INNATE IMMUNE RECOGNITION BY HUMAN TOLL-LIKE RECEPTOR 10 AND OTHER MEMBERS OF THE TOLL-LIKE RECEPTOR 2 SUBFAMILY

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology in the Graduate College of the University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

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ABSTRACT

Toll-like receptors (TLRs) are central receptors of the innate immune system which drive host inflammation and adaptive immune responses upon detection of invading microbes. Drugs targeting TLRs are of considerable interest as potential inflammatory regulators, vaccine adjuvants, and novel immunotherapeutics. Among human TLRs, TLR10 is the only remaining family member without a defined agonist or function. Phylogenetic analysis reveals that TLR10 is most related to TLR1 and TLR6, both of which mediate immune responses to a variety of microbial and fungal components in cooperation with TLR2. Knowledge gained of this orphan receptor is useful to fully understand the function of TLR2 subfamily, which comprises TLRs 2, 1, 6 and 10. The primary objective of this doctoral dissertation research is to define the innate immune sensing function of TLR10 and understand the mechanisms responsible for ligand recognition within the TLR2 subfamily.

Chapter One introduces the general field of innate immunity and reviews the current knowledge on TLRs, including structure and function, signaling pathways as well as signaling regulation of these receptors with an emphasis on the TLR2 subfamily.

Chapter Two describes a high-throughput chemical library screen developed to uncover the ligands of TLR10 and novel TLR2 agonists. A synthetic chemical library of 24,000 compounds was screened using an IL-8 driven-luciferase reporter in cells expressing TLRs 2, 1, 6 and 10. The screen failed to discover TLR10 ligands, but yielded several novel TLR2 dependent activators that utilize TLR1, TLR6, or both as co-receptors. These novel small molecule compounds are aromatic in nature and structurally unrelated to any known TLR2 agonists. Two of the most potent compounds exhibit
species specificity and are inactive toward murine peritoneal macrophages. Mutational analysis reveals that while the central extracellular region of TLR1 is required for stimulation, there are subtle differences in the mechanism of stimulation mediated by the synthetic compounds in comparison to natural lipoprotein agonists. The three most potent compounds stimulate TNF-α production form human peripheral blood monocytes. The implication of these results and the potential importance of the novel TLR2 agonists are also discussed (Guan et al. *Journal of Biological Chemistry*. 2010 July 30, 285: 23755-23762).

Chapter Three focuses on the identification of TLR10 ligands and characterization of sensing mechanisms by this receptor. The generation and analysis of chimeric receptors, containing the extracellular recognition domain of TLR10 and the intracellular signaling domain of TLR1, revealed that TLR10, in cooperation with TLR2, senses triacylated lipopeptides and a wide variety of other microbial-derived agonists shared by TLR1, but not TLR6. TLR10 requires TLR2 for innate immune recognition and these receptors colocalize in the phagosome and physically interact in an agonist dependent fashion. Computational modeling and mutational analysis of TLR10 show preservation of the essential TLR2 dimer interface and lipopeptide binding channel found in TLR1. Co-immunoprecipitation experiments indicate that, similar to TLR2/1, TLR2/10 complexes recruit the proximal adaptor MyD88 to the activated receptor complex. However, TLR10, either alone or in cooperation with TLR2, fails to activate typical TLR-induced signaling including NF-κB, IL-8 or IFN-β driven reporters (Guan et al. *Journal of Immunology*, 2010 May 1;184(9):5094-103). This finding explains why I was unable to discover
synthetic ligands of TLR10 in Chapter Two. Interestingly, the novel chemical compounds are agonists recognized only by TLR2/1 and not by TLR2/10.

Chapter Four describes the generation of monoclonal antibodies against the extracellular domain of TLR10. A total of 15 hybridoma lines were established that secrete monoclonal antibodies that bind strongly to TLR10, but to neither TLR 1 nor TLR6. The purified anti-TLR10 antibody was used to examine endogenous TLR10 expression in human peripheral blood. I found that granulocytes, natural killer (NK) cells, monocytes and T cells lack detectable expression of TLR10. However human B cells express high cell surface levels of this receptor, suggesting that TLR10 plays a functional role in the B cell lineage.

Chapter Five summarizes major findings from the dissertation research, assesses their contribution and potential implications to the TLR field, and explores future research directions for understanding the biological roles of TLR10.
ACKNOWLEDGEMENTS

This dissertation marks a great milestone in my life. I owe everlasting gratefulness to many people who warmly supported me in undertaking this project. I am especially grateful to Richard Tapping, a great advisor and mentor. None of this would have been possible without his insightful guidance, persistent encouragement, patience and understanding throughout my graduate study. He has taught me critical thinking about the literature, creative design and scientific analysis of a project, optimistic attitude towards crisis situations, and a lot more than I could ever give him credit for here. And, to me, he definitely means more than an academic advisor.

I am very grateful to my graduate committee members, James Slauch, Peter Orlean, Rachel Whitaker, and Joanna Shisler. Each of them has offered me professional guidance and provided important advice toward completing my project over these years. I especially thank James Slauch for his guidance regarding how to scientifically design an experiment during my first year rotation in his laboratory.

I would like to thank my current and former lab mates for creating a wonderful work environment and helping me make sense of my research results, and more importantly for their friendship. In particular, I thank Katherine Ayoade not only for teaching me almost all the technical details of mammalian tissue culture but also for helping me gain confidence in my first year in the States. Sincere thanks also go to Diana Rose Ranoa, Song Jiang and Xinyan Li for their expert contributions to my project. I am especially grateful to Diana Rose Ranoa for her carefully commenting and editing several chapters of this dissertation.
I am also deeply grateful to my friends who support me to overcome the obstacles encountered in my graduate study as well as in my life. I really value their friendship and appreciate their faith in me.

Finally, I wish to thank my parents, Yuying Zhang and Yongshun Guan, and my brother Shikun Guan, who always offer me love and support. Most importantly, thanks from the bottom of my heart to my husband, Hua Qin, who accompanied me in this long journey of graduate study, and my baby boy Andrew. Both of them give me endless inspiration and motivation in the pursuit of my objectives and happiness of life.
Dedicated to Hua and Andrew

My Wonderful Boys
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LIST OF ABBREVIATIONS

TLR, Toll-like receptor
IL, interleukin
TNF, tumor necrosis factor
LRR, leucine rich repeat
ECD, extracellular domain
TIR, Toll/IL-1 receptor
PAMPs, pathogen-associated molecular patterns
PRRs, pattern-recognition receptors
APC, antigen-presenting cells
CLR, C-type lectins
RIG, retinoic acid-inducible gene I
RLRs, retinoic acid-inducible gene I (RIG)-like receptors
NOD, nucleotide-binding oligomerization domain ()
NLRs, NOD-like receptors
LPS, lipopolysaccharide
IFNs, interferons
MD2, myeloid differentiation factor 2
MyD88, myeloid differentiation antigen protein 88
TIRAP/Mal, TIR domain-containing adapter/MyD88 adapter like
TRIF, TIR domain-containing adapter inducing interferon-β
TRAM, TRIF-related adapter molecule
IRAKs, IL-1R-associated kinases
TRAF6, TNFR-associated factor 6
MAPK, mitogen-activated protein kinases
TAK1, TGF-β-activated kinase 1
TAB1, TAK1-binding protein 1
IKK, inhibitor of NF-κB kinases
IRFs, interferon response factors
TANK, TRAF-family-member-associated NF-κB activator
TBK1, TANK binding kinase 1
CREB, cAMP response element binding protein
PKB, protein kinase B
GSKs, glycogen synthase kinase 3
ST2, suppressor of tumorigenicity
SIGIRR, single immunoglobulin IL-1 related protein
SARM, sterile α and Heat-Armadillo motif
pDC, plasmacytoid dendritic cell
NK cells, natural killer cells
CHAPTER ONE
INTRODUCTION

The Innate Immune System

Vertebrates are constantly exposed to microorganisms present in the environment and yet only occasionally develop perceptible diseases. Most invading pathogens are eliminated by protection mechanisms, the immune system, which evolved for this purpose. The mammalian immune system is divided into the innate and adaptive branches. The adaptive system, comprised of T and B lymphocytes, is remarkable in its capability to generate a specific response against virtually any foreign antigen. This is achieved through a huge repertoire of lymphocytes each bearing a unique antigen receptor, so that the entire pool of antigen receptors is very large and highly diverse. In the event of infection, an individual lymphocyte that encounters a proper antigen proliferates and differentiates into effector cells capable of binding to antigens or secreting a specific antibody. Clonal selection, together with long-lived memory cells produced to increase protection against subsequent re-infection with the same agent, makes the adaptive immune system absolutely necessary for the generation of a robust immune response (Janeway et al., 2005). However, there is a delay of 4-7 days before a sufficient population of antigen-specific cells is produced to provide protection. During this time, pathogens can flourish inside the body and damage the host if unchecked. The innate immune components, which include macrophages, dendritic cells, neutrophils, anti-microbial peptides and the complement system, constitute the first line of defense which provides immediate recognition and responses to invading microorganisms. Microorganisms that cross the epithelial barrier of the body are met immediately by the cells and molecules that can induce a rapid innate immune response. Phagocytic
macrophages offer direct killing of pathogens and also mediate the release of proinflammatory chemokines and cytokines that recruit more innate immune cells and proteins from local blood vessels to the site of infection, thus initiating the process of inflammation. In addition, clear evidence has shown that the innate immune response makes a crucial contribution to the activation of adaptive immunity (Medzhitov and Janeway, 2000).

The innate immune response provides an initial discrimination between self and non-self. Innate immune recognition is mediated by a number of germline-encoded receptors which detect relatively invariant molecular patterns present on viruses, microbes, fungi, protozoans and helminths. These structures are called pathogen-associated molecular patterns (PAMPs), and the receptors have been denoted as pattern-recognition receptors (PRRs) (Akira et al., 2006). PRRs are expressed on a variety of innate immune effector cells, and once they sense a PAMP, inflammatory responses are immediately triggered which accounts for the rapid innate immune response. Moreover, the signaling from activated receptors induces the expression of co-stimulatory molecules on macrophages and dendritic cells (antigen-presenting cells, APCs) which subsequently generate effective adaptive immune responses. In the past two decades, several families of PRRs have been characterized and the understanding of PAMP recognition is rapidly growing. We will first take a closer look at PRRs in the innate immune system, a subset of which will be the focus of this thesis.

**Pattern Recognition Receptors**

Cellular PRRs can be broadly classified into transmembrane proteins such as Toll-like receptors (TLRs) and C-type receptors (CLR), and cytoplasmic proteins such as
Retinoic acid-inducible gene I (RIG)-like receptors (RLRs) and NOD-like receptors (NLRs). Here we briefly review their ligands and function (Takeuchi and Akira, 2010).

The best-characterized PRR family is the TLR family. Toll, the founding receptor of the TLR family, was originally described in the fruitfly as a component of embryonic development (Hashimoto et al., 1988). Later, it was shown to trigger the formation of antimicrobial peptides in response to fungal infection in the adult fly (Lemaitre et al., 1996). It is now known that the Toll homologues, termed TLRs, are present from lower invertebrates to mammals and have a critical role in the defense against various infections. To date, ten TLRs have been identified in humans and twelve in mice. All TLRs are type I transmembrane proteins and are expressed either on the plasma membrane or endosomal membrane. They are characterized by an extracellular domain (ECD) containing leucine-rich repeats (LRRs), and a transmembrane region followed by a cytoplasmic signaling domain homologous to that of the interleukin-1 (IL-1) receptor and termed the Toll/IL-1R (TIR) domain (Takeda et al., 2003). Different TLRs recognize distinct molecular patterns of microorganisms (Fig. 1.1). Cell-surface TLRs sense conserved microbial cell wall constituents, such as lipoproteins of bacteria and mycoplasma (TLRs 2, 1, and 6), lipopolysaccharide (LPS) of Gram-negative bacteria (TLR4) and flagellin of bacteria (TLR5). Endosomal TLRs, comprising TLRs 3, 7, 8 and 9, detect nucleic acid ligands derived from viruses and bacteria, including double-stranded RNA (dsRNA) (TLR3), single-stranded RNA (ssRNA) (TLR7) and dsDNA (TLR9). Recognition of the ligands by TLRs triggers signaling pathways leading to the activation of transcription factors such as NF-κB, AP-1 and IRFs, which regulate the production of pro-inflammatory cytokines and type-I interferons (IFNs). TLRs can also
trigger activation of adaptive immune responses, including up-regulation of co-stimulatory molecules essential for T cell activation, T cell differentiation and antibody responses (Pasare and Medzhitov, 2004). Individual TLRs activate different signaling cascades depending on the distinct combination of adapters involved (Brikos and O'Neill, 2008). The ligand-TLR interaction and TLR signaling pathways will be expanded in the next section.

CLRsa are another class of transmembrane PRRs which are characterized by the presence of a carbohydrate-binding domain. CLRs recognize mannose, fucose and glucan carbohydrates on the surfaces of microorganisms such as viruses, fungi and bacteria. Following ligand sensing, CLRs trigger internalization of the pathogen, expression of specific cytokines and subsequent antigen presentation which drives T cell differentiation (Geijtenbeek and Gringhuis, 2009). Two examples of CLRs are dectin-1 and the mannose receptor, which are highly expressed in macrophages and dendritic cells and function directly as phagocytic receptors.

The pathogens that invade the cytosol are detected by cytoplasmic PRRs, including RLRs and NLRs. Both receptor families have received recent attention because of their roles in detection of viral and bacterial infection as well as the activation of inflammatory protein complexes in response to pathogens. The RLR family is composed of three members: retinoic acid-inducible gene I (RIG-1), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP-2) (Takeuchi and Akira, 2009). These proteins all possess a central helicase/ATPase domain which is responsible for the recognition of specific RNA patterns generated during viral infection, such as ss/ds RNA structure patterns associated with ssRNA viruses and some
dsDNA viruses. Ligand engagement of RLRs leads to the activation of IRFs and NF-κB, which coordinate the production of type 1 IFNs (Rehwinkel and Reis e Sousa, 2010). On the other hand, the NLR family is composed of more than 20 members that detect intracellular pathogens and stress signals. The receptors in this family share the characteristic arrangement of a C-terminal LRR domain, a central nucleotide-binding oligomerization domain (NOD) and an N-terminal effector domain (Kanneganti et al., 2007). Among this family, NOD1 and NOD2 detect portions of bacterial peptidoglycan and subsequently activate NF-κB. Another set of NLRs, upon activation, induce the assembly of the inflammasome complex and drive processing of inflammatory cytokines (Ting et al., 2010). While cytoplasmic PRRs have emerged as critical components of the innate immune response (most notably, the processing of pro-IL-1β), several questions, including mechanisms of ligand sensing, receptor structure, signaling pathways, as well as the interplay with TLRs, remain to be clarified in the near future.

**TLR Structure and Function**

Our knowledge of the TLR family has been growing rapidly over the past decade. Structural studies have begun to reveal how individual TLRs can distinguish different PAMPs, and the dissection of signaling pathways has shown how TLRs elicit inflammatory responses. Here we will highlight these recent advances.

The ECDs of TLRs, which are responsible for binding various PAMPs, contain 19-25 tandemly arranged LRRs. Each LRR is 20-30 amino acid-long and contains a conserved LxxLxLxxN motif with the remaining residues being more variable. The LRRs of TLRs form a solenoid structure with β strands and either loops or helical structures on the concave and convex surface, respectively (Bella et al., 2008). As mentioned
previously, different TLRs recognize structurally- and chemically- diverse molecular patterns of microorganisms. The recently solved crystal structures of ligand-bounded ECDs of several TLRs provide clues to the mechanisms of ligand recognition.

Most TLRs form homodimers upon ligand binding. The well-studied TLR4, together with an accessory molecule called myeloid differentiation factor 2 (MD2), recognizes LPS of Gram negative bacteria. The lipid A portion of enteric bacterial LPS is the biologically active part and is composed of phosphorylated diglucosamine with four primary and two secondary acyl chains (Jin and Lee, 2008). The crystal structure of TLR4/MD2/LPS complex revealed that LPS binding induces the formation of a receptor multimer with two complexes of TLR4/MD2/LPS arranged symmetrically in an m-shaped structure. Five of the lipid chains of LPS are accommodated by a hydrophobic pocket of MD2 and the sixth one is in contact with both TLR4 and MD2 (Park et al., 2009).

Among the TLRs localized in endosomal compartments, only the crystal structure of TLR3 bound to its ligand, dsRNA, has been solved to date. The sugar phosphate backbone of a dsRNA, which is 40-50 bp in length, binds to both N-terminal and C-terminal portions of the glycan-free convex surface of TLR3. The dsRNA bridges together two molecules of TLR3 which form a stable m-shaped dimer juxtaposed at the C-terminal ends (Liu et al., 2008a).

Unlike the homodimer configuration for most TLRs, TLR2 forms heterodimers with either TLR1 or TLR6 and mediates responses to a wide range of agonists. TLR1 and TLR6 enable TLR2 to discriminate subtle structural differences within the agonists and therefore greatly increased the repertoire of agonists for TLR2. The TLR2/1 pair
recognizes bacterial triacylated lipoprotein. In contrast, the TLR2/6 heterodimer is responsible for sensing mycoplasma diacylated lipoprotein (Takeuchi et al., 2001; Takeuchi et al., 2002). The crystal structure of the ECDs of TLRs 2 and 1 in complex with a synthetic triacylated lipopeptide Pam$_3$CSK$_4$ reveals an m-shaped heterodimer of TLR2 and TLR1 coordinately bound to the ligand Pam$_3$CSK$_4$. Two acyl chains of Pam$_3$CSK$_4$ insert into an internal pocket of TLR2, and the remaining amide-bound lipid chain occupies the hydrophobic channel in TLR1 (Fig. 1.2) (Jin et al., 2007). Recently, the crystal structure of TLR2/TLR6/diacylated lipopeptide complex revealed that the overall structure is very similar to that of TLR2/TLR1/ Pam$_3$CSK$_4$ except that the lipid channel in TLR6 is blocked by two bulky amino acids which may explain why the TLR2/6 complex is unresponsive to triacylated lipoproteins (Kang et al., 2009). More specifically, the ECDs of TLRs 2, 1 and 6 can be divided into three subdomains, N-terminal, central and C-terminal. The central and partial C-terminal domains (LRR5-12) are unusual in that they lack the asparagine ladder needed to maintain the standard β-sheet twist (the ladder is formed by strictly preserving relative positions of the conserved asparagines in each LRR), and the length of their LRRs is more variable. These unique LRR motifs create the lipopeptide-binding sites in TLR2 and TLR1, both of which are located at the heterodimer interface. Domain swapping experiments between TLR1 and TLR6 has also indicated that the central LRRs of these receptors are required for discrimination of different lipopeptide agonists (Omueti et al., 2005). Taken together, all structural studies have indicated that the sites of interaction between ligands and their respective TLRs are very different from one to another, but they all form a stable m-shaped dimeric arrangement between two subunits upon ligand binding.
Ligand-induced dimerization of ECDs brings together the two intracellular TIR domains of the receptors. TIR domains are comprised of a five-stranded parallel β-sheet (βA-βE) surrounded by five α-helical segments (αA-αE) (Xu et al., 2000). Multiple mutational and structural modeling studies have shown that the BB loop (connecting βB and αB) and the DD loop (connecting βD and αD) are important for driving the interaction between TLRs as well as between TLRs and adapters. The most prominent example is a proline-to-histidine mutation in BB loop of TLR4 is responsible for rendering C3H/HeJ mice insensitive to LPS (Poltorak et al., 1998). Similarly, the replacement of the homologous Pro 681 with His in the BB loop of human TLR2 disrupts signal transduction in response to zymosan and Gram-positive bacteria without disturbing the overall structure of the TIR domain (Underhill et al., 1999).

**TLR Signaling Pathways**

The TIR-TIR structure creates a scaffold for the recruitment of adapter proteins which mediate TLR signaling (Gay and Gangloff, 2008). Adapter proteins are a group of TIR domain-containing molecules, which includes MyD88 (myeloid differentiation antigen protein 88), TIRAP/Mal (TIR domain-containing adapter/MyD88 adapter like), TRIF (TIR domain-containing adapter inducing interferon-β), and TRAM (TRIF-related adapter molecule). Different combinations of adaptor molecules are activated by different TLRs resulting in various signaling outputs (Toshchakov et al., 2005). The TLR pathways are roughly categorized as MyD88-dependent and TRIF-dependent.

MyD88 is the adapter employed by all TLRs except for TLR3. TLR2 and TLR4 signaling requires TIRAP for the recruitment of MyD88 to the receptors. MyD88 associates with IL-1R-associated kinases (IRAKs) to form signaling complexes. The
recently determined crystal structure of the MyD88-IRAK4–IRAK2 complex showed that 6 MyD88, 4 IRAK4 and 4 IRAK2 molecules assemble to form a helical signaling tower, called a Myddosome complex, in which MyD88 recruits IRAK4 and the MyD88-IRAK4 complex recruits IRAK2 (Lin et al., 2010). Following multiple phosphorylation events, IRAKs dissociate from MyD88 and interact with TNFR-associated factor 6 (TRAF6), a protein which mediates the activation of NF-κB and the MAPK (mitogen-activated protein kinases) cascades. TRAF6 interacts with the ubiquitin-conjugating enzymes comprised of Uev1A and Ubc13 and itself becomes polyubiquinated, which consequently activates a complex of TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3. When TAK1 becomes ubiquinated, it phosphorylates the inhibitor of NF-κB kinase (IKK) complex, composed of IKK-α, IKK-β and NF-κB essential modulator (NEMO). Subsequently, the IKK complex phosphorylates IκB, an NF-κB inhibitory protein. This event frees NF-κB to translocate into the nucleus and activate the genes responsible for inflammatory responses. The TAK1 complex also interacts with the MAPK cascades, in which p38 and JNK are activated. They in turn lead to activation of another transcription factor complex, AP-1, which targets inflammatory-response genes. In addition, the MyD88-dependent pathway also activates interferon response factors (IRFs) from activated TLR7 and TLR9 signaling. In this setting, when MyD88 forms complexes with IRAKs and TRAF6, the signaling cascade can proceed to the phosphorylation of different IRFs in a receptor and cell-specific way. The phosphorylated form of IRFs translocate to the nucleus to activate the expression of type 1 interferons, which induce hundreds of genes involved in antiviral responses (Brikos and O'Neill, 2008).
TRIF is an additional proximal adaptor for TLR3 and TLR4 signaling. TLR4 requires a bridging adaptor, TRAM, for activating TRIF. TRIF associates with two IKK-related kinases, TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) and IKKe. The complex then phosphorylates IRF3 and IRF7, leading to their activation and translocation into the nucleus, resulting in the induction of IFNs. TRIF has also been shown to activate the NF-κB pathway through TRAF3 and TRAF6 (Brikos and O'Neill, 2008).

Aside from the inflammatory pathways mentioned above, TLR signaling has been shown to regulate cytokine production as well as cell proliferation via MAPKs and PI3K (phosphoinositide 3-kinase). The MAPKs regulated by TLRs activate cAMP response element binding protein (CREB), which is a major transcription factor involved in cell metabolism. Moreover, several TLRs, including TLRs 2, 3 and 5, have been shown to interact with PI3K, whose downstream kinases are Akt/PKB (protein kinase B) and GSKs (glycogen synthase kinase 3). These kinases are involved in multiple cellular activities and appear to affect TLR signaling in both negative and positive ways depending on the cell type as well as the kind of cytokines produced (Li et al., 2010).

**Negative Regulation of TLR Signaling**

TLR activation is a double-edged sword. The immune response triggered by TLR engagement is indispensable for elimination of invading microorganisms and activation of adaptive immunity. However, excessive or inappropriate activation of TLR signaling can lead to severe inflammatory diseases and autoimmune diseases, such as septic shock and rheumatoid arthritis. Therefore, TLR signaling must be tightly controlled. More than
20 molecules have recently been shown to be involved in negative regulation of TLR signaling (Lang and Mansell, 2007), a few of which are briefly described here.

Expression of natural soluble decoy receptors, which are proteins lacking the transmembrane and cytoplasmic domains of TLRs, have been described for TLR2 and TLR4. The soluble forms of these receptors have been shown to attenuate TLR2 and TLR4 signaling in response to bacterial lipopeptides and LPS, respectively, presumably by competing with membrane-bound receptors for TLR ligands or by blocking the interaction between receptors and accessory molecules such as CD14 and MD2 (Iwami et al., 2000; LeBouder et al., 2003).

There also exist transmembrane protein regulators such as ST2 (suppressor of tumorigenicity) and SIGIRR (single immunoglobulin IL-1 related protein). Both contain a TIR domain in their intracellular portion, and have been found to inhibit MyD88-dependent signaling activated by TLR agonists. Current evidence suggests that ST2 acts as an inhibitor by sequestering the adaptors MyD88 and TIRAP (Brint et al., 2004). SIGIRR exerts its negative regulatory function possibly by interfering with the formation of receptor complexes and/or by attenuating the recruitment of signaling molecules, IRAK and TRAF6, to TLRs or IL1R (Wald et al., 2003).

A number of intracellular negative regulators are capable of inhibiting TLR signaling at the adapter level. Examples include MyD88s, SARM (sterile α and Heat-Armadillo motif), and IRAKM. MyD88s is an alternatively spliced, short form of MyD88 and the inhibition of signaling is due to its failure to recruit IRAK4 (Burns et al., 2003). SARM is known to interact with TRIF and thereby prevent, either directly or indirectly,
its signaling (Carty et al., 2006). IRAKM is a member of the IRAK family and can inhibit the association of IRAK with TRAF-6 (Kobayashi et al., 2002).

Another way to control TLR signaling is by regulating the expression level of receptors. Previous studies in the Tapping laboratory have shown that TLRs 2, 1 and 6 expression levels are modulated upon ligand stimulation. Interestingly, in response to TLR agonists, TLR2 mRNA and protein level were rapidly up-regulated in human monocytes, while coreceptors TLRs 1 and 6 mRNA were down-regulated (Johnson and Tapping, 2007). This suggests a possible negative regulation of TLR2 activation. The increase in TLR2 expression could lead to the production of the soluble form of TLR2, which acts as a decoy receptor to dampen responses, while the decrease in coreceptor TLR1 and TLR6 expression would prevent cells from responding to TLR2 agonists (Johnson, 2008).

**The TLR2 Subfamily**

The TLR2 subfamily comprises TLRs 1, 2, 6 and 10. As previously mentioned, TLRs 2, 1 and 6 need to cooperate as TLR2/TLR1 or TLR2/TLR6 heterodimers to mediate signals in response to various agonists. Besides bacterial lipoproteins, TLR2 is also responsive to a wide variety of microbial structural components including zymosan yeast particles, lipoteichoic acid and peptidoglycan of Gram-positive bacteria, atypical LPS derived from *Porphyromonas gingivalis*, membrane lipoarabinomannans from mycobacteria, and type-II heat-labile enterotoxins from *Escherichia coli*, as well as a wide range of unknown agonists from a variety of pathogenic microbes (Hajishengallis et al., 2005; Takeda et al., 2003). TLR2 has also been reported to detect diverse molecules of host origin associated with cell death and tissue damage, such as heat shock proteins,
hyaluronan fragments (degradation products of the extracellular matrix) and amyloids. However, for some TLR-stimulating ligands it is difficult to completely rule out possible contamination with other potent TLR agonists, such as lipoproteins or endotoxins (Erridge, 2010).

There are additional co-receptors that can enhance TLR2 function in recognition of agonists and initiation of immune responses. For example, the myeloid receptor CD14 transfers lipopeptides to TLR2 complexes, and increases cellular sensitivity to low concentrations of agonists (Nakata et al., 2006). The scavenger receptor CD36 acts as a TLR2/TLR6 coreceptor for sensing LTA and MALP-2 (macrophage-activating lipopeptide 2) (Hoebe et al., 2005). Integrin β3 is shown to form a complex with TLR2 and contributes to the initiation of TLR2 responses to lipopeptides, LTA and zymosan (Gerold et al., 2008). Dectin-1 and mannose binding receptor have also been shown to collaborate with TLR2 in recognition of microbial components (Ip et al., 2008).

TLRs 2, 1 and 6 are predominantly expressed on macrophages, monocytes, dendritic cells and B cells, along with other cells associated with innate immunity, such as endothelial and epithelial cells. Stimulation of these cells with TLR2 ligands induces the production of proinflammatory cytokines such as TNF-α, IL-8 and IL-6. A recent study has shown that TLR2 expressed on inflammatory monocytes can be activated by viruses leading to the induction of type I IFN (Barbalat et al., 2009). Such reactions were shown to occur only in response to viral ligands but not bacterial ones, and the signaling is unique to inflammatory monocytes. In addition, the induction of IFN requires internalization of TLR2, whereas signals leading to the production of TNF and IL-6 occur at the cell membranes.
Numerous studies have examined the function of the TLR2 subfamily. TLR2 knock-out mice have an increased susceptibility to infection with *Streptococcus pneumonia*, *Streptococcus aureus*, *Listeria monocytogenes* and other bacteria, suggesting a general role of TLR2 in the protection of the host from infectious diseases (Wetzler, 2003). Furthermore, receptor polymorphisms within the TLR2 subfamily are associated with various infections. For example, the R753Q variant of TLR2 is linked to increased susceptibility to tuberculosis as well as an acute rheumatic fever in children (Ogus et al., 2004). The I602S variant of TLR1 is impaired in cell-surface trafficking, but provides protection against leprosy in a Turkish population (Johnson et al., 2007). Given the role of TLR2 in infectious diseases, TLR2 ligands have constituted important vaccine adjuvants to modulate immune responses. Synthetic lipopeptides, such as Pam3CKS4 and Malp-2, are currently under development as adjuvants. The Lyme disease vaccine contains an outer-surface lipopeptide A (OspA) from *Borrelia burgdorferi*, which is sensed primarily through TLRs 2 and 1 (Alexopoulou et al., 2002). Additional examples of antigens that are also TLR2 agonists include zwitterionic polysaccharides derived from group B Streptococcus and PorB porin from a nonpathogenic *Neisseria* strain (Gallorini et al., 2009; Liu et al., 2008b). In addition, some natural ligands of TLR2, when conjugated with a carrier protein antigen, have been shown to greatly enhance vaccine immunogenicity (Jackson et al., 2004).

TLR2-dependent reactions also contribute to chronic inflammation. Studies in TLR2 knock-out mice support a role for TLR2 in the promotion of atherosclerosis, type 1 diabetes, asthma, arthritis and ischemic reperfusion injury. The potential ligands that stimulate TLR2 in these diseases could be microbial antigens as well as endogenous
products. In humans, a higher expression level of TLR2 has been demonstrated in patients with type 1 diabetes, rheumatoid rhinitis and inflammatory bowel diseases compared to healthy donors (Drexler and Foxwell, 2010; Ospelt and Gay, 2010). Taken together, these studies suggest that TLR2 may be a useful therapeutic target in preventing some of these diseases. Research on TLR2 antagonists and inhibitors of TLR2 signaling should provide more clues to the mechanisms by which TLR2 contributes to disease development.

**Human TLR10**

Despite extensive studies on TLRs in the past decade, TLR10 has remained an orphan member of the human TLRs whose ligand and function are not known. The studies described in this thesis mainly focus on this orphan receptor. TLR10 belongs to the TLR2 subfamily and is most closely related to TLR1 and TLR6 (Fig. 1.3). The genes for TLRs 10, 1 and 6 are tandemly arranged on chromosome 4. Phylogenetic analysis shows that the three genes arose from tandem gene duplication events of an ancestral gene. Phylogenetic analysis also predicts that TLR10 arose before the emergence of TLR1 and TLR6 (Roach et al., 2005). Studies on chicken TLRs have revealed that several chTLR1s share a phylogenetic clade with human TLR1-6-10 and when expressed, respond to lipopeptides with the cooperation of chTLR2 (Keestra et al., 2007; Temperley et al., 2008), which suggested that the ligand recognition function predates the mammalian divergence of the TLR1-6-10 gene cluster. Thus, TLR10 has been speculated to be a potential partner for TLR2. In addition, given the fact that the TLR2 subfamily has evolved under strong purifying selection, the independent maintenance of the TLR10 gene suggests a distinct function for this receptor (Roach et al., 2005).
The ECD of TLR10 contains 19 sequential LRR motifs flanked by an N-terminal cap and a C-terminal cap (Bella et al., 2008). Similar to TLR1 and TLR6, the sequence analysis of the TLR10 LRRs has revealed that the asparagine ladder is absent from LRR5-12 along with variable LRR lengths in this region, suggesting that TLR10 also has the three-domain architecture (N-term, central and C-term) in its extracellular portion (Jin and Lee, 2008).

A recent crystal structure of the TLR10 TIR domain revealed a dimer in the asymmetric crystal packing unit. The dimer interface is mainly composed of the residues from the BB loop, and this configuration was predicted to provide an extensive interface for binding to adapter molecules (Nyman et al., 2008). However, it is uncertain if the homodimeric structure of the TLR10 TIR domain seen in the crystal corresponds to a biologically relevant arrangement since it exists as a monomer in solution. Moreover, the TIR domains of TLR1 and TLR2 were each solved as monomeric units. A docking study performed on the TIR domains of TLR1 and TLR2 proposed that the DD loop in TLR2 is in close contact with the BB loop of TLR1 to form the TIR-TIR heteodimer. In this configuration, the residues Gly 676, His 646 and Tyr 737 in TLR1 appear to interact with residues in the TIR domain of TLR2 (Gautam et al., 2006). Interestingly, these three residues are conserved in TLR10’s signaling domain. On the other hand, a sequence alignment of TLRs 1, 6 and 10 reveals that there are also substantial sequence differences in the TIR domains of TLR10 compared to the other two receptors, including significant changes within the BB loop as well as a number of substitutions and insertions. These analyses imply that TLR10 might be a partner for TLR2 but may possess a distinct signaling function.
TLR10 has a unique expression profile compared to TLR1 and TLR6, as measured by TLR10 mRNA. Unlike the wide expression pattern of TLR1 and TLR6, TLR10 expression appears to be more tissue- and cell type-specific, indicative of the functional divergence of TLR10 from the other two receptors. TLR10 mRNA is highly expressed in lymphoid tissues such as the spleen, lymph nodes, thymus, tonsils, and lung (Chuang and Ulevitch, 2001). The analysis in isolated cell types has shown a high level TLR10 expression in the B cell lineage and weak expression in plasmacytoid dendritic cells (pDC), a cell type known to produce large amounts of IFNs upon recognition of viral components by TLRs (Bourke et al., 2003; Hasan et al., 2005; Hornung et al., 2002). A recent study has indicated that TLR10 is highly expressed in human regulatory T cells and that its expression is regulated through the cooperation between the transcription factors FOXP3 and NF-AT (Bell et al., 2007). Moreover, the expression level of TLR10 is elevated in B cells following cell activation through the B-cell receptor, or following stimulation with microbial products (Bourke et al., 2003).

The stimulation of B cells with TLR ligands, such as lipopeptides, LPS and CpG DNA, have been shown to induce cell proliferation, differentiation and IgM production (Ganley-Leal et al., 2006; Genestier et al., 2007). TLR signaling in B cells has also been shown to be required for inducing isotype switching in response to T-dependent antigens (Jegerlehner et al., 2007). Together, the expression of TLR10 on B cells and up-regulation upon cell activation suggest a potential role for this receptor in B cell biology.

Another unusual aspect about TLR10 is that it does not possess a mouse homologue due to the interruption of the mTLR10 gene by gaps and retroviral gene insertion (Hasan et al., 2005). However, TLR10 is conserved in rat, pig, cow and
macaque, among other mammals. The fact that TLR10 is a pseudogene in mice has prevented a functional assessment of this receptor using classical gene knock-out approaches.

**Thesis Outline**

Over the past 13 years, the importance of TLRs in driving innate and adaptive immune responses has been well established. TLR10 remains the only uncharacterized receptor among the human TLR family. Given its unusual expression profile and clear phylogenetic relationship with TLRs 2, 1 and 6, knowledge gained on this orphan receptor is useful to fully understand the function of the TLR2 subfamily and its possible linkage to adaptive immunity. The specific objectives of this study were to discover the ligand for TLR10, examine the mechanisms by which TLR10 recognizes its ligand, and analyze the role of TLR10 in innate immunity.

Chapter Two of this thesis describes a high-throughput chemical library screen developed to discover synthetic ligands for TLR10 as well as novel TLR2 agonists. This screen examined IL-8 promoter induction of a luciferase reporter gene as a measure of receptor activity. Additionally, TLR10, together with TLRs 2, 1 and 6, was expressed on SW620 cells to maximize the chances of identifying TLR10 activators. Although the screening system failed to discover TLR10 ligands, it did successfully uncover several novel TLR2-dependent activators that utilize TLR1, TLR6, or both as co-receptors. These novel small molecule compounds are structurally unrelated to any known TLR2 agonists. Functional analysis of various TLR mutants reveals that there are subtle differences in the mechanism of stimulation mediated by the synthetic compounds in comparison to natural
lipoprotein agonists. The functional implication of these results and the potential biologic importance of the novel TLR2 agonists are also discussed.

In studies described in Chapter Three of this thesis, I proceeded to define TLR10 ligands using different approaches. The generation and analysis of chimeric receptors, containing the extracellular recognition domain of TLR10 and the intracellular signaling domain of TLR1, revealed that TLR10, in cooperation with TLR2, senses triacylated lipopeptides and a wide variety of other microbial-derived agonists shared by TLR1, but not TLR6. Interestingly, two of the most potent novel chemical compounds discovered in Chapter Two were found to be agonists recognized only by TLR2/1 but not by TLR2/10. Computational modeling and mutational analyses defined the mechanism by which TLR10 recognizes lipopeptide ligands. The results show that TLR10 preserves the TLR2 dimer interface and the lipopeptide binding channel found in TLR1. However, TLR10, either alone or in cooperation with TLR2, failed to activate typical TLR-induced signaling including NF-κB, IL-8 or IFN-β driven reporters, which explains why I was unable to discover synthetic ligands of TLR10 in Chapter Two. Based on the results, it is concluded that human TLR10 cooperates with TLR2 in the sensing microbial products but possesses a unique signaling function.

Chapter Four of this thesis describes my efforts to examine the expression of endogenous TLR10 protein. Through the development of anti-TLR10 monoclonal antibodies, a flow cytometric analysis of TLR10 in human whole blood cells was undertaken in these studies. The expression profile observed could be very useful for predicting an immune function for TLR10. Finally, Chapter Five integrates research
findings from previous chapters, and presents contributions and implications of this work for the TLR field.
Figures

Figure 1.1. Human TLRs and their ligands\textsuperscript{1}.

\textsuperscript{1} Thanks to Dr. Richard I. Tapping for the figure.
Figure 1.2. Structure of the TLR2/1/Pam3CSK4 complex (Jin et al., 2007). The m-shaped structure of the TLR2/1/Pam3CSK4 complex. Image was generated using PDB file 2z7x through PyMol software.
Figure 1.3. Phylogenetic analysis of hTLR family members. The phylogenetic tree was derived from an alignment of the amino acid sequences for hTLR members.
CHAPTER TWO
IDENTIFICATION OF NOVEL SYNTHETIC TOLL-LIKE RECEPTOR 2 AGONISTS BY HIGH THROUGHPUT SCREENING

Introduction

Cellular innate immune responses drive the immediate release of pro-inflammatory mediators that enable leukocytes to access the site of infection as well as responses of professional antigen presenting cells essential for generating effective adaptive immunity (Fearon, 1997; Medzhitov and Janeway, 1997b). The primary triggers of these responses are a family of pattern recognition receptors known as Toll-like receptors (TLRs). Humans possess 10 TLR family members, numbered 1 through 10, subsets of which are expressed in leukocytes and the epithelial cells of mucosal surfaces (Muzio et al., 2000; Zaremerber and Godowski, 2002). There are two major types of TLRs, those that reside in intracellular compartments and sense viral and bacterial nucleic acids and those that are expressed on the cell surface and sense outer membrane components of bacteria, fungi and protozoan organisms (Akira et al., 2006; Iwasaki and Medzhitov, 2004). TLRs also recognize numerous molecules arising from damage to self tissues and this mode of TLR activation appears to play a central role in a number of non-infectious chronic inflammatory conditions (O'Neill et al., 2009; Zhang and Schluesener, 2006).

While there are marked differences in signaling and gene induction across various TLRs, all cell surface TLRs engage a core signaling pathway culminating in the activation of NF-κB and the production of proinflammatory chemokines, cytokines and cell adhesion molecules (Kawai and Akira, 2007; O'Neill and Bowie, 2007).

TLR2 is a cell surface receptor that senses a remarkable variety of bacterial, fungal and viral products as well as inflammatory self components. Among TLR2 agonists, bacterial lipoproteins are by far the most potent (Aliprantis et al., 1999; Brightbill et al., 1999). Additional TLR2 agonists comprise a diversity of structures including bacterial and fungal lipids, acylated sugars and proteins, unmodified protein complexes as well as certain polysaccharides (Miyake, 2007; Tapping, 2009). TLR2 needs to form heterodimers with either TLR1 or TLR6 to generate signals and these TLR2/1 and TLR2/6 complexes discriminate different microbial products (Ozinsky et al., 2000). For example, triacylated bacterial lipoproteins (mimicked by the lipopeptide Pam3CSK4) and diacylated lipoproteins (including the lipopeptide MALP-2, from mycoplasma) activate cells through TLR2/1 and TLR2/6 heterodimers, respectively (Takeuchi et al., 2001; Takeuchi et al., 2002). We have recently found that similar to TLR1, TLR10 also cooperates with TLR2 in the recognition of triacylated lipopeptides (presented in Chapter Three) (Guan et al., 2010). Additional co-receptors are essential for recognition of certain TLR2 agonists and thereby serve to increase the repertoire of agonists for this receptor (Miyake, 2007; Tapping, 2009).

Given their therapeutic potential, there is considerable interest in pharmaceuticals that modulate TLR activation. TLR antagonists hold great clinical promise for the treatment of numerous inflammatory conditions and are under investigation for the treatment of viral infections, redirecting allergic helper T cell responses, and as anticancer therapeutics (Kanzler et al., 2007; Makkouk and Abdelnoor, 2009; O'Neill et al., 2009). Some TLR agonists also have proven safety and efficacy in humans as vaccine adjuvants and are currently in use in Europe (Casella and Mitchell, 2008; Kanzler et al.,
2007). Synthetic lipopeptide agonists for TLR2 exhibit strong adjuvant activity when either mixed or directly conjugated to various antigens (Eriksson and Jackson, 2007; Moyle and Toth, 2008). In addition to lipopeptides, a variety of other natural TLR2 agonists exhibit adjuvant activity including zwitterionic polysaccharides from Group B Streptococcus (Gallorini et al., 2009), Type IIb heat labile enterotoxin from enteropathogenic Escherichia coli (Liang et al., 2009b), and porin B from pathogenic Neisseriae spp. (Liu et al., 2008b).

Despite their potential clinical utility, no high throughput screens for synthetic compounds that stimulate TLR2 have been reported. In this chapter, the identification of novel synthetic TLR2 agonists by chemical library screening is presented. The structures of these small agonists are unrelated to any known natural agonists for TLR2. Mutagenesis studies indicate that TLR2/1 recognizes the compounds through mechanisms different from that of microbial lipopeptides. The compounds induce cytokine production in human peripheral blood monocytes suggesting they are worthy of further clinical development.

**Materials and Methods**

*Reagents-* D-Luciferin and coenzyme A trilithium salt were purchased from Sigma-Aldrich Corporation. Luciferin solution and luciferase assay buffer have been described elsewhere (Pazzagli et al., 1992). The synthetic bacterial lipopeptides Pam3CSK4, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteiny(lysyl)3-lysine and PamOct2CSK4, N-palmitoyl-S-[2,3-bis(octanoyloxy)-propyl]-(R)-cysteiny(lysyl)3-lysine were obtained from EMC Microcollections. R isomer of MALP-2, S-[2,3-bis(palmityloxy)-propyl]-(R)-cysteiny-GNNDESNIKFKEK (macrophage-activating
lipopeptide-2) was purchased from Alexis Biochemicals. Chemical compound A (N-methyl-4-nitro-2-[4-(4-nitrophenyl)-1H-imidazolyl]aniline), Compound B (methyl 2-[(anilinocarbonothioyl)amino]-4,5,6,7-tetrahydro-1-benzo thiophene-3-carboxylate), Compound C (ethyl 2-([(4-methoxyphenyl)amino]carbonothioyl]amino)-4,5,6,7-tetrahydro-1-benzo thiophene-3-carboxylate), Compound E (N1-(4-chlorobenzyl)-N2-(4-methylphenyl)-N2-(methylsulfonyl)glycinamide) and Compound F (N2-(4-bromophenyl)-N1-(4-methoxybenzyl)-N2-(phenylsulfonyl)glycinamide) were individually purchased from Chembridge Corporation. Compounds were dissolved and diluted in dimethyl sulfoxide (DMSO).

**Plasmid Constructs-** A common variant of human TLR1 represented by NCBI accession number AAI09095 is referred to as wild-type TLR1 in this paper and was used as the basis for generating various chimeric variants and mutants. The generation of TLR1 and TLR6 chimeric receptors were described previously (Omueti et al., 2005). The P315L polymorphic variant of TLR1 was generated as previously described based on the technique of overlap extension PCR (Omueti et al., 2007). TLR1 point mutants were generated by random mutagenesis using error-prone PCR (Cirino et al., 2003). Briefly, the central region of TLR1 was excised from a modified FLAG-tagged TLR1 construct with XbaI (underlined letters) sites flanking LRRs 9-12 (Omueti et al., 2005). Error prone PCR was performed on this fragment using forward 5’-CCAATCTAGAAACAACCTTGGAATTCTTTCAATTAGGATCC-3’ and reverse 5’-CCAATCTAGATTTTAAGGTAAGACCCCTTTTGAGTTTGG-3’ primers. Error prone fragments were reinserted into TLR1 and the clones were screened by Clone Checker (Invitrogen, Carlsbad, CA) and verified by DNA sequencing. Screening of the library
revealed several TLR1 variants (V311E, F314D, Q316K, Y320N, E321V, I328N, V399D) with reduced responses to Pam3CSK4 (Guan et al., 2010; Liang et al., 2009a).

**Cell Culture-** All cultured cells were grown at 37°C in a humidified environment containing 5% CO2. Human colonic epithelial SW620 cells and murine macrophage RAW 264.7 cells were cultured in RPMI 1640 medium containing 10%(v/v) fetal bovine serum (FBS) and 2mM L-glutamine. Murine peritoneal macrophages were cultured in RPMI 1640 medium supplemented with 10%(v/v) FBS, 2mM L-glutamine, 100μg/ml streptomycin and 100U/ml penicillin. Human peripheral blood monocytes were cultured in RPMI 1640 medium supplemented with 10% autologous plasma and 2mM L-glutamine.

**Chemical Library Screening-** The chemical library was composed of approximately 24,000 compounds, among which 9,000 were from a private collection of the Department of Chemistry at the University of Illinois, and the rest were from Chembridge Corporation. All the compounds were kept in dimethylsulfoxide (DMSO) at 10mM in a 384-well plate format. The human colonic epithelial cell line SW620 was transiently co-transfected with TLRs 1, 2, 6 and 10 along with a firefly luciferase reporter driven by the IL-8 promoter in a 10-cm tissue culture dish. One day post transfection, cells were trypsinized and reseeded into 384-well plates (Corning Corp., Corning, NY) at a density of approximately 10^4 cells per well in a total volume of 20 μl. The next day chemical compounds were added into each well using a 384-pin transfer apparatus which delivered 0.2~1μl of each compound at an approximate concentration of 100μM. Each plate contained wells treated with DMSO alone as a negative control as well as Pam3CSK4 and MALP-2 as TLR2 agonist positive controls. The cells were incubated
with individual compounds and controls for at least 6 hours followed by cell lysis in a volume of 20μl. Luciferase values were normalized to DMSO-treated cells to determine relative cell activation levels. Compounds with greater than 2.5 fold activity over DMSO alone were selected for a second round of screening. A total amount of 217 compounds were retested for TLR-dependent activity in SW620 cells transfected with the TLRs against those transfected with the empty expression vector pFLAG-CMV.

*Transient transfection assays*- SW620 cells were cotransfected with various TLR combinations along with an IL-8 promoter driven firefly luciferase reporter and a Renilla luciferase transfection control reporter. The amount of DNA used for transfection was 20ng/ml for TLR2, 180ng/ml for TLR1, TLR6, TLR10 or TLR1 variants, 150ng/ml for IL-8 driven luciferase gene and 50ng/ml for Renilla control. Transfection was mediated by using Fugene6 (Roche Applied Science, Indianapolis, IN) at a lipid volume to DNA weight ratio of 4:1. Two days after transfection, cells were stimulated with indicated agonists for at least 6 hours and cell lysates were collected. Luciferase enzyme activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI). The values of firefly luciferase were first divided by that of Renilla luciferase values to normalize the transfection efficiency in different wells. All the values were then normalized to those of unstimulated cells with empty pFLAG-CMV vector and reporters to determine the relative luciferase activity.

*Stimulation of mouse peritoneal macrophages*- All animal experiments were conducted in accordance with a protocol approved by the University of Illinois Institutional Animal Care and Use Committee. Wild Type C57BL6 mice were purchased from The Jackson Laboratory. TLR deficient mice were kind gifts of Dr. Shizou Akira
Murine peritoneal macrophages were isolated from the mouse peritoneal cavity. Peritoneal cells were suspended in the medium described above and incubated in tissue culture plates for 2 hours. The non-adherent cells were removed by medium change. Adherent cells were initially seeded at $2 \times 10^5$ cells per well in 96-well cell culture plates. Cells were treated with indicated compounds, lipopeptides or DMSO controls for 16 hours. The concentration of mouse IL-6 or TNF-α in culture supernatants were determined by ELISA according to manufacturer’s instructions (Invitrogen, Carlsbad, CA).

**Stimulation of human peripheral blood monocytes** - Blood was obtained from healthy donors in accordance with a protocol approved by the University of Illinois Institutional Review Board. Human peripheral blood mononuclear cells were isolated from blood of healthy donors by Ficoll gradient centrifugation. CD14+ monocytes were further purified by magnetic negative selection (Miltenyi Biotec, Gladbach, Germany). The purified monocytes were cultured in the medium described above at density of $1 \times 10^5$ cells per well in 96-well plates. Cells were treated with indicated compounds, lipopeptides or DMSO controls for 16 hours. The concentration of human TNF-α in culture supernatants were determined by ELISA according to manufacturer’s instructions (Invitrogen, Carlsbad, CA).

**Results**

**Identification of novel TLR2 agonists by chemical library screening** - The Tapping laboratory has previously shown that SW620, a human colonic epithelial cell line lacking endogenous expression of TLRs 1, 2, 6, and 10, can be used to reconstitute receptor activity following transient transfection (Omueti et al., 2005). In these transfection assays,
a firefly luciferase gene driven by the promoter of the IL-8 provides a robust reporter of TLR2/1 and TLR2/6 activity. To identify novel chemical compounds that activate TLR2 heterodimers and discover the synthetic ligands for TLR10, I adapted this assay (co-transfection of SW620 with TLRs 1, 2, 6 and 10 as well as the reporters) to a 384-well format and screened a chemical library comprised of 24,000 individual synthetic compounds. The primary screen, based on a 2.5 fold enhancement of luciferase activity, yielded 217 initial hits. These 217 compounds were comparatively rescreened against SW620 cells transfected with empty FLAG-CMV vector to exclude compounds that activate the reporter in a TLR-independent fashion. This rescreening identified a total of 16 compounds with reproducible TLR2-dependent activation. In order to discriminate between TLR2/1, TLR2/6, and TLR2/10 mediated activation, cells transfected with different combinations of TLRs were examined for their response to the 10 most potent compounds (Fig. 2.1A). Most of the compounds were active toward cells co-expressing TLR2 and TLR1, while compound F was active toward cells expressing TLR2 and TLR6. Compound E appeared to be an agonist for both TLR2/1 and TLR2/6 receptor pairs.

Neither TLR10 alone nor TLR10 paired with TLR2 was responsible for any cellular activation. The signaling of TLR10 was later found not to be able to activate the IL-8 promoter (see Chapter Three for details). In addition, transfection of TLR2 alone did not render SW620 cells responsive to any of the compounds supporting the idea that TLR2 requires other TLRs for activity.

All of the active compounds have low molecular masses ranging from 300 to 500 Daltons and are structurally unrelated to any known TLR2 agonists (Fig. 2.1B). Two of the most active compounds, B and C, share the same core structure consisting of 3-
carboxylbenzothiophene linked via a carbonothioylamino bridge to an anilino group. It should be also noted that compound E and F, which activate cells through TLRs 2 and 6, also contain the same core chemical structure comprised of N1-(benzyl)-N2-(phenyl)-N2-(sulfonyl)glycinamide. The independent identification of compounds with similar core structures provides confidence that my screen has identified bona-fide TLR2 agonists. The compounds represent the physically smallest agonists for TLR2 reported to date.

*Compounds A, B and C are potent and TLR specific*- Compounds A, B and C were selected for further analyses as they exhibited the highest levels of TLR-dependent stimulation. The activation of TLR2/1 was detected at compound concentrations as low as 30 nM (10 ng/ml) and stimulatory activities comparable to that of Pam3CSK4 were achieved when the concentration reached 3 μM (1 μg/ml) (Fig. 2.2). The does-response curves of compounds A and B continued to rise over a broad concentration and saturable levels of reporter activation were not observed even at 30 μM (10 μg/ml). In contrast, despite structural similarity to compound B, compound C appears to reach saturable activation levels at a concentration of 300nM (100 ng/mL). Notably, none of the three compounds exhibited any activity toward TLR2/6, even at the highest concentration tested.

To confirm the specificity of the compounds for TLR1 and TLR2, I treated murine peritoneal macrophages derived from wild type, TLR1, TLR2, and TLR6 knockout mice with the compounds and measured stimulation of IL-6 production (Fig. 2.3A). The control stimuli were triacylated lipopeptide PamOct2CSK4 and diacylated lipopeptide MALP-2, which have been identified as strong murine TLR2/1 and TLR2/6 agonists, respectively (Buwitt-Beckmann et al., 2006; Takeuchi et al., 2001). As
expected, macrophages from both TLR2 and TLR6 deficient mice were unresponsive to MALP-2 stimulation while those from TLR1 deficient mice responded well. Conversely, the triacylated lipopeptide PamOct₂CSK₄ showed reduced activity toward both TLR1 and TLR2 deficient cells but not TLR6 deficient cells. Compound A activated murine macrophages in both a TLR1 and a TLR2 dependent manner confirming its specificity toward these two receptors. In contrast, compounds B and C did not stimulate cytokine production even from macrophages derived from wild type mice. These results suggest that compounds B and C activate human but not mouse TLRs. To confirm this finding I examined the activity of the compounds toward RAW 267.4 cells (Fig. 2.3B). While compound A was active, compounds B and C failed to induce TNF-α production in this murine derived macrophage cell line. These results show that compound B and C exhibit species-specific activity for human, but not mouse TLR2/1. While inactive, neither compounds B or C were observed to have antagonistic activity for mouse TLR2 (data not shown).

*Compounds A and B do not antagonize or synergize with lipopeptides*- Given the distinct structural features between the compounds and the lipopeptide agonists, I next tested the ability of these agonists to exhibit synergy in the activation of TLR2/1. Cells co-expressing TLRs 2 and 1 were stimulated with various concentrations of Pam₃CSK₄ together with a constant concentration of compound A or compound B (Fig. 2.4A). An additive response was observed suggesting a lack of any synergistic interaction. As the concentration of Pam₃CSK₄ exceeded 10 ng/ml, the more potent stimulatory effect of Pam₃CSK₄ began to overshadow that of the compounds suggesting that the former has a higher binding affinity for TLR2/1 compared to the latter. Given their weaker activity, I
next examined whether the compounds could act as antagonists toward Pam$_3$CSK$_4$. To this end, cells were incubated with compound A or compound B for 30 minutes prior to stimulation with Pam$_3$CSK$_4$ (Fig. 2.4B). No inhibition was observed suggesting that the weaker stimulatory activity of the compounds was due to either weaker receptor affinity and/or occupation of unique binding sites on the receptors compared to the lipopeptides. While compound A and compound B are structurally distinct small-molecule activators, I have observed that they do not exhibit synergism or antagonism with respect to each other (data not shown).

*The central LRRs of TLR1 are essential for activation by the compounds*- The Tapping laboratory have previously found that the central LRRs of TLR1 and TLR6 are responsible for discriminating between Pam$_3$CSK$_4$ and MALP-2 agonists, respectively, using domain swapping experiments in which LRRs were exchanged between these two receptors (Omueti et al., 2005). In this approach, all of the chimeric receptors maintain activity toward one of the lipopeptide agonists, indicating that in all cases the solenoid structure is preserved. To define the region of the extracellular domain in TLR1 responsible for compound recognition, I examined the ability of the compounds to activate the chimeric receptor constructs in conjunction with TLR2 following transient transfection in SW620 cells (Fig. 2.5). As observed previously, wild type TLR1, in cooperation with TLR2, mediated robust responses to both compounds and Pam$_3$CSK$_4$. An N-terminal exchange construct T6(1-8)/T1, in which LRRs 1 through 8 of TLR1 were replaced with those of TLR6, was activated by the compounds and by Pam$_3$CSK$_4$. However, when the N-terminal replacement of TLR1 with TLR6 was extended to LRR12 (construct T6(1-12)/T1), responses to all the agonists were completely lost. Additionally,
the reverse chimera T1(1-8)/T6, in which the first 8 LRRs of TLR6 were replaced with those of TLR1, was completely inactive toward all the agonists. Only when the first 12 N-terminal LRRs of TLR1 were replaced with those of TLR6, did the resulting chimera T1(1-12)/T6 exhibit any activity toward the compounds and Pam3CSK4. It is worthy to note that the activation of T1(1-12)/T6 by compounds B and C was barely detectable. Finally, an internal swap chimera T1(6-17)/T6, in which LRRs 6 through 17 of TLR6 replaced those of TLR1, restored activity to all of the agonists. Taken together, these results indicate that similar to Pam3CSK4, the central extracellular domain comprising LRRs 9 through 12 of TLR1 is required for recognition of compound agonists.

Compound-mediated activation of TLR2/1 is distinct from that of Pam3CSK4-
Sequential LRR motifs form spring-like structures in which each LRR contributes a single turn with hydrophobic residues buried in the interior of the solenoid (Bella et al., 2008; Jin and Lee, 2008). The crystal structure of the human TLR2/TLR1/Pam3CSK4 complex reveals that the TLR1 and TLR2 solenoids form an m-shaped heterodimer in which the Pam3CSK4 ligand is coordinately bound. In the complex the two acyl chains of the diacyl glycerol unit of Pam3CSK4 are bound to a hydrophobic pocket of TLR2, whereas the third amide-bound acyl chain of the ligand interacts with a more narrow hydrophobic channel within the central region of TLR1 (Fig. 6A). In addition to sharing ligand binding, the receptors themselves are predicted to make direct contacts in this central region through a number of hydrogen bonds and hydrophobic interactions (Jin et al., 2007).

Given the importance of the central LRRs in the TLR2/1/lipopeptide complex, a random mutagenesis library was generated in which TLR1 variants with single amino
acid changes were created. Through screening of the library several TLR1 variants were identified that affect responses to Pam3CSK4 (Liang et al., 2009a). To further explore the mechanisms by which TLR recognition of the small compounds and Pam3CSK4 leads to cellular activation, I compared the responses of TLR1 point mutant receptor variants to the different TLR2/1 synthetic agonists. Tyr320 of TLR1 is predicted to make hydrophobic interactions with residues Leu324 and Tyr323 of TLR2. There are also predicted ionic interactions between Glu321 of TLR1 and Arg321 of TLR2 (Fig. 2.6A). The individual substitution of these TLR1 residues results in an overall partial loss of responses to both Pam3CSK4 and the compounds (Fig. 2.6B). A charged amino acid substitution at Val339 of TLR1, which engages the center of a hydrophobic patch of TLR2 comprised of Phe322, Phe 349 and Leu371, resulted in a dramatic and uniform loss of receptor activity to all the agonists. These results suggest that unimpaired receptor dimer interaction is required to mediate efficient responses to both the chemical compounds and to the lipopeptides.

Several of the point mutants involve amino acid changes at positions 311 to 316 of TLR1. These residues form a loop that contributes to the receptor dimer interface and also reside at sites of interaction with the lipopeptide (Fig. 2.6A). In the TLR2/1/lipopeptide complex, Val311 of TLR1 appears to make hydrophobic contacts with a hydrophobic patch of TLR2 contributed by Leu350, Pro352 and Tyr376. A TLR1 variant in which Val311 of the loop is replaced with Glu retained activity toward Pam3CSK4 but exhibited highly attenuated responses to compound A and partial activity to compounds B and C (Fig. 2.6B). When Phe314, which appears to orient the protein backbone of the loop through intramolecular contacts with TLR1, is substituted with a
charged Lys residue the responses to either lipopeptide or compound A are almost completely abolished, but those to either compound B or compound C are partially preserved. Pro315Leu is a naturally occurring polymorphism of TLR1 that exhibits highly attenuated responses to Pam₃CSK₄ (Omueti et al., 2007). This proline residue resides in the channel that accommodates the peptide and is predicted to engage TLR2 through hydrophobic interactions (Jin et al., 2007). Similar to lipopeptides, this receptor variant also displayed a highly attenuated activity to compound A, but responses to compounds B and C were partially retained (Fig. 2.6B). In addition to the acyl chains, TLR1 also interacts with Pam₃CSK₄ by forming a hydrogen bond between the side chain of Gln316 and the amide oxygen of the lipopeptide. The fact that substitution of Gln316 with lysine abrogated Pam₃CSK₄ stimulation, indicates that this is an important receptor-ligand interaction. Interestingly, this TLR1 variant retained greater than half maximal responses to all the chemical compounds. Taken together, the results suggest that while the gross configuration of the TLR2/1 heterodimer in similar for the different agonists, there are subtle differences at the receptor interface important for driving cellular activation.

The compounds stimulate TNF-α production from human peripheral monocytes I next evaluated the ability of compounds A, B and C to activate human monocytes isolated from peripheral blood of healthy donors (Fig. 2.7). I found that the compounds dose dependently activated TNF-α production in human monocytes. While all three compounds induced TNF-α release at micromolar concentrations, compounds B and C exhibited weaker activity than compound A. The fact that the compounds are able to
activate primary human cells suggests that they may have clinical utility, perhaps as novel vaccine adjuvants.

**Discussion**

In this study, I have adapted a cell based TLR2 activity assay to a format that permits screening of small chemical libraries for novel TLR2 agonists. The assay utilizes an SW620 epithelial cell line expressing TLRs 1, 2, 6 and 10 as well as a luciferase reporter driven by the promoter of the IL-8. This assay remains sensitive and robust, exhibiting a 30-50 fold increase in luciferase activity in response to TLR2 lipopeptide agonists, despite adaptation to a 384-well format in which measurements are derived from as few as 5,000 cells per well. An initial screen of 24,000 compounds provided 217 hits and a secondary screen, which permits as assessment of TLR2 dependency, revealed 5 compounds with greater than 5 fold activity. All 5 compounds share 5 or 6 atom-ring structures including phenol, thiophene and imidazole groups, which are absent in TLR2 natural agonists. Compounds B and C share a core (anilinocarbonothioyl) amino-benzothiophene structure. Similarly, compounds E and F are both sulfonylglycinamides with terminal phenyl groups. These compounds are the smallest TLR2 agonists identified to date and only slightly larger than the imidazoquinoline agonists for TLR7 and TLR8. The independent identification of structurally similar compounds from a diverse chemical library serves to validate the screening assay and demonstrates that it presents a robust method of identifying novel TLR2 agonists.

TLR activation involves agonist-induced receptor dimerization. This event brings together two receptor signaling domains which serve as platforms for the recruitment of adaptor molecules required to initiate signaling (Monie et al., 2009; O'Neill and Bowie,
The structure of the TLR2/1/Pam\textsubscript{3}CSK\textsubscript{4} complex reveals coordinate binding of the lipopeptide by both receptors in which two acyl chains are embedded within a hydrophobic pocket of TLR2 with the third amide linked acyl chain bound within a hydrophobic channel of TLR1 (Jin et al., 2007). Additionally, hydrogen bonds between the glycerol and peptide backbone of Pam\textsubscript{3}CSK\textsubscript{4} and the TLRs, as well as direct contacts between the receptors themselves at the receptor dimer interface, contribute to stable heterodimer formation. As diacylated lipopeptide agonists of TLR2/6 lack the third amide linked acyl chain, the TLR2/6/Pam\textsubscript{2}CSK\textsubscript{4} complex relies more heavily on these latter interactions. Indeed, the crystal structure reveals that the hydrophobic channel of TLR6 cannot accommodate long acyl chains due to blockage by two phenylalanine residues (Kang et al., 2009).

Assessment of both domain exchange and point mutants revealed that, similar to lipopeptides, activation by the compounds requires the central LRRs of TLR1. Strikingly, TLR1 residues Tyr320, Glu321 and Val339, which are farther removed from the Pam\textsubscript{3}CSK\textsubscript{4} binding site and appear to interact with TLR2 at the receptor dimer interface, make similar contributions to receptor activity independent of the agonist used. Taken together, these results suggest that the orientation and interactions between TLR1 and TLR2 in the activated heterodimer complex are similar irrespective of the agonist.

Despite these similarities, the lipopeptide and chemical agonists differ in their ability to activate TLR1 variants that possess individually altered residues located in the loop of LRR11. These residues line the lipopeptide binding pocket and many are presumed to interact with TLR2 at the receptor dimer interface. The stronger inhibitory effect of the Gln316 mutation on Pam\textsubscript{3}CSK\textsubscript{4} activation versus the compounds is
supported by the crystal structure which predicts a hydrogen bond between this residue and the peptide backbone of the lipopeptide. However the differential effects of individual TLR1 mutations at Val311, Phe314 or Pro315 are harder to explain and suggest that the contribution of these residues, either to a ligand binding pocket or to interactions at the receptor dimer interface, is subtly different dependent upon the agonist. As expected however, the effect of TLR1 mutations on the activity of the structurally related compound B and C agonists is similar. Interestingly, compounds B and C were inactive toward murine cells and thus exhibit species specificity for human but not mouse TLR2/1. To my knowledge, the only other species specific TLR2 agonist reported to date is a tri-lauroylated lipopeptide which stimulates mouse but not human TLR2 (Grabiec et al., 2004).

The activity of the TLR1 point mutants, and the fact that the bulky phenyl groups of the compounds are too large to be accommodated by the hydrophobic channel of TLR1, suggests that the compounds bind directly within the interface of the TLR2/1 heterodimer. It is important to note that even the most active compounds identified in my screen are approximately 3 orders of magnitude less potent in the TLR2 activation assay than the lipopeptide control agonists (Fig. 2.2). This is perhaps not surprising given that none of the screened chemical libraries contained compounds with long acyl chains which are known to contribute to ligand binding and heterodimer formation. Similarly, a wide variety of naturally occurring agonists which lack acyl chains are far less potent TLR2 activators than bacterial lipopeptides and lipoproteins (Miyake, 2007; Tapping, 2009). Since TLR2 mediates responses to such a wide range of agonists, additional ligand docking analyses, mutagenesis studies and crystal structures will be required to fully
understand the full scope of interactions that can ultimately drive heterodimer formation and activation of TLR2 complexes.

The incorporation of TLR agonists in vaccine development represents a promising mechanism to boost immune responses to infectious agents and tumor antigens. However, inflammatory toxicity associated with strong TLR agonists limits their broad application (Hauguel and Hackett, 2008; Lahiri et al., 2008). The TLR2-dependent adjuvants under development include lipopeptides, zwitterionic polysaccharides and larger bacteria-derived proteins (Eriksson and Jackson, 2007; Gallorini et al., 2009; Liang et al., 2009b; Liu et al., 2008b; Moyle and Toth, 2008). While I have not tested compounds A, B and C for adjuvanticity, I have observed that these synthetic TLR2 agonists weakly induce TNF-α production from human monocytes (Fig. 2.7). I have also found that even at high concentrations the compounds fail to activate TLR2 as potently as lipopeptides (Fig. 2.2). This may be of clinical benefit as such weak or partial agonists often avoid the toxicity associated with strong inflammation. For example, the lipopolysaccharide analogue monophosphoryl lipid A is a weak or partial TLR4 agonist and an effective adjuvant in Hepatitis B vaccine formulations approved in Europe and Argentina. The small size and defined chemistry of the compounds also favors direct conjugation to antigen, an approach with lipopeptide that elicits robust antibody responses to tumor antigens in mice (Ingale et al., 2007).

In conclusion, the high-throughput screening assay developed in this study has uncovered novel synthetic small molecule TLR2 agonists. Next generation analogues of the compounds may exhibit pharmacologic and clinically favorable characteristics and
will enable a closer examination of the structure-function relationship between agonists and TLR2 complexes.
**Figures**

*Figure 2.1. Activity and structure of novel chemical agonists for TLR2.* (A) SW620 cells were co-transfected with various combinations of TLRs and an IL-8 driven luciferase gene and seeded into a 384-well plate. Two days after transfection, cells were stimulated with the indicated compounds at a concentration of approximately 100 μM for at least 6 hr. Cells were then lysed and luciferase activities were measured. All values were normalized to those of unstimulated cells with reporter and empty FLAG-CMV vector. Each bar represents the average of two independent experimental values. (B) The chemical structures of the novel TLR2 agonists. Dashed lines shown the common structures identified for certain pairs of compounds.
Figure 2.2. Compound A, B and C are potent TLR2/1 agonists. SW620 cells were cotransfected with vectors encoding the indicated TLRs, an IL-8 driven firefly luciferase gene and a Renilla luciferase control. Two days after transfection, cells were stimulated with different concentrations of compounds A, B or C for 6 hours and cell lysates were analysed for dual luciferase activity. After correcting for transfection efficiency using Renilla luciferase, all values were normalized to those of unstimulated cells transfected with empty FLAG-CMV vector. Error bars represent the standard deviation of three independent wells stimulated with the indicated concentration of agonist.
Figure 2.3. Compound A, but not B and C, induces IL-6 production in murine macrophages in a TLR1 and 2 dependent manner. (A) Murine peritoneal macrophages prepared from wild type, TLR1-/-, TLR2-/- and TLR6-/- were plated at the density of 1x10^5 cells per well. Cells were then stimulated with 10 μg/ml of either compound A, B or C, 100 ng/ml of PamOct2CSK₄ or 100 ng/ml of MALP-2 for 16 hours. IL-6 release was measured in the culture supernatant by ELISA. (B) RAW cells were plated at a density of 0.8x10^5 per well and were stimulated with 5 μg/ml of either compound A, B or C for 16 hours, and TNF-α release was measured in the culture supernatant by ELISA. Error bars represent the standard deviation of values obtained from 3 independent wells.
Figure 2.4. *Compound A and B do not synergize or antagonize Pam3CSK4 activity.* SW620 cells were transfected with TLR2, TLR1, IL-8 reporter and Renilla transfection control. (A) Two days after transfection, cells were stimulated with increasing concentrations of Pam3CSK4 alone or Pam3CSK4 along with either compound A or B (1 μg/ml) for 6 hours. (B) Two days after transfection, cells were pre-treated with compound A or B (1 μg/ml) for 30 min, followed by stimulation with increasing concentrations of Pam3CSK4 for 6 hours. Luciferase activity was measured as described before. The error bars represent the standard deviation of values obtained from three independent wells.
Figure 2.5. The central extracellular region comprised of LRRs 9-12 of TLR1 is required for compound-mediated cell activation. SW620 cells were cotransfected with TLR2 and the indicated TLR1 chimeras, an IL-8-driven luciferase gene, and a Renilla transfection control. Cells were stimulated with 5 μg/ml of compound A, B or C or 20 ng/ml of Pam3CSK4 for 6 hours. Luciferase activity was measured as described before. Error bars represent the standard deviation of values obtained from three independent wells.
Figure 2.6. Compound-mediated activation of TLR2/1 is distinct from that of Pam3CSK4. (A) Electrostatic surface representations of the TLR1 and TLR2 heterodimer interface based upon the crystal structure of the TLR2/1/Pam3CSK4 complex (Jin et al., 2007). PyMol software was used to split open the TLR2/1/lipopeptide complex and each TLR was rotated 90 degrees to display the dimer interfaces of TLR1 and TLR2. Negative and positive charges are shown in red and blue, respectively. The lipopeptide agonist Pam3CSK4 is repeated in stick form in both images with carbons, nitrogens, oxygens, and sulfur are colored in green, blue, red and yellow, respectively. Mutated amino acid residues of TLR1 along with corresponding residues on TLR2 that are presumed to make interactions are indicated. (B) SW620 cells were cotransfected with TLR2 and the indicated TLR1 mutants, an IL-8-driven luciferase gene, and a Renilla transfection control. Cells were stimulated with 5μg/ml of compound A, B or C or 20ng/ml of Pam3CSK4 for 6 hours. Luciferase activity was measured as described before. The error bars represent the standard deviation from three independent wells. The experiment was repeated three times without significant differences between replicates. A representative experiment is shown.
Figure 2.7. Compounds A, B and C induce TNF-α production from human monocytes. Human peripheral blood monocytes were cultured at a density of $1 \times 10^5$ cells per well with increasing concentrations of compounds A, B or C for 18 hours. TNF-α release was measured in the culture supernatant by ELISA. Error bars represent the standard deviation of values obtained from three independent wells.
CHAPTER THREE
HUMAN TOLL-LIKE RECEPTORS 10 AND 1 SHARE COMMON
MECHANISMS OF INNATE IMMUNE SENSING BUT NOT SIGNALING³

Introduction

The innate immune system is the first line of defense in response to an invading pathogen. Cellular innate immune defenses are necessary for the direct killing of pathogens, and they also mediate the immediate release of pro-inflammatory mediators that enable immune cells to access the site of infection, as well as the responses of professional antigen presenting cells essential for generating effective adaptive immunity (Fearon, 1997; Medzhitov and Janeway, 1997a). Primary triggers of inflammatory and adaptive responses are the TLRs, a family of cell surface innate immune sensors that exist in organisms ranging from lower invertebrates to higher mammals. Mammalian TLRs alert the host to the presence of infection through direct recognition of conserved structural components of viruses, bacteria, fungi and protozoans (Akira et al., 2006; Iwasaki and Medzhitov, 2004). In addition to microbial and viral products, TLRs also sense molecules associated with damage to self tissues as well as host products of inflammation (Zhang and Schluesener, 2006).

Humans possess 10 TLR family members, numbered 1 through 10, subsets of which are expressed in leukocytes and the epithelial cells of mucosal surfaces (Faure et al.; Muzio et al., 2000; Zarember and Godowski). In accordance with their role in host defense, monocytes/macrophages express most TLRs (Muzio et al., 2000), while the

³ Adapted from Guan, Y., Ranoa, D.R.E., Jiang, S., Mutha, S.K., Li, X., Baudry, J., Tapping, R.I. *Journal of Immunology* 2010 May 1;184(9):5094-103.
expression of individual TLRs in dendritic cells depends upon their subtype (Barton and Medzhitov; Kadowaki et al., 2001). The microbial agonists for TLRs include a wide variety of structures. For example, TLRs 3, 7, 8 and 9 respond to nucleic acids from both bacteria and viruses and these intracellular family members induce the expression of type-1 interferons that possess potent antiviral activities (Krieg and Vollmer, 2007; Uematsu and Akira, 2007). In contrast, TLRs 1, 2, 4, 5 and 6 sense outer membrane components of bacteria, fungi and protozoan organisms and these receptors are expressed on the surface of mammalian cells. Although most TLRs signal as homodimers, TLR2 requires either TLR1 or TLR6 for activity (Ozinsky et al., 2000). TLR1/2 and TLR6/2 heterodimers discriminate between different microbial products which include bacterial lipoproteins, bacterial lipoteichoic acids, non-enteric bacterial lipopolysaccharides, fungal phospholipomannans, protozoan glycoprophatidylinositol anchors and mycobacterial lipomannans and phosphatidylinositolts (Akira et al., 2006). The cell surface TLRs engage a core signaling pathway leading to activation of NF-κB, AP-1 and other transcription factors that drive the production of proinflammatory chemokines, cytokines and cell adhesion molecules (O'Neill and Bowie, 2007).

Despite extensive research on the TLRs, human TLR10 has remained an orphan receptor without a known agonist or function. TLR10 was initially cloned in 2001 and shares highest homology with both TLR1 and TLR6 (Chuang and Ulevitch, 2001). In mammals, TLRs 10, 1 and 6 genes are tandemly arranged and appear to have arisen from duplication events. Phylogeny supports the idea that TLR10 arose before the gene duplication that generated TLR1 and TLR6 (Roach et al., 2005; Zhou et al., 2007). Chickens possess two TLRs that share ancestral origins with the TLR1-6-10 cluster and
two additional TLRs that share origins with TLR2 (Temperley et al., 2008). The fact that these chicken TLRs mediate responses to lipopeptides through cooperative interactions indicates that this recognition function predates the mammalian expansion of the TLR1-6-10 cluster (Hughes and Piontkivska, 2008). The TLR2 subfamily is thought to have evolved under positive and balancing selection and among primates TIR domains appear to have undergone purifying selection (Ferrer-Admetlla et al., 2008; Nakajima et al., 2008). The independent maintenance of TLR10 and its associated TIR domain suggest a distinct biological role for this receptor (Hughes and Piontkivska, 2008; Roach et al., 2005).

TLR10 is a somewhat unusual family member in that it is highly expressed in the B cell lineage suggesting that it plays a critical role in B cell function. The presence of sequence gaps and retroviral insertions reveals that mouse TLR10 is a pseudogene, a situation that precludes the generation and phenotypic assessment of a TLR10 knock-out mouse. In this chapter I report that human TLR10 shares a variety of agonists with TLR1 including a number of cell surface components of bacteria and fungi. Similar to TLR1 and TLR6, TLR10 requires TLR2 for recognition. However, I have found that TLR10 lacks downstream signaling typically associated with other TLR2 family members.

**Materials and Methods**

*Ethics Statement*- Veterinary care was provided by the clinical and technical staff of the Division of Animal Resources at the University of Illinois at Urbana Champaign which is an AAALAC accredited facility. All animal experiments were approved by the University of Illinois Institutional Animal Care and Use Committee.
Reagents- Synthetic bacterial lipopeptides, \( N\)-palmitoyl-\( S\)-[2,3-bis(palmitoyloxy)-propyl]-\( (R)\)-cysteiny1-\( (lysyl)3\)-lysine (Pam\(_3\)CSK\(_4\)), \( S\)-[2,3-bis(hydroxy)-propyl]-\( (R)\)-cysteiny1-\( (lysyl)3\)-lysine (PamCSK\(_4\)), \( N\)-palmitoly-\( S\)-[2-hydroxy-3-\( (palmitoyloxy)\)propyl]-\( (R)\)-cysteiny1-\( (lysyl)3\)-lysine (PamCysPamSK\(_4\)), and \( S\)-[2,3-bis(palmitoyloxy)-propyl]-\( (R)\)-cysteiny1-GNNDESNI SKEK (macrophage-activating lipopeptide-2 (MALP-2)) were purchased from EMC Microcollections (Tuebingen, Germany). The microbial-derived agonists, LPS derived from *Porphyromonas gingivalis*, heat-killed *Acholeplasma laidlawii* (HKAL), lipoteichoic Acid from *Staphylococcus aureus* (LTA-SA), and lipomannan from *Mycobacteria smegmatis* were purchased from Invivogen (San Diego, CA). Zymosan particles and heat-killed *Staphylococcus aureus* (HKSA) were purchased from Invitrogen Life Technologies (Carlsbad, CA). *Mycobacteria* membrane fractions were received from National Institute of Allergy and Infectious Diseases Contract N01 AI-75320 entitled "Tuberculosis Research Materials and Vaccine Testing". The *Escherichia coli* type II heat-labile enterotoxin LT-IIa B subunits (LT-IIaB) was from Dr. T. Connell (University at Buffalo, State University of New York, Buffalo, NY).

The monoclonal anti-FLAG antibody, HRP-conjugated anti-Flag (M2) mAb and anti-hemagglutinin (HA) affinity gel were purchased from Sigma-Aldrich (St. Louis, MO). HRP-conjugated anti-HA Ab were purchased from Miltenyi Biotec (Auburn, CA). The anti-human TLR1 antibody (clone GD2.F4) and anti-human TLR2 antibody (clone T2.5) were obtained from eBioscience (San Diego, CA). The secondary antibody, Biotin-conjugated donkey anti-mouse IgG (H+L) and a streptavidin-conjugated fluorophore
tertiary antibody were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

*Plasmid constructs* - The primers for construction of the plasmids in this study are listed in Appendix Table 1. All constructs were verified by complete sequencing of both strands of all recombinant insertions.

CD4-TLRs—the CD4-TLR4 construct was a kind gift from Dr. Charles Janeway Jr (Yale University, New Haven, CT). The coding region of mouse CD4 extracellular domain (ECD) was fused with that of the transmembrane (TM) and intracellular portions of TLR1, TLR2 or TLR10. Both PCR products were cloned together into pCDNA3.1.

ENA-78 reporter—the promoter region (approximately 660bp) of human neutrophil-activating peptide (ENA-78) gene was amplified from human genomic DNA (Corbett et al., 1994). The PCR product was then cloned into the pGL3 basic vector (Promega, Madison, WI).

TLR10 chimeric receptors and internal chimeras— TLR1-10, TLR10-1 and TLR10-6 were created by overlap extension PCR (Horton et al., 1990). TLR10 internal chimera T10 (6-17)/T1 and T10 (6-17)/T6 were generated using the unique TLR1 and TLR6 constructs made previously in which unique restriction sites were engineered at the end of LRR5 (Omueti et al., 2005). The LRR 6-17 region of TLR10 was amplified with appropriated enzyme sites at both ends and ligated into the digested products of TLR1 or TLR6.

TLR-ECD-Fc Fusions--Pure soluble forms of TLR1, TLR2, and TLR10 were produced in large quantities using the hybrid LRR technique developed by Jin *et al.* (Kim et al., 2007). In short, a truncated portion of the TLR ectodomain lacking the C-terminal
cap was fused to the highly conserved LRR C-terminal capping module of VLRB.61, a hagfish variable lymphocyte receptor. With the aid of the computer software DNAWorks (Bethesda, MD) (Hoover and Lubkowski, 2002), six adjacent sets of ~60-bp overlapping oligonucleotides were used to synthesize the sequence encoding amino acids 133-200 of the hagfish VLRB.61 clone (amino acid 133-200). Using overlap extension PCR, the VLR coding region was fused to the 3’-end of TLR 1, 2, and 10 ECD encompassing amino acids 22-476, 17-508, and 20-474, respectively. The PCR products were cloned as a BgIII/NheI fragment into a modified pDisplay vector containing an HA-tag upstream of the BgIII site and an Fc domain of the human IgG1 downstream of the NheI site (kindly provided by Dr. David M. Kranz, Department of Biochemistry, University of Illinois at Urbana-Champaign). A thrombin cleavage site (LVPRGS) was also added at the 3’-end of the TLRvlr hybrid to allow cleavage of the soluble TLR from the Fc fusion protein. A Flag-tagged TLR2vlr-Fc construct was also engineered. (The constructs of TLR-ECD-Fc Fusions were generated by another graduate student, Diana Rose Ranoa, in the Tapping laboratory.)

TLR10 mutants— Site-directed mutagenesis of the TLR10 mutants was performed in TLR10 chimeric receptor TLR10-1. Oligos encoding amino acid changes were designed and used to introduce mutations into the TLR10 template through PCR.

TLR Retroviral vectors—The retrovirus vector pMXs-IRES-GFP (Cell Biolabs, San Diego, CA) was modified by inserting the preprotrypsin signal sequence followed by a FLAG linker into BamH1 and Not1 site to generate pMX-preprotrypsin-FLAG plasmid. The coding sequences of TLR1, TLR10 and TLR10-1 were directly inserted into the modified retrovirus vector to generate pMX-TLR1, pMX-TLR10 and pMX-TLR10-1.
(The constructs of TLR Retroviral vectors were generated by a post-doctoral researcher, Xinyan Li, in the Tapping laboratory.)

Cell culture and stable cell lines- The human colonic epithelial cell line SW620 was cultured in RPMI 1640 media containing 10%(v/v) fetal bovine serum (FBS) and 2mM L-glutamine. 293T cells were cultured in DMEM medium supplemented with 10%(v/v) FBS, 2mM L-glutamine. The human TLR10 expressing, stable RAW 264.7 cell lines were obtained by nucleofection (Lonza, Basel, Switzerland) of HA-tagged TLR10 plasmid or an empty vector control into RAW 264.7 cells. After selecting in G418 (0.3 mg/ml) for 3 weeks, stable batch cell lines expressing high level of TLR10 were verified by both RT-PCR and flow cytometry using anti-HA antibody. Cells were cultured at 37°C in a humidified environment containing 5% CO2. (Stable RAW 264.7 cell lines were generated by another graduate student, Song Jiang, in the Tapping Laboratory.)

Stable HEK 293F cell lines expressing recombinant soluble forms of each TLR ECD-Fc fusion protein were generated by transfection of the recombinant TLR plasmids using the cationic lipid transfection reagent, 293fectin™ (Invitrogen Life Technologies). Cells were then cultured under G418 selection (0.25 mg/ml) for 3-4 weeks, and individual stable clones expressing high levels of recombinant TLR protein were isolated using limiting dilution cloning and selected by western blotting. Clones were cultured at 37°C, 8% CO2 in serum-free Freestyle 293F expression medium (Invitrogen Life Technologies). (Stable HEK 293F cell lines were generated by another graduate student, Diana Rose Ranoa, in the Tapping Laboratory.)

Transient transfection and dual luciferase reporters assay- SW620 cells or 293T cells were cotransfected with various TLRs combinations along with an experimental
promoter driven firefly luciferase reporter and a Renilla luciferase transfection control reporter (pRL-Null). Transfection was mediated by using Fugene6 (Roche Applied Science, Indianapolis, IN) at a lipid to DNA ratio of 4:1. Two days after transfection, cells were stimulated with indicated agonists for at least 6 hours and cell lysates were collected. Luciferase enzyme activities were measured using Dural-luciferase reporter assay system (Promega). The values of firefly luciferase were first divided by those of Renilla luciferase values to normalize the transfection efficiency among different wells.

*Preparation of MEFs and Retroviral transduction-* TLR1 deficient mice were a kind gift of Dr. Shizou Akira (Osaka University, Osaka, Japan). Murine embryonic fibroblasts (MEFs) were prepared from 13.5-day-old embryos and cultured in DMEM supplemented with 10% fetal bovine serum. Cells at passage 3, 4 or 5 were utilized for the experiments. For retrovirus infection, the 293T Amphi packaging cell line was transfected with retroviral vectors using Fugene 6 reagent (Roche Applied Science). The viral supernatant was harvested 48 hours post transfection and used to infect MEF cells by incubating with MEFs cells under 10ug/ml polybrene (Sigma-Aldrich) for 24 hours. After culturing cells in fresh DMEM medium for another 24 hours, the infected MEFs were collected and plated in 96-well plate at the density of 1x10^4 cells per well and cultured for 12 hours. For stimulation, cells were treated with increasing concentrations of Pam3CSK4 for 24 hours. The concentration of mouse IL-6 in the culture supernatant was measured by ELISA using paired antibodies (Invitrogen). (This work was performed by a post-doctoral researcher, Xinyan Li, in the Tapping laboratory.)

*Confocal Microscopy-* Stable cell lines of RAW 264.7 cells expressing HA-tagged TLR10 were grown on chambered microscope slides (Lab-Tek, Nalge Nunc,
Rochester, NY) and incubated with 2x10^6 zymosan particles/ml for 10 minutes. Cells were fixed in 4% paraformaldehyde for 20 min and permeabilized in acetone for 5 minutes at -20°C. Non-specific sites were then blocked by incubating for 30 minutes at 4°C in blocking buffer (PBS/10% rabbit serum/0.03% NaN3). HA-tagged TLR10 was detected by using mouse anti-HA monoclonal antibody (Sigma-Aldrich), biotin-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories), and Streptavidin conjugated Alexa 555 (Molecular Probes, Eugene, OR). Cells were then co-stained for mouse TLR2 using a directly conjugated TLR2.5-Alexa 488 antibody (eBioscience). All images were obtained with 40X objective and oil immersion using a Zeiss LSM510 (Carl Zeiss, Thornwood, NY) confocal microscope at the School of Molecular and Cellular Biology Imaging Facility (University of Illinois, Urbana, IL). Vector control cell lines did not exhibit staining for the HA tag. (This work was performed by another graduate student, Jiang Song, in the Tapping laboratory.)

**Immunoprecipitation and Western Blot-** HEK 293T cells were transfected in 10-cm tissue culture dishes with specific TLRs along with MyD88 (0.75 ug of HA-tagged TLR2, 2.25 ug of FLAG-tagged TLR1 or TLR10, 1 ug of FLAG-tagged MyD88). 24 hours post transfection, half the dishes were treated with Pam3CSK4 (200ng/ml) for 10min and all cells were lysed using RIPA buffer. Cell extracts were incubated with anti-HA affinity gel for 1 hour at 4 °C followed by extensive washes of the beads with lysis buffer. Samples were separated on 7.5% PAGE and transferred to Hybond-P membrane (GE healthcare, Piscataway, NJ). Western blotting was performed using HRP-conjugated anti-FLAG antibody (M2) or HRP-conjugated anti-HA antibody.
Protein purification- 293F cell lines stably expressing TLR-ECD-Fc fusion proteins were seeded at 0.3x10^6 cells/ml in serum-free medium and incubated with shaking for 5 days. Recombinant protein G sepharose beads (GE Healthcare; 1ml 50% slurry) were added to 1L of filtered culture supernatants, and stirred at 4°C overnight. Protein G beads were recovered by centrifugation at 3000xg for 15 minutes at 4°C and subsequently packed in a glass column connected to the AKTA prime purification system (GE Healthcare). The beads were washed with 30 ml binding buffer (20mM sodium phosphate buffer pH 7.0) at a flow rate of 1.0 ml/min, and the fusion protein was eluted with 0.1M glycine-chloride (pH 2.3) in 1.0-ml fractions into tubes containing 100ul of neutralizing buffer Tris-Cl (pH 9.0). The eluted protein was dialyzed overnight against PBS (pH 7.4) at 4°C. Proteins were concentrated using Amicon ultra centrifugal filter device with 10,000 nominal molecular weight limit (Millipore, Bedford, MA). The protein concentration was measured using bicinchoninic acid assay (BCA).

The TLRvlr-Fc fusion protein was then incubated with thrombin (Novagen, Gibbstown, NJ) at 25°C for 16-18 hours to facilitate the removal of the Fc tag, at an optimized concentration of 1U thrombin per 0.25 mg fusion protein. After thrombin cleavage, protein G beads were added to the protein samples to remove Fc fragments as well as uncut Fc-tagged TLRvlr in solution. After 2 hours incubation at 4°C, the slurry was passed through a spin filter (Novagen) to separate the beads containing Fc fragments and uncut proteins from the TLRvlr proteins. The purity of the hybrid proteins was determined by mass spectrometry (Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois at Urbana-Champaign) using MALDI as ionization
technique and sinapinic acid as a calibration matrix. Final protein concentration after thrombin cleavage was measured using the BCA assay.

Receptor-binding assays- HA-tagged TLR1vlr and TLR10vlr hybrid proteins (10μg/mL in PBS pH 7.4) were coated in microtiter wells overnight at 4°C. For all ELISAs binding steps were performed at room temperature in MES buffer (pH 7.5) and wells were washed with PBS containing 0.05% Tween-20. After blocking with a commercially-available blocking buffer (Pierce, Rockford, IL) for 2 hours, wells were washed and incubated with an equimolar amount of Flag-tagged TLR2vlr that had been pre-incubated for 2 hours at 25°C with various concentrations of the synthetic ligand Pam$_3$CSK$_4$, MALP-2, and Ac$_2$CSK$_4$, in MES buffer (pH 7.5). One set of preincubation reactions contained 20μg/ml blocking anti-TLR2 antibody (T2.5). After washing, wells were incubated with HRP-conjugated anti-Flag (M2) mAb (Sigma-Aldrich). Detection was performed by the addition of o-phenylenediamine (OPD) tablet (Sigma-Aldrich) dissolved in 0.05M Phosphate-citrate buffer (pH 5.0) containing 0.05% H$_2$O$_2$. The colorimetric signal was stopped with 4N H$_2$SO$_4$ and absorbance at 490nm was read using an ELISA plate reader. (This work and protein purification was performed by another graduate student, Diana Rose Ranoa, in the Tapping laboratory.)

Modeling of the TLR2/10/lipopeptide complex- The program MOE version 2006 (Chemical Computing Group, Montreal, Quebec, Canada) was used for all the modeling work. The amino acid sequence of the TLR1 ECD was aligned with the sequence of the TLR1 sequence as in the TLR10/TLR2/lipopetide complex (NCBI Protein Databank entry 2Z7X (Jin et al., 2007)), using the BLOSUM62 scoring matrix as implemented in MOE, and 10 models were generated using the HOMOLOGY function provided in the
MOE program. Each of these 10 models was submitted to an energy minimization using the CHARMM force field (Brooks et al., 2009) version 27 as implemented with MOE, with solvent effect implicitly described using distance-dependant dielectric and a cutoff for non-bonded interactions between 8 Å and 10 Å. During the homology modeling, the structure of TLR10 was modeled with the explicit inclusion of TLR2 and lipopeptide structures.

Results

TLR10 signaling is distinct from that of other TLR2 subfamily members- All TLRs are characterized by an N-terminal extracellular domain (ECD), comprised of leucine rich repeat motifs (LRRs), followed by a single spanning transmembrane domain (TM) and a C-terminal intracellular Toll/Interleukin-1 receptor (TIR) signaling domain. To assess the function of TLR10 without knowledge of the ligand, the ECD domain of the receptor was replaced with that of CD4; an approach that has previously been used to generate constitutively active forms of the TLRs (Medzhitov et al., 1997; Ozinsky et al., 2000). Following transfection, a CD4-TLR10 fusion failed to induce reporter gene expression from a variety of promoters including NF-κB, IL-8, and IFN-β (Fig. 3.1). In contrast, a CD4-TLR4 positive control activated expression of all of these reporter constructs. Since TLR10 is most highly related to TLRs 1 and 6, I hypothesized that, similar to these receptors, it may be a heterodimeric partner for TLR2. As expected, cotransfection of CD4-TLR1 and CD4-TLR2 induced activation of NF-κB and IL-8-driven luciferase reporters. In contrast, CD4-TLR10 in combination with CD4-TLR2 failed to activate any of the reporters (Fig. 3.1). Notably, cell surface expression of all the CD4-TLR chimeras was confirmed (Appendix Fig. A.1). Taken together, these results
suggest that CD4-TLR10, either alone or in combination with TLR2, fails to activate reporters commonly associated with TLR signaling.

_TLR10 is a partner for TLR2 and shares a variety of agonists with TLR1_- TLR1 and TLR6 enable TLR2 to discriminate between different microbial products including bacterial lipoproteins (Takeuchi et al., 2001; Takeuchi et al., 2002). For instance, the TLR2/1 dimer mediates responses to the triacylated lipopeptide Pam₃CSK₄, whereas the TLR2/6 dimer is required for detection of diacylated lipopeptides such as MALP-2. Functional analyses demonstrated that the receptor ECD is responsible for lipopeptide recognition and structural studies showed that the Pam₃CSK₄ ligand is coordinately bound by TLR1 and TLR2 (Jin et al., 2007; Omueti et al., 2005; Sandor et al., 2003). Given the phylogenetic relationship between TLRs 1, 6 and 10, I hypothesized that lipopeptides could also be agonists for the latter receptor. To investigate this hypothesis chimeric receptors were generated in which the ECD and TM domains of human TLR10 and TLR1 were swapped (Fig. 3.2A). After confirming the expression of the chimeric receptors on the cell surface (Appendix Fig. A.2), I assessed their ability to mediate cellular responses to lipopeptides using SW620 cells, a human epithelial cell line in which the activities of TLR2 subfamily members can be reconstituted due to lack of endogenous expression (Omueti et al., 2005). As expected, TLR1 enabled cells co-expressing TLR2 to respond to Pam₃CSK₄ (Fig. 3.2B). However, neither TLR10 nor the reciprocal TLR1-10 chimera, comprising the TLR1 ECD and TM domain fused to TLR10 signaling domain, could reconstitute lipopeptide responses when co-expressed with TLR2 (Fig. 3.2B). Importantly, TLR10-1, a chimeric receptor in which the ECD and TM domain of TLR1 was replaced with that of TLR10, mediated full responses to
Pam$_3$CSK$_4$ in cooperation with TLR2. Taken together, these results demonstrate that TLR10 cooperates with TLR2 in sensing of triacylated lipopeptides. The lack of response of TLR10/2 to Pam$_3$CSK$_4$ supports the idea that signaling from this receptor complex differs from that of related family members.

To fully confirm the role of TLR10 in sensing microbial lipopeptides, reconstitution experiments were performed using mouse embryonic fibroblasts (MEFs) derived from TLR1-deficient animals. The TLR1 deficient MEFs were transduced with retroviral vectors expressing human TLR1, TLR10-1, and wild type TLR10 and then cultured in the presence of varying concentrations of Pam$_3$CSK$_4$ (Fig. 3.2C). As reported previously, TLR1-deficient cells exhibit very weak responses to Pam$_3$CSK$_4$ (Takeuchi et al., 2002). As expected, retroviral expression of hTLR1 enabled higher sensitivity and robust responses of MEFs to Pam$_3$CSK$_4$. Importantly, TLR1 deficient MEFs reconstituted with chimeric TLR10-1 receptor also exhibited sensitivity and responses to Pam$_3$CSK$_4$ comparable to that of TLR1 transduced cells. These results demonstrate that chimeric TLR10-1 rescues the responses of TLR1 deficient cells to triacylated lipopeptides. Interestingly, production of IL-6 in TLR1 deficient MEFs transduced with virus expressing wild type TLR10 exhibited slightly enhanced dose-dependent responses to Pam$_3$CSK$_4$ compared to empty virus, suggesting that the cytoplasmic domain of TLR10 may weakly activate IL-6 production in these cells.

To better define the agonist specificity of TLR10, cells coexpressing TLR10-1 and TLR2 were stimulated with synthetic lipopeptide compounds and a variety of natural microbial agonists with differing specificity toward TLR1/2 or TLR2/6. PamCysPamSK$_4$ and PamCSK$_4$ are TLR2/1 specific agonists that are missing either one or both lipids of
the diacyl glycerol group of Pam3CSK4 respectively (Omueti et al., 2005). In contrast, MALP-2 is a TLR2/6 specific agonist that possesses the diacyl glycerol group but lacks the third acyl chain on the N-terminal cysteine residue. In combination with TLR2, TLR10-1 mediated responses to TLR1-specific, but not TLR6-specific, synthetic lipopeptides (Fig. 3.3A). Similarly, cellular responses to zymosan, atypical LPS and heat-killed microbes were mediated by TLR2 in combination with either TLR1 or TLR10-1. In contrast, cellular responses to lipoteichoic acid were instead enabled by TLR2 and TLR6, established receptor pairs for this agonist (Fig. 3.3B). Taken together, the data indicate that TLR10, coupled with TLR2, is able to detect a variety of microbial components and suggest that TLR10 shares agonist specificity with TLR1 but not with TLR6. A TLR10-6 chimeric receptor yielded results identical to those obtained with TLR10-1 supporting the idea that agonist specificity is dictated by the extracellular domains of the receptors (data not shown). Co-expression of wild type TLR10 with TLR2 did not reconstitute responses to any of the TLR2 agonists, further suggesting a unique signaling function for this receptor complex.

**TLR2 and TLR10 physically interact and colocalize in phagosomes**- TLRs 1, 2 and 6 are recruited to phagosomes where they sense and mediate phagocyte responses to incoming microbial cargo (Ozinsky et al., 2000; Underhill et al., 1999). To examine TLR10 trafficking during phagocytosis, HA-tagged human TLR10 was stably expressed in the mouse macrophage cell line RAW264.7. In the absence of the stimulus, TLR10 and TLR2 appear to be dispersed on the plasma membrane (Fig. 3.4). Upon incubation with zymosan particles both receptors are highly enriched and colocalize in early phagosomes. These results support the findings that TLR2 and 10 cooperate in mediating
responses to zymosan and show that both receptors localize to the phagosome during ingestion of whole microbes.

To determine if TLR2 and TLR10 physically interact, co-immunoprecipitation studies were performed using HEK cells transiently expressing affinity tagged versions of different TLR pairs. TLR10 was observed to co-immunoprecipitate with TLR2 and this apparent association between the receptors increased with the addition of Pam3CSK4 ligand (Fig. 3.5). As expected, a similar ligand-enhanced association was observed between TLR2 and TLR1 (Jiang et al., 2006; Johnson et al., 2007; Tapping and Tobias, 2003). In addition, the recruitment of the proximal signaling adaptor MyD88 to the receptor complexes was assessed in these experiments. The co-immunoprecipitation of MyD88 with TLR2 alone was barely detectable and was greatly enhanced by co-expression of either TLR1 or TLR10 in HEK cells (Fig. 3.5). The addition of Pam3CSK4 ligand greatly enhanced the apparent recruitment of MyD88 to either TLR2/1 or TLR 2/10 heterodimers. These results suggest that similar to TLR2/1 and TLR2/6 heterodimers, TLR2/10 signaling involves the recruitment of MyD88 to the activated receptor complex.

To further investigate ligand-induced physical interactions between TLRs, soluble forms of TLRs 1, 2 and 10 were produced using a hybrid LRR technique in which the ECD of each TLR was fused to a C-terminal LRR motif from hagfish (Jin et al., 2007). The physical association of purified TLR2 with TLR10 ECDs was studied using microtiter plate assays where TLR10 was immobilized on the plate followed by the addition of TLR2 alone or with various ligands. As shown in Fig.3.6A, TLR2 exhibited weak interaction with TLR10 which was greatly enhanced by the addition of Pam3CSK4
in a dose dependent fashion. Addition of an antagonistic anti-TLR2 antibody T2.5 (Meng et al., 2004) dose-dependently inhibited ligand-induced complex formation between TLR2 and TLR10. In contrast, the non-stimulatory control compound Ac2CSK4, lacking both the acyl- and amide-bound long chain fatty acids of Pam3CSK4, did not enhance physical association between TLR2 and TLR10 showing the requirement of lipid chains in formation of a stable heterodimer. Moreover, incubation with MALP-2, the diacylated lipopeptide agonist for TLR2/6, had no ability to induce a TLR2/10 complex. Collectively, these results demonstrated that the ECDs of TLR2 and TLR10 associate with each other to form a stable complex in a ligand-dependent manner. As expected, identical results were obtained using TLR1 as a control in place of TLR10 (Fig. 3.6B).

*Modeling of the TLR2/10/lipopeptide complex reveals structural similarity to the TLR2/1/lipopeptide complex*- Similar to TLR1 and TLR6, the ECD of TLR10 is comprised of 19 sequential LRR motifs. Structurally, LRRs form a solenoid or spring in which each LRR motif comprises a single turn with the leucines, or other appropriately spaced hydrophobic residues, packed within the interior (Bella et al., 2008; Jin and Lee, 2008). The solved crystal structure of the TLR2/1/lipopeptide complex reveals that the TLR2 and TLR1 solenoids form a heterodimer in which the lipid chains of Pam3CSK4 are coordinately bound (Jin et al., 2007). Two acyl chains of the diacyl glycerol group are accommodated by a hydrophobic pocket of TLR2 and the third amide-linked lipid chain occupies a hydrophobic channel in TLR1. The heterodimer is further stabilized by additional interactions at the dimer interface (Jin et al., 2007). Since TLR1 and TLR10 share 43% amino acid identity at the ECDs, identical lipopeptide specificity, and TLR2 as a coreceptor, computational modeling was deemed to be a reasonable approach for
gaining insights into the structure of the TLR2/10/lipopeptide complex. The computational model of TLR2/10/lipopeptide exhibits the same overall structure as that of the TLR1 containing complex (Fig. 3.7A). Similar to TLR1, the model predicts a hydrophobic channel on the convex surface of TLR10 which accommodates the amide-linked lipid chain of the Pam₃CSK₄ lipopeptide.

Prior to the solution of the TLR2/1/lipopeptide structure, domain swapping experiments between TLR1 and TLR6 successfully defined the central LRRs of these receptors as the region required for lipoprotein discrimination (Grabiec et al., 2004; Omueti et al., 2005). Since the ligand-binding and receptor dimerization region are predicted by the model to comprise the central LRRs of TLR10, the effects of exchanging LRRs 6-17 of TLR1 and TLR6 with that of TLR10 was examined as a first approach for validating the model and defining the region of the ECD required for lipopeptide recognition. As before, the ability of the chimeric receptors, in collaboration with TLR2, to mediate responses to different lipopeptides was assessed in SW620 cells (Fig. 3.7B). As observed previously, in conjunction with TLR2, TLR1 and TLR10-1 enabled cellular responses to Pam₃CSK₄, but not to MALP-2, whose activity was dependent upon TLR6. When LRRs 6-17 of TLR1 were replaced with the corresponding region of TLR10, T10(6-17)/T1), the receptor retained lipopeptide specificity. Additionally, when LRRs 6-17 of TLR6 were replaced with those from TLR10, the resulting chimeric receptor, T10(6-17)/T6, exhibited full activity toward Pam₃CSK₄, but complete loss of activity toward MALP-2. These results demonstrate that LRRs 6-17 of TLR10 are responsible for defining the lipopeptide specificity of the receptor and support the molecular model of the TLR2/10/lipopeptide complex.
TLR10 and TLR1 interactions with TLR2 are similar but not identical- The residues which contribute to the formation of the binding pocket are conserved between TLR1 and TLR10 and preserve the overall shape of the lipid-binding channel (Fig. 3.8A and 3.8B). Hydrogen bonds between the peptide portion of Pam3CSK4 and TLR1 are also conserved in the TLR10 model (Fig. 3.8B). To further validate and explore the structural model, site-directed mutagenesis was performed on the residues in the ECD that appear to be crucial for ligand binding and TLR2 dimer formation. All of the receptor mutants were expressed on the cell surface (Appendix Fig. A.3) and their ability to mediate cellular responses to Pam3CSK4 were assessed. To provide a functional readout in the SW620 epithelial cell system, the TLR10 mutants were generated within the TLR10-1 chimeric receptor. Corresponding amino acid residues in TLR1 were also assessed for comparative purposes. The model predicts that similar to TLR1, a loop comprising amino acids 311 to 316 contributes to the dimer interface and the entrance of the hydrophobic lipid binding channel (Fig. 3.8B and 3.8C). The orientation of the loop is important especially as the backbone oxygen between amino acids 313 and 314 forms a hydrogen bond with the lipoprotein (Fig. 3.8B). Phe314 of TLR1 and Ile314 of TLR10 are buried and make hydrophobic intramolecular contacts that appear to orient the protein backbone of the loop. Substitution with a charged lysine residue at position 314 almost completely abrogated the function of both receptors (Fig. 3.9A and 3.9B). In both TLR1 and TLR10, Val311 and Phe312 are positioned at the entrance of the channel; however substitution of either amino acid with a charged residue had little effect on the activity of either receptor to Pam3CSK4. Nearly complete loss of activity was observed following mutation of Gln316 whose side chain in both receptors forms a hydrogen bond with the amide oxygen
of the Pam₃CSK₄ lipid chain. The structural requirements for recognition of PamCysPamCSK₄ by either TLR1 or TLR10 is identical to that of Pam₃CSK₄ and as expected, the various TLR1 and TLR10 mutants exhibit indistinguishable responses to these two ligands (Fig. 3.9A and 3.9B).

Both TLR1 and TLR10 possess an extensive TLR2 binding interface comprised of a hydrophobic core surrounded by hydrogen bonding and ionic interaction networks (Jin et al., 2007) (Fig. 3.8C). In both receptor complexes, the hydrophobic core overlaps with the entrance of the lipid binding channel, however the surrounding polar and charged residues that make H-bonds and ionic interactions with TLR2 are less conserved between TLR1 and TLR10 (Fig. 3.8C). Hydrophobic interactions with TLR2 contributed by Pro315, Tyr320, Val339 and Leu359 of TLR1 are contributed by Leu342, Tyr320, Pro339, and Ile359 of TLR10, respectively (Fig. 3.8C). Substitution of either Tyr320, Pro339 or Ile359 with polar or changed amino acids dramatically reduced TLR10 activity towards Pam₃CSK₄ (Fig. 3.9A). Similarly, a Val339 mutant of TLR1 also exhibited greatly attenuated activity. In contrast to TLR10, the Tyr320 mutant of TLR1 retained most of the receptor activity. This residue is predicted by the model to make more intimate contact than TLR1 with the hydrophobic face of TLR2. Pro315 resides at the center of the hydrophobic core of TLR1 and interacts with Tyr323 of TLR2 (Fig. 3.8C). Substitution of Pro315 with leucine, which constitutes a naturally occurring polymorphic variant of TLR1, greatly attenuates receptor activity as reported previously (Omueti et al., 2007). Conversely, substitution of Glu315 of TLR10 with leucine, which is not predicted by the model to contribute to heterodimer formation, retained more than half of the receptor activity (Fig. 3.9). In conclusion, the structural model of the
TLR2/10/lipopeptide complex is consistent with and supported by the mutational studies. While strikingly similar in overall structure, the TLR1 and TLR10 containing complexes appear to possess subtle differences at the TLR2 dimer interface.

**Discussion**

TLRs play a central role in host defense by driving appropriate inflammatory and adaptive responses following infection. The early identification of microbial agonists for the TLRs was greatly facilitated by the systematic generation and phenotypic assessment of knock-out mice and provided the first insights into TLR function (Akira et al., 2006). However, this approach is not feasible for defining the function of TLR10 as multiple gaps and insertions have rendered the receptor a pseudogene in mice as evidenced by sequences from a number of inbred strains (Hasan et al., 2005). I have found that *Mus caroli*, which emerged approximately 4 million years ago and predates the *Mus musculus* group, also possesses a TLR10 pseudogene characterized by numerous gaps and insertions (data not shown). The absence of TLR10 in mice, coupled with a lack of understanding of TLR10 signaling, has precluded the identification of synthetic or natural agonists and have rendered this receptor the only remaining orphan human TLR.

I have performed experiments using chimeric receptors to overcome these obstacles based upon phylogenetic evidence that TLR10 is most related to TLR1 and TLR6, both of which independently cooperate with TLR2 in the sensing of a variety of microbial and fungal components. Through this approach, I report that TLR10 shares a number of microbial agonists with TLR1, but not TLR6, and utilizes TLR2 as a co-receptor. Notably, expression of a chimeric receptor, which replaces the TLR1 ECD with that of TLR10, fully reconstitutes responses of TLR1-deficient macrophages to
triacylated lipopeptide. TLR10 and TLR2 colocalize in the early phagosome and the ECDs of the two receptors physically interact in a ligand-dependent manner. A computational model of the TLR2/10/lipopeptide complex and mutagenesis studies reveal similarities, as well as some subtle differences, in the ligand binding and dimer interface in comparison with the TLR2/1/lipopeptide crystal structure. Both analyses have showed that the residues contributed to TLR2 dimer interaction are less conserved between TLR10 and TLR1. Therefore, it is not surprising that TLR10, in cooperation with TLR2, mediated limited responses to the novel synthetic TLR2/1 agonists discovered in Chapter Two. These low-molecular-weight agonists are predicted to bind directly within the interface of the TLR2/1 dimer (see details in Chapter Two). Only at concentrations higher than 10 µM, compound A exhibited some activity toward TLR2/10-1, while its activation of TLR2/1 was detected at a concentration of 30 nM (Appendix Fig. A.4 and Fig. 2.2). Neither compound B nor compound C exhibited any ability to activate the cells expressing the TLR2 and TLR10-1 even at the highest concentration tested, indicating these compounds are TLR1 specific agonists and not sensed by TLR10.

The idea that agonist recognition drives receptor dimer formation is supported by the finding that artificial chimeric receptors which replace the TLR ECD with either CD4 or integrin pairs, that form natural dimers, exhibit constitutive activation (Medzhitov et al., 1997; Ozinsky et al., 2000; Zhang et al., 2002). I have found that expression of CD4-TLR10, either alone or with CD4-TLR2, was unable to activate NF-κB, IL-4, IL-8, ENA-78 or IFN-β driven promoters in HEK293 or SW620 cells (Fig. 3.1 and data not shown). These findings are in contrast to a report showing that CD4-TLR10 activated NF-κB, IL-
4 and ENA-78 driven promoters in HEK cells (Hasan et al., 2005). Despite the ability to appropriately activate these promoters using either CD4-TLR4 or a combination of CD4-TLR1 and CD4-TLR2, I have been unable to detect TLR10-dependent activation of any of the reporter constructs (Fig. 3.1). In support of this work, an integrin-TLR10 chimera, either alone or with an appropriate integrin-TLR2 partner, does not activate NF-κB despite the fact that other integrin-TLR combinations are able to do so (Zhang et al., 2002). In addition, I found that either TLR1, TLR6 or TLR10-1, but not TLR10, induces NF-κB or IL-8 driven promoters in response to lipopeptide when coexpressed with TLR2 (Fig. 3.2).

TLR signaling is mediated by an intracellular Toll/IL-1 receptor (TIR) domain, so named because it shares homology with that of IL-1 receptor (O'Neill and Bowie, 2007; West et al., 2006). The appropriate association of two intracellular receptor TIR domains enables the recruitment of specific TIR domain-containing adaptor molecules which ultimately induce the expression of appropriate immune response genes (Kawai and Akira, 2007; O'Neill and Bowie, 2007; Sheedy and O'Neill, 2007; West et al., 2006). The crystal structures of the solved TIR domains of TLR1, TLR2 and TLR10 exhibit a conserved fold composed of five parallel β-sheets surrounded by five α-helical segments (Nyman et al., 2008; Xu et al., 2000). Multiple studies have shown that 2 exposed segments, designated the BB loop and the DD loop, are critical for dimer formation and adaptor recruitment which drive downstream signaling (Gautam et al., 2006; Poltorak et al., 1998; Toshchakov et al., 2005; Xu et al., 2000). The TLR10 TIR domain crystallizes as a dimer with juxtaposed BB loops (Nyman et al., 2008), however the idea that this constitutes a physiologically relevant form of the receptor is based upon receptor activity
experiments that I cannot reproduce (Hasan et al., 2005). Instead of BB loop interactions, rigid body protein-protein docking studies between the TIR domains of TLR1 and TLR2 reveal a different orientation which involves an interaction between the BB loop of one monomer and the DD loop of another (Gautam et al., 2006). In this configuration His646, Asn700, Gly676 and Tyr737, which are perfectly conserved between TLR1 and TLR10, comprise the TIR domain dimer interface with TLR2 (Gautam et al., 2006).

Despite conservation, there are substantial sequence differences between TIR domains of TLR10 and TLR1. Most notably, TLR1 and TLR6 have identical RNFVPG BB loop sequences while that of TLR10 consists of SYFDPG and also includes a two amino acid insertion in the preceding α-helix. These and other differences within the TLR10 signaling domain may contribute to a distinct charge distribution in TLR2/10 surface that confers a unique specificity for adapter binding and downstream signaling (Dunne et al., 2003). I have observed that the adaptor MyD88 co-immunoprecipitates with the TLR2/10 heterodimer and that this interaction is enhanced by lipopeptide agonist (Fig. 3.5). However, TLR2/10 fails to activate signals typically associated with MyD88 activity, including canonical activation of NF-kB, suggesting that recruitment of this adaptor to TLR2/10 is distinct from that of other TLRs including the closely related TLR2/1 and TLR2/6 heterodimers. The idea that recruitment of MyD88 can differ among TLR2 heterodimers is not without precedence as an I179N point mutation in this adaptor abolishes MyD88-dependent signaling of all TLR complexes, including TLR2/1, while having no effect on MyD88-dependent TLR2/6 signaling (Jiang et al., 2006).

In addition to phylogenetic evidence, the unique expression pattern of TLR10 further suggests a functional divergence from TLR1 and TLR6. In comparison to these
latter two receptors, TLR10 expression appears to be more restricted with RNA message found predominantly in lymphoid tissues including the spleen, lymph nodes, thymus and tonsils (Chuang and Ulevitch, 2001; Opsal et al., 2006; Shinkai et al., 2006). An analysis of isolated cell types reveals that high levels of TLR10 expression are restricted to the B cell lineage with weaker expression in plasmacytoid dendritic cells (Bourke et al., 2003; Hasan et al., 2005; Hornung et al., 2002). While TLR expression levels are generally low in naïve B cells, they are highly induced upon BCR stimulation (Bernasconi et al., 2003; Bourke et al., 2003). A variety of TLR agonists have been shown to act directly on B cells to induce antibody production to T-independent antigens (Vasilevsky et al., 2008) and to enhance proliferation, isotype switching and differentiation in response to T-dependent antigens (Meyer-Bahlburg et al., 2007; Pasare and Medzhitov, 2005; Ruprecht and Lanzavecchia, 2006). TLR7 and TLR9 agonists are generally the focus of many of these studies, especially given their potential utility as therapeutics (Kanzler et al., 2007) and their established role in the autoimmune condition lupus (Marshak-Rothstein, 2006). However, TLR2 agonists can also provide stimulatory signals to human B cells (Chiron et al., 2008; Ganley-Leal et al., 2006; Mansson et al., 2006). Memory B cells constitutively express a number of TLRs, including TLR2 and TLR10, and can be induced by TLR agonists to proliferate and differentiate to plasma cells (Bernasconi et al., 2003; Bourke et al., 2003). Concordantly, cell lines derived from mature B cell neoplasias express high levels of TLR10 and a variety of TLR ligands, including lipopeptides, enhance the survival of both multiple myeloma and chronic lymphocytic leukemia cells (Bohnhorst et al., 2006; Bourke et al., 2003; Muzio et al., 2009).
In an analogous manner, TLRs have also been shown to play a direct costimulatory role during T cell activation (Marsland and Kopf, 2007). Human T cells express high levels of TLR2 following T cell engagement and these cells produce elevated levels of cytokine in response to lipopeptide (Komai-Koma et al., 2004). TLR2 is constitutively expressed in memory T cells which exhibit enhanced responses to lipopeptides. Taken together with the observations in B cells, the results suggest that TLR10 plays an ancillary role in lymphocyte stimulation especially where the expansion and controlled maintenance of memory B and T cells are concerned. Further evidence that TLR10 plays a role in the control of adaptive immune responses is the finding that this receptor is expressed by regulatory T cells through transcription factors Foxp3 and NF-AT (Bell et al., 2007). It is worthy to note that TLR10 genetic variants have been associated with susceptibility to both extrapulmonary tuberculosis and to asthma (Lazarus et al., 2004; Ma et al., 2007), two diseases in which TLR2-mediated adaptive immunity is thought to play a major role.

The discovery that TLR10 is orthologous to TLR1 in ligand recognition but not signaling calls for a re-evaluation of studies where lipoproteins or other TLR2/1 agonists have been used in the stimulation of human and other non-murine mammalian systems. In vivo, the competition of TLR1 and TLR10 for both ligand and coreceptors such as TLR2 is likely to have significant functional consequences given the different expression patterns and signaling outputs of these two receptor complexes. In addition to providing a means to study TLR10 function, the discovery of lipopeptides and other microbial agonists for this receptor has important implications for the future therapeutic targeting of TLR2.
**Figure 3.1.** The TLR10 homodimer and TLR2/TLR10 heterodimer do not induce activation of NF-κB, IL-8 and IFN-β promoters. HEK 293 cells were cotransfected with indicated CD4-TLR constructs (400 ng/ml), various luciferase reporters (150 ng/ml) and a Renilla luciferase transfection control (50 ng/ml). Luciferase activities were measured 48 hours post transfection. Values represent the level of constitutive reporter activation over that of vector alone whose activity was taken as one. The error bars represent the standard deviation of three independent values.
Figure 3.2. TLR10 cooperates with TLR2 and senses synthetic lipopeptides. (A) Schematic diagrams of wild type or TLR1 and TLR10 chimeric constructs. (B) SW620 cells were cotransfected with the dimeric constructs (180ng/ml) along with TLR2 (20ng/ml), an IL-8 driven luciferase reporter and a Renilla luciferase transfection control as indicated. Two days after transfection cells were stimulated for 6 hours with 20ng/ml Pam3CSK4 followed by measurement of luciferase activity. Values represent the level of reporter activation over that of vector alone whose activity was taken as one. (C) MEFs were derived from TLR1 knock-out mice and transduced with retroviruses encoding the indicated constructs. The cells were subsequently incubated with increasing concentrations of Pam3CSK4 for 12 hours and IL-6 concentrations in the culture supernatant were measured by ELISA. Error bars represent the standard deviation of three independent values.
Figure 3.3. **TLR10 shares agonist specificity with TLR1.** SW620 cells were cotransfected with indicated combinations of TLRs, an IL-8 promoter driven luciferase reporter and Renilla transfection control reporter. Cells were stimulated for 6 hours with (A) 1µg/ml PamCSK₄, 20ng/ml PamCysPamCSK₄, 20ng/ml Malp-2, or (B) 10⁷ particles/ml zymosan, 2µg/ml LTA-SA, 20ng/ml *P. gingivalis* LPS (P.G. LPS), 200µg/ml heat-killed *S. aureus* (HKSA) or 10⁷ particles/ml heat-killed *A. laidlawii* (HKAL) followed by luciferase activity assays. Values represent the level of reporter activation over that of vector alone whose activity was taken as one. Error bars represent the standard deviation of three independent values.
Figure 3.4. TLR2 and TLR10 co-localized in phagosomes. RAW 264.7 cells stably expressing HA-TLR10 were either untreated or incubated with zymosan particles at 2x10^6/ml for 10 minutes before fixing. Cells were co-stained for endogenous TLR2 (Alexa 488) and HA-tagged TLR10 (Alexa 555) followed by confocal fluorescence microscopy.
**Figure 3.5. TLR10 interacts with TLR2 and the receptor complex recruits MyD88.** HEK 293T cells were co-transfected with indicated TLRs and FLAG-tagged MyD88. One day after transfection, cells were either untreated or treated with Pam₃CSK₄ at 200ng/ml for 10min. Cell lysates were immunoprecipitated with anti-HA antibody and samples were analyzed for the presence of TLR10, TLR1 and MyD88 using anti-FLAG antibody. Whole cell lysates (WCE) were analyzed to verify the expression of transfected TLRs and MyD88.

| HA-TLR2 | + + + + + + |
| FLAG-TLR1 | - - + + - - |
| FLAG-TLR10 | - - - - + + |
| FLAG-MyD88 | + + + + + + |
| Pam₃CSK₄ | - + - + - + |

**IP: Anti-HA**

- TLR10
- TLR1

**WB: Anti-FLAG**

- MyD88

**WCE**

- Anti-HA
  - TLR2
  - TLR10
  - TLR1

- Anti-FLAG
  - MyD88
Figure 3.6. Binding of TLR2 to either TLR10 or TLR1 is induced by Pam3CSK$_4$. (A) HA-tagged TLR10 ECD or (B) HA-tagged TLR1 ECD was immobilized on microtiter wells, followed by incubation with Flag-tagged TLR2 with increasing concentrations of Pam3CSK$_4$ with or without T2.5 Ab, MALP-2, or Ac$_2$CSK$_4$ as indicated. The amount of protein binding was detected through an HRP-conjugated anti-Flag antibody. Data are representative of at least three independent replicates.
Figure 3.7. Modeling of the TLR2/10/Pam₃CSK₄ complex and functional analyses of receptor chimeras reveal a role for the central LRRs of TLR10 in Pam₃CSK₄-mediated activation. (A) A computational model of TLR10 (yellow) in the complex with TLR2 (blue) and Pam₃CSK₄ (red) based upon the crystal structure of the TLR2/1/lipopeptide complex. (B) SW620 cells were cotransfected with the indicated combinations of TLRs, an IL-8 promoter driven luciferase reporter and a Renilla luciferase control. Cells were stimulated with 20ng/ml Pam₃CSK₄ or 20ng/ml Malp-2 followed by measurement of luciferase activity. Values represent the level of reporter activation over that of vector alone whose activity was taken as one. Error bars represent the standard deviation of three independent values.
Figure 3.8. The predicted lipopeptide-binding sites and receptor dimer interface of TLR2/TLR10 complex. (A) Sequence alignment of LRRs 9 through 14 of TLR1 and TLR10. Consensus sequences are shown in red. The mutated sites in TLR1 or TLR10 are labeled with asterisks. (B) The modeled lipid-binding channel in TLR10 and the main dimerization interface between TLR10 and TLR2. The side chains of TLR10 residues (gold) that interact with residues in TLR2 (blue) are shown along with carbon (grey), nitrogen (blue), oxygen (red) and sulfur (yellow) atoms of the lipopeptide. Hydrogen bonds are depicted as straight pink lines. Lipopeptide elements are shown in gray (carbon), blue (nitrogen), red (oxygen) and yellow (sulfur). All three-dimensional illustrations were produced with UCSF Chimera (Pettersen et al., 2004) (C) Residues involved in the receptor dimer interface in TLR2/10/lipopeptide model or the TLR2/1/lipopeptide structure are shown and interactions are depicted by dotted lines (Jin et al., 2007).
Figure 3.9. Mutation of TLR10 residues essential to TLR2/TLR10/lipopeptide complex formation affects receptor function. SW620 cells were cotransfected with TLR2, (A) various TLR10 mutants or (B) various TLR1 mutants as indicated along with an IL-8 promoter driven luciferase reporter and Renilla luciferase control. Cells were stimulated with 20ng/ml Pam$_3$CSK$_4$ or 20ng/ml PamCysPamSK$_4$ for 6 hours followed by luciferase activity assays. Results are presented as the percentage of the wild type receptor response and error bars represent the standard deviation of three independent values.
CHAPTER FOUR

GENERATION OF MONOCLONAL ANTIBODIES AGAINST TOLL-LIKE RECEPTOR 10 AND CHARACTERIZATION OF THE RECEPTOR EXPRESSION PROFILE IN HUMAN BLOOD CELLS

Introduction

Among human TLRs, TLR10 is the only remaining family member without a defined function. Phylogenetic analysis has shown that TLR10 belongs to the TLR2 subfamily which includes TLRs 1, 2, 6 and 10. TLR10 is most closely related to TLR1 and TLR6 and these three genes share a tandem locus on chromosome 4p14 (Beutler and Rehli, 2002). TLR1 or TLR6 cooperates with TLR2 to function and the heterodimeric TLR2/TLR1 or TLR2/TLR6 complexes mediate responses to a great variety of agonists, including bacterial lipoproteins, lipoteichoic acid, zymosan from yeast cell walls, peptidoglycan of Gram-positive bacteria, atypical LPS derived from non-enteric bacteria, and membrane lipoarabinomannans from mycobacteria. (Akira et al., 2006; Takeda et al., 2003). Cooperation with either TLR1 or TLR6 enables TLR2 to discriminate the subtle differences between tri- and di- acylated lipopeptides. TLR2/1 recognizes triacylated Pam\textsubscript{3}CSK\textsubscript{4}, while TLR2/6 responds to diacylated MALP-2. Crystal structure studies have revealed that ligand recognition involves direct contact of the lipopeptide with both receptors in the heterodimer (Jin et al., 2007; Kang et al., 2009). In Chapter Three, I reported that TLR10 also cooperates with TLR2 in the recognition of triacylated lipopeptides and other microbial-derived agonists shared by TLR1. The structural model of TLR2/TLR10/lipopeptide predicted the presence of a lipid-binding channel in TLR10.
However, TLR10 failed to activate the signaling typically associated with other TLR2 family members (Guan et al., 2010).

Members of the TLR family are differentially expressed on leukocytes and endothelial cells. In general, monocytes/macrophages and dendritic cells express most TLR subsets (Kadowaki et al., 2001; Muzio et al., 2000; Zarember and Godowski, 2002). The characterization of TLR expression in various tissues and cell types is crucial to understanding TLR function. Previous studies have shown that the presence of TLR10 mRNA is more tissue- and cell- type specific than that of TLR1 and TLR6, an indication of its functional divergence from the latter two receptors. The mRNA of TLR10 is highly expressed in lymphoid tissues such as spleen, lymph nodes, thymus and tonsils (Chuang and Ulevitch, 2001). Analyses of isolated cell types demonstrated that TLR10 transcripts are specifically limited to B cell lineages and are weakly expressed in plasmacytoid dendritic cells (pDC) (Bourke et al., 2003; Hasan et al., 2005; Hornung et al., 2002). Moreover, the expression level of TLR10 is dramatically up-regulated by activation of resting B cells (Bourke et al., 2003). TLRs expressed in B cells have been shown to be involved in proliferation, differentiation and production of specific antibodies in response to TLR agonists (Lanzavecchia and Sallusto, 2007; Pasare and Medzhitov, 2005). For example, naïve B cells respond to lipopeptides and CpG DNA (a TLR9 ligand), with enhanced cell proliferation, differentiation and IgM secretion (Ganley-Leal et al., 2006; Ruprecht and Lanzavecchia, 2006). pDCs, also known as interferon-producing cells, are a subset of dendritic cells with B cell morphology. pDCs are specialized in producing large amounts of type I interferons, particularly IFN-α, upon stimulation with viral
nucleic acid-containing complexes recognized by TLR7 and TLR9 present in these cells (Lande and Gilliet, 2010). The role of TLR10 in these cells has yet to be uncovered.

The expression of TLR10 has so far only been assessed at the messenger RNA level, and my earlier attempts to detect the expression of the TLR10 receptor on the cell surface have been hampered by the lack of anti-TLR10 antibodies that recognize properly folded receptors. The commercially available anti-TLR10 antibodies were developed against a short synthetic TLR10 peptide and have a low affinity for the surface-expressed receptor. To evaluate the endogenous expression and facilitate functional studies of TLR10, here I report the generation and characterization of monoclonal antibodies (mAbs) against the extracellular domain of the receptor. I also measured TLR10 expression in various cell types isolated from human peripheral blood and found that granulocytes, natural killer (NK) cells, monocytes and T cells lacked detectable expression of TLR10, but human B cells expressed high cell surface levels of the receptor. The monoclonal antibodies generated in this study will serve as powerful experimental tools to further characterize TLR10 expression in various tissues and cell types, and ultimately define this orphan receptor’s biological functions.

**Materials and Methods**

*Plasmid and protein purification*- The TLR10 extracellular portion containing aa 20-474 was cloned into a modified pDisplay vector for expression of the TLR10 extracellular domain. Plasmid construction and protein purification were described in detail in the Chapter Three. (This work and protein purification was performed by another graduate student, Diana Rose Ranoa, in the Tapping laboratory.)
Generation of anti-TLR10 mAb through mice immunization and establishment of hybridoma clones. This work was performed in part by the Immunological Resource Center (University of Illinois at Urbana-Champaign). Five BALB/c mice were immunized by intraperitoneal (i.p.) injection of 50 µg TLR10 protein. Three weeks after initial priming, mice were boosted twice in 3-week intervals by i.p. injection of 25 µg of TLR10 protein. On week 8, small volumes of blood were collected from each immunized mouse to evaluate antibody responses through ELISA and flow cytometric staining of HEK 293T cells expressing TLR10 on their cell surface. The mouse with the highest antibody titer and receptor staining potential was selected by performing a final boost through i.p. injection of 25µg of TLR10 protein. On week 11, spleenic cells were collected and fused with myeloma cells.

When viable hybridoma colonies were apparent (approximately 600 clones were isolated), culture supernatant from mature clones was screened for antibody production by ELISA. 100 µl of supernatant from hybridoma cells were added to microtiter plates that were coated overnight with 5 µg/ml of either TLR10 protein or TLR1 protein, and incubated for 1h at room temperature. After plate washing at the end of the incubation, detection of antibody-antigen binding was performed by the addition of horse radish peroxidase (HRP)-conjugated donkey anti-mouse (Jackson ImmunoResearch laboratories, West Grove, PA) at a concentration of 0.15 µg/ml for additional 30 min incubation. The colorimetric signal was measured after addition of o-phenylenediamine substrate and hydrogen peroxide at 490 nm.

The hybridoma clones with positive ELISA results were further screened by flow cytometric analysis. HEK 293T cells were transfected with a vector carrying FLAG-
tagged TLR10, FLAG-tagged TLR1 and empty vector respectively in a 10-cm tissue culture dish by using the calcium chloride transfection method. Two days post-transfection, cells were removed from the plate and transferred to 96-well PCR plates at a density of approximately $2 \times 10^5$ cells per well. Cells were then incubated on ice with 50 µl of each hybridoma supernatant for 30 min followed by incubation with FITC-conjugated donkey anti mouse IgG (Jackson ImmunoResearch laboratories). Cells were then washed, fixed, and analyzed for surface TLR expression using a FACSCanto (BD Bioscience, San Jose, CA).

Selected hybridoma clones were expanded and subclones were established by limiting dilution. The subclones were screened by using the same processes described above. The isotype of the mAbs was determined using the mouse ELISA rapid antibody isotyping kit (Thermo Scientific, Rockford, IL).

**Blocking antibody screening** - The human colonic epithelial cell line SW620 was transiently co-transfected with the chimeric receptor TLR10-1, TLR2, an IL-8 promoter driven firefly luciferase reporter and a Renilla luciferase transfection control reporter in a 10-cm tissue culture dish (see Chapter Three for details). One day post-transfection, cells were trypsinized and re-seeded into 96-well plates with a total volume of 100 µl. There are enough cells growing on a 10-cm tissue culture dish to approximately re-seed 1.5 96 well plates. The following day, 50 µl of culture media were replaced with the same amount of the hybridoma supernatant and the anti-TLR10 antibodies in the supernatant were allowed to bind to TLR10-expressing cells for 30 minutes prior to stimulation with Pam$_3$CSK$_4$ at 20 ng/ml for 6 hours. At the end of stimulation, cells were washed with
PBS buffer and subsequently lysed in 25 µl of passive lysis buffer for 20 minutes. 15 µl of the cell lysate were analyzed for luciferase activity as described previously.

Antibody purification- The selected hybridoma clones were cultured at 37°C, 7.5% CO₂ in DMEM containing 10% FBS, 2mM L-glutamine and aminopterin (the medium was purchased from the IRC Facility, UIUC). For small-scale batch purification, 100 µl recombinant protein G Sepharose beads (GE healthcare, Piscataway, NJ) were added to 6 ml of filtered culture supernatants, and incubated at 4°C overnight. Protein G beads were recovered by centrifugation at 3000 x g for 15 minutes at 4°C. The beads were washed with 3 ml binding buffer (20mM sodium phosphate buffer pH 7.0), and the antibody was eluted with 50 µl of 0.1M glycine-chloride (pH 2.5) in a 50µl volume followed by addition of 20 µl neutralizing buffer Tris-Cl (pH 9.0). The eluted antibody was dialyzed overnight against PBS (pH 7.4) at 4°C and the concentration was measured using bicinchoninic acid assay (BCA).

When the growth of hybridoma clones reached high confluence, the media was switched to Hyclone SFM4MAb-Utility supplemented with L-Glutamine, a serum-free hybridoma growing medium. The culture supernatant was then collected and filtered to remove the cell debris. A volume of 200 ml of the supernatant was loaded on a pre-packed1 ml protein G column using the AKTA Prime purification system (GE Healthcare) at a flow rate of 1.0 ml/min. The column was then washed with 15 ml binding buffer and the antibody was eluted with 0.1M glycine-chloride (pH 2.5) in 3.0-ml fractions into tubes containing 100ul of neutralizing buffer 1M Tris-Cl (pH 9.0). Antibodies were concentrated using an Amicon ultra centrifugal filter device with 10,000 nominal
molecular weight limit (Millipore, Bedford, MA) followed by dialysis and concentration measurement.

Detection of endogenous TLR10 from blood leukocytes by flow cytometric analysis- Blood samples were obtained from normal healthy volunteers under a protocol approved by the University of Illinois IRB. Peripheral blood leukocytes were prepared by applying erythrocyte lysing buffer (BD Bioscience) to whole blood followed by cell wash using PBS buffer. The sample size was maintained at 2-3 x 10^5 cells per treatment.

A FACSCanto (BD Bioscience) was used for one- or two-color flow cytometry and data were analyzed using FCS Express software. Cell surface expression of endogenous TLR10 was measured using mAb clone 3C10C5. Isotype control antibody mouse IgG1 and FITC-conjugated anti-human CD3, CD14, CD16 and CD19 were purchased from eBioscience (San Diego, CA). To minimize FcR-mediated mAb binding, 2% rabbit serum was included in the flow cytometric staining buffer. A three-step staining protocol was performed to detect surface TLR10 on primary cells, which involves incubation of cells with an anti-TLR10 antibody at a concentration of 20 µg/ml followed by biotinylated donkey anti-mouse IgG, and finally with allophycocyanin-conjugated streptavidin (Jackson ImmunoResearch laboratories). For co-staining of surface TLR10 and cell surface markers, cells were further incubated with FITC-conjugated antibodies following surface TLR10 staining.

Results

Production of antibodies against human TLR10 in mice- Like other TLRs, TLR10 is characterized by an extracellular domain (ECD), containing 19 leucine-rich repeat motifs responsible for ligand recognition, followed by a transmembrane domain and an
intracellular TIR signaling domain. To investigate the biological function of TLR10, I developed monoclonal anti-TLR10 antibodies using purified and properly folded human TLR10 ECD protein as the immunizing antigen. This purified TLR10 ECD, expressed in HEK 293F cells as a soluble protein, comprised aa 20-474 (LRR 1-18) which includes the essential region required for ligand recognition and protein-protein interaction. To generate antibodies against TLR10, five BALB/c mice were immunized three times within 8 weeks by i.p. injection of TLR10 protein. Blood samples were taken from these mice to assess anti-TLR10 antibody titer in the serum. The reactivity between TLR10 and its antiserum was measured by adding serially-diluted mouse antiserum or a control serum from a non-immunized mouse to microtiter plates coated with purified TLR10 protein. Using ELISA, the binding of the antibody in the serum to the TLR10 antigen was detected by the addition of HRP-conjugated anti-mouse polyclonal antibody. As shown in Fig. 4.1A, the antisera from mice 0, LL and RR exhibited high reactivity to the antigen even when diluted 6400 fold. Slightly lower antibody titers were observed in mice L and R. As expected, the control serum did not show specificity toward TLR10 protein.

The anti-TLR10 sera were further examined for their ability to detect the full-length receptor expressed on cell surface. Using each antiserum as a primary antibody, flow cytometric staining of 293T cells was performed following transfection of Flag-tagged TLR10. As a positive control, cells were stained by anti-FLAG antibody. Flow cytometry analysis revealed that the antisera from the five immunized mice showed variable binding to surface TLR10 (Fig. 4.1B). The data are shown as a quantification of fold increase in mean fluorescence intensity values between TLR10-expressing cells and those transfected with an empty vector. The anti-TLR10 antiserum from mouse LL
exhibited the highest cell surface staining of TLR10 among all the antisera. The serum from mouse RR showed a similar antibody titer as that for mouse LL for the ELISA assay, but limited cell surface staining ability when compared with the serum from mouse LL. It is worth noting that the fluorescence signal obtained with mouse LL serum as the primary antibody is 2.5 fold higher than surface staining using the anti-FLAG antibody. Fig. 4.1C shows a typical histogram profile of surface TLR10 staining by the antiserum from mouse LL which created a larger fluorescence shift than that of anti-FLAG antibody, indicating the anti-TLR10 antibodies in the serum strongly bind to full-length TLR10.

In addition, I wished to screen for an inhibitory antibody to TLR10; one that is capable of abrogating TLR10ECD binding to ligand and/or the TLR2 coreceptor. To this end, the chimeric TLR10 receptor TLR10-1, together with TLR2, were expressed in SW620 cells to provide a functional readout. Cells were incubated with varying amounts of each anti-TLR10 antiserum for 30 minutes prior to stimulation with lipopeptides. A partial inhibitory effect of the antiserum from mouse LL on TLR10-mediated cell activation was observed (data not shown). Finally, mouse LL was chosen for a final booster shot before sacrifice and fusion of spleen cells to generate hybridoma clones.

_Establishment of hybridoma subclones expressing anti-TLR10 mAb-_ Splenocytes from the mouse LL were fused with myeloma cells, and the resulting hybridomas were screened for the secretion of anti-TLR10 antibodies by ELISA. To exclude antibodies that cross-react with TLR1, which shares the highest sequence homology and ligand specificity with TLR10, two sets of ELISAs were performed using plates coated with TLR1 protein or TLR10 protein, respectively. Hybridoma supernatants from 52 out of 600 wells were found to contain antibodies that were specific to TLR10 but not to TLR1.
These 52 hybridoma clones were further screened for binding to surface-expressed TLR10 using flow cytometric analysis. This screening identified a total of 17 hybridoma clones that exhibited specific reactivity to TLR10. However, the supernatants of these clones failed to effectively inhibit TLR10-dependent cell activation in the bioassay described above. Five out of the 17 clones (P2G3, P3C10, P5C2, P3G10, P5H9), whose supernatants showed strong immunostaining ability, were subcloned by limiting dilution. The subclones generated were again tested for TLR10 specificity by ELISA and flow cytometric analysis. A total of 15 subclones (3 per clone), which met the criteria in the two aforementioned assays and exhibited no cross-reactivity to TLR1, were propagated and established as anti-TLR10 hybridoma cell lines. Fig. 4.2 shows a summary of the detection of surface-expressed TLR10 using the supernatant of established hybridoma subclones. All 15 subclones efficiently recognize TLR10 but not TLR1. In comparison, the cell surface expression of TLR1 was readily measurable using the control antibody anti-FLAG.

Characterization of anti-TLR10 mAb purified from hybridoma subclones- With established hybridoma lines, I next characterized the mAbs purified from the supernatant of the 15 subclones. Using an immunoglobulin (Ig) isotyping kit, the mAb expressed by all 15 subclones were identified as mIgG1 subclass with kappa light chain. To detect the titer of the mAbs in ELISA, various concentrations of individual mAbs were added to TLR10 protein immobilized on microtiter plates. As shown in Fig. 4.3A, the mAbs derived from the same cellular origin exhibited uniform reactivity to the antigen. All mAbs recognize the TLR10 protein at a concentration as low as 0.1 µg/ml and robust ELISA signals are achieved when a 1 µg/ml concentration is used. Clones P2G3, P3C10
and P5C2 appear to be more suitable for detecting the immobilized TLR10 protein than clones P5G10 and P5H9 since the colorimetric signals generated from the former group were stronger than those of the latter group.

The amount of each mAb required for detection of surface-expressed TLR10 was also examined. Various concentrations of individual mAbs were used as primary antibodies to bind to TLR10 expressed on the surface of transiently-transfected HEK 293T cells. Flow cytometry analysis revealed that the anti-TLR10 mAbs are capable of detecting surface TLR10 at a concentration of 10µg/ml (Fig. 4.3B). The antibodies derived from subclones P3C10, P5C2, P5G10, and P5H9 generated a stronger staining signal compared to anti-FLAG antibody binding, which was added at a higher concentration (20 µg/ml). The antibodies from subclones of P2G3 produced a similar fluorescence signal as the anti-FLAG antibody. To further confirm the specificity of these mAbs, TLR1- or TLR6-transfected 293T cells were assessed for staining with these anti-TLR10 antibodies. As expected, no cross-reactivity was observed indicating that these mAbs are highly specific for TLR10 (data not shown). Altogether, the data demonstrated that the 15 batch-purified mAbs are suitable for both ELISA and flow cytometry analysis.

Detection of endogenous TLR10 on human peripheral blood cells- To characterize the expression profile of endogenous TLR10 protein, I investigated the receptor expression profile in human peripheral blood cells by flow cytometry. After removal of red blood cells, white blood cells were incubated with either anti-TLR10 mAb (purified from clone P3C10C5) or a mouse IgG1 isotype control antibody, followed by biotin-streptavidin staining steps. A dot plot of white blood cells forward and side scatter signals shows three distinctive cell populations representing granulocytes, monocytes and
lymphocytes (Fig. 4.4A). The expression of TLR10 was analyzed by gating on each cell population. Fig. 4.4B reveals that TLR10 is not present on the surface of monocytes and granulocytes. The gate was verified by staining cells with antibodies against cell surface markers CD14 and CD16, which are known to be expressed on monocytes and granulocytes/natural killer (NK) cells, respectively (data not shown). In contrast, the anti-TLR10 mAb stained a small portion of the lymphocyte population. To further identify the subpopulation of lymphocytes that express TLR10, I performed dual color flow cytometry staining (Fig. 4.4C). Co-staining of blood cells with an anti-TLR10 mAb and an antibody against a known lineage marker for either B cells (CD19) or T cells (CD3) revealed that the anti-TLR10 mAb clearly stained all CD19+ cells, but not CD3+ cells. Taken together, the result suggests that TLR10 receptor expression is highly restricted to B cells, while granulocytes, NK cells, monocytes and T cells lacked detectable amounts of endogenous TLR10 on their surface.

Discussion

Since the discovery of mammalian TLRs, a wide variety of antibodies against TLRs 1-9 have been generated and applied to numerous studies aimed at assessing expression levels in different cell types, regulation of expression and functional analyses. Human TLR10 was first cloned in 2001; however, it has remained as an orphan member of the human TLRs due to lack of understanding of its natural ligands and signaling pathways. The commercially-available anti-TLR10 antibodies poorly detect the receptor expressed on cell surface and are more suitable for use in immunoblot assays. The goal of this study was thus to generate an effective anti-TLR10 monoclonal antibody to allow
better understanding of expression of TLR10 and to facilitate more directed functional studies of the receptor.

Genetic studies reveal that the mouse TLR10 is a pseudogene due to the presence of gaps and retroviral gene insertions (Hasan et al., 2005). This allowed me to develop anti-human TLR10 antibodies in mice. Purified TLR10ECD was used as the antigen to immunize mice and the resulting hybridoma clones, as well as subclones, were screened for production of antibodies that were specific for human TLR10 by performing both ELISA and flow cytometric staining of TLR10-transfected 293T cells. To exclude clones with cross-reactivity to TLR1, purified TLR1 ECD and HEK 293T cells expressing surface TLR1 were included in ELISA and cell staining assays, respectively. An initial ELISA screen of 600 hybridoma clones provided 52 positives and surface protein staining revealed that 17 out of 52 clones were capable of detecting TLR10 expressed on the cell surface. All 17 clones secrete mAbs that can specifically react with TLR10 protein, but not with TLR1 or TLR6. The affinity measurements performed by another graduate student, Diana Rose Ranoa, in the Tapping laboratory revealed that the affinity of the anti-TLR10 mAb P3C10C5 for purified TLR10 ECD has a dissociation constant \(K_D\) of \(5.09 \times 10^{-10}\), which indicates strong binding between the antibody and antigen. It should be noted that two commercially available antibodies (2A11 from GeneTex, and 386A from Imgenex) were approximately 5 fold less sensitive than the mAb developed in this study in detecting TLR10 ECD immobilized on microtiter plates and clone 2A11 failed to detect TLR10 expressed on the cell surface (data not shown).

Additional screening for blocking antibodies was performed using a bioassay in which individual hybridoma supernatant or purified antibody was incubated with SW620
cells transfected with TLR2 and TLR10-1 prior to stimulation with lipopeptide agonists. Unfortunately, the screening failed to yield an antibody that effectively inhibits lipopeptide-mediated TLR2/10 heterodimerization. The results suggest that the epitope of the established anti-TLR10 antibodies is located outside of the region for ligand binding and receptor dimerization in TLR10.

Using the purified antibody, I then examined TLR10 expression in human peripheral blood cells and detected a robust and exclusive expression of TLR10 in B cells. Previous studies have investigated the mRNA of TLR10 in various cell types, showing that the level of TLR10 message is high in B cells (Bourke et al., 2003; Hornung et al., 2002). Using the anti-TLR10 mAb I have developed, the present study has shown that human B cells express high endogenous levels of cell surface TLR10. The B cell pool in the peripheral blood of healthy adults consists of approximately 60% naïve B cells and 40% memory B cells (Klein et al., 1998). Data shown in Fig. 4.4C indicate that the anti-TLR10 mAb stained the entire B cell population in the peripheral blood of this donor, suggesting that TLR10 is present in both naïve and memory B cells. In support of this finding, TLR10 mRNA has been found in all stages during B cell differentiation with the exception of pre-B cells (Bourke et al., 2003; Hasan et al., 2005). In addition, another study of B cells derived from human peripheral blood has shown that the TLR10 mRNA is detected at relatively low levels in naïve B cells with higher levels in memory B cells (Bernasconi et al., 2003). I have observed that a small subpopulation of the B cells appears to possess extremely high levels of surface TLR10 expression represented by the intensive fluorescence signal after TLR10 staining which may correspond to the memory B cells. Additional flow cytometric costaining of blood cells with anti-TLR10 mAb and
lineage markers CD19 as well as CD27, a marker for somatically mutated B cells, are required to verify the expression of TLR10 protein in blood B cell subpopulations (Bohnhorst et al., 2001).

In the analysis of TLR10 expression in human peripheral blood cells, I observed variation in the percentage of TLR10 positive cells detected in peripheral blood from donor to donor, ranging from 2.9% to 9.8% of the lymphocytes. Some blood donors possess limited surface TLR10-expressing cells even after the amplification of flow cytometric signal by a biotinylated secondary antibody and a fluorophore-conjugated streptavidin. Co-staining for known cell markers in these low TLR10-expressing cells revealed that TLR10 was expressed only on a small fraction of the B cell population, and was undetectable on the rest of the B cells as well as other cell types. This observation suggests that certain genetic or environmental factors influence the levels of surface expressed TLR10. The anti-TLR10 mAbs established in this study are very useful tools to further define both surface and intracellular TLR10 expression, which will help uncover the mechanisms behind the difference in TLR10 expression among donors.

In addition to the readily detectable TLR10 transcripts in B cells, previous studies on the expression pattern of TLRs have shown that the TLR10 mRNA level in resting B cells was quickly induced after BCR engagement or treatment with B cell mitogens (Bernasconi et al., 2003; Bourke et al., 2003). An increase in TLR10 expression has also been observed in naïve B cells derived from infected tonsils (Mansson et al., 2006). The mAbs generated in this study will enable us to evaluate the regulation of TLR10 expression at the protein level. Together, the expression of TLR10 on B cells and its up-
regulation upon cell activation suggest that this receptor likely plays an important role in B cell function.

Low levels of TLR10 mRNA on pDCs have been reported in a few studies. pDCs are located between the small lymphocytes and monocytes in a dot plot with scatter signals of human blood cells, and accounts for 0.2%-0.6% of all white blood cells (Dzionek et al., 2000). While a slight fluorescence increase in TLR10 staining compared to the isotype control was observed in such a cell population, the present study was unable to clearly reveal TLR10 expression in pDCs possibly due to the low expression level of the receptor and/or scarcity of pDCs in blood cells. Additional flow cytometric staining using purified pDCs and/or pDC-specific cell surface marker BDCA-2 will be helpful for identifying TLR10 protein expression in this cell type.

In conclusion, high affinity mAbs against the extracellular domain of TLR10 were generated in this study and used to detect physiological receptor expression. The antibodies provide powerful experimental tools to further characterize TLR10 expression and explore molecular interactions and pathways in which TLR10 functions. All these should contribute to a better understanding of the immune function of human TLR10.
Figure 4.1. Screening for the presence of anti-TLR10 antibody in serum isolated from mice immunized with TLR10 protein. (A) Screening by ELISA. Serially-diluted sera from five immunized mice were added to microtiter plates coated with purified TLR10 and incubated for 1 hour. Antibody binding was detected by the addition of HRP-conjugated anti-mouse and OPD substrate, and the colorimetric reaction was measured at 490nm. (B) Screening by flow cytometry. HEK 293T cells transfected with either Flag-TLR10 vector or an empty vector were incubated with anti-flag (M2) or serum diluents from immunized mice, followed by incubation with biotin-conjugated anti mouse antibody and finally with PE-conjugated streptavidin. After labeling, cells were analyzed by flow cytometry to screen for mouse sera that contain anti-TLR10 antibodies. Data are presented as the fold increase in fluorescent signal from TLR10-expressing cells relative to empty vector expressing cells. The values represented the average of two independent staining. (C) Flow cytometry histogram profile of HEK 293 cells transfected with either Flag-TLR10 (solid line) or empty vector (dashed line) and stained with anti-Flag or serum from mouse LL as indicated.
Figure 4.2. Summary of flow cytometry staining of cell surface TLR10 (black bars) or TLR1 (white bars) using the supernatant from the 15 selected hybridoma subclones. HEK 293T cells were transfected with empty vector FLAG-CMV, FLAG-TLR10 or FLAG-TLR1 respectively. After 48 hours, cells were incubated with anti-FLAG (M2) or the supernatant of the indicated hybridoma subclones followed by staining with biotin-conjugated anti-mouse Ig antibody and PE-conjugated streptavidin. After labeling, cells were analyzed by flow cytometry for surface expression. Data are presented as the fold increase in fluorescent signal from TLR10- or TLR-1 expressing cells relative to non-TLR (CMV) expressing cells. All 15 subclones efficiently bound TLR10, and showed no cross reactivity toward TLR1.
Figure 4.3. The detection limit of purified anti-TLR10 antibodies to TLR10 protein in ELISA (A) and flow cytometry (B) assays. (A) TLR10 protein-coated wells were incubated with indicated antibodies at different concentrations. The amount of antibody binding was detected through an HRP-conjugated anti-mouse Ab. (B) HEK 293T cells were transfected with either empty vector FLAG-CMV or FLAG-TLR10. After 48 hours, cells were incubated with anti-FLAG (20 µg/ml) or batch-purified mAbs from 15 subcolones at 10 µg/ml, 1 µg/ml and 0.1 µg/ml. Subsequently, cells were incubated with FITC-conjugated anti mouse antibody. After labeling, cells were analyzed by flow cytometry for surface expression TLR10. Data are presented as the fold increase in fluorescent signal from TLR10-expressing cells relative to non-TLR10 (CMV) expressing cells. A concentration of 10 µg/ml was the minimum amount needed for detection of TLR10 expression on the cell surface.
Figure 4.4. Detection of endogenous TLR10 in human peripheral blood cells. (A) Scatter profile of various white blood cell populations isolated from peripheral blood of a healthy donor. (B) Blood cells were stained with anti-TLR10 mAb (20 µg/ml) (red) or isotype control (black), and subsequently incubated with biotin-conjugated anti-mouse Ig antibody and APC-conjugated streptavidin. In each histogram, cells were gated at the indicated population. (C) Dual color staining for TLR10-expressing cells in the lymphocyte population. The blood cells were stained with anti-TLR10 mAbs in a three-step staining protocol described above followed by staining with FITC-conjugated CD19 or CD3. Dot plots were gated in the lymphocyte population. Numerical values indicate the percentage of cells in the respective quadrant.
CHAPTER FIVE

CONCLUSION

Summary

This dissertation research explores the innate immune sensing function of TLR10 and uncovers novel synthetic agonists for TLR2. I have defined agonists for TLR10, the only remaining orphan human TLR, uncovered alternative mechanisms of activating TLR2 subfamily members, and provided insights for designing TLR-targeted immunotherapies and vaccine adjuvants. This chapter will summarize the major findings of the dissertation, assess their contribution, and explore future research directions.

Chapter Two of this thesis presents the identification of novel synthetic TLR2 agonists by screening a chemical library. The initial objective of the screening included a search for TLR10 ligands as well as novel TLR2 agonists. The high throughput screen of 24,000 synthetic compounds identified 16 novel TLR2 agonists that utilize TLR1, TLR6 or both as coreceptors. These compounds represent the smallest agonists for TLR2 to date and their aromatic structural features are unrelated to any known TLR2 agonists. The observations led me to carefully characterize the three most potent compounds from different perspectives. I first established that these compounds activate cells in the nanomolar range and are highly specific for TLR1 and TLR2. Although the compounds possess distinct structural features from known lipopeptide agonists, they do not exhibit synergistic activity, nor do they act as antagonists toward natural TLR2 activators, which suggests that the compounds occupy binding sites different from those of lipopeptides on the receptors. Motivated by this finding, I further studied the mechanisms by which TLR1 recognizes the compounds. Domain-swapping experiments in which LRRs were
exchanged between TLR1 and TLR6 showed that, similar to lipopeptides, the central region comprised of LRRs9-12 of the extracellular domain of TLR1 is essential for recognizing the chemical compounds (Omueti et al., 2005). Detailed analysis was carried out using TLR1 variants which have been shown to affect responses to lipopeptides. The data revealed that while unimpaired TLR2/1 dimer interaction is required to mediate efficient responses to both chemical compounds and lipopeptides, different residues contribute to lipopeptide-mediated and compound-mediated cell activation. Given the structural traits of the compounds and the differences observed in TLR1 variants, it is proposed that the compounds bind directly within the interface of the TLR2/1 heterodimer. Finally, the compounds were shown to activate human peripheral blood monocytes to produce cytokines, which suggests that they may have therapeutic applications as vaccine adjuvants. It also should be noted that compounds with similar structures have been independently identified in the chemical library. This finding validates the screening assay in this study as a powerful approach for identifying novel TLR2 agonists.

It remains unclear why the search failed to yield a compound that activates TLR10. This question was answered in Chapter Three in which I found that TLR10 fails to activate typical TLR-induced signaling outputs, including NF-κB-, IL8- and IFN-β-driven reporters. Thus, the high-throughput chemical library screening which used an IL-8-driven reporter could not have been activated by TLR10 alone or in combination with TLR2. In Chapter Three, I continued to define a ligand for TLR10 through creation of a chimeric receptor TLR10-1 which contains the ECD of TLR10 and the intracellular signaling domain of TLR1. The key assumption is that lipopeptides could also be
agonists for TLR10 based upon the phylogenetic evidence that TLR10 is most related to TLR1 and TLR6. Using the chimeric receptor, I demonstrated that TLR10 cooperates with TLR2 in the sensing of lipopeptides as well as a variety of microbial-derived agonists shared by TLR1, but not TLR6. This finding was confirmed by showing that the chimeric receptor TLR10-1 fully restored the response of TLR1-deficient macrophages to triacylated lipopeptides. Furthermore, the requirement of TLR2 in TLR10-mediated ligand recognition was verified by the following evidence:

1) TLR10 and TLR2 colocalize in early phagosomes in response to stimulation with zymosan particles.

2) TLR10 coimmunoprecipitates with TLR2, and the association between the receptors increases with the addition of tri-acylated lipopeptides.

3) Purified ECDs of TLR2 and TLR10 physically interact with each other in a ligand-dependent manner.

I then characterized and compared the mechanisms responsible for ligand recognition by TLR10 and TLR1. A computational model of TLR2/10/lipopeptide was built based on the solved crystal structure of TLR2/1/lipopeptide (Jin et al., 2007). The model shows that TLR10 preserves the TLR2 dimer interface and the lipopeptide binding channel found in TLR1. The relevance of the model is supported by a domain-swapping experiment which demonstrated that the LRRs 6-17 of TLR10 ECD are responsible for lipopeptide specificity. Additionally, site-directed mutagenesis of key amino acid residues, predicted by the model to be functionally important, led to an attenuated response to lipopeptides. When compared to the corresponding residues in TLR1, I found that the interactions of the two receptors with lipopeptides are similar, but they appear to
possess subtle differences at the TLR2 dimer interface. Interestingly, the novel chemical compounds which likely fit in the interface of the TLR2/1 heterodimer showed limited or no activity towards the TLR2/10 heterodimer. Finally, a proximal signaling adapter MyD88 was shown to be recruited to the TLR2/10 complex, and this association was enhanced by the addition of lipopeptides. The result suggests that the interaction of MyD88 with the TLR2/10 heterodimer is distinct from that of other TLR2 family members which may account for the failure of this complex to induce signals typically associated with MyD88 activity.

Chapter Four describes the generation and characterization of anti-TLR10 monoclonal antibodies that will facilitate functional studies of TLR10. A purified and properly folded human TLR10 ECD protein was used as the antigen to immunize five mice for production of antibodies against TLR10. The resulting hybridoma clones were screened for specific binding using both ELISA with purified TLR10 protein as antigen and flow cytometric staining of 293T cells transfected with Flag-tagged TLR10. A total of 17 hybridoma lines were isolated that secrete monoclonal antibodies that bind strongly to TLR10, but to neither TLR1 nor TLR6. Five out of the 17 clones were subcloned by limiting dilution and screened in the two aforementioned assays. Finally, a total of 15 subclones were established as anti-TLR10 hybridoma cell lines. All mAbs recognize the purified TLR10 ECD at a concentration as low as 0.1 µg/ml and detecte surface expressed TLR10 at a concentration of 10 µg/ml. The antibodies derived from most of these subclones generated a stronger TLR10 staining signal against Flag-tagged TLR10 than that of the anti-Flag antibody. The purified mAbs were used to examine endogenous TLR10 expression in human peripheral blood. Granulocytes, NK cells, monocytes and T
cells lack detectable expression of TLR10, but human B cells express high cell-surface levels of the receptor, which represents a unique expression profile among mammalian TLRs.

**Significance and Future Work**

TLRs are evolutionarily conserved receptors serving as sensors for a wide variety of pathogen-associated patterns (PAMPs) from viruses, bacteria, fungi and protozoa. Upon recognition of their agonists, TLRs mediate immediate protection for the host and modulate the action of the adaptive immune system (Medzhitov and Janeway, 1997a; Takeda et al., 2003). Unlike most TLRs which signal as homodimers, TLR2 requires either TLR1 or TLR6 for activity and the cooperative complexes enable TLR2 to recognize various bacterial and fungal cell wall components. TLR1 and TLR6 share highly homologous intracellular domains and TLR2/1 and TLR2/6 complexes mediate identical signaling pathways (Farhat et al., 2008). TLR2 is expressed on various cells of both the innate and adaptive immune system. The engagement of TLR2 complexes with their ligands leads to the activation of NF-κB, AP-1 and other transcription factors which induce pro-inflammatory responses. Stimulation with TLR2 agonists also provides specific signals to modulate B cell responses to antigens (Brikos and O'Neill, 2008; Chiron et al., 2008).

Studies using TLR2 knockout mice have indicated a role of TLR2 in protecting the host from infectious diseases (Echchannaoui et al., 2002; Takeuchi et al., 2000). Receptor polymorphisms within the TLR2 subfamily have also been associated with various microbial infections (Misch and Hawn, 2008; Ogus et al., 2004). In addition, inappropriate or prolonged inflammation triggered through TLR2 has been implicated in
the development of chronic inflammatory disorders including arthritis, atherosclerosis and asthma (Kormann et al., 2008; Palsson-McDermott and O'Neill, 2007). Indeed, TLR2 antagonists hold great promise in the treatment of chronic inflammatory diseases. On the other hand, TLR2 agonists are under investigation as vaccine adjuvants to modulate immune responses (O'Neill et al., 2009).

The novel synthetic TLR2 activators described in Chapter Two represent the smallest molecules known to activate the TLR2/1 heterodimer. The characterization of these compounds identified an alternative mechanism of activation of the TLR2/1 complex which may provide novel approaches for the TLR2-based design of vaccine adjuvants. Future work concerning the novel TLR2 activators could be directed at the generation of a secondary chemical library composed of analogues of the most potent compounds. The screening of the secondary library may yield compounds with stronger activity and pharmacologically favorable features.

The TLR2 subfamily comprises TLRs 1, 2, 6 and 10. The genes encoding TLRs 1, 6 and 10 share the same locus on the chromosome and are likely to have risen from gene duplication events. Based on phylogeny, TLR10 predates the generation of TLRs 1 and 6. As evolutionarily conserved receptors, TLRs evolved under strong purifying selection (Beutler and Rehli, 2002; Kimbrell and Beutler, 2001). The independent maintenance of TLR10 suggests a distinct biological role for this receptor. Defining the innate sensing and the immune function of TLR10 will contribute to a full understanding of the TLR2 subfamily function.

The finding that TLR10 senses triacylated lipopeptides and other microbial-derived agonists shared by TLR1, but not TLR6, reveals for the first time the innate
sensing function of TLR10. The results described in Chapter Three firmly demonstrate that TLR10 requires TLR2 for innate immune recognition. Biological, biophysical and structural evidence was provided to support the long-standing prediction that TLR10 is a heterodimeric partner of TLR2. The fact that TLR10 cooperates with TLR2 in ligand sensing, but fails to induce typical TLR associated signaling events, suggests that TLR10, rather than further expanding the already wide ligand spectrum of TLR2, contributes to a signaling function distinct from that of other TLR2 subfamily members. In addition, protein expression studies using anti-TLR10 mAbs described in Chapter Four confirm that the high level of TLR10 protein is restricted to B cells, an expression pattern different from that of TLR1 and TLR6, which further suggests a functional divergence from the latter two receptors.

The discovery that TLR10 shares ligand specificity with TLR1 calls for the re-evaluation of previous studies using lipoproteins or other TLR2/1 agonists for the stimulation of human B cells. The treatment of purified B cells with the triacylated lipopeptide Pam3CSK4 induced IL-6 secretion and IgM secretion as well as the initiation of germinal center formation (Ganley-Leal et al., 2006; Mansson et al., 2006). Human multiple myeloma cell lines and chronic lymphocytic leukaemia cells respond to Pam3CSK4 with an enhanced survival rate (Bohnhorst et al., 2006; Muzio et al., 2009). It is very likely that TLR10 plays a role in these lipopeptide-mediated reactions given its high and regulatable expression in B cell lineages. It is also worth noting that the novel chemical compounds identified in Chapter Two are highly specific to TLR2/1, and do not activate TLR2/10. Therefore, these compounds may be used to distinguish between TLR1- and TLR10-mediated effects.
Although the expression of TLR10 in cell types other than B cells was not detected in this study, TLR10 transcripts have been found in pDCs and regulatory T cells (Bell et al., 2007; Hornung et al., 2002). In addition, the Tapping laboratory and other researchers have shown that TLR10 mRNA is inducible in macrophages upon differentiation or exposure to certain nanoparticles (Lucarelli et al., 2004). These cell types also express other TLR2 subfamily members to varying degrees. As pointed out in Chapter Three, the competition of TLR1 and TLR10 for ligand and coreceptors is likely to have significant functional consequences for host immune defense and inflammation, given the different signaling outputs of these two receptor complexes. The preliminary studies in the Tapping laboratory indicate that TLR10 acts as a decoy receptor to dampen cell responses to lipopeptides in myeloid cells.

Moreover, TLR10 is a nonfunctional pseudogene in mice, but intact TLR10 sequence has been identified in rat, bovine and porcine chromosomes (Opsal et al., 2006; Shinkai et al., 2006). The future design of vaccine adjuvants or TLR2 antagonists should be tested in an animal model with a functional TLR10 gene, so as to have more relevance to human responses.

Finally, the structural model of TLR2/10/lipopeptide complex and functional studies of TLR10 have provided a molecular basis for the rational design of TLR10-specific agonists. The unique expression pattern of TLR10 in B cells suggests a potential use of TLR10 in selective targeting of B cells during immunotherapy.

A logical extension of this study is to explore the role of TLR10 in human immune defense. The directions currently being pursued in the Tapping laboratory include: assessing the ability of TLRs 1, 6 and 10 to compete for the coreceptor TLR2
and their agonists using soluble forms of the receptors in biophysical assays, determining
the signaling function of TLR10 through transient transfection studies as well as the
generation of myeloid cells lines that stably express TLR10, and exploring the in vivo
function of TLR10 in whole animal models by generating TLR10 transgenic mice. Based
on the preliminary studies, we propose that TLR10 dampens classical TLR2-mediated
activation in myeloid cells presumably through recruitment of the proximal signaling
adapter MyD88 in a manner distinct from that for TLR2/1 and TLR2/6 complexes.
Similarly, TLR10 may also play an inhibitory role in B cell proliferation and
differentiation. Further work on the identification of a TLR10 specific ligand and the
generation of an inhibitory anti-TLR10 antibody should help verify the model we have
proposed.
REFERENCES


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Figure A.1. Surface expression of CD4-TLRs constructs. 293T cells were transfected with indicated CD4-TLR constructs (solid line) or an empty vector (dotted line) at the concentration of 1 µg/ml. Cells were stained sequentially with a monoclonal antibody for mouse CD4, a biotin-conjugated donkey anti-mouse secondary antibody and a streptavidin-PE-conjugated tertiary antibody. Surface expression was analyzed by flow cytometry.
Figure A.2. Surface expression of TLR10 chimeric constructs. (A) 293T cells were transfected with a FLAG-tagged wild-type TLR10, a FLAG-tagged TLR10-1 or an empty vector at the concentration of 2.5 µg/ml. Cells were stained sequentially with the anti-FLAG mAb (M2), a biotin-conjugated donkey anti-mouse secondary antibody and a streptavidin-PE-conjugated tertiary antibody. (B) 293T cells were transfected with a FLAG-tagged wild-type TLR1 and a FLAG-tagged TLR1-10 and an empty vector at the concentration of 2 µg/ml. Cells were stained sequentially with the anti-FLAG mAb (M2) and FITC-conjugated donkey anti-mouse secondary antibody. Surface expression was analyzed by flow cytometry.
Figure A.3. Surface expression of TLR10 mutants. 293T cells were transfected with indicated FLAG-tagged TLR10-1 (solid line), FLAG-tagged TLR10 mutants (solid line) or an empty vector (dotted line) at the concentration of 2.5 µg/ml. Cells were stained sequentially with the anti-FLAG mAb (M2), a biotin-conjugated donkey anti-mouse secondary antibody and a streptavidin-PE-conjugated tertiary antibody. Surface expression was analyzed by flow cytometry.
Figure A.4. TLR10 fails to sense chemical compound-agonists. SW620 cells were cotransfected with vectors encoding the indicated TLRs, an IL-8 driven firefly luciferase gene and a Renilla luciferase control. Two days after transfection, cells were stimulated with different concentrations of compound A, B or C for 6 hours and cell lysates were analysed for dual luciferase activity. After correcting for transfection efficiency using Renilla luciferase, all values were normalized to those of unstimulated cells transfected with empty FLAG-CMV vector. Error bars represent the standard deviation of three independent wells stimulated with the indicated concentration of agonist.
### Table A.1 Cloning Primers.

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<tr>
<th>Primer Name</th>
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<td><strong>CD4-TLRs constructs</strong></td>
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<tr>
<td>CD4 Forw*</td>
<td>GGA AGC TTA CCA CCA TGT GCC GAG CCA TC</td>
</tr>
<tr>
<td>CD4 Rev*</td>
<td>AAC TCG AGC ACT GTC TGG TTC ACC CTG GAT AAA ACC TGG</td>
</tr>
<tr>
<td>TLR1 Forw*</td>
<td>ATT CTC GAG ATA ACT CTG ATC GTC ACC ATC</td>
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<td>TLR1 Rev*</td>
<td>GCG TCT AGA CTA TTT CTT TGC TGT CTC TGT CAG C</td>
</tr>
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<td>ATT CTC GAG ACA GCA CTG GTG TCT GTG GAC ATG</td>
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<td>ENA-78 Rev*</td>
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<td><strong>TLR10 chimeric constructs</strong></td>
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<td>Forward</td>
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</tr>
<tr>
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Table A.1 (continued)

| TLR10-VLR Forw | CGAGAACTAAATATTGCATCCAAACCAGCTGAAGAGCGTGCCTGATGGCA TTTT |
| VLR Rev       | TTGGCTAGCGGAGCCCCCTTGGGACCAGAGTGGGGCAAATAATGGACCTC ACTGTTTTTC |

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<tr>
<td>F312D Forw</td>
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<tr>
<td>F312D Rev</td>
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<td>I314D Forw</td>
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<tr>
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<td>Q316K Forw</td>
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<tr>
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<tr>
<td>I359D Forw</td>
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<td>I359D Rev</td>
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* indicates the engineered restriction sites.
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