE. Cells were (un)stimulated with pI:pC (50 μg/ml) for 3 hours. Immunoblots of whole cell lysates were probed for α-phosphoS396-IRF3, α-IRF3, α-HA or α-actin antibodies. Blots are representative of two independent experiments. Densitometry was calculated using ImageJ software. Each band was corrected for background scatter and then the phospho-IRF3 and IRF3 bands were normalized to their respective actin bands. Ratio of phospho-IRF3:total IRF3 is reported under corresponding bands.
plot revealed a downward, concave curve, similar to the double reciprocal plot of IRF3 varied, TBK1 assays (Figure 4.13B).

4.3.8 SIKE is able to interact with a multitude of proteins

Following the discovery that SIKE was a novel substrate of TBK1, experiments were then focused on possible roles for SIKE downstream of TBK1. The first approach to identifying a SIKE function was to examine SIKE’s interaction network. A co-immunoprecipitation experiment of FLAG-SIKE, transiently over-expressed in HEK293 cells, was used to isolate SIKE interaction networks +/- dsRNA stimulation for three hours. These samples were subjected to tandem MS/MS analysis to identify proteins within the solution. This analysis revealed that SIKE formed a network with at least 13 other proteins in the cell (Figure 4.14 and Table 4.4). These proteins could be broadly classified as transcription-associated (p15 and CLE), RNA transport (ALY), ribosomal (NPM1, Treacle, eEF1A), chaperones (HSP70, HSP80, cyclophilin A), cytoskeletal (γ-actin isoform, keratin), and enzymes (α-enolase and glyoxylase I). The presence of keratin may be specific or could be due to contamination during sample preparation. Surprisingly, the SIKE interaction network did not appear to be altered following dsRNA stimulation. Under these conditions, SIKE should become serine phosphorylated by TBK1-mediated interactions. We hypothesize that the similar interaction map +/- dsRNA was due to heterogeneous levels of SIKE phosphorylation.

To determine if the post-translational modification state of SIKE altered the SIKE interaction network, the experiment was repeated with the S6A (phospho-knockout) and S6E (phospho-mimetic) SIKE mutants (Table 4.5). While this analysis identified 27 interaction partners, the interaction networks were again nearly identical regardless of SIKE modification
Figure 4.13 SIKE is a substrate of TBK1. A. Michaelis-Menten plot of TBK1 mediated phosphorylation of SIKE with saturating ATP (100 μM), pre-activated TBK1 (4.93 nM) and SIKE varied from 0.043-8.4 μM. Data were fit to a 2-parameter rectangular hyperbola (SigmaPlot). B, C. Lineweaver-Burk plots of (B) TBK1-SIKE assays with SIKE varied from 0.043-8.4 μM, ATP (100 μM) and (C) TBK1-IRF3 assays with IRF3 varied from 0.042-20.8 μM, ATP (100 μM). TBK1 was at 4.93 nM in both assays. Data for 0.042-0.42 μM (O) and 1.7-20.8 μM (●) IRF3 and 0.043-0.23 μM (O) and 0.43-8.4 μM (●) SIKE were fit to a linear polynomial equation (SigmaPlot).
**Figure 4.14 SIKE interaction partners in vivo.** HEK293 cells, transiently transfected with FLAG-SIKE, were (un)stimulated with 50 μg/mL pI:pC and harvested three hours later. A FLAG co-immunoprecipitation experiment removed SIKE and any interaction partners from whole cell lysates. Stimulate and un-stimulated SIKE samples were run on a 10% SDS-PAGE gel and analyzed via Coomassie/Silver stain. Heavy band at 28 kDa is SIKE. Red arrows indicate interaction partners that were pulled-down with SIKE in the co-IP experiment.
### Table 4.4 Tandem mass spectrometry analysis of WT SIKE immunoprecipitants from HEK293 cells

<table>
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<th>Bait</th>
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<th>Protein</th>
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<th>drRNA - Peptides Identified</th>
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<td></td>
<td>IPI00640404</td>
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<tr>
<td>Prey</td>
<td>IPI00221222</td>
<td>RNA polII coactivator p15</td>
<td>Activated RNA polymerase II transcriptional coactivator p15</td>
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<td></td>
<td>IPI00792100</td>
<td>CLE7, C14orf168</td>
<td>modulate RNA polII, component of tRNA splicing complex</td>
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<tr>
<td></td>
<td>IPI00328840.9</td>
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</table>

*, potential contaminant during sample preparation. Blue, over 95% probability; orange, 50-76% probability
state or dsRNA treatment. Interestingly, while ALY, an eEF protein, NPM1, and \( \gamma \)-actin were again identified, the majority of the remaining proteins, which were found to interact with SIKE, were RNA binding proteins (ribosomal and ribonucleoproteins), histones and a mitochondrial ATP synthase subunit. These results led us to question whether SIKE had the ability to directly bind RNA.

**4.3.9 SIKE does not bind dsRNA but interacts with a variety of proteins**

As SIKE functions downstream of a dsRNA receptor and may form interactions with several RNA binding proteins, we performed preliminary SEC experiments to determine whether SIKE associated with RNA. SEC experiments (Figure 4.15) revealed that SIKE did not associate with pI:pC, a synthetic RNA analogue composed of double- and single-stranded RNA.

In parallel with these experiments, fluorescence microscopy to examine SIKE localization with a SIKE YFP construct (JKB unpublished data) had been completed (Figure 4.16). These studies showed that SIKE was excluded from the nucleus. This data suggested that SIKE interactions with nuclear proteins (i.e. p15, CLE, histones, etc.) either occurred in the cytosol as these proteins are being translated or these interactions occurred during sample preparation when the integrity of the nucleus was compromised. To determine if these nuclear protein interactions were an artifact of sample preparation, we isolated the cytosolic fraction and then proceeded with the co-immunoprecipitation / tandem MS/MS experiments with the S6E/S6A SIKE constructs (Table 4.6). No dsRNA stimulated samples were analyzed by MS for this experiment. Under these conditions, we identified 19 interaction partners, again including HSP, \( \gamma \)-actin, eEF proteins, \( \alpha \)-enolase, and NPM1. Underway are experiments to confirm MS identified interaction partners (reciprocal co-immunoprecipitation, immunofluorescence, co-
localization assays, etc) focusing on the five proteins identified in multiple experiments (Figure 4.17).
Table 4.5 Tandem mass spectrometry analysis of S6A and S6E SIKE immunoprecipitants from HEK293 cells.

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* Potential contaminant during sample preparation. Blue, over 95% probability; orange, 50-79% probability; red, 20-49% probability.
Figure 4.15 SEC experiments reveal that SIKE does not bind dsRNA. A. Chromatograph of pI:pC (100 μg) separated on a Superdex 200 10/300 GL column. Peak fractions were analyzed via SDS-PAGE, Coomassie/silver stain. B. Chromatograph of WT SIKE72 (1mg) separated on a Superdex 200 10/300 GL column and analyzed as above. C. Chromatograph of pI:pC (100μg) and WT SIKE72 (1mg) were incubated together on ice for 30 minutes. Following incubation, the solution was separated and analyzed as in A. and B. Red line under peaks indicates fractions analyzed by SDS-PAGE.
**Figure 4.16** Fluorescence microscopy experiments reveal SIKE is localized to the cytoplasm. HEK293 cells were transiently transfected with mTurq SIKE72 (A&B) or SIKE72 sYFP2 (C&D). Cells were viewed under white light (A&C) or Hg source with a 488nm excitation, 515 long pass emission cube filter set (B&D) 24 hours post-transfection (JKB unpublished data).
Table 4.6 Tandem mass spectrometry analysis of S6A and S6E SIKE immunoprecipitants from cytosolic fractions of HEK293 cells

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* potential contaminant during sample preparation. Blue, over 95% probability; green, 88-94% probability; orange, 50-79% probability; purple, 20-49% probability; no fill, 0-19% probability.

Accession numbers lacking a protein designate are associated with the preceding protein heading. In these cases, SEQUEST identified a protein group (peptide shared between >1 protein, but each protein also had unique peptides) that were then clustered as a set of proteins with overlapping peptide evidence that can be treated as a proxy for a single identification.
Figure 4.17 Venn Diagram of MS/MS data from SIKE interaction experiments. Results of co-immunoprecipitation experiments are detailed from WT SIKE (Table 4.4), S6A and S6E SIKE WCL (whole cell lysates) (Table 4.5) and S6A and S6E SIKE cytoplasmic fractions (Table 4.6). Shared interactions between all experiments include; γ-actin, eukaryotic elongation factors (eEF protiens), heat shock proteins (HSP proteins) and nucelophosmin.
CHAPTER V
DISCUSSION

The innate immune system is the body’s first line of defense against viral and bacterial challenge. Essential to this safeguard is the initial recognition of invading microorganisms. In this dissertation, the focus is on the TLRs, a highly conserved subset of PRRs that are known for their ability to recognize a variety of PAMPs [14, 29, 151]. Pathogen recognition by the TLRs initiates signaling cascades that lead to the modulation of gene expression involved in orchestrating an anti-microbial response. Interestingly, while the concept of innate immunity was discovered in the late 19th century, much is still unknown regarding this critical defense mechanism as is evident by new discoveries made daily in the field. To that end, this dissertation focuses on three growing areas in innate immune research: pathogen recognition, through studies based on the adjuvant potential of dsRNA (Chapter 2); immune pathway activation, through studies based on the effects of receptor clustering in TLR3 signal transduction (Chapter 3); and regulation of PRR signal transduction, through studies based on an endogenous inhibitor of the
TLR3 response (Chapter 4). While these three topics seem to examine disparate facets in the innate immune response, they are tied together by the fact that the coordinated activation and regulation of innate immunity is critical to maintaining host viability. Over-activation of an immune response is associated with autoimmune disorders, breast and prostate cancer, obesity and glaucoma while non-response leads to pathogen control of host and potential death [103, 152, 153]. By examining the mechanisms that initiate and control the innate immune response, this dissertation sought to identify possible regulatory targets as well as novel immune defense strategies used by a host to thwart pathogen challenge.

The adjuvant potential of dsRNA

It is a well-established fact that TLR3 is activated by the viral genomic material dsRNA leading to a robust innate immune response [88, 89]. From this, we postulated that dsRNA would be a viable candidate for adjuvant studies as a major goal of vaccination, and the co-administration of adjuvants, is the generation of a strong immune response that can provide long-term protection against infection. The major discovery of this work is that a 139bp dsRNA ligand is a viable candidate for future vaccine adjuvant studies. These results were confirmed by a paper published shortly after the onset of our preliminary data collection and further revealed that dendritic cell activation and adjuvant effectiveness increased with increasing dsRNA length [143].

Interestingly, however, through the evaluation of a ligand’s ability to successfully mature iDCs to DCs based on the gain of CD80/CD86 cell surface receptor expression, we showed that while LPS-treated positive control cells exhibited a 100% gain in both receptors, cells treated with 139bp dsRNA ligand only exhibited an 80% gain in CD80 expression and a 25% gain in CD86 expression (Figure 2.4). While DC maturation was evident, the incompleteness of this
event, in response to dsRNA, raises the possibility that other dsRNA ligands, targeted to different dsRNA receptors, may also induce potent immune responses and hence be more viable vaccine adjuvant candidates. Jelinek et al., authors of the study confirming that dsRNA is a viable candidate for adjuvant studies directed at TLR3, also showed data supporting the possibility of other dsRNA receptors being potent adjuvant targets. A 540bp dsRNA ligand in their investigations, shown previously to induce a robust TLR3 response, did, in fact, induce a 25-fold increase in CD86 expression in FLT3 ligand-generated DCs (FL-DCs) [143]. However, this same ligand was only able to increase CD86 expression by two fold in splenic DCs and failed to induce CD86 expression in GM-CSF-generated DCs (GM-DCs). Interestingly however, their data showed that pI:pC was able to induce CD86 expression in GM-DCs of TLR3-/− mice suggesting that other receptors might be responsible for the GM-DC response to pI:pC [143].

Taking these experiments one step further, Jelinek et al. investigated the possibility that MDA5 may be required for the induction of CD86 expression in response to pI:pC as it had been previously shown that MDA5 is able to respond to pI:pC [154]. In their studies, deletion of MDA5 abrogated the induction of CD86 expression of GM-DCs in response to pI:pC suggesting that pI:pC enters the cytoplasm in GM-DCs where it activates MDA5 [143]. As the authors of the paper were focused on the adjuvant potential of dsRNA as it pertained to the TLR3 response, they did not investigate the fold increase in CD86 expression upon stimulation by synthetic dsRNA ligands directed towards MDA5. However, it would be interesting to investigate whether dsRNA directed toward MDA5 (dsRNA > 1-2kb in length [154]) may induce a potent DC maturation event as this would suggest that long pieces of dsRNA and pI:pC exert a qualitatively different effect on the immune system than shorter dsRNA oligonucleotides (≤ 139bp).
The preferred strategy for the development of new generation vaccines is to add highly purified synthetic adjuvants, which activate only the elements of the immune response required for protection and will not trigger a more generalized activation of the immune response [155]. This makes dsRNA a perfect candidate for adjuvant studies as a select set of receptors recognize and initiate a response from dsRNA. Furthermore, dsRNA has several properties that render it advantageous in adjuvant potential studies. First, compared to other potential adjuvants such as QS21, a purified plant extract that enhances the ability of the immune system to respond to vaccine antigens but is highly unstable due to hydrolyses [156], or ssRNA, which is rapidly degraded in many hosts [157], dsRNA is stable as it is resistant to digestion by ubiquitous RNases [158, 159]. Second, dsRNA oligonucleotides are soluble, homogenous molecules that can be manufactured in a consistent fashion [143]. This is in contrast to many other potential adjuvants such as polyI:C12U [134], polyI:CLC [160] or PIKA [161], which are heterogeneous (vary between samples in both chemical structure and biological function) and promiscuous in their activation of multiple immune response pathways with unpredictable consequences [162]. Finally, dsRNA recognition appears to depend upon length, as observed in multiple investigations [88, 154], making its recognition more easily adaptable in adjuvant studies. This is in contrast to TLR9-based adjuvant studies in which specific nucleotide sequences of CpG oligodeoxynucleotides are required for the induction of an immune response [163]. Taken together, it may therefore be possible to manipulate the adjuvant properties of dsRNA simply by varying the lengths of the oligonucleotides which would effectively direct the ligands towards different dsRNA receptors in the cell. This should produce highly specific immune responses that will effectively enhance vaccine antigen potency. While experiments are required to validate this hypothesis, the ease with which dsRNA length can be manipulated to target different
receptors in the cell and thus induce specific pathway activation, makes it an ideal candidate in experimental vaccination systems.

Through this study, we have shown the ability to produce milligram quantities of 48bp-, 139bp- and 540bp-dsRNA ligands (Figure 2.1) that can be used to test the adjuvant potential of dsRNA as it pertains to the immune response. Furthermore, we have shown the ability to generate iDCs (Figure 2.2, Figure 2.3) to be used in the evaluation of dsRNA ligands as adjuvants. Finally, we have shown that a 139bp dsRNA ligand does have the ability to partially mature iDCs to DCs (Figure 2.4) and is a viable candidate for future adjuvant studies. Through this work, we have also raised the possibility that other dsRNA receptors [154], particularly RIG-I (dsRNA longer than 23 nucleotides with a linear structure and uridine- or adenosine-rich ribonucleotide sequences bearing a 5’-triphosphate [164]), MDA-5 (dsRNA greater then 1-2kb [154]), or PKR (dsRNA greater then 33bp in length or containing secondary structural imperfections [165]) may be viable vaccine adjuvant candidates and should be explored further due to the ease with which each dsRNA ligand can be adapted to target each of these receptors.

**Elucidating the Role of Receptor Clustering in TLR3 Signal Transduction**

Previous cell-based studies of the TLR3:dsRNA complex had shown that a minimum of two signaling units were required to initiate endosomal TLR3 signaling [88]. This led us to postulate that a clustering of signaling unit complexes, dependent upon the size of the dsRNA ligand being recognized, was required for competent TLR3 signaling. Structural and functional studies of TLR3 with neutralizing antibodies, in this study, provide evidence for a new TLR3 signaling model in which dsRNA:TLR3 signaling units laterally cluster to achieve efficient signaling (Figure 3.6). Furthermore, in cell lines transiently expressing TLR3 (Figure 3.1) or endogenous TLR3 in normal human epithelial cells (Figure 3.5), data indicated that blocking
signaling unit lateral clustering (by Fab12/mAb12) is nearly as effective in inhibiting TLR3 signal transduction as blocking dsRNA-mediated dimerization of TLR3 (by Fab15/mAb15). Taken together, this study indicated that signaling unit – signaling unit lateral clustering is essential for efficient TLR3 signaling induced by dsRNA.

Interestingly, in the studies presented here, TLR3hi cells, stably expressing TLR3, are responsive to 49bp dsRNA (Figure 3.7) while HEK293 cells, transiently expressing TLR3, are not responsive to the same ligand (Figure 3.1). These differences, I postulate, could be due to recently reported TLR3 proteolytic processing events described in Chapter 1.6 [71, 72]. As discussed previously, dsRNA binds to the TLR3-ECD in two regions, one near the N-terminus (LRR-NT to LRR3) and the other at the C-terminus (LRR19 to LRR21) [30, 87, 88]. The location of these binding sites is important as multiple groups have now shown that TLR3 cleavage, by Cathepsins B and H, into N- and C-terminal fragments, occurs prior to ligand recognition and pathway activation [71,72]. While investigators debate whether cleavage events are essential for TLR3 signaling, data has shown that proteolytic processing leads to an accumulation of cleaved peptides [72], of which, the C-terminal fragments have a longer half-life, as compared to un-cleaved protein, and are able to signal in response to dsRNA [71, 72]. Taken together, I postulate that these cleavage events occur so that smaller, more stable C-terminal fragments of the TLR3-ECD can more efficiently recognize short dsRNA ligands (≤48bp). As discussed by Luo et al., short dsRNA ligands (~21bp) are long enough to form a complex with TLR3 with only the C-terminal binding sites engaged [166]. Accumulation of the C-terminal cleaved TLR3-ECD fragments, I postulate, leads to improved recognition of short dsRNA. Subsequent formation of cleaved signaling units (2TLR3-ECD-C-terminal-fragments:1dsRNA), I further hypothesize, leads to lateral signaling unit clustering as detailed
for TLR3 signaling unit binding to long dsRNA ligands (Figure 3.6). These lateral clusters of cleaved signaling units can then be disrupted with Fab1068 and Fab12 binding which is compatible with data shown in Figure 3.7. Interestingly, prior to proteolytic processing, investigators have shown a requirement for the presence of a transporter protein, UNC93b1, and the addition of complex glycans in the Golgi complex [71]. Taken together, I suggest that the ideas postulated here would explain why TLR3hi cells respond to 49bp dsRNA while HEK293 cells, transiently expressing TLR3, have no response. While TLR3hi cells would undergo normal transport and glycan modification followed by processing events that lead to recognition of 49bp dsRNA ligands and subsequent response, HEK293 cells, transiently over-expressing the TLR3 receptor, would not contain the components necessary to fully modify or process an over-expressed protein. This would in turn prevent the accumulation of C-terminal TLR3-ECD fragments necessary for signaling unit formation and competent TLR3 signaling. Furthermore, the ideas postulated here would also explain why a controversy exists as to the importance of the proteolytic processing of TLR3 as groups have only examined TLR3 processing events in response to pI:pC, a long dsRNA ligand. However, while these postulated ideas may address many questions, numerous experiments need to be done to validate these assertions.

Previous reports have also shown that TLR3:dsRNA signaling unit formation relies on stabilization conferred by each individual component (TLR3-ECDs do not efficiently dimerize without additional stabilization conferred by dsRNA binding, and dsRNA binding is weak without stabilization by homotypic interactions between TLR3-ECDs) [88]. Furthermore, these reports postulate that additional stabilization might be conferred by interactions between pairs of TLR3-ECDs on long dsRNA molecules as they show that binding affinities increase with dsRNA length beyond the 40-50bp required for dimer formation [88]. However, molecular
modeling results presented in this study (Figure 3.6) did not identify any additional interactions between TLR3-ECDs of multiple signaling units. This data, combined with Fab1068 data showing that shorter separations (≤ 125 Angstroms) of lateral signaling units still permit the partial induction of TLR3s signaling (Figure 3.2 and Figure 3.6), suggests that intracellular factors must play a role in bridging TIR dimers for signaling unit lateral clustering and efficient TLR3 signaling. This would not be unusual as investigations into MyD88-dependent TLR signaling pathways found that signal transduction was mediated by the formation of an intracellular Myddosome, a 14-subunit left-handed helical oligomer consisting of six molecules of MyD88, four molecules of IRAK4 and four molecules of IRAK2.

We postulate that a likely candidate to facilitate the intracellular assembly of TIR dimers in TLR3 signaling unit lateral clustering events is TRIF, the intracellular adaptor protein that mediates TLR3 signaling [167]. The TIR dimer of TRIF is known to interact with the TLR3 TIR dimer and recent data has suggested that TRIF dimerization is required for TLR3 function [90]. If TRIF does play a role in bridging TIR dimers of TLR3-ECDs participating in lateral clustering, this would define yet another role for an already important adaptor molecule in TLR3 signal transduction.

Already found to mediate downstream NF-κB and IRF3 activation as well as to directly interact with caspase 8 to induce apoptosis [168, 169], TRIF is a critical adaptor molecule in efficient TLR3 signal transduction. Due to its importance in the TLR3 signaling cascade, numerous pathogens, from Vaccinia virus (VACV) and Hepatitis C virus to Salmonella and Shigella, have been shown to produce proteins that target the TIR domain of TRIF [170, 171]. Researchers have postulated that this viral-mediated interaction prevents downstream activation of the NF-κB and IRF3 pathways and inhibits caspase 8 interactions. However, if our hypothesis
is correct and TRIF does, in fact, mediate the ligand-induced lateral clustering of TLR3 signaling units, pathogens may target TRIF for additional reasons than those already postulated. By targeting the TIR domain of TRIF, pathogens would prevent TRIF dimerization which, from previous studies [90], would seem to be required for efficient lateral clustering of TLR3 signaling units. By preventing lateral clustering, activation upon pathogen recognition would be abolished and a host response would be negligible. Further experiments would certainly be required to prove the validity of this hypothesis.

In summary, through the characterization of three TLR3-ECD specific monoclonal antibodies (Figure 3.2-3.4), we have provided structural and biochemical evidence that lateral signaling unit clustering (Figure 3.6) is necessary for productive TLR3 signaling. Furthermore, I postulate that proteolytic processing of the TLR3-ECD could mediate short dsRNA (≤49bp) recognition and competent TLR3 signaling. We further postulate that TRIF is required for lateral clustering events and competent TLR3 signaling. Taken together, our data suggests that lateral clustering seems to be a mechanism that could unify the detection and activation of TLR3 by both short and long dsRNA ligands.

**SIKE: A novel TBK1 substrate and endogenous regulator of the type-I interferon response**

TANK binding kinase 1 (TBK1) serves as a key convergence point in multiple innate immune signaling pathways [94, 95, 101]. In response to receptor-mediated pathogen detection, TBK1 phosphorylation of downstream substrates promotes production of pro-inflammatory cytokines and type I interferons. Although a central player in the innate immune system’s defenses that shapes the downstream innate immune response, mechanisms by which the host controls TBK1 activity are not well understood. To address this issue, we sought to define,
mechanistically, how an endogenous inhibitor of TBK1, SIKE, blocks the TBK1 mediated anti-viral response.

The major discovery of this work is that SIKE is not only an endogenous inhibitor of TBK1-mediated phosphorylation of IRF3, but is also a TBK1 substrate. SIKE has a 6.5 fold lower $K_M$ (0.41 $\mu$M), as compared to IRF3, with a comparable $V_{max}$ suggesting that changes in SIKE concentration could effectively manipulate TBK1 function through altered substrate selection. Using kinetic analyses and protein interactions assays, we characterized how this novel TBK1 substrate effectively inhibits type I interferon production.

In our kinetic studies, we examined the effect of SIKE on TBK1-mediated IRF3 phosphorylation using a macromolecular IRF3 substrate (residues 173-427). Previous studies on TBK1 kinetic mechanism, using an Inhibitor of κBα peptide as substrate [116], showed that the enzyme functioned via a rapid equilibrium, random order (RERO) mechanism. Our data obtained with macromolecular substrates are consistent with this mechanism, but reveal evidence (non-linear, downward concave double reciprocal plot (Figure 4.4E-F)) suggesting the existence of negative cooperativity in substrate binding or the presence of a regulatory site for protein substrate. The equilibria for this mechanism with respect to IRF3 phosphorylation are given in Figure 5.1A, and is described by Dalziel’s generalized rate equation [172]:

$$\frac{e}{V_o} = \Phi^{\circ} + \frac{\Phi_1}{ATP} + \frac{\Phi_2}{IRF3} + \frac{\Phi_{12}}{ATP:IRF3}$$

where $e$ is TBK1 concentration in the reaction, $V_o$ is the initial velocity of the reaction, and $\Phi^{\circ}$ are Dalziel coefficients that, for an RERO mechanism, pertain to the concentrations of specific
complexes in the reaction mechanism (\(\gamma_1 = \text{TBK1:IRF3}, \gamma_2 = \text{TBK1:ATP}, \gamma_{12} = \text{TBK1} \) and \(\gamma_0 = \text{TBK1:ATP:IRF3}\)), labeled in Figure 5.1A.

We first determined the baseline kinetic parameters for IRF3 and ATP as summarized in Table 4.1. The double reciprocal plot of IRF3 varied substrate was not linear, but a downward concave curve (Figure 4.13). This type of plot is indicative of one of three phenomena: i) substrate activation, ii) non-identical active sites or iii) negative cooperativity. In examining these three explanations, we assumed from both the related IKK\(\beta\) [173] and TBK1 crystallographic structure [112] and our own size exclusion chromatography (data not shown) that TBK1 functions as a dimeric species (required for ii & iii above). Substrate activation entails a substrate binding site (with lower affinity than the active site) that when bound leads to activation of the reaction. For non-identical active sites, the dimeric TBK1 would have a high and low affinity active site for substrate \textit{a priori}. Finally, negative cooperativity between TBK1 active sites would occur if binding of substrate at the first active site lowered the affinity for substrate at the second active site by allosteric interactions between the subunits. Alone, the IRF3 varied assay data (Figure 4.4A) does not allow us to discern which mechanism is at play. Luckily, SIKE also functioned as a macromolecular TBK1 substrate. The double reciprocal plot of the SIKE varied assays also exhibited this characteristic downward concave curvature (Figure 4.13). As SIKE and IRF3 share no sequence homology and are distinct macromolecular substrates, the substrate activation or non-identical active site mechanism seem improbable as these macromolecular substrates must be similarly recognized by the kinase at a site independent of the active site or with equally reduced affinity at a non-identical active site. Rather, negative cooperativity induced by recognition of IRF3’s and SIKE’s shared phosphorylation motif could explain the non-linear double reciprocal plots. Negative cooperativity requires communication
Figure 5.1 Models suggesting SIKE and TBK1 function. A. Diagram of rapid equilibrium-random order (RERO) enzyme mechanism of TBK1 with binding of I (SIKE) to each enzyme complex. B. Schematic of mechanisms to interpret non-linear LWB plots of TBK1:IRF3 and TBK1:SIKE data and SIKE inhibition of TBK1-mediated phosphorylation of IRF3. Ribbon diagram of TBK1 (pdbcode 4IM0) shows overall arrangement of dimeric kinase.
between active sites. Interestingly, in IKKβ, the two active sites appear to be independent of one another with dimerization mediated by the SDD (scaffold dimerization domain). In contrast, the TBK1 structure revealed three additional dimerization interfaces between the kinase and ubiquitin-like domains of one subunit to the SDD of the contralateral subunit (Figure 5.1B). These additional subunit interfaces identified in the TBK1 structures may provide a means of communicating substrate binding between active sites. Cell based and biophysical studies of a kinase:substrate complex (mutating residues at the interfaces to block proposed subunit communication) to complement the reported kinase:ATP analog inhibitor structures [110-112] would directly address this hypothesis.

Additionally, in a RERO mechanism, the $K_M$ value of IRF3 is the $K_d$ for IRF3 binding to the E-ATP complex. From the two linear regions of IRF3’s double reciprocal plot, we calculated the $K_M$ value at low (0.042-0.42μM) versus high (0.42-20.8μM) substrate concentrations as 50 nM and 3.5 μM, respectively. Similarly, the two linear regions of SIKE’s double reciprocal plot were 55.9 nM and 395 nM at low (42-208 nM) and high (208-8,000 nM) substrate concentrations, respectively. This data suggests that binding of substrate, either IRF3 or SIKE, to the first active site occurs with near equal affinity. The ~10 fold difference in $K_M$ values at high substrate concentration favoring the TBK1:SIKE complex would suggest that SIKE functions primarily as a competitive inhibitor of IRF3 phosphorylation.

For a simple RERO mechanism, an alternative substrate (as we have shown SIKE to be) should give competitive inhibition with respect to IRF3 and mixed (or noncompetitive) inhibition with respect to ATP [174]. Analysis of SIKE’s inhibitory effects on TBK1-mediated phosphorylation of IRF3 revealed a mixed-type inhibitor (Figure 4.4 and Table 4.1) with respect to ATP with ~2-3 fold decreases in $K_M$ and $V_{MAX}$ (Fig. 4.4D and Table 4.1), as expected for a
RERO mechanism. Surprisingly, SIKE inhibition in IRF3 varied experiments did not exhibit a competitive inhibition pattern, but rather displayed primarily as a non-competitive inhibitor, reducing $V_{\text{MAX}}$ by 3 fold (Fig. 4.4C and Table 4.1). To further interpret these results, we examined SIKE inhibition of the TBK1 reaction in terms of the generalized linear rate equation for a RERO mechanism developed by Dalziel [172]. In the RERO mechanism, SIKE could in theory interact with any TBK1 complex and would affect the appropriate $\Phi$ parameter. The rearranged generalized linear rate equation with ATP as the varied substrate is:

$$\frac{e}{V_o} = \frac{1}{ATP} \left[ \Phi_1 + \frac{\Phi_{12}}{IRF3} \right] + \left[ \Phi_o + \frac{\Phi_2}{ATP} \right]$$

In this equation, the slope effects result from SIKE interactions with E and/or E-IRF3, while intercept effects result from the interaction of SIKE with E-ATP and/or E-ATP-IRF3. From the $K_{i,\text{app}}$ values determined from the slopes and intercepts of the double reciprocal plots, we observed that the $K_{i,\text{app}}$ derived from the intercept is $\sim$56 nM, whereas the $K_{i,\text{app}}$ derived from the slopes is $\sim$420 nM. This suggests that the apparent affinity of the E-ATP or E-ATP-IRF3 complexes for SIKE is greater than the affinity of E alone or the E-IRF3 complex. Rearranging the generalized equation for IRF3 as the varied substrate gives:

$$\frac{e}{V_o} = \frac{1}{IRF3} \left[ \Phi_2 + \frac{\Phi_{12}}{ATP} \right] + \left[ \Phi_o + \frac{\Phi_1}{ATP} \right]$$

Here, the slope effects can be attributed to the interaction of SIKE with E and/or E-ATP, while intercept effects are attributable to SIKE interactions with E-IRF3 and/or E-ATP-IRF3. The $K_{i,\text{app}}$ values determined from slope and intercept were 111 nM and 98 nM, respectively, equivalent between the two grouped complexes. Since we have shown that SIKE is directly phosphorylated by TBK1 (Figure 4.7 and Figure 4.8), SIKE would be expected to bind to free
enzyme or enzyme-ATP complexes, but not IRF3 containing complexes as no interaction between SIKE and IRF3 have been observed (Figure 4.5). The fact that SIKE alters either or both \( \gamma_0 \) or \( \gamma_1 \) suggests that these effects must arise from either allosteric interactions between active sites or the existence of a separate regulatory site for SIKE. Both of these possibilities are consistent with the observed downward curvature of the double reciprocal plots with either SIKE (Figure 4.13A) or IRF3 (Figure 4.4C) as the varied substrate. Since IRF3 and SIKE are quite different overall structures, these studies suggest that TBK1 substrates bind with negative cooperativity between otherwise identical sites rather than the existence of an IRF3/SIKE binding site separate from the active sites in the dimer.

Interestingly, while also investigating the TBK1-mediated phosphorylation sites of SIKE, we noticed that four of the six phosphorylated serines of SIKE align well with the phosphorylation sites of IRF3 (Figure 4.9) [119]. The strongest homology was evident at the SIKE sequence \( ^{184}L-S-I-S-S-E^{189} \) comprised of a cluster of three phosphorylation sites. When TBK1 substrates are compared, the phosphorylation pattern of TBK1 substrates has three forms; singly as in Akt [103], insulin receptor [175] and optineurin [105], multiple sites but not clustered as in DDX3 [115], or multiple clustered sites as in IRF3 [176], IRF7 [82] and, as we show here, SIKE. In IRF3/7, these clustered phosphorylation sites, when modified, alter protein function, activating the transcription factor. We explored the role of these clustered, SIKE phosphorylation sites in the context of inhibition of TBK1-mediated IRF3 phosphorylation. When replaced with the phosphomimetic mutations, Ser to Glu, SIKE’s inhibitory activity (\( K_{\text{I, app}} \) ~900 nM versus WT \( K_{\text{I, app}} = 350 \) nM) and interaction with TBK1 are greatly diminished (Figure 4.10 and Figure 4.11). As the introduced negative charge grossly mimics SIKE’s product form, these results are consistent with product release from an enzyme. Initial studies of SIKE held
that SIKE maintained TBK1 in an inactive state prior to TBK1 activation at which point SIKE was released from the TBK1 complex. Here, we attribute the TBK1:SIKE interaction to SIKE’s high affinity for the enzyme and SIKE’s release to its post-translational modifications.

Similar to IRF3 where individual phosphorylation sites have been attributed to specific function, we probed the six SIKE phosphorylation sites to determine if a subset of residues was essential to TBK1’s preferential binding to SIKE over IRF3. With a complete phosphoknockout mutant (Ser to Ala), SIKE’s inhibitory activity increased 4.5 fold, consistent with retained TBK1 recognition. The S2A versus S4A mutants further defined the cluster of phosphorylation sites centered at the \( ^{185}SxSSxS^{190} \) motif as essential to phosphorylation dependent release of SIKE from TBK1, whereas the phosphorylation state of the S2A sites did not contribute to SIKE’s release from TBK1. The single mutation, S185A, alone could enhance inhibition of TBK1-mediated IRF3 phosphorylation. In fact, the \( K_{i_{app}} \) values for the S6A (77 nM) and the single, S185A (41 nM) mutants are in line with the Michaelis constant at low SIKE concentrations (55.9 nM, Figure 4.13). Additional interactions between TBK1 and SIKE or the structural context of the phosphorylation motif may also contribute to the high affinity TBK1:SIKE interaction. This is supported by the ability of SIKE 72-184 to inhibit TBK1 function and SIKE 1-112 to form an interaction with TBK1 (Figure 4.11 and Figure 4.12). SIKE72-184 retains a single phosphorylation site (S133) that when mutated to Ala (S2A mutant) does not alter SIKE inhibitory activity. Therefore, inhibition by this mutant is attributed to not only S133 docking to TBK1’s active site, but also interactions between SIKE and TBK1 outside of the active site. Similarly, SIKE 1-112, as it contains no phosphorylation sites, must form a TBK1:SIKE interaction exclusive of the active site. These interactions independent of the active site are consistent with the non-competitive/mixed type inhibition mediated by SIKE.
Taken together, SIKE’s original characterization as an endogenous inhibitor of the TBK1-mediated antiviral response only partially explains SIKE function. Comparison of the pseudo $K_d$ for the E-ATP complex showed that SIKE is preferred as a TBK1 substrate 6.5 fold over IRF3. At equivalent concentrations, SIKE would appear to inhibit IRF3 phosphorylation. This would not be the first time a TBK1 substrate had initially been defined as an inhibitor. Optineurin (OPTN), an autophagy receptor, was classified as an endogenous TBK1 inhibitor. Subsequent studies defined OPTN as a TBK1 substrate [177]. Phospho-OPTN has increased affinity for LC3 (microtubule associated protein light chain 3), thereby promoting selective autophagy of ubiquitin-coated cytosolic bacteria and providing a mechanism by which TBK1 functions in maintaining xenophagosomes [105, 106]. In a similar manner, while SIKE has the ability to endogenously regulate TBK1, it appears that TLR3 pathway activation and subsequent release of SIKE from the TBK1 kinase complex, leads to its interaction with other binding partners effectively promoting other functions in different areas of the cell.

In fact, co-immunoprecipitation experiments aimed at detecting interaction partners of SIKE revealed that this 207 amino acid protein is able to interact with at least 13 other proteins in the cell (Figure 4.14 and Table 4.4). Surprisingly, the SIKE interaction network did not appear to be altered following dsRNA stimulation (Figure 4.14 and Figure 4.15) which should mediate the TBK1 phosphorylation of SIKE (Figure 4.12). Similar results were observed when phosphoknockout (S6A) and phosphomimetic (S6E) SIKE constructs were used. Also surprising was the fact that neither TBK1 nor any of the scaffold proteins, which interact with TBK1 in the TLR3 kinase complex (TANK, SINTBAD, or NAP1) were found to associate with SIKE. While the scaffold proteins, which I postulate are, in fact, high affinity substrates of TBK1 due to data showing that NAP1 can also be phosphorylated by TBK1 (data not shown), have never been
shown to associate with SIKE, the TBK1:SIKE interaction is essential in the TLR3 mediated innate immune response. Taken together, these results lead me to postulate that in interaction experiments, the transient over-expression of SIKE in HEK293 cells is causing us to elucidate SIKE interactions with only the most prominently expressed proteins in the cell. While we are identifying a number of interaction partners, I postulate that this over-expression is causing us to miss a number of other interaction partners that could potentially identify further functions of SIKE in vivo. To solve this problem, we will either have to create cell lines stably expressing a tagged version of SIKE, or use a SIKE antibody (already developed), and repeat the interaction experiments in order to investigate endogenous interactions in the cell. While experiments may not have elucidated every interaction partner of SIKE in vivo, multiple interaction experiments (Table 4.4-4.6) revealed four proteins that were found in each investigation (Figure 4.17).

The first SIKE interaction partner found in all co-immunoprecipitation experiments was nucleophosmin (NPM1), a protein known to be associated with nucleolar ribonuceloprotein structures, involved in the biogenesis of ribosomes, and assist small basic proteins in their transport to the nucleolus [178]. While SIKE has never been found in the nucleus (Figure 4.16), the regulation of NPM1 through SUMOylation suggests another post-translational modification that may affect SIKE function and its binding affinity to multiple substrates in the cell.

The second interaction partner, found in all SIKE interaction experiments, was a class of proteins known as the heat shock proteins (HSP). This family of functionally related proteins is involved in the folding and unfolding of other proteins and has been shown to be involved in the up-regulation of stress as well as in immunity [179]. While its role with SIKE is not yet apparent, further microscopy experiments will elucidate the role of this interaction in the cell as it pertains to SIKE function.
The third interaction partner common in all SIKE binding experiments was again a class of proteins, all known as eukaryotic elongation factor proteins (eEF). The members of this family are involved in protein synthesis as they facilitate translational elongation, from the formation of the first peptide bond to the formation of the last [180]. Interestingly, elongation factors are also known to be targets for pathogens which attempt to alter protein function in a host so as to go unrecognized and stay alive [180]. While SIKE may interact with this protein in a regulatory role, similar to its interaction with TBK1, various kinetic and interaction based experiments will be required to identify the fundamental reason for this interaction.

The final interaction partner, found in all SIKE binding experiments, was γ-actin. This ubiquitously expressed protein is required for reinforcement and long-term stability of cytoskeletal structures making it an essential building block of the cytoskeleton in all non-muscle cells [181]. Interestingly, FGFR1OP2, discussed in Chapter 1.8 for its 56% sequence homology to SIKE, associates with actin cytoskeleton networks in oral wound fibroblasts [129]. Taken together, we postulate that SIKE also functions as an actin cytoskeleton associated protein participating in cytoskeleton rearrangement (cell migration (DCs), phagocytosis (macrophages), xenophagy). This function would be in response to pathogen challenge through its phosphorylation by TBK1. Further cell based assays and fluorescence microscopy experiments are necessary to validate this hypothesis but SIKE does appear to associate with cytoskeleton structures (Figure 4.16) and structural rearrangement proteins (Table 4.4 – Table 4.6) at a high frequency.

In further examining SIKE function and post-translational modifications, questions were raised regarding other potential modifications that could affect SIKE. Investigations revealed that the E3 ligases, TRAF2 and TRAF5, are able to poly-ubiquitinate SIKE in the presence of S1P
(data not shown). Taken together, from the discovery of multiple SIKE interaction partners along with both phosphorylation and ubiquitination events targeting SIKE in the cell, I postulate that SIKE has multiple functions in the cell which are mediated by specific post-translational modifications targeting the protein at distinct points in time. These modifications, I further postulate, affect the affinity of SIKE for specific substrates thereby modifying its interaction partners and changing its function. To examine this hypothesis, further post-translational modifications affecting SIKE, from acetylation and methylation to glycosylation and SUMOylation, would need to be identified and confirmed in vivo. From that point, individual interaction assays with SIKE and each of its binding partners would need to be performed to elucidate potential roles for the post-translational modifications and their effects on the affinity of SIKE for each of its interaction partners.

Finally, just as the human body needs to regulate the inflammatory response to prevent chronic inflammation and degenerative diseases, viruses need to subvert the immune response to stay alive in their host. While SIKE interacts with TBK1 to endogenously regulate type-I interferon production in the TLR3 response, viruses target TBK1 to prevent TLR3 pathway activation. Viruses such as HCV and VACV produce proteins, NS3 and NIL, which associate with TBK1 and inhibit its ability to phosphorylate IRF3 [22]. Other viruses, such as Hantaviruses, disrupt TBK1 interactions with upstream components, such as the TRAF adaptor proteins, which are required for TBK1 to identify TLR3 pathway activation. By defining the mechanism by which an apparent endogenous inhibitor of the TLR3 pathway is able to regulate the TLR3 response, we postulated that we would identify potential sites of pathway modulation. In doing so, we wondered if viruses had also exploited this approach. As discussed previously, from our studies into the regulatory mechanism of SIKE, we discovered that TBK1
phosphorylated SIKE on six serine residues (Figure 4.8). Of these residues, we found that four, present in an $^{185}\text{SxSSxS}^{190}$ motif, were critical to the TBK1:SIKE interaction as their mutation to a phosphoknockout construct (S4A) decreased the $K_{\text{app}}$ of SIKE by more than 3-fold compared to WT (Table 4.3). Interested in examining whether this TBK1 phosphorylation motif was conserved in SIKE, we performed a sequence alignment analysis of SIKE constructs from human to zebrafish (Figure 5.2) and found significant conservation. Taking this analysis one step further, we questioned whether viral proteins, from human parainfluenza virus 2 and 5 to RBP and the mumps virus, known to inhibit TLR3 signaling by acting as TBK1 substrates, share this TBK1 phosphorylation consensus sequence with SIKE (Figure 5.2). Although the exact site of TBK1 phosphorylation is unknown in these viral proteins, we did identify an SxS motif in all viral substrates. As a strong conservation was observed, we postulate that this motif may be key in targeting substrates to TBK1 and may be critical to viral subversion of the type I interferon response. Further phosphopeptide mapping experiments are required to confirm or refute the SxS site as a target of TBK1. Additional kinetic experiments, along with the creation of knockout constructs, will be required to investigate the importance of this SxS motif in viral substrates if it is, in fact, a target of TBK1.

In summary, this study describes the mechanism by which SIKE inhibits TBK1-mediated phosphorylation of IRF3, essential to type I interferon production. Kinetic analyses showed that SIKE not only inhibits IRF3 phosphorylation (Figure 4.4), but is also a high-affinity, TBK1 substrate (pseudo $K_D=410\text{nM}$) (Figure 4.13). With respect to IRF3 phosphorylation, SIKE is shown to function as a mixed-type inhibitor ($K_{\text{app}}=350\text{nM}$) rather than, given its status as a TBK1 substrate, as a competitive inhibitor (Figure 4.4). Further analysis revealed that TBK1 phosphorylation of IRF3 and SIKE displayed negative cooperativity. Additionally, both
Figure 5.2 TBK1 phosphorylation consensus motif is conserved throughout evolution in SIKE and is apparent in viral substrates of TBK1. Sequence alignment of known (red) and predicted (green) TBK1-mediated phosphorylation sites.
substrates are shown to share a similar $K_M$ at low substrate concentrations (~50nM), but deviate >8 fold at higher substrate concentrations (IRF3=3.5mM; SIKE=0.4mM). TBK1:SIKE interactions are further shown to be modulated by SIKE phosphorylation, clustered in SIKE’s C-terminal portion (Serine residues 133, 185, 187, 188, 190, 198) (Figure 4.5). These sites exhibit striking homology to IRF3’s phosphorylation motif (Figure 4.9). Mutagenic probing further revealed that phosphorylation of S185 controled TBK1:SIKE interactions (Figure 4.10). Taken together, our studies demonstrate for the first time that SIKE functions as a TBK1 substrate and inhibits TBK1-mediated IRF3 phosphorylation by forming a high affinity TBK1:SIKE complex. These findings provide key insights into endogenous control of a critical catalytic hub that is achieved, not by direct repression of activity, but by redirection of catalysis through substrate affinity. Furthermore, in addition to the previously identified preference of S-$\phi$ ($\phi$= hydrophobic residue) for TBK1 substrates, we have found that the SxSS motif is conserved in SIKE, IRF3 and many viral protein substrates. From these findings, we postulate the existence of a TBK1 phosphorylation consensus motif that is a critical regulatory motif in both endogenous regulation and viral subversion of the type-I interferon response.

Conclusions

In culmination, the chapters in this dissertation provide novel insight into different facets of the innate immune response that can be used to understand mechanisms by which a host thwarts pathogen challenge. While adjuvant potential studies in Chapter 2 characterized a 139bp dsRNA ligand as a viable vaccine adjuvant, further data led us to postulate that by varying dsRNA length, we could target different receptors in the cell thereby creating a tailored immune response. Similarly, while receptor clustering studies in Chapter 3 characterized a new TLR3
signaling model in which dsRNA:TLR3 signaling units laterally cluster to achieve efficient signaling, further data led us to postulate the importance of TRIF in pathogen recognition as we suggest that this intracellular adaptor protein mediates the receptor clustering events required for competent TLR3 signaling. Finally, the studies in Chapter 4 demonstrate, for the first time, that SIKE functions as a TBK1 substrate and inhibits TBK1-mediated IRF3 phosphorylation by forming a high affinity TBK1:SIKE complex. The data presented also provides key insights into the endogenous control of a critical catalytic hub that is achieved, not by direct repression of activity, but by redirection of catalysis through substrate affinity. Furthermore, through the elucidation of a potential TBK1 phosphorylation consensus motif, these studies also define a potential regulatory motif that can be targeted for therapeutic development to modulate TBK1 activity. Taken together, these studies establish a foundation for building long-term studies on the function, regulation and viral subversion of the innate immune response.
On the following pages are chromatographs with corresponding SDS-PAGE analysis of peak fractions from TLR3-ECD:Fab:dsRNA studies detailed in Chapter 3. Methods are described in Chapter 3, section 3.2.3.
Figure A1. TLR3-ECD alone in T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A2. 139bp dsRNA ligand alone in T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A3. Fab12 alone in a T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A4. Fab15 alone in a T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A5. Fab12 + Fab15 run in a T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A6. TLR3-ECD and 139bp dsRNA run in a T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A7. TLR3-ECD and Fab12 run in a T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A8. TLR3-ECD and Fab15 run in a T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A9. TLR3-ECD + Fab12 + Fab15 run in a T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A10. TLR3-ECD + 139bp dsRNA + Fab12 run in a T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A11. TLR3-ECD + 139bp dsRNA + Fab15 run in a T+R+F12+F15 SEC experiment (Figure 3.3 analysis).
Figure A12. TLR3-ECD + 139bp dsRNA + Fab12 + Fab15 run in a T+R+F12+F15 SEC experiment. (Figure 3.3 analysis).
Figure A13. TLR3-ECD alone run in a T+R+F1068 SEC experiment (Figure 3.5B analysis).
Figure A14. 49bp dsRNA ligand alone run in a T+R+F1068 SEC run (Figure 3.5B analysis).
Figure A15. Fab1068 alone run in a T+R+F1068 SEC experiment (Figure 3.5B analysis).
Figure A16. 49bp dsRNA + Fab1068 run in a T+R+F1068 SEC experiment (Figure 3.5B analysis).
Figure A17. TLR3-ECD and Fab1068 run in a T+R+F1068 SEC experiment (Figure 3.5B analysis).
Figure A18. TLR3-ECD and 49bp dsRNA run in a T+R+F1068 SEC experiment (Figure 3.5B analysis).
Figure A19. TLR3-ECD + 49bp dsRNA + Fab1068 run in a T+R+F1068 SEC experiment (Figure 3.5B analysis).
Figure A20. TLR3-ECD alone run in a T+R+F12 SEC experiment (Figure 3.5C analysis).
Figure A21. 49bp dsRNA ligand alone run in a T+R+F12 SEC experiment (Figure 3.5C analysis).
Figure A22. Fab12 alone run in a T+R+F12 SEC experiment (Figure 3.5C analysis).
Figure A23. 49bp dsRNA ligand + Fab12 run in a T+R+F12 SEC experiment (Figure 3.5C analysis).
Figure A24. TLR3-ECD and Fab12 run in a T+R+F12 SEC experiment (Figure 3.5C analysis).
Figure A25. TLR3-ECD + 49bp dsRNA ligand run in a T+R+F12 SEC experiment (Figure 3.5C analysis).
Figure A26. TLR3-ECD + 49bp dsRNA + Fab12 run in a T+R+F12 SEC experiment (Figure 3.5C analysis).
APPENDIX B
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VITA

James Dennis Marion Jr. was born June 19, 1984 in Ridgewood, NJ to the parents of James and Mary Lou Marion. He earned a Bachelor of Science degree in Biochemistry and Molecular Biology with minors in History and Journalism from the University of Richmond, Richmond, VA in 2006. While at the University of Richmond, James worked in the Laboratory for Structural Biology, Biophysics and Bioinformatics of Dr. J. Ellis Bell for four years. Having presented his research at numerous national scientific meetings and having earned a number of awards including three summer internship fellowships, James went on to work as a research assistant and lab manager in the same laboratory from 2006 to 2008. In the fall of 2008, James started the PhD program in the Department of Biochemistry at Virginia Commonwealth University, Richmond, VA. He joined the laboratory of Dr. Jessica K. Bell in the spring of 2009 for his dissertation work. In 2011, James attended the American Society for Biochemistry and Molecular Biology annual meeting and received a graduate/post-doctoral travel fellowship. In 2012, James attended the Protein Society annual meeting and received a 2012 Finn Wold travel award. In his tenure at Virginia Commonwealth University, James was inducted into the Phi Kappa Phi national honor society and the Alpha Epsilon Lambda national honor society. James was an honorable mention for the Phi Kappa Phi national scholarship and was awarded a 2012/2013 VCU Thesis/Dissertation Assistantship award. Through his dissertation work, James contributed to three journal articles and produced two first author manuscripts. The work documented here is the product of five years of experimentation and experience culminating into one thesis. James will continue his training as a postdoctoral fellow in the laboratory of Elizabeth Komives at the University of California – San Diego.