TLR1 I602S: TRAFFICKING DYNAMICS AND ROLES IN RESISTANCE TO MYCOBACTERIAL INFECTION

BY

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DISSERTATION

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Abstract

Innate immunity provides defense mechanisms required for protection against constant exposure to potentially pathogenic microorganisms. Essential to this role is the ability to rapidly recognize the presence of foreign components or organisms indicative of infection. Toll-like receptors (TLR), a ten-member family of innate immune proteins, serve as major pattern recognition receptors which sense conserved constituents of bacteria, fungi, and viruses. TLR activation results in the production of proinflammatory cytokines and co-stimulatory molecules required for the establishment of local inflammation and adaptive immunity. Toll-like receptor 1 (TLR1), in cooperation with TLR2, is essential to the innate immune sensing of bacterial cell wall components, such as triacylated lipoproteins, lipoglycans and, in particular, many mycobacterial-associated molecules. The other eight TLRs recognize a diverse array of microbial ligands, including lipopolysaccharide, flagellin, and nucleic acids. TLRs are expressed by various immune cells and can be both displayed on the cell surface or in intracellular compartments. Following protein synthesis, these receptors must traffic to appropriate subcellular locations in order to have maximum access to their particular microbial ligand. If improper distribution occurs, TLRs may be rendered nonfunctional due to the inability to sense the presence of these ligands.

We have previously identified a frequent single nucleotide polymorphism in TLR1 (I602S) which greatly inhibits trafficking of the receptor to the cell surface. Individuals homozygous for the serine allele at amino acid position 602 have low to undetectable levels of plasma membrane TLR1. Conversely, the TLR1 602I variant can be readily detected on the cell surface. This striking phenotype is strictly caused by a trafficking defect and not a difference in total cellular expression of each receptor. The absence of surface TLR1 confers hyporesponsiveness of innate immune cells to TLR1 agonists whereby cells homozygous for TLR1 602S have deficient secretion of proinflammatory cytokines in response to extracellular ligands. It could be expected that individuals deficient in recognition of bacterial TLR1 ligands may be vulnerable to infection. Interestingly however, homozygosity for TLR1 602S protects against leprosy and tuberculosis: diseases caused by the intracellular pathogens *Mycobacterium leprae* and *Mycobacterium tuberculosis*, respectively. This dissertation describes our investigation of the TLR1 I602S polymorphism in the context of receptor trafficking and host defense against mycobacterial infection. My thesis project has sought to determine the
mechanisms underlying the protective roles of TLR1 I602S in infection and immunity, and how differential trafficking and signaling contribute to these functions. To apply this phenotype more globally to TLR biology, we have also used the I602S polymorphism as a valuable model system for studying both TLR trafficking dynamics and the role of TLR1/2 in immune defense.

Chapter 1 of this thesis serves as a general introduction to the field of immunology and in particular, mechanisms of innate immunity. The concept of pattern recognition receptors is discussed, with a major focus on the Toll-like receptor family of proteins including their structure, ligands, and signaling functions. Also in Chapter 1, the concept of how genetic variation within TLRs contributes to individual resistance or susceptibility to infectious disease, especially mycobacterial disease, is introduced. Here I highlight single nucleotide polymorphisms in TLR1, 2, 4, and 9 which affect resistance to these types of infections, and link TLR genotype to phenotypic effects on disease susceptibility. Since TLR1 and TLR2 are the primary sensors of mycobacterial products, I focus on SNPs in these receptors with particular focus on the I602S polymorphism. Chapter 2 describes the characterization of processes controlling TLR1 trafficking and attempts to shed light on the mechanisms responsible for the trafficking deficiency exhibited by TLR1 602S. Specifically it discusses how mutagenesis was used to identify a short trafficking motif within the TLR1 primary amino acid sequence, which is interrupted by the 602S polymorphism. In addition two endoplasmic reticulum chaperones, PRAT4A and PRAT4B, were shown to differentially regulate TLR1 surface trafficking based upon the presence of the polymorphism.

A number of studies have identified several mechanisms by which mycobacteria subvert TLR1/2 signaling to create immunosuppressed environments conducive for bacterial replication. I hypothesized that since these immunosuppressive effects are caused by mycobacterial stimulation of TLR1/2, then the lack of TLR1 602S on the cell surface would prevent these inhibitory signals. Chapter 3 goes on to discuss experiments based on this hypothesis and highlights several mechanisms by which homozygosity for TLR1 602S protects individuals against mycobacterial disease. Such mechanisms include differential resistance to inhibitory effects on macrophage activation and microbicidal functions.

Finally, Chapter 4 serves as an overall summary of this research’s implications for the field of innate immunity and anti-mycobacterial defense. It describes the possible future directions this thesis project could take and proposes clinical significance of the work.
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Table of Contents

Chapter 1: Innate Immunity, Toll-like Receptors, and Mycobacterial Disease ........................................1
Overview ................................................................................................................................................1
Pattern Recognition ............................................................................................................................2
Toll-like Receptors and Ligands ..........................................................................................................3
Toll-like Receptor Trafficking ............................................................................................................8
Toll-like Receptor Signaling ..............................................................................................................9
Genetic Variation of TLRs and Infectious Disease ...........................................................................11
Mycobacterial Disease .......................................................................................................................12
TLR-Mediated Recognition of Mycobacteria ...................................................................................14
Subversion of TLR Signaling by Mycobacteria ...............................................................................16
TLR Polymorphisms and Mycobacteria .............................................................................................19
The TLR1 I602S Polymorphism and Mycobacteria ........................................................................22
Thesis Outline ....................................................................................................................................24
Figures ...............................................................................................................................................26
References ..........................................................................................................................................35

Chapter 2: Cell Surface Trafficking of TLR1 is Differentially Regulated by the
ER Chaperones PRAT4A and PRAT4B ..........................................................................................49
Introduction .........................................................................................................................................49
Experimental Procedures ..................................................................................................................50
Results .................................................................................................................................................52
Discussion ..........................................................................................................................................59
Acknowledgements ............................................................................................................................61
Figures ...............................................................................................................................................62
References ..........................................................................................................................................82

Chapter 3: The TLR1 602S Polymorphism Protects Against Leprosy and
Tuberculosis by Overcoming Mycobacterial Immune Subversion ..............................................86
Introduction .........................................................................................................................................86
Experimental Procedures ..................................................................................................................88
Results .................................................................................................................................................90
Discussion ..........................................................................................................................................95
Acknowledgements ............................................................................................................................99
Figures ...............................................................................................................................................100
References ..........................................................................................................................................110

Chapter 4: Summary and Future Directions ..................................................................................116
Clinical Implications ..........................................................................................................................116
Unanswered Questions and Future Experimentation .......................................................................119
References ..........................................................................................................................................123
Chapter 1
Innate Immunity, Toll-like Receptors, and Mycobacterial Disease

Overview

On a daily basis, humans are exposed to thousands of microorganisms. These microbes, mostly in the form of bacteria, viruses, and fungi, are present in every breath of air taken, upon all surfaces encountered, and in daily consumed food and drink. In addition, humans are inoculated with vast quantities of beneficial microflora, ten times the number of cells comprising our own organs and tissues, populations of which must be strictly controlled and tolerated. The very nature of these microorganisms drives them to replicate at an incredibly high degree of efficiency, using whatever surrounding molecular resources to do so. The human body is a warm and moist collection of such organic and inorganic resources and, if given the chance, many of these microbes would rapidly convert our tissues into countless replicas of themselves. Fortunately for humans and most other multicellular forms of life, evolution has provided defense systems of impermeability, containment, and clearance, whose primary purposes are to dispose of that which is not recognized as self. The resulting collection of these mechanisms composes the host immune system.

Immune defenses primarily come in one of three forms: physical barriers, cellular immunity, and humoral immunity. The best method to prevent an invading microorganism from posing a threat is to block its access to vulnerable areas of the body. Physical barriers, mainly in the form of skin epithelium and mucosa, often adequately serve this purpose. However, accidental breaches in these barriers through injury or dehydration easily permit entry of microbes into bodily environments conducive for replication. At this point, cellular and humoral immunity become essential. Resident white blood cells, primarily in the form of tissue-resident macrophages and dendritic cells, begin to engulf and destroy invading microbes. Along with facilitating direct microbial clearance, these leukocytes send chemical signals to recruit additional cells to the site of infection in an attempt to aid the restoration of tissue homeostasis. Furthermore, resident dendritic cells migrate from their initial sites to the nearest secondary lymphoid tissues, where they activate and recruit T helper cells which in turn enhance the microbicidal functions of macrophages. Also present in these secondary lymphoid tissues are B cells which, upon activation, secrete components of humoral immunity in the form of antibodies. Released antibodies, in addition to complement serum proteins circulating peripherally in the blood, leak out from dilated vessels at the site of infection and combine to
both neutralize and directly kill microbes. Many other cellular players are involved in these intricately orchestrated means of defense. However, the indispensible event which must take place in order to initiate an immune response to potentially dangerous microorganisms is the initial recognition of the danger itself.

**Pattern Recognition**

Organisms from all three domains of life share fundamental processes required for sustaining the physical integrity, survival, and replication of the cell. These reactions require the interplay between complex biological molecules and, although the results of processes like DNA replication and cell wall synthesis are similar, often the molecules involved possess characteristics that are distinct from one type of organism to another. These distinctions or patterns, along with their physical location, are used by the immune system to sense the presence of cellular components not designated as typical tissue constituents, such as those belonging to infecting microorganisms.

It is the rapid recognition of non-self molecules which gives the innate immune system its abilities to trigger acute inflammation and protect the host with high efficacy. This process is directed by a family of germline encoded proteins known as pattern recognition receptors (PRRs). PRRs are expressed by an ever-increasing repertoire of known cell types. These proteins are primarily expressed by innate immune cells, such as monocytes, macrophages, dendritic cells, and neutrophils, but extensive research has identified many others, including adaptive immune cells, epithelium, endothelium, fibroblasts, and even nerve cells.

Pattern recognition receptors are organized mainly by the conserved microbial ligands they sense. PRRs which recognize carbohydrates distinct to microorganisms like yeast and prokaryotes are classified as C-type lectins. Examples of these are the mannose receptor and dectin receptors, which bind mannosylated and glucan-rich components of bacterial and fungal cell walls, respectively (57, 149). Importantly, beta-glucan and terminal mannosylation of glycoproteins are not present in humans, a fact which represents the premise for innate pattern recognition. Scavenger receptors are another class of PRRs and sense a variety of ligands including acetylated low-density lipoproteins and polyanionic molecules. Mannose, dectin, and scavenger receptors are all expressed on the cell surface, and are comprised of an extracellular ligand binding domain, a single pass transmembrane domain, and a signaling domain which
initiates the receptors’ functions upon ligand recognition (83). These PRRs mainly serve as trigers of phagocytosis, a classical innate immune mechanism for cell-mediated destruction of pathogens. In addition to phagocytosis, activation of these PRRs can lead to expression of pro-inflammatory mediators, such as cytokines and chemokines, which are required for orchestrating a full immune response.

Other examples of PRRs are the nod-like receptors (NLRs) and RIG-like receptors (RLRs). Unlike their phagocytic counterparts, these PRRs are expressed intracellularly in the cytoplasm. NLRs are composed of a nucleotide oligomerization domain (NOD), an effector caspase activation and recruitment signaling domain (CARD), and a ligand binding domain composed of leucine rich repeats (LRRs) (40, 146). These PRRs recognize various Gram positive and negative bacterial components including peptidoglycan and RNA. Upon ligand binding they recruit and activate caspase enzymes, which cleave pro-interleukin-1 to functional interleukin-1β (IL-1β), allowing secretion of this important pro-inflammatory leukocyte mitogen. RLRs ligand binding domains consist of RNA helicases, which allow them to serve as sensors of RNA viruses (150). Instead of controlling the production of IL-1, this receptor’s CARD domain initiates expression of type-1 interferons, cytokines essential to antiviral defense. All of the above described PRRs are capable of promoting induction of inflammatory mediators to a limited extent, but none of these groups of receptors are capable of recognizing as diverse array of ligands or induce as large a variety of cytokines as the Toll-like receptor (TLR) family of pattern recognition receptors. The expression and function of the TLRs will remain the focus of this dissertation.

Toll-like Receptors and Ligands

The theory of how pattern recognition bridges innate and adaptive immunity was first proposed by Charles Janeway in 1989 where, at an annual Cold Spring Harbor Symposium, he predicted the presence of a group of germline encoded receptors capable of recognizing conserved microbial molecules (60). It wasn’t until almost a decade later that his lab cloned and characterized the first human Toll-like receptor in 1997-1998 (93).

The importance of human TLRs in the context of innate immunity was first realized upon the discovery that the Drosophila Toll protein not only was vital for dorsoventral pattern formation during embryonic development, but that a deficiency in Toll lead to dramatically increased sensitivity to fungal infection (82). Since then TLRs, the human orthologs of Toll, have
been increasingly recognized as playing a vital role in regulating a wide array of inflammatory-related processes: including antimicrobial immunity, tissue repair and homeostasis, autoimmunity, and cancer. TLRs regulate tissue repair by inducing kinases which control cell division and by controlling the release of chemoattractive cytokines which recruit macrophages responsible for clearance of damaged tissues (116). Autoimmune reactions to self-tissues can be initiated or exacerbated via TLR recognition of several known endogenous human ligands (94). Similarly, tumor formation is known to be both promoted and inhibited by differential TLR activation, as inflammation can both initiate cell growth and lead to anti-tumor immunity (117).

Not surprisingly, Toll-like receptors are important targets for pharmaceutical research. Newly developed synthetic TLR agonists are frequently arising to serve as drug treatments which take advantage of the inflammatory properties of TLR activation (54). Examples include the synthetic TLR7 agonist, imiquimod, which has been shown to exhibit potent anti-basal cell carcinoma properties, in addition to a TLR4-based agonist currently in use as a vaccine adjuvant. Many synthetic antagonists which block TLR activation during autoimmune reactions are similarly being developed.

The TLR family contains combined aspects of all the previously mentioned PRRs. Like C-type lectins, they are type-1, single-pass transmembrane receptors with an N-terminal ligand binding domain and a C-terminal signaling domain. TLRs are expressed on the cell surface, like scavenger receptors, but some members are strictly expressed in intracellular endosomal compartments (Fig. 1.1). Like NLRs, TLR ligand binding domains are composed of repetitive amino acid sequences rich in leucine residues (LRRs). These LRRs form curved solenoids, whose cooperative binding of ligand facilitate dimerization of two receptors (Fig. 1.2). Activation of TLRs leads to the production of many proinflammatory chemokines and cytokines including both interleukins and interferons.

Like other PRRs, TLRs are essential to the innate immune sensing of evolutionarily conserved microbial components. These components are referred to as pathogen associated molecular patterns (PAMPs), or more generally, microbial associated molecular patterns (MAMPs). These molecular signatures are primarily specific to microbes and typically absent in normal host tissues. Upon infection however, surveillance of the extracellular milieu provided by the TLRs senses molecules shed or displayed by microorganisms.

Humans possess ten genes encoding Toll-like receptors, each recognizing a particular agonists or group of related agonists (Fig. 1.3). TLR3, 7, 8, and 9 are subdivided based on their
shared recognition of bacterial and viral nucleic acids. These nucleic acids come both in the form of pathogen-associated genomic DNA and RNA, but also in the form of actively transcribed mRNAs, which differ in post-transcriptional modifications from host RNAs. These TLRs are primarily expressed intracellularly in endosomal compartments. This strategy takes advantage of the release of nucleic acids upon phagocytosis and breakdown of bacteria or viruses. Also, viral replication events require internalization of virions into various subcellular compartments, which may contain endosomally expressed TLRs. Furthermore, intracellular expression of nucleic acid sensing TLRs protects the host against accidental autoimmunity, which would otherwise result from surface TLR recognition of host DNA and RNA upon cellular damage or apoptosis.

Nucleic acids take on various conformations, all of which are collectively recognized by the TLR3, 7, 8, and 9 sub-group. TLR3 recognizes doubled-stranded RNA derived from both dsRNA viral genomes or actively replicating ssRNA genomes (4). Single-stranded RNA is also able to activate TLR3 to a certain extent. Thanks to the research endeavors studying TLR biology over the past 14 years, synthetic agonists are currently available for most of the TLRs. Polyinosine:polycytosine (poly I:C) serves as a synthetic dsRNA analog and TLR3-specific ligand. Mice deficient in various TLRs are known to have increased sensitivity to infection or decreased clearance of microbes normally recognized by the receptors. For example, TLR3 knockout mice show impaired cytokine secretion and survival when challenged with the ssRNA virus, encephalomyocarditis virus (47). TLR7 and 8 are highly homologous and primarily recognize single-stranded RNA derived from viral genomes, and bacterial or viral mRNAs. They were originally shown to bind the antiviral nucleoside analog, resiquimod, and subsequently, synthetic agonists like imiquimod and loxoribine have become available (101, 64). Finally, TLR9 binds unmethylated, double-stranded DNA rich in cytosine-phosphate-guanine (CpG) repeats (12, 55). These motifs are distinct to microbial-associated DNA, as 70-80% of CpG repeats in human DNA are methylated. Synthetic CpG oligonucleotides are readily available, and sequence variation within these DNAs is known to confer diverse levels of TLR9 activation.

Of these four intracellular TLRs, only the structure of TLR3 has been solved by x-ray crystallography (25). Similarly to all TLRs, the extracellular domain of TLR3 forms a curved, horseshoe-like solenoid structure composed of beta sheet repeats on the internal face of the curve and loops on the external face (Fig. 1.2). This domain is glycosylated at several sites but the agonist-binding domain remains relatively sugar-free. Upon ligand binding, TLR3
homodimerizes by sandwiching dsRNA between the lateral faces of both ectodomains. This dimerization event situates the intracellular signaling domains of the two TLRs into close proximity, allowing signal transduction to occur. TLR signaling will be discussed in further detail in the next section.

Unlike the nucleic acid sensing TLRs, the remaining receptors (TLR1, 2, 4, 5, 6, and 10) are predominantly expressed at the plasma membrane, where they are best available to contact microbial components present in the extracellular milieu. TLR5 recognizes highly conserved regions in bacterial flagellin, a protein component of almost all motile bacteria (53). Both Gram positive and negative bacterial flagella are composed of a helical filament, which is largely composed of flagellin monomers arranged in a hollow polymer. Flagellin from multiple bacterial species is easily purified and commercially available for studying TLR5 homodimer activity. TLR5 appears to be highly important to immunity in the human gut. There, expression strictly limited to the basolateral side of the intestinal epithelium discriminates between commensal Escherichia coli versus invasive enteric infections, like those caused by Salmonella species (43).

TLR4 was the first human TLR identified and remains the most characterized and studied of the Toll-like receptors (93). TLR4 is the sensor of the Gram negative endotoxic lipoglycan, lipopolysaccharide (LPS) (26, 59, 114). LPS, a major outer membrane component of most Gram negative organisms, has classically been recognized for its potent immunostimulatory properties. The structure of LPS can vary between organisms, but share fundamental organization. The outermost O-antigen is composed of a repetitive glycan polymer, followed by the core oligosaccharide which links the O-antigen to lipid A, a phosphorylated glucosamine disaccharide decorated with varying numbers of fatty acids. This lipid A component represents the agonist for TLR4 and serves as a major immune pyrogen. The TLR4 system requires a unique set of co-receptors and lipid carrier proteins for maximum activity. LPS-binding protein (LBP) and CD14 disaggregate and deliver lipid A to another lipid chaperone, MD2. Two LPS-bound MD2 monomers, along with two TLR4 molecules, form the final functional TLR4 tetramer complex (76). Expression of each component in the system is tightly regulated, whereby inflammation triggering the acute phase dramatically increases serum levels of these proteins. TLR4 knockout mice exhibit LPS insensitivity and resistance to endotoxic shock but are more susceptible to Gram negative infections caused by Klebsiella, Haemophilus, and Pasteurella species (17, 23, 59, 145). The importance of TLR4’s discovery and characterization was recently
recognized when Bruce Beutler was awarded a shared 2011 Nobel Prize in Physiology and Medicine for his work with the receptor.

TLR1, 2, 6, and 10 form a unique subfamily within the Toll-like receptors. Not only do they share high levels of homology and recognize related agonists, they are also the only TLRs to heterodimerize upon ligand recognition. TLR2 heterodimerizes with TLR1 and TLR6 to recognize a diverse array of lipidated molecules present in bacterial and fungal cell membranes and walls (106). TLR1/2 heterodimers primarily sense triacylated lipoproteins present in most bacteria, but can also bind lipoteichoic acid from Gram positive bacteria, lipoarabinomannan and lipomannan from mycobacteria, and zymosan particles derived from fungal cell walls (5, 91, 124, 134, 138, 151). TLR2/6 heterodimers mainly sense diacylated lipoproteins derived from mycoplasma cell membranes (135, 136). Synthetic triacylated and diacylated lipopeptides are commercially available for use as TLR1/2 and TLR2/6 agonists; palmitoly-3-cysteine-lysine-4 (PAM₃CSK₄, PAM3) and macrophage activating lipopeptide-2 kDa (MALP2) are classic synthetic TLR1 and TLR6 agonists, respectively. Crystal structures for both TLR1/2 and TLR2/6 heterodimers reveal the mechanism for differential sensing of acyl chain numbers (61, 67) (Fig. 1.2). Two lipid chains from PAM3 insert into a hydrophobic pocket within the TLR2 ectodomain, while the third resides within a similar pocket in TLR1 (Fig. 1.2B). Interestingly, although TLR1 and TLR6 share high amino acid sequence identity, a phenylalanine (343F) residue blocks the putative hydrophobic channel of TLR6, limiting its ability to bind triacylated lipoproteins (167) (Fig. 1.2C). Similarly, a naturally occurring polymorphism in TLR1 (P315L) which lies near the hydrophobic ligand binding region reduces sensitivity of TLR1 for PAM3 (105). Like TLR4, TLR1, 2, and 6 dimers are known to utilize co-receptors, including CD14 and CD36, to enhance or specify activity, depending on the ligand. As expected, mice lacking TLR2 are highly susceptible to Gram positive Staphylococcus aureus and, when challenged with Mycobacterium tuberculosis, TLR2 mutants produce lower levels of TNFα, IL-1, and IL-2, compared to wildtype controls (130, 133). TLR1 is also known to cooperate with TLR2 to recognize mycobacteria. TLR1-deficient mice are not only hyporesponsive to synthetic triacylated lipopeptides but also to a major mycobacterial lipoprotein, LpqH (19 kDa lipoprotein) (134). Differential recognition of mycobacterial ligands by TLR1 is a major focus of my thesis research and will be a highlight of Chapter 3.

TLR10 is also phylogenetically related to TLR1 and TLR6, however, sequence homology is where the similarities end. The natural ligand for TLR10 is currently unknown, but is of great
interest to the Tapping laboratory. It has been shown that TLR10 lacks signaling elements and functions shared by other TLRs, such as common intracellular adaptor molecules and the ability to induce proinflammatory cytokines. Recent research in the Tapping lab has revealed that chimeric TLR10-TLR1 proteins are capable of recognizing agonists similar to TLR1, although the dissimilarities been sequence in the cytoplasmic domains of the two receptors suggest a unique signaling function (44). The ten TLRs, as well as their dimer partners and ligands, are displayed in Figure 1.1.

**Toll-like Receptor Trafficking**

Receptor trafficking and subcellular localization are vital for proper recognition of conserved molecular patterns by their respective TLRs. Distribution of TLRs within the cell is widespread and varies according to both the stage of TLR synthesis and the activation state of the cell. Multiple cellular compartments may contain TLRs simultaneously, including the endoplasmic reticulum (ER), Golgi apparatus, trafficking vesicles, plasma membrane, endolysosomal membrane, and phagosomal compartments.

Similar to other type-1 transmembrane proteins, translation of TLR mRNA begins in the cytoplasm but, once the short signal peptide is exposed, a signal recognition protein (SRP) binds the N-terminus of the growing polypeptide chain and directs the ribosome to the rough ER. A translocon protein complex then directs the growing amino acid chain through the ER membrane into the lumen. Translocation into the ER continues until a stop-transfer sequence is translated which directs the remaining polypeptide to be synthesized in the cytoplasm. Thus, the TLR’s extracellular, transmembrane, and signaling domains are properly oriented. The signal peptide bound by the translocator complex is cleaved, allowing the lumen-exposed extracellular domain to be modified by glycosyltransferases and properly folded by relevant chaperones. Depending on the TLR, a newly synthesized receptor may traffic out the ER by anterograde transport to other internal compartments or to the cell surface.

Regulation of TLR trafficking is an important mechanism by which cell responsiveness to microbial agonists can be controlled (90). At the most basic level, heighten or lowered gene transcription influencing TLR expression on the plasma or endosomal membranes can respectively increase or decrease inflammatory processes. In addition, complex regulation of TLR transport out of the ER would effectively modify receptor access to agonists. Mechanisms
controlling the final destination of each TLR are not fully understood but two important components have been identified as being important mediators of TLR trafficking: ER-resident chaperones and internal TLR trafficking motifs.

To date, four TLR-specific trafficking chaperones have been identified: glycoprotein 96 (gp96), protein associated with Toll-like receptor 4-A (PRAT4A), PRAT4B, and the human homolog of Caenorhabditis elegans uncoordinated 93 protein, UNC93B1 (3). These chaperones have been shown to act as specific mediators of Toll-like receptor subcellular distribution. Gp96 is a ubiquitous chaperone in the cell and expressed to high levels. It was observed that B cell lines deficient in gp96 showed hyporesponsiveness to microbial stimuli, an effect linked with subsequent lack of TLR surface expression (119). Other studies revealed that PRAT4A deficiency displayed similar phenotypes and a single mutation in the chaperone can block TLR trafficking and cellular responses to receptor stimulation (73, 132, 144). UNC93B1 displays specificity for the nucleic acid sensing TLRs, as a single mutation in this chaperone inhibits ligand recognition by endosomally expressed TLRs (131). Finally, little is known about PRAT4B except that it is required for TLR4 surface trafficking and cellular response to LPS (74). All of the TLRs, with the possible exception of TLR10, are known to rely on at least one of these chaperones for proper localization and cell sensitivity to respective agonists. TLRs also rely on internal amino acid sequence to serve as subcellular addresses, directing them to locations accessible to agonists. Trafficking motifs for TLR1, 3, 4, 7, 8, 9 have previously been identified (11, 48, 66). Amino acid sequence within the transmembrane domains of TLR7, 4, and 9, and sequences within the intracellular domain of TLR3 and 8 have been previously shown to influence proper receptor distribution. Chapter 2 will describe my studies characterizing the TLR1 trafficking motif, which combines sequence from both the transmembrane and intracellular domains. The direct mechanisms by which these sequences direct localization remain uncharacterized. Further details of TLR trafficking will be discussed in Chapter 2.

Toll-like Receptor Signaling

As with most mammalian receptors, TLR signal transduction involves a complex cascade of protein-protein interactions, phosphorylation, and cleavage events (Fig. 1.4) (18, 72). Ligand binding is believed to bring the signaling domains of two TLRs into close enough proximity as to provide a conformational platform supporting a complex of adaptor proteins (Fig. 1.2A). The
Toll-interleukin-1 receptor (TIR) domain is the conserved motif present in these two immune receptors which direct intracellular signaling. This domain facilitates protein-protein interaction between the TIR portion of the TLR and TIR-containing cytoplasmic adaptor proteins recruited to the dimerized receptors. It is not surprising that, since the ten TLRs recognize diverse agonists and traffic to varying subcellular compartments, they utilize multiple signal pathways to facilitate differential functions of host defense. Myeloid differentiation primary response 88 gene (MyD88) is the key adaptor protein used by all but one of the TLRs to initiate a common signal cascade but, depending on the TLR, an array of various proteins are subsequently recruited to the signaling complex. For example, TLR1, 2, 6, and 4 recruit a secondary adaptor protein, the TIR adaptor protein (TIRAP).

A primary outcome of TLR1, 2, 4, 5, and 6 signaling is activation of the transcription factor, nuclear factor kappa-B (NFκB) (70). Serving as a master regulator of the immune response, NFκB induces transcription of a host of pro-inflammatory cytokine and chemokines, such as tumor necrosis factor-alpha (TNFα), interleukin-1 beta (IL-1β), IL-6, IL-8, and IL-12, which direct early inflammation by recruiting and activating leukocytes at the site of infection. In addition to cytokines, NFκB upregulates surface molecules, such as major histocompatibility complex II (MHCII) and CD86, which in turn enhance antigen presentation and co-stimulation of recruited adaptive cells. These enhancements are key mechanisms by which TLRs not only recognize microbial patterns but actively compose an effective adaptive immune response to infection.

The pathway leading to NFκB activation involves many players, only a few of which will be described here. MyD88 binding to activated TLR dimers rapidly recruit interleukin-1 receptor associated kinases, IRAK1, 2, and 4, which leads to the phosphorylation and activation of the TNF receptor associated factor, TRAF6 (71). This E3 ubiquitin ligase eventually leads to the induction of the inhibitor of NFκB kinase (IKK), which phosphorylates the inhibitor of NFκB alpha (IκBα). NFκB is normally sequestered in the cytoplasm by way of binding and masking of its nuclear localization sequence by IκBα. Upon phosphorylation by IKK, IκBα is targeted for ubiquitination and subsequent degradation via the proteasome, freeing NFκB to translocate into the nucleus and induce target genes.

Although TLR7, 8, and 9 also use MyD88 and are capable of activating NFκB, sensing of viral nucleic acids by these receptors also induce type-1 interferons by a separate TRAF3-dependent pathway (69). Secreted interferons act in an autocrine and paracrine fashion to
induce antiviral states in cells by both shutting down protein translation and activating cytoplasmic RNases. Type-1 interferons, which include interferon-alpha (IFNα) and IFNβ, are induced by the transcription factors interferon regulatory factor 3 (IRF3) and IRF7. Like NFκB, the IRFs translocate to the nucleus to regulate target genes, including interferon-activated genes. Alternatively, TLR3 uses the TIR-containing adaptor inducing interferon beta (TRIF) in place of MyD88, but still uses TRAF3 in the same manner to TLR7, 8, and 9. Interestingly, both TLR2 and TLR4 are capable of inducing interferons by recruiting TRIF, although this mechanism requires the internalization of ligand-bound TLR4 into endosomal compartments (9, 65). In addition to NFκB and IRFs, TLRs activate the mitogen activated protein kinase (MAPK) pathway, c-Jun kinase (JNK) pathway, and activator protein-1 (AP-1), factors important for cell division, differentiation, and apoptosis.

Genetic Variation of TLRs and Infectious Disease

Recognition of pathogens and the establishment of a cytokine profile at the site of infection are fundamnet to microbial clearance and restoration of tissue homeostasis. It is not surprising that genetic variation within TLRs affecting either protein expression or function can influence susceptibility to infectious organisms. While there are no complete human TLR knockouts, there are known mutations which dramatically affect receptor function.

Phylogenetic divergence amongst the TLRs is a heavily researched topic and recent studies have provided insights into the co-evolution between TLRs and pathogenic microorganisms. Population genetic analyses reveal that human TLRs have evolved into two distinct groups separated by function (20). The intracellular nucleic acid sensing receptors, TLR3, 7, 8, and 9, were shown to have been undergoing strong purifying selection. Selection such as this ensures that particularly important residues are conserved and resist mutation. This sort of sensitivity indicates that these receptors play an important, non-redundant role in host survival, most probably against viral infections. Alternatively, the mutational constraint observed for the remaining cell surface TLRs is much more relaxed. Over 20% of individuals have at least one damaging missense mutation in TLR1, 2, 4, 5, 6, or 10, with 16% possessing at least one nonsense mutation (20). Interestingly, the TLRs with the two highest rates of missense mutations in the general population are dimer partners TLR1 (13%) and TLR2 (5%) (20). It is theorized that displaying a higher flexibility for mutation within these TLRs may, in certain
scenarios, enhance protective immunity. Indeed it has been shown that there is a high degree of positive selection within the TLR1-6-10 gene cluster which may be adapting certain mutations within these receptors to confer greater protection against specific pathogens (10).

Genetic variation in the form of single nucleotide polymorphisms (SNPs) is abundant in the human genome, with an estimated 10 million common SNPs present per genome (80). Numerous, common SNPs have been identified in human TLRs, many of which have been associated with differential susceptibility to infectious diseases (96). A SNP at nucleotide position 1174 (TLR5 392-STOP), an early stop mutation in TLR5, creates a truncated version of the receptor which decreases alveolar macrophage sensitivity to flagellin and increases susceptibility to Legionnaire’s disease (52). Well characterized nonsynonymous coding alterations in TLR4, D299G and T399I, have been associated with abrogated responsiveness to LPS by alveolar macrophages and airway epithelium, and studies correlate the 299G and 399I alleles with an increased susceptibility to malaria (6, 97). A dominant negative SNP of TLR3 (P554S) in heterozygous form was shown to predispose pediatric patients to herpes simplex virus-associated encephalitis (13). Although many TLR SNPs associate with heightened susceptibility to pathogens, others exhibit protective qualities. The same TLR4 alleles have been shown to increase resistance to Legionnaire’s disease, while a SNP deleterious to TLR2 signaling (R753Q) decreases incidence of chronic Lyme disease (122). There is increasing evidence in the literature that certain species of bacteria, especially mycobacteria, are capable of subverting TLR signaling as a means of immune evasion, and that deficiencies in TLR activity may provide protection against these inhibitory effects.

Mycobacterial Disease

Members of the genus Mycobacterium, such as M. leprae, M. tuberculosis, and M. avium, represent evolutionarily ancient pathogens of historical and worldwide prevalence. The earliest known human case of M. leprae infection has been traced back to over 4000 years ago from East Indian skeletal remains (120). Human infection by M. tuberculosis is arguably even more ancient, as evidenced by 9000 year old bone samples from Neolithic settlements testing positive for TB DNA (58). This thesis will focus on TLR1 in relation to the mycobacterial diseases leprosy and tuberculosis, with M. avium serving as a BSL2 model mycobacterial organism used for experimental analyses.
Mycobacterium tuberculosis was identified as the causative agent of tuberculosis in 1882 by the microbiology pioneer, Robert Koch. This disease was, and still is today, a global killer, with one third of the world population estimated to be infected by *M. tuberculosis* (Mtb) bacilli. Droplets containing Mtb cells are aerosolized by coughs or sneezes from infected individuals, where they are subsequently inhaled into the lungs of others and reach their primary cellular tropism, alveolar macrophages. Upon phagocytosis, Mtb cells begin a complex program of host immunosuppression, which blocks normal antimicrobial processes of the infected macrophage and establishes a suitable niche for intracellular replication. Infected macrophages secrete proinflammatory cytokines and chemokines, which recruit monocytes and helper T cells. These cells ultimately conglomerate to wall off the site of infection, creating characteristic granulomas or tubercles.

Identified in 1873 by Gerhard Armauer Hansen as the causative agent of leprosy, *M. leprae* are fastidious, intracellular bacilli which have been studied extensively for their complex pathogenesis and host interactions. Cellular tropisms for *M. leprae* principally include tissue-resident macrophages, especially in the skin and upper respiratory tract, and Schwann cells of the peripheral nervous system. The intricate spectrum of clinical manifestations which exists for the disease is illustrated by two polar responses, tuberculoid or lepromatous leprosy, and various intermediate or borderline forms (98). The tuberculoid manifestation is typically less severe and is characterized by lower leprosy burden and containment of bacilli by distinct granulomas. This form exhibits few hypopigmented skin lesions and scarce thickening of peripheral nerves, leading to loss of sensation in extremities and the skin. Lepromatous leprosy constitutes the opposite polar reaction to infection where high, diffuse bacterial load can cause extensive skin plaques, nodules and thickening, and numerous anesthetic zones due to pervasive peripheral nerve damage.

Surprisingly little genotypic variation exists between strains of *M. leprae*, a fact inconsistent with the high degree of variability in virulence and disease penetrance between individuals. This suggests that success of infection and leprosy progression rests in large part upon the patient’s immune response and genetic complement. Strikingly, the variable spectrum of leprosy outcome correlates tightly with the balance of T helper cell-mediated immunity responding to *M. leprae* infection. A robust T\(_{h1}\) host response, featuring appreciable production of interferon-gamma (IFN\(_\gamma\)) and interleukin-12 (IL-12) along with macrophage and CD4\(^+\) T cell activation, is typically represented in the contained, tuberculoid form. Alternatively a strong T\(_{h2}\)
response, characterized by an intense but non-protective humoral reaction, in addition to high IL-4 and IL-10 release, coincides with the lepromatous manifestation. A system of regulation controlling such a delicate balance is certain to be complex, but undoubtedly must begin with pathogen recognition at the sites of host inoculation.

**TLR-Mediated Recognition of Mycobacteria**

The majority of TLRs (TLR3, 4, 5, 7, 8, and 9) signal via homodimerization in the presence of agonist binding. Members of the TLR2 subfamily, which includes TLR1, 2, 6, and 10, are unique in their ability to form heterodimeric complexes which sense an extremely diverse group of microbial molecules. Interestingly, the cell wall and membrane of mycobacteria are especially rich in TLR1/2 agonists. The lipoglycans lipomannan (LM) and mannose or arabinose-capped lipoarabinomannan (ManLAM and AraLAM) are major virulence factors in mycobacterial species. ManLAM and AraLAM in particular are potent TLR1/2 agonists, and have been shown to contribute both to macrophage activation and immunomodulation of host responses (96, 138). These molecules behave in large part as scavengers of reactive oxygen species deployed by the phagosomal oxidative burst (22). In addition, stimulation of macrophages with purified *M. leprae* and *M. tuberculosis* LAM molecules has been shown to decrease IFNγ-inducible microbial killing by macrophages, reduce T cell proliferation and activation, and inhibit protein kinase C, a key transduction molecule in IFNγ and respiratory burst signaling (22, 68, 124, 126). Several mechanisms by which recognition of mycobacterial products by the TLRs counterintuitively promotes microbial growth will be discussed in the next section.

Although TLR2 has the ability to dimerize with multiple TLR co-receptors, TLR1/2 heterodimers are the primary sensors by which immune cells recognize mycobacterial lipoproteins (75, 88). Significant examples of such mycobacterial agonists include the *M. leprae* and *M. tuberculosis* 19kDa lipoprotein orthologs, ML1966 and LpqH (92). These membrane-anchored proteins possess adhesin properties and serve as major mycobacterial surface antigens (32). Like the cell wall lipoglycans, LpqH in particular provides robust immunosuppressive functions. The impressive ability of these triacylated lipoproteins to deactivate macrophages has been well characterized and shown to be dependent on engagement of TLR2 (8, 39, 42, 79, 88, 103, 108, 109). The *M. leprae* annotated genomic sequence predicts 31 different lipoprotein genes, including the enzymatic machinery for ligation of acyl chains. Orthologous partners for all of these genes exist in *M. tuberculosis*, which
possesses an additional 60 genes encoding putative lipoproteins. In addition to the well-studied 19kDa antigen, *M. leprae* also encodes a 33kda (ML0603) lipoprotein with TLR2 immunostimulatory activity. It is conceivable that many of the triacylated lipoproteins displayed by both leprosy and tuberculosis bacilli could signal via TLR1/2 heterodimers, and may possess similarly immunosuppressive functions to those of the 19kDa antigen.

Non-lipidated molecules derived from mycobacteria are also capable of binding and signaling through TLR1/2. The 6kDa early secreted antigenic target (ESAT-6) of *M. tuberculosis* is a highly potent CD4+ T cell antigen and is absent in most non-tuberculosis complex mycobacteria (46, 110, 127). A commonly used diagnostic test for TB, approved by the FDA in 2005, is based upon whole blood stimulation with purified ESAT-6 in an IFNγ release assay. Unlike classical proinflammatory TLR agonists, it has been shown that ESAT-6 engages TLR2 in a way that inhibits MyD88-dependent TLR signaling including activation of NFκB and interferon regulatory factors (110). *M. leprae* possesses an ESAT-6 ortholog with 36% identity, ML0049, and both monocytes and T cells from leprosy patients respond to L-ESAT-6 by secreting IFNγ (41). At this time no direct evidence is available to support an immunosuppressive role for ML0049 similar to that of ESAT-6.

While innate immune responses to both *M. leprae* and *M. tuberculosis* rely heavily on TLR1/2 stimulation by various cell wall components, TLR4 has also been shown to play a role in detecting mycobacteria. The classical ligand for TLR4 is lipopolysaccharide (LPS), derived from the outer cell membrane of Gram-negative bacteria. Despite the fact that LPS is absent from mycobacterial membranes, studies in transfected cells and murine macrophages have shown that *M. leprae* and *M. tuberculosis* are both recognized by TLR4 (91). For *M. tuberculosis*, this effect was linked to MD-2-mediated TLR4 recognition of secreted heat shock protein 65 (HSP-65) and chaperonin 60 protein (19, 21). Another secreted tuberculosis protein, the adhesin heparin-binding hemagglutinin (HBHA), was found to bind TLR4, induce maturation, and activate proinflammatory cytokine secretion in dendritic cells (63). *M. leprae* encodes orthologs of HSP60, HSP65, and HBHA, but no studies have examined whether these proteins possess similar TLR4 stimulating qualities to those of *M. tuberculosis*. It has been reported however that LPS binding by TLR4 can be blocked with heat-killed *M. leprae*, inhibiting monocyte secretion of IL-1β and IL-6 (16).

TLR9 mediates recognition of unmethylated CpG elements in viral and bacterial DNA, a motif relatively rare in vertebrate genomes (27). Unlike TLR1, 2, and 4, which traffic to the cell
surface, TLR9 is predominantly expressed intracellularly in the endoplasmic reticulum (81). Upon pathogen stimulation, TLR9 localizes to endosomal compartments, where the receptor gains access to genomic DNA released from the degradation of phagocytized bacteria or internalized virus (2). Primary human monocyte-derived macrophages and murine dendritic cells respond to stimulation with CpG DNA derived from various strains of \textit{M. tuberculosis} (H37Rv and H37Ra) and \textit{M. bovis} (wildtype and BCG) by increasing transcription of TNFα and IFNα (7). Interestingly, DNA from the attenuated strains induces a much more vigorous TNFα response than DNA from virulent mycobacteria (75).

**Subversion of TLR Signaling by Mycobacteria**

Based upon the established role of TLRs in host defense, polymorphisms which negatively affect receptor function would be expected to increase susceptibility and worsen outcomes to infection. This generality holds true for a number of TLR polymorphisms in various infectious disease settings and simply reflects the host’s inability to properly recognize and respond to bacterial agonists. However, a number of functionally deficient polymorphisms in TLR1 and TLR4 have been found to confer protection to mycobacterial infection. This finding suggests that, during the course of evolution, mycobacteria have subverted the TLR system in ways that are advantageous to establishing and maintaining infection. The extreme success for a pathogenic organism such as mycobacteria requires either high rates of transmission or powerful immunoevasive strategies. Transmissibility for tuberculosis is not particularly profound and only occurs during relatively rare active phases. The likelihood of passing leprosy is equally low, yet these pathogens are consistently endemic worldwide. Over many decades, the exquisite mechanisms by which mycobacteria neutralize and even subvert host defenses have been uncovered, potentially explaining the impressive effectiveness of these pathogens. Much of the experimental evidence supporting this idea in the context of TLRs is provided below.

Several studies have revealed that prolonged mycobacterial stimulation via TLR1/2 causes macrophages to become refractory to IFNγ (Fig. 1.6) (81, 84-91). IFNγ, a type-2 interferon primarily secreted by T cells and natural killer cells, is a Th1-skewing cytokine essential to the containment of mycobacterial infection. This secreted dimeric glycoprotein is a classical activator of innate immune effectors and plays a key role in the induction of macrophage microbicidal functions, including phagolysosome maturation and oxidative burst. In addition,
IFNγ facilitates a priming function by upregulating the cellular machinery required for antigen presentation, phagocytosis, and T cell co-stimulation. Mice deficient in cytokine production or the IFNγ receptor develop disseminating and ultimately fatal mycobacterial disease (28). Also, a human mutation linked to IFNγ signaling associates with susceptibility mycobacterial disease and disseminated infection following vaccination with the Calmette-Guérin bacillus strain of *M. tuberculosis* (29).

TLR activation plays an important role in IFNγ production by T cells. Active NFκB provided by TLR ligation on macrophages or dendritic cells induces expression and secretion of IL-12, a cytokine essential to the Th1 skewing of CD4+ T helper cells. Once exposed to IL-12, these cells begin secreting IFNγ, establishing a positive IFNγ/IL-12 feedback loop (Fig. 1.6). However, stimulation with the *M. tuberculosis* 19kDa lipoprotein is sufficient to greatly reduce induction of IFNγ-regulated genes in many cell types, including murine RAW264.7 cells, mouse bone marrow derived macrophages, human THP-1 cells, and primary human monocytes (Fig. 1.6). Microarray analysis has shown an inhibition of IFNγ-inducible transcription of IL-12 receptor mRNA upon stimulation with mycobacterial lipoproteins. This provides direct interference with the IFNγ-IL-12 signaling axis. Further subversion of TLR1/2 signaling is illustrated by abrogated IFNγ-dependent upregulation of macrophage activation markers MHCII, CD64, and CD86 in cells stimulated with either whole mycobacteria or purified triacylated lipoproteins. MHCII is a receptor essential for the activation of adaptive immunity by professional antigen presenting cells (APCs). Forced downregulation of MHCII by mycobacteria corresponds to reduced antigen processing in murine bone marrow derived macrophages and associates with a curtailed ability to activate T cells. Phagocyte-expressed CD86 (B7.2) provides co-stimulatory signals which, in addition to MHCII, are essential for CD4+ T helper cell activation and survival. Simultaneous inhibition of CD86 and MHCII by TLR1/2 activation would impart a major hindrance on recruitment of adaptive help at the site of infection. Finally, CD64 (FcγRI), a key phagocytic receptor specific for IgG-opsonized pathogens, facilitates pathogen internalization and induction of the oxidative burst. Downregulation of this receptor could potentially reduce phagocytic uptake of mycobacteria by macrophages, dendritic cells and neutrophils, thereby decreasing pathogen clearance and generation of peptides for antigen presentation.

*M. tuberculosis* also appears to subvert host adaptive immunity through secretion of ESAT-6. The engagement of TLR2 by ESAT-6 inhibits IFNγ induction of CTIIA, the transcriptional
co-activator required for MHCII expression (78). Furthermore, the TLR2-dependent inhibition of IFNγ signaling by mycobacterial lipoproteins was shown to act through a similar mechanism, whereby inhibition of CIITA blocks induction of many macrophage activation markers (111, 112).

Another recent TLR2-dependent immunoregulatory mechanism used by mycobacteria has been identified which affects the macrophage respiratory burst (31). Inducible nitric oxide synthase (iNOS) generates nitric oxide (NO), a major component of the phagolysosomal oxidative burst. NO reacts with superoxide, a product of NADPH oxidase, to generate peroxynitrite, a powerful oxidant capable of damaging diverse biological molecules within phagocytized pathogens. iNOS requires cellular pools of L-arginine to drive catalysis and the availability of this substrate has been shown to be a rate limiting step in production of NO.

Arginase-1, a metabolic enzyme important in the urea cycle, shares arginine as a substrate. This enzyme participates in the final step of the cycle by metabolizing L-arginine to L-ornithine and urea. Curiously, it has been reported that during *Mycobacterium bovis* (BCG) infection of J774.1 mouse macrophages, the levels of urea rise with increasing bacterial replication (137). A recent study has linked these observations to mycobacterial pathogenesis by showing that stimulation of primary mouse macrophages with BCG upregulates levels of both arginase-1 mRNA and protein (34). Surprisingly, TLR2 and MyD88 knockout mice resist upregulation of arginase-1 (34). In addition, Arginase-1 knockouts exhibit enhanced NO production in BCG infected macrophages and lower bacterial burdens in an aerosol infection model of *M. tuberculosis* (34).

Another report found that cell supernatants from BCG infected macrophages are capable of inducing arginase-1 in uninfected neighboring macrophages, an effect dependent on the production of IL-6 and IL-10 (115). These data suggest that mycobacterial stimulation of TLR2 induces arginase-1, which inhibits the oxidative burst and allows increased survival within the macrophage endosome (Fig. 1.7). Similar to the inhibition of IFNγ signaling, Arginase-1 mediated immunosuppression was found to be dependent on CEBP-β (34).

Colonization of Schwann cells by *M. leprae* is known to stimulate granuloma formation and cell-mediated nerve injury (128). The influx of numerous adaptive and innate immune cells proximal to an infected peripheral nerve may produce direct Schwann cell damage and killing, or indirect death via pressure-induced ischemia. Immunohistochemistry of skin biopsies from both lepromatous and tuberculoid leprosy patients reveals TLR1 and TLR2 surface expression by multiple cell types, including Schwann cells (77, 104). Schwann cell TLR2 is functional as indicated by the activation of NFκB by synthetic bacterial lipopeptides (77, 104). TLR2-
dependent activation by the 19kDa lipoprotein of \textit{M. leprae} has also been shown to induce apoptosis in primary human Schwann cells. This effect was reduced when cells were incubated with blocking anti-TLR2 antibodies (104). Additionally, nerve injury promotes massive upregulation of TLR1, many fold over most other Schwann cell-expressed TLRs (45). Since \textit{M. leprae} is capable of activating apoptosis in a TLR2, and presumably TLR1-dependent manner, it is possible that upregulation of TLR1 by nerve damage may establish a positive feedback loop for Schwann cell deterioration (Fig. 1.8). Apoptotic properties have also been noted for the \textit{M. tuberculosis} 19kDa antigen. Lopez \textit{et al.} revealed a TLR2-dependent induction of apoptosis in monocyte-derived macrophages stimulated with the triacylated lipoprotein (84).

\textbf{TLR Polymorphisms and Mycobacteria}

Genetic association studies have identified a number of single nucleotide polymorphisms (SNPs) in TLR genes which associate with susceptibility or resistance to bacterial and viral infection. Given the importance of TLRs in mediating host responses to pathogenic mycobacteria, it is not surprising that genetic variation effecting expression or function of these receptors influences host susceptibility to leprosy and tuberculosis. Due to the high relevance of host genetic factors in the outcome of \textit{M. leprae} and \textit{M. tuberculosis} infection, decades of research have been devoted to identifying key polymorphisms which associate strongly with the predisposition to clinical leprosy manifestation, spontaneous clearance, and to the spectrum of disease progression. The following section will review specific TLR polymorphisms which are associated with \textit{M. leprae} and \textit{M. tuberculosis} infection. This section will focus on the potential mechanistic basis for their effects on pathogenesis and host response based on the known subversive effects described in the previous section.

An insertion/deletion polymorphism in the TLR2 promoter lies at position -196 to -174 bp upstream of the start codon. This polymorphism has been studied in the context of TLR2 expression in both asthma and hepatitis C infection (100, 102). An \textit{in vitro} reporter construct carrying the deletion allele of the TLR2 promoter induces lower luciferase activity than the insertion allele, suggesting that the former possesses inherently reduced transcriptional activity (102). In addition, primary human monocytes from individuals carrying the deletion allele produce significantly less IL-8 upon stimulation with peptidoglycan (100). A subsequent disease association study of Caucasian (OR=0.41, p=<0.001) and African (Guinea-Bissau)
(OR=0.70, p=0.02) tuberculosis cohorts revealed protection via homozygosity for the fully functional insertion allele (143).

A microsatellite marker located between -162 and -100 bp in the promoter region of TLR2 contains two adjacent variable number tandem repeats of CT and TG which vary in length from 280-290 bp (15, 148). An investigation of Ethiopian leprosy patients by Bochud et al. revealed a lower frequency of the 290 bp repeat allele in disease cases versus healthy controls (OR=0.62, p=0.02) (16). In addition, a 288 bp allele was observed less frequently in lepromatous versus tuberculoid leprosy patients (OR=0.49, p=0.02). However, this same allele was also shown to greatly increase susceptibility to reversal reaction (OR=5.83, p=0.001) in a subgroup of patients which had been followed for 8 additional years (15). Using an in vitro reporter assay, Yim et al. revealed that variability in the number of microsatellite repeats effected TLR2 promoter function in response to cytokine stimulation (148).

No phenotypic effects on TLR2 function have been linked to a synonymous SNP (N199N, C597T) in the extracellular domain of this receptor. However, the 8 year leprosy reaction followed-up by Bochud et al. identified a protective role for TLR2 597T against reversal reaction (OR=0.34, p=0.002) (15). This polymorphism was also shown to be highly relevant in a Vietnamese cohort of TB patients where progression of disease was more than two fold higher and dissemination of bacilli to the brain was 3 fold higher in individuals homozygous for the 597C allele (140).

The G2258A SNP (R753Q) in the cytoplasmic domain of TLR2 has been well characterized as a functionally deficient variant with reduced responses to bacterial lipoproteins and synthetic di- and triacylated lipopeptides (85, 122). Most reports on TLR2 R753Q have shown inhibition of signaling in response to Borrelia burgdorferi lipoproteins, such as OspA and whole cell lysates. One report observed a lower frequency of TLR2 753Q in Lyme disease patients than healthy controls (OR=0.39, p=0.033) and an even stronger protective effect was conferred in late stage Lyme disease patients (OR=0.16, p=0.018) (122). Interestingly, the converse result was obtained when a Turkish cohort of tuberculosis patients was genotyped for the TLR2 polymorphism. In this study, the risk of developing tuberculosis was increased over 6-fold for TLR2753Q/Q individuals and 1.6 fold for heterozygotes (107). Another study of Turkish pediatric patients also associated the TLR2 R753Q heterozygotes with TB, citing an over 5-fold increased risk of infection (OR=5.05, p=<0.001) (30). Taken together these results indicate that a functionally deficient TLR2 variant plays opposite roles in two different infectious diseases;
protection in the context of Lyme disease and susceptibility in the context of TB. Although no disease association studies have been performed between TLR2 R753Q and leprosy, it would be very interesting to see if the results observed in the context of TB would also extend to *M. leprae*.

Numerous studies of two TLR4 polymorphisms, D299G (G896A) and T399I (C1196T), have revealed increased risk of infection with several Gram-negative organisms as well as septic shock (1, 50, 86). These SNPs, which alter amino acids in the extracellular domain of TLR4, appear to affect receptor function depending upon the experimental system under investigation. Some studies have reported a decrease in LPS-induced IL-12 and IL-10 in asthma patients, reduced cytokine secretion in an inhaled LPS human model, and inhibited signaling in cell-based transfection models (36, 121, 125). However, other studies have found no functional deficits exhibited by primary human monocytes and PBMCs obtained from individuals who are either heterozygous or homozygous for these TLR4 variants (33, 35, 142).

Similar to TLR2, deficient TLR4 function is generally associated with increased susceptibility to mycobacterium infection. For example, a significant increase in the frequency of the TLR4 299G allele was observed in pulmonary tuberculosis patients in an Asian Indian cohort (OR=2.1, *p*=0.001), with highest bacillary loads observed in homozygous individuals (99). Other studies in HIV/TB co-infected patients indicate that 299G is a risk factor for active tuberculosis in Mediterranean Caucasians (OR=2.0) and Tanzanian patients (OR=2.8, *p*=0.06), but at borderline statistical significance (37). No statistically significant findings were associated with TLR4 T399I for TB (99, 37). In contrast to *M. tuberculosis*, a protective role for TLR4 D299G has been identified in association with *M. leprosy* infection. Bochud et al. examined an Ethiopian population and observed lower frequencies of TLR4 D299G (OR=0.34, *p*<0.001) and T399I (OR= 0.16, *p*<0.001) among leprosy patients (14). Conversely, another investigation found TLR4 D299G in a Malawi cohort of leprosy patients afforded no protection, although their cohort was half the size of the previous study (38).

Velez et al. investigated the prevalence of several TLR9 polymorphisms in tuberculosis patients of Caucasian, African American, and African (Guinea-Bissau) descent (143). No functional studies have been performed examining the effects of these polymorphisms on TLR9 signaling. However, two of these gene-flanking polymorphisms, rs352139 and rs5743836, appear to confer protection to TB. rs352139 was observed less frequently in Caucasian (OR=0.53, *p*=0.017) and African American (OR=0.58, *p*=0.029) TB patients, while rs5743836 provided
protection in African Americans (OR=0.54, p=0.024) and in Caucasians (OR=0.50, p=0.015) (143). No statistically significant reduction in risk was associated with members of the Guinea-Bissau cohort.

The TLR1 I602S Polymorphism and Mycobacteria

A SNP involving a thymine to guanine transversion (T1805G) in TLR1 results in a nonsynonymous substitution at amino acid position 602 (I602S); a position residing in the cytoplasmic region proximal to the receptor’s transmembrane domain (52, 62). Although western blotting and intracellular staining illustrate equivalent protein expression of each variant, both transfection and primary human monocyte studies reveal a trafficking deficiency for TLR1 602S, causing the receptor to be absent from the plasma membrane (62). In transfected cell lines and monocytes from TLR1 602S/S homozygotes, the subsequent lack of surface TLR1 602S induces a state of hyporesponsiveness to TLR1/2 agonists, including bacterial lipoproteins and synthetic triacylated lipopeptides (52, 62). These results serve to highlight the importance of TLR localization in receptor function.

The allelic distribution of TLR1 I602S varies greatly depending on racial ancestry. For example, Caucasian individuals possess the 602I allele at ~25% and the 602S allele at ~75%, while African American individuals possess a reciprocal allele frequency of ~75/25 percent for I/S (34, 51). Hispanic, Turkish and Nepalese individuals have I/S ratios of ~70/30, 57/43, and ~94/6 percent, respectively (34, 46, 51). The TLR1 602S allele appears to be virtually absent in East Asian individuals with a 602I allele frequency of >99% (62).

Peripheral blood mononuclear cells from TLR1 602S/S individuals were shown to have greatly diminished cytokine production when stimulated with whole irradiated *M. leprae* and *M. tuberculosis*, as well as membrane and cell wall fractions (52, 62, 95). It would therefore be expected that individuals homozygous for TLR1 602S would have increased susceptibility to mycobacterial infection. However, several disease association studies have revealed a protective role for the deficient TLR1 602S variant against the development of both clinical leprosy and tuberculosis (52, 87, 95, 147). The study performed by Johnson et al. observed that a cohort of Turkish leprosy patients had a significantly higher allele frequency for TLR1 602I versus healthy controls. Conversely, the TLR1 602S allele was significantly underrepresented
amongst leprosy patients, with an odds ratio for infection of 0.48 (p<0.05) among TLR1 602S/S homozygotes (62).

\( T_{H2} \)-directed lepromatous or borderline forms of leprosy are potentially unstable disease states and may quickly revert to a \( T_{H1} \) type adaptive response during a so-called reversal reaction. This can result in a sudden and exacerbated increase in acute T cell-mediated immunity and create more intense tuberculoid symptoms along with local tissue damage. Misch et al. observed that the TLR1 602S allele confers protection against leprosy reversal reaction (OR= 0.51, p=0.01) (95). TLR1 I602S also has been shown to influence risk of tuberculosis. Ma et al. observed a significant increase in the rate of extrapulmonary TB infection in African American patients possessing the 602I/I genotype (OR= 2.5, p<0.001) (87). Striking evidence of this polymorphism’s role in leprosy was established when an extensive and unbiased genome-wide array of 2092 genes in 1500 individuals identified TLR1 602S (OR=0.31, p<0.001) as one of two alleles that afforded the greatest protection against leprosy; the other being the MHCII allele HLA-DRB1/DQA1 (OR=0.43, p<0.001) (147). A second polymorphism in TLR1, N248S (A743G), is in strong linkage disequilibrium with the TLR1 602I allele which may explain the finding, observed in a Bangladesh cohort of leprosy patients, that M. leprae infection associates with the TLR1 248S/S genotype (OR=1.34, p=0.02) (52, 95, 123).

Collectively, the above studies convincingly highlight the role of TLR1 and TLR2 in mycobacterial pathogenesis. Based on the established immunosuppressive activity of the TLR1/2 complex following recognition of mycobacterial agonists, it is not difficult to imagine why a functionally deficient allele of TLR1 would be advantageous. If TLR1 602S is unable to gain access to the cell surface, where it is proximal both to its co-receptor and bacterial ligand, immune evasion through the TLR1/2 signaling complex would not be achieved by mycobacteria (Fig. 1.6-1.8). One could take this a step further to suggest that TLR1 602S/S homozygous individuals infected with \( M. leprae \) are more likely to develop the less severe tuberculoid form of the disease. It is intriguing to note that similar to TLR1, but unlike TLR2, deficient TLR4 functions are generally associated with increased resistance to \( M. leprae \). If the D299G and T399I SNPs do indeed confer hyporesponsiveness to TLR4 agonists, the protection to mycobacteria infection is analogous to the trafficking deficient TLR1 I602S. At this time no groups have examined the potential for mycobacteria-derived TLR4 agonists to induce immunosuppressive effects similar to that of TLR1/2 ligands. Such studies could provide a mechanism by which deficient TLR4 signaling provides resistance to mycobacteria.
Through many functional and genetic studies it can be concluded that single nucleotide polymorphisms in TLRs have the ability to both modify receptor function and associate with risk of leprosy and tuberculosis (Fig 1.9). Some investigations have identified SNPs which negatively affect TLR signaling and correspondingly induce hyporesponsiveness in immune cells to these bacteria. In some cases, such as TLR2 in the context of TB and leprosy and TLR4 in the context of TB, this abrogated function correlates unsurprisingly with increased susceptibility of the host to infection. Unexpectedly however, this trend is not universal. The I602S polymorphism of TLR1 inhibits trafficking of the receptor to the cell surface thereby abrogating innate recognition of mycobacterial agonists. This functional deficiency in TLR1 is not associated with an increased risk of infection but instead reduces the likelihood of developing clinical leprosy by over half. Chapter 3 will describe a study which provides mechanistic insights for this unexpected protective phenotype.

**Thesis Outline**

This dissertation focuses on the characterization of the aforementioned human single nucleotide polymorphism, TLR1 I602S. Conversion of an isoleucine to a serine, whose position lies intracellularly near the TLR1 transmembrane domain, greatly inhibits surface expression of the receptor to levels undetectable by flow cytometric analysis (62). Associated with the aberrant surface display of the I602S polymorphism is an inability of primary monocytes and macrophages to respond to TLR1/2 agonists, such as bacterial lipoproteins and lipoglycans (52, 62). Previous work by Johnson et al. also revealed a protective role for TLR1 602S against mycobacteria, in which the incidence of leprosy in homozygous individuals was significantly reduced (145). Subsequent studies have strongly supported these results and have further illustrated protection conferred by the trafficking-deficient TLR1 602S allele against leprosy reversal reaction and tuberculosis (87, 95, 141, 147).

The TLR1 602 polymorphism and its various alleles have been associated with not only mycobacterial infection but also other infectious diseases including Chlamydia, Lyme disease, inflammatory bowel disease, urinary tract infection, and even differential BCG and Lyme vaccination efficacy (51, 113, 118, 129, 139).

The allele frequencies of 602I and 602S in the human population appear to be heavily dependent on racial ancestry. The allele frequency for 602S is 75% in Caucasians, 26% in
peoples of African descent, and is extremely rare (<1%) among individuals of East Asian ancestry. The combined genotyping data from the National Center for Biotechnology Information SNP database and the Johnson et al. study show that over half of white individuals were homozygous for the aberrantly displayed TLR1 allele (TLR1 602S/S), while the majority of peoples of African and Asian descent were homozygous for the surface-expressed allele (TLR1 602I/I). Organized allele frequencies for several ancestral backgrounds can be found in Figure 1.5.

My thesis research has sought to link genotype to phenotype by answering two main questions: why does a single amino acid change in TLR1 abrogate surface trafficking of the receptor and what is the mechanism by which TLR1 602S/S individuals resist mycobacterial disease? To answer the first question, I used PCR mutagenesis to create point, truncation, and chimeric TLR mutants to determine what primary amino acid sequence is required for surface trafficking of TLR1. I hypothesized that the serine at position 602 was interrupting a potential localization motif or a domain required for efficient interaction with components of the cell’s protein trafficking machinery. Other approaches I used to answer this question involved finding stimuli which could either rescue the trafficking phenotype of TLR1 602S or to mimic the deficiency with TLR1 602I. The results for these experiments revealed not only that the 602S position lies within the trafficking sequence for TLR1 but also highlighted two important and opposing trafficking functions for the ER chaperones, PRAT4A and PRAT4B. These results will be discussed in greater detail in Chapter 2.

Chapter 3 examines the mechanisms by which the TLR1 602S protects against mycobacteria. It is known that mycobacteria have a vast arsenal of immunomodulatory strategies, some of which involve usurpation of TLR1/2 signaling to inactivate monocytes and macrophages. Because these cell types play a central role in anti-mycobacterial immunity, we hypothesized that the deficiency in TLR1/2 signaling observed in TLR1 602S/S individuals could offer protection against TLR-dependent immunosuppression. I will describe how TLR1 602S/S macrophages resist the downregulation of important macrophage activation markers, resist inhibition of IFNγ signaling, block upregulation of enzymes which normally abrogate the oxidative burst, and how they potentially display enhanced killing of mycobacteria. Finally, Chapter 4 will summarize the results developed over my graduate tenure and will propose future directions.
Figure 1.1 Subcellular organization of the TLRs.

The known human TLRs, their relative subcellular localization, dimer partners, and respective general ligands are shown here. TLR1, 2, 4, 5, 6, and 10 are predominantly expressed on the cell surface, while TLR3, 7, 8, 9 are distributed to intracellular endosomal compartments. A function common to all TLRs is, upon ligand-mediated dimerization, signaling events lead to activation of a master regulator of the immune response, NFκB.
Figure 1.2 Crystal structures of ligand-bound TLR1, 2, and 6.

(A) Shown is a plasma membrane-expressed TLR2 (blue)/TLR1 (green) heterodimer with paired intracellular TIR signaling domains. (B) TLR1 (orange) and TLR2 (pink) heterodimerize to sense the synthetic triacylated lipopeptide, PAM3CSK4 (black). (C) Similarly, TLR2 (pink) and TLR6 (silver) heterodimerize to bind a synthetic diacylated lipopeptide, PAM2CSK4 (purple). Phenylalanine 343, which blocks the hydrophobic channel used by TLR1 to bind a third acyl chain, is labeled (adapted from refs 61, 67).
<table>
<thead>
<tr>
<th>TLRs</th>
<th>Localization</th>
<th>Endogenous Ligands</th>
<th>Synthetic analogs</th>
<th>Fully synthetic molecules</th>
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<tr>
<td>TLR1</td>
<td>Plasma membrane</td>
<td>Lipopeptides (Bacteria and Mycobacteria), Soluble factors (A. mlengitis)</td>
<td>HSPB6, HSPB7</td>
<td>Tricyclic lipopeptides</td>
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**Figure 1.3 List of Known Natural and Synthetic TLR Ligands.**

Listed are the ten human TLRs, their subcellular localization, and ligands (89).
Figure 1.4 The Toll-like Receptor Signaling Pathways.

TLR signaling involves a complex network of adaptors, kinase, and ubiquitin ligases to transmit signal upon ligand-binding. Cartooned are examples of surface (TLR4) versus intracellular (TLR3) receptor signaling pathways. TLR activation can not only lead to release of proinflammatory cytokines via NFκB, but also induces interferons using IRF (24).
Figure 1.5 TLR1 I602S Allele Frequencies Among Several Ancestral Backgrounds.

Frequencies for the serine or isoleucine alleles at position 602 in TLR1 are shown. Peoples of African descent possess an I:S frequency of 75:25%; Asian, 99:1%; Caucasian, 25:75%; Hispanic 70:30%; Nepalese, 96:4%; Turkish, 47:53% (49).
Figure 1.6 Mycobacterial inhibition of the IFNγ/IL-12 axis.

Stimulation of macrophage TLRs by bacterial products activates NFκB which drives transcription of interleukin-12, along with other cytokines and chemokines that subsequently recruit and instruct T cells. Secreted IL-12 stimulates T cells to produce IFNγ, which serves to further stimulate the macrophage. Activation of macrophages by IFNγ leads to induction of transcription factors such as STAT1 and CBP, which facilitate production of transactivators, including CIITA. This results in the transcription of multiple IFNγ-regulated genes, including MHCII, CD86, and CD64. MHCII is required for antigen presentation and, along with CD86, is used by macrophages to activate CD4+ T helper cells. Antibody-opsonized particles are recognized by the phagocytic receptor, CD64, which binds the Fc portion of IgG. TLR1 602I traffics to the cell surface where it forms heterodimers with TLR2 to sense microbial agonists. Binding of the mycobacterial 19kDa lipoprotein to TLR1/2 has been shown to block STAT1-CBP activity, thereby dampening the induction of IFNγ-regulated genes. It is possible that the trafficking deficient TLR1 602S allele (dashed lines) confers protection against leprosy and TB by preventing the subversion of IFNγ signaling by these mycobacterial agonists (158).
Arginine (R) is required as a substrate for inducible nitric oxide synthase (iNOS) in the production of nitric oxide. Nitric oxide contributes to microbial killing by combining with reactive oxygen intermediates in the phagolysosome to produce highly toxic peroxynitrite. Activation of surface TLR2 and TLR1 602I by mycobacterial products, including the 19kDa lipoprotein, induces transcription of arginase-1 in a CEBPβ-dependent manner. Arginase-1 reduces substrate availability for iNOS by breaking down arginine, reducing the production of reactive oxygen species and favoring mycobacterial survival. TLR1 602S does not traffic to the cell surface (dashed lines) and therefore does not form a TLR1/2 signaling complex which would normally dampen the oxidative burst (158).
Schwann cells displaying TLR1 602I and TLR2 are stimulated by the *M. leprae* 19kDa lipoprotein to commence programmed cell death. Damage to peripheral nerves, by either direct infection or inflammatory ischemia, induces TLR1 expression which, in combination with TLR2 and mycobacterial products, can amplify apoptotic signals. Upregulation of TLR1 from damage caused by either direct infection or inflammatory ischemia could predispose nerves to further injury, resulting in classical leprosy anesthesis. Cells expressing the trafficking-deficient TLR1 602S may resist this mechanism of nerve damage (158).
<table>
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<td></td>
<td>0.50</td>
<td>Caucasian (80)</td>
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**Figure 1.9 Summary of TLR SNPs associated with mycobacterial disease.**

TLR polymorphisms are organized by receptor, genotype, phenotype, and cohort. The odds ratio (OR) measures the strength of association between genotype and disease. A ratio less than 1 depicts the degree of protection and a ratio greater than 1 depicts the factor of increased susceptibility. Reference numbers are included with the associated cohort. RR, reversal reaction; del, deletion; ins, insertion; VNTR, variable number tandem repeat (158).
References


Chapter 2

Cell Surface Trafficking of TLR1 is Differentially Regulated by the ER Chaperones PRAT4A and PRAT4B

Introduction

Toll-like receptors (TLRs) are central elements of the innate immune system that provide a first line of immune defense against infectious agents. The direct recognition of bacterial, fungal or viral components induces TLR activation and results in the cellular expression and release of immune mediators (2). TLRs play indispensable roles in bridging the innate and adaptive immune systems by inducing the expression of genes encoding cell adhesion molecules, proinflammatory cytokines and chemokines, as well as the co-stimulatory molecules and antigen presentation machinery required for T cell activation (9).

Humans possess 10 TLR family members, numbered 1 through 10, subsets of which are expressed in leukocytes and a wide variety of tissue types. TLRs can be broken down into two main groups based upon their function and phylogenetic relationship. TLRs 3, 7, 8, and 9 are grouped according to their ability to recognize various bacterial and viral nucleic acids. The remaining TLRs predominantly sense bacterial and fungal cell surface components. For example, TLR4 and TLR5 are sensors of bacterial LPS and flagellin, respectively. The TLR2 subfamily, comprised of TLRs 1, 2, 6, and 10, mediates immune responses to a variety of microbial cell wall components including lipoproteins and glycolipids. Cell signaling by all TLRs is initiated by the coordinate binding of ligand which induces receptor dimer formation. While most TLRs signal as homodimers, TLR2 signals by forming heterodimers with either TLR1, 6, or 10 in an agonist dependent fashion. For example, cell activation by triacylated bacterial lipoproteins occurs through coordinate binding by TLR1 and TLR2.

The subcellular localization of each TLR is vital for the appropriate recognition of microbial components leading to cell activation (4, 18). TLR3, 7, 8, and 9 are endosomally localized, providing proximity to bacterial and viral nucleic acids generated during viral replication or released following microbial degradation. Conversely, TLR1, 2, 6, and 10 are displayed on the plasma membrane where they are best available to contact bacterial and

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1 The following represents research originally published by Bryan E. Hart and Richard I. Tapping. “Cell Surface Trafficking of TLR1 is Differentially Regulated by the Chaperones PRAT4A and PRAT4B.” Journal of Biological Chemistry. 2012.
fungal cell wall constituents (31). TLR localization is dynamic and surface TLRs traffic to phagosomes during uptake of particulate microbes (34).

A variety of receptor regions have been identified as trafficking signals which direct TLR transport from the endoplasmic reticulum to either the cell surface or endosomal compartments. These trafficking motifs include the transmembrane domains of TLR4, 7, and 9, and the cytoplasmic linker region of TLR3 (1, 3, 13 21). Interruption of these sequences and the associated trafficking mechanisms prevents proper localization of TLRs, thereby disrupting accessibility to ligands and blocking recognition and responses to invading pathogens. In addition, several ER-resident chaperones have been shown to control subcellular receptor distribution (1). Gp96, PRAT4A, PRAT4B, and UNC93B1 are examples of such chaperones and their disruption can ablate proper localization of specific TLRs and induce hyporesponsiveness to their respective agonists (14, 25, 29, 30, 35, 39).

We have recently identified a frequent single nucleotide polymorphism in TLR1 (I602S) which greatly inhibits trafficking of the receptor to the cell surface (11). Aberrant surface display of the I602S variant is associated with a marked inability of cells to respond to soluble TLR1 agonists (7, 11). Interestingly, disease association studies have identified TLR1 602S as a key protective allele against both tuberculosis and leprosy (11, 17, 19, 27, 28, 33, 38).

In this paper, the trafficking deficiency of TLR1 602S is used as a model to identify the regulatory elements and chaperones required for TLR1 trafficking. We have found that the transmembrane domain and a short intracellular region interrupted by the 602S polymorphism is necessary for plasma membrane localization of TLR1, and is sufficient to drive surface display of TLR9. Additionally, we have found that the ER chaperones PRAT4A and PRAT4B differentially regulate trafficking of all surface-displayed TLRs, and that the physical interaction between TLR1 602S and PRAT4B may be responsible for the aberrant localization of this common TLR1 variant.

**Experimental Procedures**

**Cell Culture:** HEK 293T and Cos7 cells were grown in RPMI-1640 media (Cellgro) supplemented with 10% fetal bovine serum (Thermo Scientific), penicillin/streptomycin (Cellgro), and 20 mM L-glutamine (Cellgro). Primary human peripheral blood mononuclear cells were isolated by ficoll-paque (GE Healthcare) gradient centrifugation. Following thirty minutes of monocyte attachment to tissue culture plates in RPMI-1640 media, extraneous leukocytes
were washed and discarded. Primary human monocyte-derived macrophages were generated by stimulating monocytes with 50 ng/ml M-CSF (R&D Systems) for seven days. During cytokine induction studies, monocytes were stimulated with 20 ng/ml IFNγ (Miltenyi Biotec, Thermo Scientific) for 24 hours.

**DNA Constructs and PCR Mutagenesis** - TLR constructs were cloned into pFLAG-CMV-1 (Sigma Aldrich) or HA-epitope pDisplay (Invitrogen) expression plasmids. Receptor point mutants and truncation mutants were generated by site-directed mutagenesis and PCR amplification. Chimeric receptors were created using PCR SOEing, described previously (8, 23). Truncation mutants are named according to the missing portion of the receptor (i.e. TLR1Δ603-C is a receptor in which the region comprising amino acids 603 to the end of the C-terminus are deleted). Chimeric receptors are named according to the portion of TLR9 which is replaced with TLR1 sequence. For example, TLR9/1 TM is a TLR9 mutant in which the transmembrane domain of TLR9 has been replaced with that of TLR1. TLR9/1 TM-Cyto replaces TLR9 residues 810-1032 with that of the transmembrane domain and cytoplasmic domains of TLR1 (amino acids 571-786). TLR9/1 TF replaces amino acids 844-849 of TLR9 with that of the trafficking motif of TLR1 (amino acids 602-607). TLR9/1 TM replaces amino acids 823-843 of TLR9 with that of TLR1 (amino acids 581-601). Finally, TLR9/1 TM-TF replaces aa823-849 of TLR9 with the transmembrane and trafficking motif of TLR1 (amino acids 581-607).

PRAT4A and PRAT4B-FLAG cDNA clones were expressed from within the pCMV6-XL5 vector (OriGene). Two anti-PRAT4B shRNAs were synthesized (Integrated DNA Technologies) and cloned into pSilencer 3.1-H1 Neo vectors (Ambion) targeting the following: Sequence 1, AAGAGGAAGAGACACGTGCCT; Sequence 2, AAACCTGGACTGGAAAGGAGA. Respective scrambled control sequences were: Scramble 1, GCGGGAAACGGCGATAATACA; Scramble 2, GAGAGGGGAACAACGGTATAAT. Cells were transfected using Fugene 6 (Roche) or TransIT 2020 (Mirius) reagents overnight, followed by replacement with fresh media for 24 hours before harvesting cells for flow cytometry or immunoblotting. Batch-derived, stable cell lines were generated by transfecting HEK 293 cells with pSilencer vectors encoding neomycin resistance, followed by selection with 500 µg/ml G418 for four weeks.

**Flow Cytometric Analysis** - Surface expression of TLRs was measured using a triple-step staining procedure. Cells were blocked with flow buffer (10% rabbit serum and 0.3% NaN₃ in
PBS), then stained using a primary mouse anti-FLAG M2 antibody (Sigma Aldrich) or mouse anti-HA.11 (Covance), followed by biotinylated anti-mouse Fab fragment (Jackson ImmunoResearch), and finally, streptavidin-conjugated Alexa Fluor-647 or Alexa Fluor-488 (Invitrogen). Staining steps were performed for 30 minutes on ice, followed by a wash with flow buffer. Intracellular expression was assessed by fixing cells in 4% paraformaldehyde, permeabilizing in 0.25% Triton X, and using the above staining protocol. Stained cells were analyzed using a BD FACS Canto flow cytometer, using FCS Express V3 (De Novo Software).

**Microscopy**- Cos7 fibroblasts were seeded onto chambered slides (Lab-Tek), transfected, and stained using the above method. The endoplasmic reticulum was visualized using pDsRed2-ER (Invitrogen). Images were acquired using a Carl Zeiss LSM510 laser-scanning confocal microscope using appropriate filter sets.

**ELISA**- Enzyme linked immunosorbent assays of IL-6 from macrophage supernatants were performed using an IL-6 CytoSet kit (Invitrogen) according to the manufacturer’s protocol. Cells were stimulated with TLR agonists for 24 hours.

**Immunoblotting and Co-immunoprecipitation**- Lysates were prepared by detaching HEK 293T cells using ice-cold 10mM EDTA, followed by lysis in RIPA buffer. Protein samples were run in 10% SDS PAGE gels. For Western blotting, primary mouse anti-FLAG (Sigma), mouse anti-HA, rabbit anti-human PRAT4A (Imgenex), rabbit anti-human PRAT4B (Santa Cruz Biotechnologies), or rabbit anti-human actin (Thermo Scientific) antibodies were incubated 1:1000 in 5% milk-TBST for one hour. Secondary HRP-conjugated anti-isotype antibodies were incubated 1:10,000 for one hour. Proteins were detected by chemiluminescence (Pierce). Anti-FLAG pull-downs were conducted using M2 affinity gel (Sigma).

**Results**

**TLR1 I602S exhibits impaired TLR1 surface trafficking**- We have previously demonstrated that TLR1 602S exhibits hyporesponsive function compared to the TLR1 602I variant (11). This study revealed that this phenotype resulted from impaired surface trafficking and was not due to a reduction in total cellular TLR1 602S protein expression (Fig. 2.1A, B, D).
The deficiency in surface trafficking is not restricted to the monocyte cell type, as staining of whole blood leukocytes shows an identical phenotype for TLR1 602S in lymphocytes and granulocytes (Fig. 2.2).

To define the subcellular localization of both TLR1 variants, Cos7 cells were transfected with vectors encoding N-terminal FLAG-tagged versions of either TLR1 602I or TLR1 602S, along with red fluorescent protein fused to an endoplasmic reticulum retention motif (ER-RFP). Transfected cells were subsequently permeabilized, stained for TLR1, and visualized by confocal microscopy. Both receptor variants exhibit high levels of intracellular expression in a perinuclear compartment and both variants co-localize with the endoplasmic reticulum (Fig. 2.1E). This result further suggests that the lack of surface expression of TLR1 602S results from improper anterograde transport from the ER to the plasma membrane.

We have previously shown that primary human monocytes homozygous for the TLR1 602S allele are deficient in mediating responses to soluble TLR1 agonists PAM3CSK4 and M. tuberculosis membrane fraction compared to monocytes that possess at least one TLR1 602I allele (11). This differential response between TLR1 variants is also observed in primary human monocyte-derived macrophages (Fig. 2.1F). However, a particulate TLR1 agonist, zymosan, induces equivalent secretion of IL-6 from macrophages of all TLR1 genotypes (Fig. 2.1F). Underhill et al. previously demonstrated that TLR2, the heterodimeric partner for TLR1, is rapidly recruited to the zymosan phagosome in murine macrophages (34). To determine if stimulation of TLR1 602S is mediated by similar phagosomal recruitment, primary human macrophages were stimulated with zymosan particles and intracellular TLR1 was visualized by confocal microscopy. As shown in Figure 2.1G, both TLR1 602I and TLR1 602S localize to the zymosan phagosome. Overall, these results suggest that phagosomal recruitment enables the TLR1 602S variant to mediate responses to particulate agonists.

An equivalent serine substitution of TLR6 mimics the trafficking deficiency of TLR1 602S – Among human TLRs, TLR1 and TLR6 are most homologous, followed by TLR10. These three TLRs are tandemly arranged on chromosome 4 suggesting that they arose from gene duplication events. Similar to TLR1, TLR6 and TLR10 heterodimerize with TLR2 in response to microbial ligands (6, 24). Sequence alignment reveals that a conserved hydrophobic amino acid (TLR6 607I and TLR10 599L) resides at a homologous position to that of TLR1 602I (Fig. 2.3A). To determine if an equivalent serine substitution has a similar phenotype to TLR1 602S, surface
expression of a TLR6 607S mutant and a TLR10 599S mutant was measured by flow cytometry. A similar point mutant was also generated in TLR2 (H606S). As shown in Figure 2.3B and C, TLR10 L599S and TLR2 H606S have unchanged surface expression, while an I to S mutation of amino acid position 606 in TLR6 abrogates plasma membrane receptor localization. Total expression of each mutant was verified by flow cytometry (Fig. 2.4). Taken together, these results show that the serine substitution of the TLR1 602S variant generates the same trafficking deficiency in TLR6 but not in more distantly related TLRs.

**Trafficking of TLR1 is sensitive to nonconserved substitutions at position 602** - To characterize the amino acid side chains of residue 602 that are required for proper surface trafficking, additional point mutants of TLR1 were created by site-directed mutagenesis and examined for cell surface expression. Substituting a conserved hydrophobic leucine residue at position 602 had little effect on TLR1 surface expression (Fig. 2.5A, B). To determine whether the trafficking defect is a result of phosphorylation at 602S, we substituted a threonine residue at this position. TLR1 602T was found to be absent on the plasma membrane. Introduction of a neutral alanine residue at position 602 also resulted in a deficiency in surface trafficking, suggesting that proper TLR1 localization is generally sensitive to nonconserved mutations at this site (Fig. 2.5A, B). All of the mutants exhibited equivalent levels of intracellular expression (Fig. 2.6).

**A short cytoplasmic domain extending from position 602 is required for surface trafficking of TLR1** - To determine the minimum sequence required for TLR1 surface trafficking, truncation mutants of the receptor’s cytoplasmic domain were generated (Fig. 2.7A). These mutants were expressed in HEK 293T cells and their surface trafficking was examined by flow cytometry. To be certain that loss of surface trafficking of the mutants was not the result of abrogated protein expression, intracellular receptor levels were verified for each mutant (Supp. Fig. 2.7). Truncation of the entire cytoplasmic domain of TLR1, beginning at position 603Y (TLR1 ∆603-C), resulted in the loss of cell surface trafficking. However, deletion of amino acids 609Y to the end of the C-terminus (TLR1 ∆609-C) has little effect on plasma membrane localization compared to the full length TLR1 602I variant. As expected, truncations which preserve larger portions of the cytoplasmic domain (TLR1 ∆616-C and TLR1 ∆623-C) also have little effect on
trafficking. These data suggest that a short cytoplasmic region proximal to the transmembrane domain and extending from position 602-608 is required for surface trafficking of TLR1.

**Cytoplasmic residues 602-607 contribute to surface expression of TLR1** - To determine the importance of individual amino acids in this short cytoplasmic region, an alanine scan was performed across residues 602-608, and the ability of the resulting point mutants to traffic to the cell surface was assessed by flow cytometry. TLR1 602A exhibited an identical trafficking defect to that of TLR1 602S, while mutation of individual residues 603-607 to alanine resulted in significantly reduced surface expression (Fig. 2.9A, B). Cell surface expression of TLR1 W608A was similar to that of TLR1 602I, suggesting that this residue is not necessary for efficient surface display. Replacement of all the residues from 603-607 with alanine (TLR1 603-607A) resulted in a fully deficient trafficking phenotype. Intracellular expression of all of the mutants was verified (Fig. 2.10). These results, in combination with the truncation mutants, highlight an essential, internal trafficking motif for localization of TLR1 to the plasma membrane.

**The transmembrane domain and adjacent residues 602-607 of TLR1 are sufficient to drive surface expression of TLR9** - To define the minimal TLR1 trafficking motif sufficient for driving surface expression, we generated chimeras between TLR9, an endosomally localized receptor, and TLR1 (TLR9/1). Surface expression of these mutants in HEK 293T cells was assessed by flow cytometry. Intracellular expression of all the chimeric receptors was verified (Fig. 2.12). As expected, wildtype TLR9 is not expressed on the cell surface (Fig. 2.11A, B). However, TLR9 can traffic to the cell surface when the transmembrane domain and entire intracellular region is replaced with that of TLR1 (TLR9/1 TM-Cyto). Neither the trafficking domain alone (TLR9/1 TF) nor the transmembrane domain alone (TLR9/1 TM) of TLR1 is sufficient to enable TLR9 to traffic to the cell surface. However, both domains of TLR1 together in the context of TLR9 (TLR9/1 TM-TF) enables surface expression. Importantly, surface expression of both TLR9/1 TM-Cyto and TLR9/1 TM-TF is lost upon introduction of the 602S mutation (Fig. 2.11). Together these results show that the transmembrane domain and trafficking motif of TLR1 is sufficient to drive surface expression of TLR9. Additionally, a chimeric receptor containing this minimally sufficient TLR1 trafficking domain retains the differential trafficking of the I602S polymorphism.
The ER chaperone, PRAT4A, positively regulates TLR surface trafficking. Protein-associated-with-TLR4 (PRAT4A) was originally identified as a positive regulator of TLR4 cell surface expression and was later shown to play a similar role in TLR1 trafficking (30, 35). Given this, we hypothesized that the deficient trafficking phenotype of TLR1 602S may result from disrupted guidance from this chaperone. To examine this hypothesis, we measured the effects of PRAT4A expression on TLR1 surface trafficking. Gp96, another ER-resident TLR chaperone shown to cooperate with PRAT4A, was also examined (14). Over-expression of gp96 in HEK 293T cells did not affect surface trafficking of either TLR1 variant (Fig. 2.13A). However, increased levels of PRAT4A boosted plasma membrane expression of TLR1 602I and enabled recruitment of some TLR1 602S to the cell surface (Fig. 2.13A, B).

We subsequently investigated the effects of PRAT4A and gp96 over-expression on other TLRs. Surface expression of both TLR2 and TLR4 were potently upregulated by PRAT4A (Fig. 2.13A, B). Interestingly, gp96 over-expression reduced TLR2 surface levels and slightly downregulated TLR4 surface expression, but did not affect the other surface TLRs (data not shown). PRAT4A could not induce surface trafficking of TLRs known to localize intracellularly, as no significant increase in surface TLR3, TLR7, TLR8, or TLR9 was detected. Surface TLR3 appeared to be slightly upregulated by gp96 over-expression (data not shown).

Since increased availability of PRAT4A overcomes the TLR1 602S trafficking deficiency, we hypothesized that the trafficking phenotype was linked to interruption of PRAT4A-TLR1 602S interaction. To assess this, both variants were transfected into HEK 293T cells and co-immunoprecipitations were performed to assess efficiency of protein-protein interaction. Pull-down of FLAG-tagged TLR1 revealed, however, that both 602I and 602S receptor variants interact equally with PRAT4A (Fig. 2.13C).

Enhancement of TLR1 surface expression by PRAT4A requires the 6 amino acid trafficking motif. To determine the minimal TLR1 sequence required for the enhancement of surface expression by PRAT4A, the truncation mutants used in Figure 2.7 were co-transfected into HEK 293T cells with or without a PRAT4A overexpression vector. Surface expression of each TLR1 mutant was measured by flow cytometry (Fig. 2.14A). As expected, PRAT4A over-expression increased detectable surface trafficking of both TLR1 602I and TLR1 602S. Surface levels of TLR1 ∆609-C, ∆616-C, and to a lesser degree TLR1 ∆623-C also were increased upon addition of PRAT4A. However, TLR1 ∆603-C and TLR1 603-607A did not traffic to the plasma
membrane when PRAT4A was over-expressed, suggesting the enhancement of surface expression by PRAT4A is affected by the presence of a complete trafficking motif. To determine if this loss of enhancement correlated with a loss of TLR1-PRAT4A interaction, the truncation mutants were transfected into HEK 293T cells and co-immunoprecipitations were performed on cell lysates pulling down TLR1 and probing for PRAT4A by Western blot. As seen in Figure 2.14B, all of the TLR1 truncation mutants still interact efficiently with PRAT4A regardless of the presence of the 6 amino acid trafficking motif.

**PRAT4B negatively regulates TLR1 surface expression** - PRAT4B, a second ER chaperone which shares 44% identity with PRAT4A, has also been identified as a regulator of TLR4 surface expression (35). To assess the role of PRAT4B in trafficking of the TLR1 variants as well as other TLRs, we performed similar experiments to those using PRAT4A. Figure 2.15A and B shows flow cytometric analyses of TLR surface expression where receptors have been co-transfected with a PRAT4B vector. Over-expression of PRAT4B potently reduced trafficking of TLR1 602I, as well as TLR2, TLR4, TLR6, and TLR10 (data not shown). Interestingly, neither PRAT4A nor PRAT4B overexpression has any effect on the surface trafficking of the TLR1/9 TMTIR chimeric receptors (Jonathan Cottrell senior thesis). PRAT4B may be regulating TLR surface trafficking by affecting intracellular protein expression, as over-expression of the chaperone greatly reduced intracellular levels of all ten TLRs (Fig. 2.17). Due to the negative regulatory role PRAT4B appears to play in TLR1 surface trafficking, we hypothesized that the deficiency in TLR1 602S localization was due to an enhanced interaction with this chaperone. To test this, we performed co-immunoprecipitations examining the level of protein-protein interaction between PRAT4B and the TLR1 variants. HEK 293T cells were transfected with PRAT4B-FLAG and either HA-tagged TLR1 602I, TLR1 602S-HA, or TLR4-HA. TLR4 was readily detected by anti-HA immunoblotting of lysates immunoprecipitated with anti-FLAG beads, confirming the previously described interaction between PRAT4B and TLR4 (Fig. 2.15C). Similarly, TLR1 602S also co-immunoprecipitated with PRATB, while TLR1 602I was almost undetectable on the anti-HA immunoblot. These results suggest that TLR1 602S exhibits a stronger interaction with PRAT4B than does TLR1 602I (Fig. 2.15C).

**Knock-down of PRAT4B rescues the TLR1 602S phenotype** - To verify the role of PRAT4B in the regulation of TLR1 trafficking, we used shRNA vectors to knock-down PRAT4B and
assessed surface localization of both TLR1 variants. Immunoblotting experiments confirmed the efficacy of two anti-PRAT4B shRNAs to knock-down PRAT4B following transient transfection (Fig. 2.16C) and in stable cell lines (Fig. 2.16D). As previously shown by Konno et al., knock-down of PRAT4B reduced surface expression of TLR4 (Fig. 2.18) (35). Conversely, surface levels of TLR1 602S were induced upon co-transfection with the anti-PRAT4B shRNAs, while TLR1 602I trafficking remained unaffected (Fig. 2.16A, B, and Fig. 2.19). Scrambled control shRNAs failed to affect trafficking of either TLR1 variant or TLR4. Taken together, these results confirm that PRAT4B positively regulates TLR4 surface expression but plays a negatively regulatory role in the surface expression of TLR1. Additionally, PRAT4B has a greater negative effect on TLR1 602S compared to TLR1 602I perhaps as a result of a stronger physical interaction with the former variant.

**Interferon-gamma upregulates TLR1 surface trafficking and PRAT4A expression in primary human monocytes** - It is well established that IFNγ primes macrophages for TLR-mediated responses and many recent studies have linked this phenotype to integration of the IFNγ and TLR signaling pathways (26). In addition, IFNγ stimulates surface expression of TLR1, TLR2, and TLR4 in human peripheral blood cells (15, 20, 22). We assessed the effect of IFNγ on surface expression of different TLR1 602 variants using primary human peripheral blood monocytes either lacking or possessing a TLR1 602I allele. As seen in Figure 2.20A and B, surface trafficking of TLR1 is induced in individuals regardless of the TLR1 602 genotype. Interestingly, surface TLR1 from 602S/S homozygotes reached levels equivalent to that of resting TLR1 602I monocytes after 36 hours of IFNγ stimulation. In addition to induction of surface TLR1 expression, flow cytometric analysis of permeabilized cells revealed an increase in intracellular TLR1, and qRTPCR analysis showed an early induction of TLR1 mRNA (Fig. 2.20C). To determine if surface-expressed TLR1 602S is capable of signaling, primary human monocytes were stimulated with PAM3CSK4 in the presence or absence of IFNγ for 24 hours, followed by measurement of TNFα release (Fig. 2.20D). As expected, PAM3CSK4 was unable to stimulate TNFα secretion from 602S homozygotes, but efficiently activated TLR1 602I monocytes. Co-treatment with IFNγ strongly induced TNFα secretion from all monocytes, with levels of TNFα production from TLR1 602S homozygotes nearing that of TLR1 602I cells. These data show that IFNγ rescues cell surface trafficking of TLR1 602S and enables the receptor to transduce inflammatory signals to soluble agonists.
Given that both PRAT4A over-expression and IFNγ stimulation are able to rescue the TLR1 602S phenotype, we examined the effects of IFNγ on PRAT4A protein expression. Primary human monocytes were stimulated with increasing concentrations of IFNγ for 24 hours, followed by a Western blot of PRAT4A in cell lysates. IFNγ stimulation greatly increased PRAT4A protein levels in a dose dependent manner (Fig. 2.20E). A similar analysis revealed no effect on expression of PRAT4B by IFNγ (Fig. 2.20F). Together, these results suggest that IFNγ rescues TLR1 602S surface trafficking through induction of PRAT4A expression.

Discussion

The subcellular distribution of each TLR is orchestrated in a way to ensure that ligand accessibility leads to cell activation. Trafficking of TLRs is dynamic, reflecting the fact that microbial agonists are diverse in terms of their accessibility and physical nature. Soluble microbial agonists in the extracellular milieu activate immune cells through cell surface receptor signaling, while particulate agonists are internalized within endosomal or lysosomal compartments where intracellular TLRs are poised to sense released nucleic acids. Importantly, the subcellular localization of TLRs also directs specific innate immune signaling pathways and responses. For example, upon LPS stimulation at the cell surface, TLR4 is recruited to lipid rafts where signaling pathways that drive classic proinflammatory responses are engaged. However, upon endocytosis, TLR4 recruits a different set of adaptors and signaling pathways which mediate production of type 1 interferons (12, 41). Similar to TLR4, TLR2 is recruited to cholesterol rich lipid rafts along with TLR1 or TLR6 and associated co-receptors, in response to soluble agonists (32). These TLRs also co-localize in phagosomes where they are thought to sample phagosomal cargo (5, 34, 37). These membrane segregation events enable efficient and coordinated signaling responses by concentrating agonists, receptors, and even signaling adaptors to defined membrane compartments (reviewed in 18, 31).

We previously reported that a common polymorphism in TLR1, I602S, prevents trafficking of TLR1 to the cell surface and is associated with greatly diminished responses to soluble TLR1 agonists (11). Here, we show that the cell surface trafficking defect persists despite high TLR1 602S expression in the ER. Surprisingly, we have found that the TLR1 602S variant is able to traffic to zymosan containing phagosomes. Accordingly, macrophages homozygous for TLR1 602S, which are unable to respond to soluble TLR1 agonists, retain the ability to respond to
particulate zymosan. The mechanism which underlies TLR1 602S trafficking to phagosomes remains unknown but could involve direct trafficking from the ER thus bypassing the need for surface expression.

We have found that amino acid position 602 is part of a short, cytoplasmic region of TLR1, which is located proximal to the transmembrane domain, and is required but not sufficient for surface localization. Mutagenesis of any amino acid in this cytoplasmic trafficking motif reduces surface trafficking, but none are as deleterious as I602S. Similar to TLR1, TLR3 contains a sequence within the region between the transmembrane and TIR domains that directs proper localization (21). However, at 23 amino acids in length, the trafficking region of TLR3 is much larger and, unlike TLR1, is sufficient on its own to direct TLR3 to endosomal compartments. In contrast to TLR3, the transmembrane domain alone is sufficient to target TLR4, TLR7, or TLR9 to their distinct subcellular locations (3, 13, 21). Using chimeric receptors, we have found that both the short 6 amino acid cytoplasmic trafficking domain and the transmembrane domain of TLR1 are required and sufficient to drive surface receptor expression. Importantly, the I602S mutation ablates surface trafficking even in the context of a chimeric TLR. Taken together, TLR1 is unique in its requirement for both cytoplasmic and transmembrane trafficking domains for proper localization.

PRAT4A is an ER resident chaperone that is required for surface trafficking of TLR4/MD2 and cellular responses to LPS (35). In PRAT4A deficient mice, cell surface expression of TLR1, TLR2, and TLR4, and endosomal localization of TLR9 are completely disrupted (30). As expected, macrophages from these mice exhibit abrogated cytokine responses to respective agonists of these TLRs, as well as ligands for TLR6 and TLR7, but not TLR3. We have observed that overexpression of PRAT4A enhances surface expression of several TLRs including TLR1 602I, TLR2, and TLR4. Surprisingly, we found that over-expression of PRAT4A rescues surface trafficking of TLR1 602S, suggesting that PRAT4A is a limiting component in the trafficking of this receptor variant to the plasma membrane. The fact that TLR1 602S retains the ability to localize to endosomal compartments suggests that the involvement of PRAT4A in this trafficking event is distinct from that of surface trafficking. In support of this idea, it was reported that proper endosomal localization of TLR9 requires an interaction with a gp96-PRAT4A complex (16) and it has been suggested that the way in which endosomal TLR9 utilizes PRAT4A may be different from that of cell surface TLRs (1).
PRAT4B, a homolog of PRAT4A, has also been reported to promote TLR4 surface expression (35). Unexpectedly, both PRAT4B over-expression and knock-down studies show that this chaperone acts to inhibit TLR1 surface expression. PRAT4B interacts more strongly with TLR1 602S than with TLR1 602I, suggesting that this chaperone could be responsible for the differential surface trafficking of these two receptor variants. How the serine polymorphism promotes a preferential interaction between PRAT4B and TLR1 602S is unclear but could involve conformational changes and/or additional, as yet unidentified, proteins in the immunoprecipitated receptor complexes. Interestingly, PRAT4B expression has been associated with increased susceptibility to sepsis, Kawasaki’s disease, and general infection (36, 40). Thus, the fact that over-expression of PRAT4B inhibits TLR trafficking may have in vivo relevance for a number of inflammatory diseases.

We and others have shown that the TLR1 602S allele is protective against mycobacterial infection, including leprosy, leprosy reversal reaction, and tuberculosis (11, 17, 19, 27, 28, 33, 38). Most strikingly, an unbiased genome-wide array of 1500 individuals identified TLR1 602S (OR=0.31, p<0.001) as one of two alleles that conferred the greatest protection against leprosy, the other being an allele of MHCII (38). IFNγ, a key component of immunity to mycobacteria and other intracellular pathogens, is released by helper T cells and potently increases microbicidal functions of macrophages, including phagosome maturation and oxidative burst. We have found that surface expression of both TLR1 602I and 602S is enhanced in primary human monocytes stimulated with IFNγ. Importantly, this induction of surface TLR1 enables monocytes from TLR1 602S homozygotes to mediate responses to soluble TLR1 agonists. This enhancement is associated with an increase in mRNA and intracellular TLR1 protein. In addition, the upregulation of TLR1 by IFNγ correlates with a large increase in intracellular PRAT4A protein expression, suggesting that induction of this positively regulating TLR chaperone may play an important role in monocyte/macrophage priming by IFNγ. The basis for the protective role of TLR1 602S in the context of mycobacterial disease is the subject of ongoing studies in our laboratory.

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**Figure 2.1 TLR1 I602S exhibits impaired TLR1 surface trafficking.**

TLR1 staining of intact (surface) and permeabilized cells (perm) was assessed by flow cytometric analysis using primary human monocytes from individuals of two TLR1 genotypes: 602 I/S (A) and 602 S/S (B). Filled histograms represent isotype controls. (C) The position of the 602 polymorphism is marked on the crystal structure of TLR1 (adapted from reference 40). (D) Cos7 cells were transiently transfected with FLAG-tagged TLR1 602I or TLR1 602S and stained to detect FLAG. Surface TLR1 (green) was visualized by scanning laser confocal microscopy. (E) Cos7 cells were co-transfected with the indicated FLAG-tagged TLR1 variants and an ER-localized, red fluorescent protein (ER-RFP, red). Cells were permeabilized and stained for intracellular TLR1 as indicated (green). Co-localization of ER and TLRs is denoted by yellow signal in the merged image. (F) Primary human macrophages from blood donors of the three different TLR1 602 genotypes were stimulated with various TLR1 agonists as indicated (N.D.; not detectable, unstim; unstimulated, PAM3; Pam3CSK4, Mtb mem; M. tuberculosis membrane fraction). IL-6 secretion was measured from culture supernatants by ELISA. (G) Intracellular TLR1 (red) was visualized in resting primary human macrophages from donors of different TLR1 602 genotypes as indicated (top). Human macrophages were also stimulated with zymosan particles (shown by arrows) to detect recruitment of TLR1 to phagosomal compartments (bottom).
Figure 2.2 The TLR1 602S phenotype is conserved in all peripheral leukocytes.

Whole blood leukocytes from donors of two TLR1 602 genotypes were stained for surface TLR1. Populations were gated on granulocytes (box 1), monocytes (box 2), and lymphocytes (box 3). Histograms from the gated populations are shown (gray line; isotype control, black line; TLR1).
Figure 2.3 An equivalent serine substitution of TLR6 mimics the trafficking deficiency of TLR1 602S.

(A) ClustalW alignment of amino acids forming the transmembrane domain (TM) and a portion of cytoplasmic domain from members of the TLR2 subfamily. Position 602 is bracketed. (B) HEK 293T cells were transfected with wildtype receptors or serine point mutants of TLR6, TLR10, and TLR2. The change in mean fluorescence intensity (ΔMFI) of transfected cells was measured by flow cytometry. Error bars represent the standard deviation of triplicate transfections. (C) Representative histograms show surface TLR expression (filled curve; empty vector, gray line; wildtype, black line; serine mutant).
Figure 2.4 Intracellular expression of TLR “602” point mutants.
HEK 293T cells were transiently transfected with the indicated FLAG-tagged TLR point mutants. Cells were subsequently fixed, permeabilized, and stained for intracellular receptor (filled curve; empty vector, black line; TLR).
Figure 2.5 Trafficking of TLR1 is sensitive to nonconserved substitutions at position 602.

(A) HEK 293T cells were transfected with the indicated TLR1 point mutants and surface expression was assessed by flow cytometry. Error bars represent the standard deviation of triplicate transfections. (B) Representative histograms show surface TLR expression (filled curve; empty vector, black line; TLR).
**Figure 2.6 Intracellular expression of TLR1 point mutants.**

HEK 293T cells were transiently transfected with the indicated FLAG-tagged TLR1 point mutants. Cells were subsequently fixed, permeabilized, and stained for intracellular receptor (filled curve; empty vector, black line; TLR).
Figure 2.7 A short cytoplasmic domain extending from position 602 is required for surface trafficking of TLR1.

(A) HEK 293T cells were transfected in triplicate with the indicated TLR1 602I truncation mutants (shown schematically). Surface expression was assessed by flow cytometry. Bars represent the mean and standard deviation of triplicate transfections. (B) Representative histograms show surface TLR expression (filled curve; empty vector, black line; TLR).
Figure 2.8 *Intracellular expression of TLR1 truncation mutants.*

HEK 293T cells were transiently transfected with the indicated FLAG-tagged TLR1 truncation mutants. Cells were subsequently fixed, permeabilized, and stained for intracellular receptor (filled curve; empty vector, black line; TLR).
Figure 2.9 Cytoplasmic residues 602-607 contribute to surface expression of TLR1.

(A) FLAG-tagged alanine scan mutants of TLR1 were transfected into HEK 293T cells as indicated and surface expression was measured by flow cytometry. The average change in mean fluorescence intensity (ΔMFI) of triplicate transfections with standard deviations is shown. (B) Representative histograms show surface TLR expression (filled curve; empty vector, black line; TLR).
Figure 2.10 Intracellular expression of TLR1 alanine scan mutants.

HEK 293T cells were transiently transfected with the indicated FLAG-tagged TLR1 point mutants. Cells were subsequently fixed, permeabilized, and stained for intracellular receptor (filled curve; empty vector, black line; TLR).
Figure 2.11 The transmembrane domain and adjacent residues 602-607 of TLR1 are sufficient to drive surface expression of TLR9.

(A) HEK 293T cells were transiently transfected with FLAG-tagged TLR9/1 chimeric receptors as indicated and surface expression was measured by flow cytometry. Schematic diagram of TLR9/1 chimeras in which regions of TLR9 (gray) are replaced by specific domains of TLR1 (black) are shown. Chimeras contain either the 602I or 602S polymorphism, as indicated (TM; transmembrane domain, TF; trafficking domain, cyto; cytoplasmic domain). Bars represent the mean and standard deviation of triplicate transfections. (B) Representative histograms show surface TLR expression (filled curve; empty vector, black line; TLR).
Figure 2.12  *Intracellular expression of TLR9/1 chimeras.*

HEK 293T cells were transiently transfected with the indicated FLAG-tagged chimeric receptors. Cells were subsequently fixed, permeabilized, and stained for intracellular receptor (filled curve; empty vector, black line; TLR).
Figure 2.13 The ER chaperone, PRAT4A, positively regulates TLR surface trafficking.

(A) HEK 293T cells were co-transfected with TLR1 602I, TLR1 602S, TLR2, or TLR4 and the ER chaperones, gp96 or PRAT4A. Surface levels of TLRs were assessed by flow cytometry. The average change in mean fluorescence intensity (ΔMFI) and standard deviation of triplicate transfections are shown. (B) Representative histograms show surface TLR expression (filled curve; empty vector, black line; TLR alone, gray line; TLR + chaperone). (C) HEK 293T cells were transfected with the indicated FLAG-tagged TLR1 variant and cell lysates were prepared. Anti-FLAG beads were used to pull down TLR1 and Western blots were probed for levels of immunoprecipitated TLR1 as well as co-immunoprecipitated PRAT4A. Actin levels in cell lysates support equivalent protein loading.
Figure 2.14 Enhancement of TLR1 surface expression by PRAT4A requires the trafficking motif.

(A) HEK 293T cells were transfected with full-length TLR1 602I or 602S or one of the TLR1 truncation mutants used in Figure 2.7 (black bars). Receptors were also co-transfected with PRAT4A (gray bars) and surface expression was measured in triplicate transfections using flow cytometric analysis. (B) HEK 293T cells were transfected with the indicated FLAG-tagged TLR1 truncation mutant and cell lysates were prepared. Anti-FLAG beads were used to pull down TLR1 and Western blots were probed for levels of immunoprecipitated TLR1 as well as co-immunoprecipitated PRAT4A.
Figure 2.15 PRAT4B negatively regulates TLR surface expression.

(A) HEK 293T cells were co-transfected with TLR1 602I, TLR1 602S, TLR2, or TLR4 and the ER chaperone PRAT4B as indicated. Surface levels of TLRs were assessed by flow cytometry. The average change in mean fluorescence intensity (ΔMFI) and standard deviation of triplicate transfections are shown. (B) Representative histograms show surface TLR expression (filled curve; empty vector, black line; TLR alone, gray line; TLR + PRAT4B). (C) HEK 293T cells were co-transfected with either TLR1 602I-HA, TLR1 602S-HA, or TLR4-HA, and PRAT4B-FLAG. Cell lysates were incubated with anti-FLAG beads to pull down PRAT4B and probed for levels of co-immunoprecipitated TLRs. Lysates confirm equivalent protein loading (actin) and equivalent production of both TLR1 variants.
Figure 2.16 Knockdown of PRAT4B rescues the TLR1 602S phenotype.

(A) TLR1 602I or TLR1 602S was transiently transfected into HEK 293T along with shRNA expression vectors targeting PRAT4B (shRNA1, shRNA2) or scrambled shRNA expression vectors (scramble1, scramble2) as indicated. TLR1 surface expression was measured by flow cytometry. Bars represent the average change in mean fluorescence intensity (∆MFI) and standard deviation of triplicate transfections. (B) Representative histograms show surface TLR expression (filled curve; empty vector, black line; TLR alone, gray line; TLR + shRNA). (C) Cell lysates from similar transient transfections were collected and probed by immunoblot for PRAT4B to assess shRNA-mediated knockdown of the chaperone. Actin levels support equivalent protein loading. (D) HEK 293 cells were transfected with empty vector (psilencer) or anti-PRAT4B shRNAs and stable batch-derived lines were selected. PRAT4B levels from cell lysates were assessed by immunoblot.
Figure 2.17  *TLR4 surface expression is reduced upon knockdown of PRAT4B.*

HEK 293T cells were transiently transfected with FLAG-tagged TLR4 with or without anti-PRAT4B shRNAs or scrambled controls. Cells were subsequently stained for surface TLR4 (filled curve; empty vector, gray line; TLR4 alone, black line; TLR4 with shRNA).
Figure 2.18 Stable knockdown of PRAT4B rescues the TLR1 602S phenotype.

HEK 293 cells were stably transfected with one of two anti-PRAT4B shRNA vectors or empty pSilencer vector as indicated. FLAG-tagged TLR1 602I or TLR1 602S were transiently transfected into the stable lines and surface expression was measured by flow cytometry (filled curve; empty vector, gray line; TLR1 602I, black line; TLR1 602S).
Figure 2.19 *PRAT4A* and *PRAT4B* affect intracellular TLR protein expression.

HEK 293T cells were co-transfected with a TLR and one of the ER chaperones, gp96 (horizontal stripes), PRAT4A, or PRAT4B as indicated. (A) Cells were permeabilized and stained to detect total TLR expression by flow cytometry. (B) HEK293T cells were transfected with either FLAG-tagged TLR1 602I or TLR2, with or without each chaperone. Lysates were probed with anti-FLAG to detect total TLR protein as well as total actin.
Figure 2.20  Interferon-gamma upregulates TLR1 surface trafficking and PRAT4A expression in primary human monocytes.

(A) Human monocytes from either TLR1 602S homozygotes or TLR1 602I individuals were stimulated with IFNγ (20 ng/ml) over a 36 hour time-course. Surface expression of TLR1 was measured by flow cytometry. Error bars represent the standard deviation from three different donors. (B) Representative histograms show monocyte surface TLR expression (filled curve; isotype control, black line; no IFNγ, silver line; 12 hr IFNγ, gray line; 36 hr IFNγ). (C) Primary human monocyte mRNA was collected over the same time-course and relative levels of TLR1 transcripts were measured by qRTPCR (compared to GAPDH mRNA). Values were normalized to that of unstimulated monocytes. Error bars represent the standard deviation from three different donors. (D) To assess the signaling competency of IFNγ-rescued TLR1 602S, monocytes were simultaneously co-incubated with the TLR1/2 agonist, PAM3CSK4 (50 ng/ml), in the presence or absence of IFNγ (20 ng/ml) for 24 hours. TNFα secretion was measured by ELISA. Error bars represent the standard deviation from three different donors. Human monocytes were isolated from blood donors and stimulated with increasing concentrations of interferon-gamma as indicated. Cell lysates were probed for PRAT4A (E) or PRAT4B (F).
References


Chapter 3

The TLR1 602S Polymorphism Protects Against Leprosy and Tuberculosis by Overcoming Mycobacterial Immune Subversion

Introduction

Over the past several decades numerous mechanisms by which mycobacteria neutralize and even subvert host defenses have been uncovered. Mycobacteria employ strategies which counteract a large majority of not only innate host defenses, such as killing by macrophage phagocytosis and oxidative burst, but also adaptive responses including T cell activation and maturation of antigen presenting cells. It is thus not surprising that one third of the world’s population is estimated to be infected with *Mycobacterium tuberculosis*. Non-tuberculous mycobacterial infections, such as those caused by *M. leprae* and *M. avium* complex species, are also important worldwide health concerns, with more than 200,000 new cases reported annually worldwide (58).

Pattern recognition receptors (PRRs) are essential to the production of proinflammatory cytokines and chemokines required for effective containment or clearance of invading mycobacteria (25, 55). Key PRRs include the ten-member family of Toll-like receptors (TLR) which serve as innate sensors of conserved microbial components, including nucleic acids and bacterial cell wall constituents (1). Although mycobacteria possess agonists for several members of the TLR family, TLR2, as a heterodimer with either TLR1 or TLR6, is the primary sensor by which immune cells recognize mycobacterial cell wall and membrane components. Mycobacterial derived agonists for the TLR1/2 heterodimer include lipomannan, lipoarabinomannan, phosphatidylinositol dimannoside, and various lipoproteins (2, 7, 13, 26, 32, 50, 51, 56). Not surprisingly, a number of TLR1 and TLR2 single nucleotide polymorphisms have been associated with mycobacterial disease (reviewed in 19, 44).

We and others identified TLR1 I602S as a common single nucleotide polymorphism in TLR1 which markedly reduces primary monocyte/macrophage responses to soluble TLR1 agonists (20, 23). Surprisingly, the TLR1 I602S polymorphism has been shown to be a key protective allele for mycobacterial diseases, including tuberculosis, leprosy, and leprosy reversal.

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2 *The following represents research originally published by Bryan E. Hart and Richard I. Tapping.*

reaction (23, 31, 33, 59). Most significantly, TLR1 602S has been identified as a major source of protection against leprosy in a genome-wide association study among 258 leprosy patients and 300 controls in New Delhi \((P = 5.7 \times 10^{-8}, \text{OR} = 0.31, 95\% \text{CI} = 0.20–0.48)\) (19). We discovered that an inability to traffic to the plasma membrane underlies the inability of the TLR1 602S variant to mediate responses to TLR1 agonists (16, 23).

Although deficient recognition of a pathogen would be expected to have detrimental effects on immune responses to infection, increasing evidence suggests that subversion of TLR1/2 signaling in macrophages represents an important immunoevasive strategy used by mycobacteria to establish and maintain chronic disease. Prolonged stimulation of macrophages with mycobacterial components is known to inhibit various aspects of macrophage antimicrobial functions in a TLR2-dependent manner (4, 16, 17, 24, 27, 28, 30, 35, 37-41, 45-47, 52, 57). Stimulation of macrophages with mycobacterial components reduces IFNγ-induced surface levels of both FcyRI (CD64) and MHCII, receptors essential for antibody-dependent phagocytosis and antigen presentation respectively (4, 16, 17, 24, 27, 28, 30, 35, 37-41, 45-47, 52, 57). In addition, mycobacterial activation through TLR2 has been shown to upregulate host arginase-1; a metabolic enzyme that depletes macrophages of intracellular arginine, which is a substrate required to produce microbicidal nitric oxide (11, 14). By subverting TLR1/2 signaling in order to abrogate cell surface MHCII, FcyRI, and the oxidative burst, mycobacteria effectively shut down local T-cell mediated immunity to infection and create highly suitable intracellular niches.

We hypothesize that individuals lacking surface TLR1, and thus exhibiting hyporesponsiveness to TLR1/2 agonists, might resist the aforementioned TLR1/2-dependent immunosuppressive mechanisms employed by pathogenic mycobacteria. Our studies have revealed that compared to TLR1 602I, myeloid cells from individuals homozygous for TLR1 602S resist down regulation of MHCIi and CD64, and also fail to upregulate arginase-1 when stimulated with soluble mycobacterial components. However, both TLR1 602S and 602I drive similar protective responses to whole mycobacteria, reflecting the ability of both variants to traffic to phagosomal compartments. Together, these results provide important insights into the mechanism by which an apparently defective TLR1 polymorphic variant provides protection against mycobacterial diseases.
Experimental Procedures

Cell Culture - Primary human monocytes and macrophages were grown in RPMI-1640 media (Cellgro) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific), penicillin/streptomycin (Cellgro), and 20 mM L-glutamine (Cellgro). Primary human peripheral blood mononuclear cells were isolated by ficoll paque (GE Healthcare) gradient centrifugation in 50 ml Leucosep tubes (Greiner Bio One). For flow cytometry and Western blotting, monocytes were purified by plate adherence (described previously, 18) or using a MACS monocyte isolation kit II (Miltenyi) according to the manufacturer’s protocol. Primary human monocyte-derived macrophages were generated by stimulating monocytes with 50 ng/ml M-CSF (R&D Systems) for at least seven days. Mycobacterium avium strain 104 pMH109 (a kind gift from Jerry Cangelosi and Trude Flo, Trondheim Norwegian University of Science and Technology, Trondheim, Norway) was cultured in 7H9 Middlebrook broth (BD Difco) supplemented with ADC enrichment (BD BBL) and 20 µg/ml kanamycin, or on 7H10 Middlebrook agar (BD Difco) plates supplemented with OADC enrichment (BD BBL) and 20 µg/ml kanamycin.

Flow Cytometric Analysis - Surface expression of macrophage activation markers was measured using a BD FACS Canto flow cytometer. To stain for surface CD64 or MHCII, cells were blocked with flow buffer (10% rabbit serum and 0.3% NaN₃ in PBS), followed by incubation with 10 µg/ml FITC-conjugated mouse anti-human CD64 (Caltag Laboratories, BD Pharmingen) or PE mouse anti-human HLA-DR (Invitrogen) on ice for 30 minutes. Cells were subsequently fixed in 4% paraformaldehyde. Flow data were gated on the appropriate monocyte/macrophage population and analyzed using FCS Express V3 (De Novo Software). Statistical analyses were performed using the Student’s t-test. Cells were stimulated with the following TLR agonists for 24 hours: PAM3CSK4 (50 ng/ml, EMC Microcollections), zymosan (1x10⁸ particles/ml, Invitrogen), PAM3CSK4-coated polystyrene beads (1x10⁷ particles/ml), H37Rv M. tuberculosis membrane fraction (500 ng/ml, BEI Resources), and gamma-irradiated H37Rv M. tuberculosis (500 ng/ml, BEI Resources).

ELISA- Quantification of TNFα and IL-6 from primary monocyte and macrophage supernatants was performed using a human TNFα or IL-6 CytoSet kit (Invitrogen), according to the manufacturer’s protocol. Cells were stimulated with TLR agonists (at above concentrations).
or co-incubated with cytochalasin D (10 µM, Sigma Aldrich) for 12 hours. An MTT assay verified no significant cytotoxic affects by the addition of the actin inhibitor.

**Immunoblotting**- Lysates were prepared by detaching monocytes using ice-cold 10mM EDTA, followed by lysis in RIPA buffer. Protein samples were run in 10% SDS PAGE gels. Primary rabbit anti-human arginase-1 (Santa Cruz Biotechnologies) and rabbit anti-human actin (Thermo Scientific) antibodies were incubated 1:1000 in 5% milk-TBST. Secondary HRP-conjugated anti-rabbit antibodies were incubated 1:10,000. Proteins were detected by chemiluminescence (Pierce).

**Microscopy**- Primary human macrophages were seeded onto chambered slides (Lab-Tek). *M. avium* cells were grown to log phase (OD≈0.6), stained with auramine-O (Sigma Aldrich) for 30 minutes, and subsequently opsonized with FBS for 30 minutes at 37°C. Macrophages were incubated with mycobacteria (10:1 MOI) for 30 minutes at 4°C to allow binding. Following incubation at 37°C for 15 minutes, macrophages were washed with PBS, fixed with 4% paraformaldehyde at 4°C for one hour, and permeabilized with acetone for four minutes at -20°C. Phagosomal TLR1 was detected using a triple-step staining protocol. Cells were blocked with flow buffer (see flow cytometry methods), then stained using a primary mouse anti-human TLR1 antibody (GD2.F4, eBioscience), followed by a biotinylated anti-mouse Fab fragment (Jackson ImmunoResearch), and finally, streptavidin-conjugated Alexa Fluor-647 (Invitrogen). Staining steps were performed for 30 minutes on ice, followed by a wash with flow buffer. Images were acquired using a Carl Zeiss LSM510 laser-scanning confocal microscope using appropriate filter sets.

**Macrophage Killing Assay**- Following differentiation for one week, primary human monocyte-derived macrophages were co-incubated with or without PAM3CSK4 (50ng/ml) or *M. tuberculosis* membrane fraction (500ng/ml) and with or without (20ng/ml) for 24 hours. A second killing assay protocol varied the order of addition, where IFNγ was added to culture media 24 hours prior to the TLR agonist. Macrophages were then infected with *M. avium* using an MOI of 10:1 (bacteria: macrophage) for 4 hours. Unbound or non-phagocytized bacteria were washed out of the culture plate three times with PBS. Macrophage killing was allowed to proceed for 2, 3, 4, or 6 days. Colony forming units which survived engulfment were recovered.
by lysing macrophages in ddH$_2$O, followed by plating of a 1:1000 dilution on Middlebrook agar for at least 7 days.

Results

*Particulate TLR1/2 agonists activate intracellular TLR1 602S following phagocytosis.*

We previously showed that peripheral blood monocytes derived from individuals who are homozygous for the 602S allele lack cell surface expression of TLR1 and exhibit blunted responses to TLR1/2 agonists compared to individuals who are either heterozygous or homozygous for the TLR1 602I allele (18, 23). Importantly, monocyte-derived macrophages retained the same expression phenotype (Fig. 3.1A, left). This result appears to be due to a receptor trafficking defect, as equivalent levels of TLR1 were observed in permeabilized cells regardless of genotype (Fig. 3.1A, right). As expected, individuals possessing the surface-expressed TLR1 602I allele responded to soluble TLR1 agonists PAM3 and Mtb membrane fraction (Mtb mem) as measured by secretion of TNFα and IL-6, while TLR1 602S/S macrophages did not (Fig. 3.1B, C). However, upon stimulation with the particulate agonists zymosan, *M. avium*, or whole *M. tuberculosis*, comparable levels of proinflammatory cytokines were produced regardless of TLR1 602 genotype. Interestingly, PAM3-coated beads were unable to elicit responses from TLR1 602S/S macrophages. Taken together, these results show that the ability of the TLR1 602S variant to mediate cell activation depends upon the physical nature of the agonist.

Given the fact that TLR1 602S activates cells in response to whole mycobacteria, we next examined the ability of TLR1 to be recruited to phagosomes containing *Mycobacterium avium*. To this end, the localization of TLR1 in primary human macrophages from individuals either homozygous for TLR1 602S or the TLR1 602I allele was followed in relation to serum-opsonized *M. avium* cells stained with fluorescent auramine-O. In resting macrophages, both receptor variants of TLR1 (stained red) concentrate intracellularly in a perinuclear compartment previously identified as the endoplasmic reticulum (Fig. 3.2A) (18). As shown in Figure 3.2B, upon phagocytosis of live *M. avium*, both variants appear to localize to the phagosome (stained green). Taken together with the cytokine release data, these findings suggest that both TLR1 602I and TLR1 602S variants are capable of driving macrophage activation from mycobacteria containing phagosomes. This result is consistent with our published finding that both variants
activate macrophages and traffic to the phagosome following ingestion of yeast zymosan particles (18). The fact that PAM3-coated beads activated macrophages through TLR1 602I but not TLR1 602S, suggests that particle recognition by additional phagocytic receptors are required to direct TLR1 602S to the phagosome, leading to cell activation.

**Phagocytosis and endosomal trafficking of particulate agonists affect activation of TLR1**  
To verify that bacterial internalization is necessary for macrophage TLR1 602S recruitment and activation, cytokine release in response to whole mycobacteria was measured in the presence or absence of the cytochalasin D; an inhibitor of actin polymerization and endocytosis. Cytochalasin D decreased the cytokine release mediated by both TLR1 602I and TLR1 602S (Fig. 3.2C, D). Interestingly, cell activation through TLR1 602I was more resistant to cytochalasin D inhibition than TLR1 602S. This increased resistance is likely due to some initial activation of TLR1 602I at the cell surface prior to endocytosis. Conversely, since TLR1 602S is not surface expressed, there is more absolute recruitment for this variant to gain access to endosomal compartments containing mycobacteria. Additionally, co-incubation of cells with the phagosome acidification inhibitor, bafilomycin A, or the microtubule polymerization/early endosome trafficking disruptor, nocodazole, gave similar results to that observed with cytochalasin D (data not shown). Together, these results confirm that internalization and trafficking of TLR1 602S to mycobacteria containing phagosomes is necessary for macrophage activation.

**TLR1 602S/S macrophages resist downregulation of MHCII in response to soluble, but not particulate, mycobacterial agonists**  
Several studies have demonstrated that pathogenic mycobacteria inhibit surface expression of class II major histocompatibility complex (MHCII), a vital component of antigen presentation and activation of CD4+ T helper cells (21-38, reviewed in 13). The suppression of surface MHCII was shown to be dependent on TLR2 recognition of a 19 kDa triacylated lipoprotein of mycobacteria, LpqH, resulting in inhibition of CIITA, a transcriptional activator of the MHCII gene (3, 37, 40, 41, 52, 57,). We hypothesized that if TLR1/2 signaling is mediating this immunosuppression by mycobacteria, a deficiency in TLR1 activity, such as that of TLR1 602S, could confer protection. To this end, we initially examined primary human monocytes from blood donors of various TLR1 602 genotypes for changes in MHCII (HLA-DR) expression after challenge with *M. tuberculosis* membrane fraction (Mtb mem).
Flow cytometric analysis verified that there was no significant difference in baseline surface MHCII based on TLR1 genotype (Fig. 3.3A). Following stimulation with Mtb mem for 24 hours, monocytes possessing a surface-expressed TLR1 602I allele exhibited an over 3-fold reduction in surface MHCII, whereas cells lacking surface-expressed TLR1 (TLR1 602S/S) displayed only marginal suppression (Fig. 3.3A, B).

To further characterize this resistance phenotype, primary macrophages were similarly examined for MHCII expression following stimulation with other TLR1/2 agonists for 24 hours. Macrophages from blood donors of the three TLR1 602 genotypes were stimulated with PAM3CSK4 (PAM3), PAM3CSK4-coated beads, *M. avium*, or Mtb mem, and surface expression of HLA-DR was measured by flow cytometry. Surface levels of MHCII did not significantly differ between either groups when macrophages were left unstimulated or were incubated with PAM3 or PAM3-coated beads for 24 hours. However, upon stimulation with live *M. avium*, macrophage surface MHCII decreased regardless of genotype (Fig. 3.3C, D). Stimulation of macrophages expressing TLR1 602I with Mtb mem also lead to a dramatic drop in detectable MHCII, while TLR1 602S/S cells largely resisted this reduction. Together, these results suggest that the absence of surface TLR1 602S may be abrogating mycobacterial-dependent suppression of MHCII surface expression. This loss of protection during stimulation with whole bacteria may be due to the fact that similarly to TLR1 602I, TLR1 602S is recruited to the *M. avium* phagosome, an event which would activate the inhibitory TLR1/2 signal independently of TLR1 genotype.

**TLR1 602S/S monocytes and macrophages resist mycobacterial inhibition of IFNγ-induced CD64**- CD64 (FcγRI), the phagocytic Fc receptor for IgG, is another marker of macrophage activation whose levels increase following stimulation with bacterial products or proinflammatory IFNγ. Like MHC II, CD64 has also been previously identified as a target of mycobacterial subversion of macrophage function, as expression is negatively modulated in cells exposed to mycobacterial products, even following IFNγ stimulation (17, 24, 47, 52). Similarly to MHCII, we hypothesized that TLR1 602S/S cells would be able to resist these inhibitory effects.

To test this, primary human monocytes from individuals of different TLR1 602 genotypes were treated with Mtb mem alone, IFNγ alone, or the two combined. Changes in surface expression of CD64 were subsequently examined by flow cytometry. As seen in Figure 3.4A, Mtb mem stimulation of TLR1 602I monocytes for 24 hours resulted in a 50% reduction of
surface CD64, whereas TLR1 602S/S cells retained levels comparable to that of unstimulated monocytes. Upon addition of IFNγ, both groups displayed a five-fold increase in plasma membrane-localized FcyRI (Fig. 3.4A). However, co-incubation of TLR1 602I macrophages with Mtb membrane markedly inhibited the IFNγ-mediated upregulation of CD64, while TLR1 602S/S homozygotes were unaffected (Fig. 3.4A, B).

To further examine the influence of other TLR1/2 agonists on CD64 expression, primary human macrophages were stimulated with PAM3, PAM3-coated beads, M. avium, or Mtb mem, and surface expression of CD64 was measured by flow cytometric analysis. As with monocytic cells, TLR1 602I expressing macrophages lost half of baseline CD64 surface expression when stimulated with Mtb membrane fraction, an effect largely resisted in TLR1 602S/S cells (Fig. 3.4C). Treatment with IFNγ induced CD64 expressed by two-fold in both groups, however, co-treatment with Mtb mem completely blocked this induction in TLR1 602I macrophages. Alternatively, as seen with primary monocytes, Mtb mem had no effect on IFNγ stimulation of CD64 in TLR1 602S/S macrophages. While soluble PAM3 or PAM3 bead stimulation had marginal effects on surface CD64, challenge with whole M. avium resulted in a significant reduction in surface CD64 in macrophages of all TLR1 602 genotypes, which could not be rescued by IFNγ treatment (Fig. 3.4C, D). Taken together, these results suggest that the lack of surface TLR1 602S protects against negative modulation of CD64 by mycobacterial stimulation. However, as with MHCII, recruitment of TLR1 602S to endosomes containing whole mycobacteria promotes the subversive effects observed following TLR1 602I stimulation.

**TLR1 602S/S monocytes resist induction of host arginase-1 when stimulated with soluble but not particulate mycobacterial agonists** - Nitric oxide has been shown to be essential for the restriction of growth and killing of intracellular mycobacteria (9, 11, 12, 21, 34, 36). Inducible nitric oxide synthase (iNOS) and the metabolic enzyme Arginase-1 compete for cytoplasmic pools of arginine as an enzyme substrate. A study by El Kasmi et al. revealed that TLR2 stimulation by mycobacteria was capable of upregulating arginase-1 in mice, leading to both a reduction in nitric oxide intermediates and an enhancement of mycobacterial survival (14). Since subversion of TLR2 signaling by mycobacteria can promote pathways inhibitory to iNOS, we hypothesized that individuals lacking surface TLR1 602S may resist mycobacterial induction of host arginase-1.
To determine if arginase-1 could be induced in human cells, primary monocytes from individuals of the various TLR1 602 genotypes were stimulated with TLR agonists for 24 hours, followed by immunoblotting of cell lysates for Arginase-1. Stimulation of monocytes with TLR1/2 agonists, PAM3 and zymosan, or TLR4 agonists, lipopolysaccharide (LPS) and live *Escherichia coli*, failed to induce host arginase-1 (Fig. 3.5A). Conversely, exposure to *M. avium* greatly enhanced protein levels of this catabolic enzyme in monocytes of all TLR1 genotypes (Fig. 3.5A, B). Similarly to the experiments involving MHCII and CD64, TLR1 602 S/S cells were unable to resist the immunomodulatory activities of whole mycobacteria. We further examined whether TLR1 602S homozygotes could prevent upregulation of arginase-1 when exposed to Mtb mem, which would only activate TLR1 602I. Strikingly, cell lysates from TLR1 602S/S monocytes stimulated with Mtb mem contained very low levels of Arginase-1, while enzyme expression was strongly induced in TLR1 602I individuals (Fig. 3.5B). This resistance was observed even under prolonged Mtb mem challenge for 48 hours (Fig. 3.6). These results support the previous observations that differential trafficking of the TLR1 602 variants contribute to protection against subversive mycobacterial strategies.

**TLR1 602S/S macrophages display enhanced killing of *M. avium*** - If iNOS-mediated killing of mycobacteria represents an important aspect of the protective TLR1 602S phenotype, we hypothesized that macrophages from TLR1 602S/S individuals may be able to display enhanced killing of mycobacteria *in vitro*. To test this, primary human macrophages derived from blood monocytes were challenged with *M. avium* and allowed to control the growth of bacteria for several time-courses. In addition, we tested the effects IFNγ or TLR1 agonist stimulation had upon killing, with the prediction that activation of surface TLR1 602I by Mtb membrane fraction could dampen nitric oxide production or suppress IFNγ-dependent microbicidal functions in TLR1 602I/I or I/S donors, but not in TLR1 602S/S individuals. Macrophages from three different blood donors representing TLR1 602I or 602S genotypes were stimulated with or without Mtb membrane fraction in the presence or absence of IFNγ for 24 hours. These macrophages were subsequently infected with *M. avium* (MOI 10:1) and bacterial killing was allowed to proceed for 2 days (Fig. 3.7A). Colony forming units (cfu) plated from macrophages lysates were finally counted and averaged. Under conditions using bacterial infection alone, TLR1 602S/S macrophages appear to control cfu counts slightly better than TLR1 602I cells, although this effect does not reach statistical significance. Incubation with IFNγ
appears to marginally enhance killing of bacteria, as denoted by fewer cfu among all donors, where TLR1 602S/S cells retain significantly fewer bacteria after two days.

Similar experiments were done over a course of 4 and 6 days of killing using two TLR1 602S/S donors and two TLR1 602I donors (Fig. 3.7B and C). A trend showing enhanced killing of *M. avium* can also be observed during these time courses, however, the sample number was not high enough to establish statistical significance. Finally, a third killing assay was performed using a different order of addition for macrophage stimulation. Primary human monocyte-derived macrophages from three different individuals of the various TLR1 602 genotypes were stimulated for 24 hours with or without PAM3CSK4 or Mtb membrane fraction following IFNγ pre-treatment for 24 hours (Fig. 3.8). These macrophages were subsequently infected with *M. avium* (MOI 10:1) and bacterial killing was allowed to proceed for 3 days. Pre-treatment of macrophages with IFNγ appeared to increase bacterial burden in macrophages regardless of genotypes. This effect is likely due to the upregulation of phagocytic receptors and increased bacterial uptake, rather than a suppression of bacterial killing. Again, a trend of lower bacterial retention can be observed in TLR1 602S/S macrophages. Co-incubation of cells with the TLR1 agonists PAM3 or Mtb membrane fraction did not significantly affect clearance of mycobacteria by either TLR1 genotype.

**Discussion**

Dynamic trafficking and differential subcellular distribution of TLRs ensures that a diverse array of microbial products is sensed by the innate immune system. Surface displayed TLRs, including TLR1, 2, 4, 5, 6, and 10, recognize a wide range of molecules shed from or present in bacterial outer walls and membranes. Ligands for these TLRs are present at appreciable concentrations in the extracellular environment of an infection site, where bacteria are actively replicating and physically engaging innate leukocytes. Recognition of pathogens and the establishment of a cytokine profile at the site of infection are essential for microbial clearance and restoration of tissue homeostasis.

We previously discovered I602S as a polymorphism which underlies a trafficking defect in TLR1 (23). Subsequent work in our lab has revealed that position 602 resides within a short 6 amino acid cytoplasmic trafficking motif which, in conjunction with the adjacent transmembrane domain, is sufficient to direct TLR1 to the cell surface (18). A serine at position 602,
representing the I602S polymorphism, interrupts this trafficking motif and prevents cell surface expression of TLR1. Importantly, monocytes derived from individuals homozygous for the 602S variant which lack cell surface TLR1 exhibit greatly attenuated responses to a variety of soluble TLR1 agonists including various mycobacterial cell wall components (20, 23).

TLR1 requires TLR2 as a heterodimeric partner in the recognition of mycobacterial cell wall components and this heterodimer initiates intracellular signaling which drives antimicrobial, inflammatory, and adaptive immune responses. The TLR1/2 heterodimer serves as a key sensor by which innate cells recognize mycobacteria and plays a critical role in host defense as evidenced by the increased susceptibility of TLR2 deficient mice to infection with pathogenic mycobacteria and the fact that numerous functionally deleterious polymorphisms in human TLR2 associate with leprosy and tuberculosis (53, reviewed in 19). These polymorphisms, which include insertion/deletions within the promoter as well as nonsynonymous single nucleotide changes in the receptor itself, result in decreased TLR2 expression or function and are associated with either an increased incidence or dissemination of mycobacterial infection (reviewed in 19). Surprisingly, we discovered that instead of being deleterious, the TLR1 cell surface trafficking defect of the 602S/S genotype associates with protection against leprosy (23). Importantly, TLR1 602S was also identified in an extensive genome-wide array as one of two alleles in humans that afford the greatest protection against leprosy (59). An independent study also revealed that the 602S variant associated with a decreased incidence of extrapulmonary tuberculosis infection, while TLR1 602I predisposed individuals to infection (31). The fact that a defective trafficking variant of TLR1 protects against leprosy and tuberculosis appears to contradict the important role of the TLR2 subfamily in host protection against pathogenic mycobacteria.

In this paper, we attempted to address the question as to how a deficient TLR1 polymorphism provides protection against mycobacterial disease. Here we report that TLR1 602S/S macrophages are unresponsive to soluble TLR1 agonists, such as PAM3 and *M. tuberculosis* membrane fraction. However, stimulation with either whole mycobacterial or fungal particles leads to co-localization of the TLR1 602S variant with the endosome along with prominent secretion of proinflammatory cytokines. As expected, pharmacologic inhibitors of vesicular trafficking prevent TLR1 endosomal localization and secretion of proinflammatory cytokines. These data suggest that although TLR1 602S is confined internally in resting cells, this receptor is a fully functional TLR1 variant in the context of whole pathogens, including
Interestingly, PAM3-coated beads do not activate proinflammatory cytokine secretion, indicating that stimulation of co-receptors which engage more complex agonists such as whole bacteria, is required for the induction of TLR1 trafficking to the endosome. In this regard, it has been previously observed that phagocytic co-receptors, such as CD36 or CD14, regulate the subcellular distribution of TLRs in response to complex agonists (54, 60). Indeed, engagement of CD36 is required for TLR2 responses to whole bacteria (49). Additionally, internalization of TLR2 by inflammatory monocytes has been shown to be a prerequisite for driving cytokine responses to both Francisella and viral particles (5, 10).

Many studies have shown that mycobacteria subvert the TLR1/2 system to their advantage. For example, prolonged stimulation of TLR1/2 by the triacylated lipoprotein LpqH induces several immunosuppressive states at both the innate and adaptive levels in myeloid cells, including resistance to IFNγ, inhibition of CIITA transactivation, reduction of surface levels of MHCI1, B7.2, and FcyRI, abrogation of antigen presentation and the oxidative burst, and even induction of apoptosis (4, 16, 17, 24, 27, 28, 30, 35, 37-41, 45-47, 52, 57). We hypothesized that resistance to mycobacterial-mediated subversion of TLR1/2, conferred by lack of TLR1 surface expression, underlies the associated resistance of homozygous TLR1 602S individuals to mycobacterial disease. In support of this hypothesis we found that primary human monocytes and macrophages from TLR1 602I blood donors exhibited dramatic reductions of surface MHCI and CD64 when stimulated with *M. tuberculosis* membrane fraction, while TLR1 602S/S cells retained significantly higher surface levels of both markers.

Arginine-dependent NO synthesis is known to be important for anti-mycobacterial defense (12, 42). The fact that mycobacterial activation of TLR2 induces host arginase-1 provides another example of subversion of this receptor system, as this enzyme depletes arginine which is required as a substrate in the production of nitric oxide (14). When stimulated with *M. tuberculosis* membrane fraction, TLR1 602S/S cells did not significantly induce arginase-1, whereas enzyme levels were potently upregulated in monocytes possessing the TLR1 602I allele. Additional examples of mycobacterial subversion of the TLR2 system include the finding that lipoarabinomannan, a major component of mycobacterial cell walls and TLR1/2 agonist, decreases macrophage microbial killing induced by IFN-γ (8, 45). Furthermore, early secreted antigenic target (ESAT-6), a small protein released by *M. tuberculosis*, inhibits MyD88-dependent signaling following engagement of TLR2 (39). While these additional subversion
mechanisms in the context of the I602S polymorphism were not explored in this paper, it would not be surprising to find protection conferred by the TLR1 602S/S genotype.

While several mechanisms of TLR2 subversion by mycobacteria exist, this receptor complex nevertheless plays a key role in host defense against this bacterium. Perhaps most importantly, through induction of a vitamin D pathway, TLR2 activation has been shown to drive upregulation of antimicrobial peptides and induction of autophagy in response to mycobacteria (15, 29). As pointed out above, the TLR1 602S variant retains the ability to traffic and signal from endosomal compartments following uptake of whole microbial particles including mycobacteria. Therefore, in the context of whole mycobacteria, these critical functions would be retained in individuals of the TLR1 602S/S genotype. In fact, in the context of whole mycobacteria we have been unable to identify a macrophage phenotype, either beneficial or detrimental, in association with the I602S polymorphism.

Since granulomas provide the primary means of containment of mycobacterial infection, it is perhaps instructive to consider their protective role in the context of the TLR1 I602S polymorphism. Granulomas consist of a core of surviving mycobacteria that is walled off by other leukocytes, primarily monocytes and effector T cells (22). As monocytes infiltrate a granuloma they are likely to be exposed to soluble mycobacterial components released by actively replicating mycobacteria and infected macrophages (6, 47). Indeed, it has been observed that mycobacteria release membrane vesicles and that those released from virulent strains contain TLR2 lipoprotein agonists (41). Similarly, the more virulent rough morphotypes of *Mycobacterium abscessus* are associated with increased lipoprotein production and subversive TLR2 engagement (43). Additionally, macrophages infected with *M. tuberculosis* release exosomes containing degraded mycobacterial components which have been shown to inhibit macrophage activation by IFNγ in a TLR2-dependent fashion (47). We hypothesize that TLR1 602S/S cells resist these effects through the absence of surface TLR1, and that subsequent lack of functional TLR1/2 heterodimers prevent subversion of TLR signals. Thus, due to exposure to soluble mycobacterial components, TLR1 602I monocytes would respond more poorly to whole mycobacteria present in the core of the granuloma compared to TLR1 602S/S cells, leading to reduced clearance and containment of the infection.

The TLR1 I602S polymorphism exhibits vastly different geographic and racial distributions. For example, the 602S allele is far more prevalent among individuals of European versus African descent with frequencies of 75 and 25 percent, respectively. This differential
distribution of the TLR1 602S allele could underlie the observation that among 25,000 nursing home patients, African Americans were found to have twice the risk of developing tuberculosis than Caucasians (48). It is interesting to note that in areas of the world with the highest incidences of mycobacterial disease, such as India and Asia where tuberculosis and leprosy are endemic, harbor the lowest frequencies of the protective TLR1 602S allele at less than 1 percent. Studies focused on additional genome-wide association and ex vivo analyses are necessary to fully elucidate the role TLR1 I602S plays in the complex host-pathogen interaction that occurs following mycobacterial infection.

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**Figure 3.1** Intracellular TLR1 602S is activated by particulate but not soluble agonists.
Figure 3.1 continued.

(A) Representative surface (left column) and permeabilized (right column, perm) staining of TLR1 in primary human monocyte-derived macrophages from a TLR1 602I (top row) and TLR1 602S (bottom row) homozygous blood donor is shown (filled histogram; isotype control, black histogram; TLR1). Primary human monocyte-derived macrophages from venous blood donors of the various TLR1 602 genotypes were stimulated with agonists, as indicated, for 24 hours and secretion of TNFα (B) and IL-6 (C) was measured from culture supernatants by ELISA (black bars; TLR1 602I/I or TLR1 602I/S donors, gray bars; TLR1 602S/S donors). Error bars represent the standard deviation of at least three donors (PAM3; PAM3CSK4, Mtb mem; M. tuberculosis membrane fraction, PAM3 beads; PAM3CSK4-coated polystyrene beads, Mtb; gamma-irradiated M. tuberculosis).
Intracellular TLR1 602S gains access to phagocytized particulate agonists in endosomal compartments.

(A) Total TLR1 staining (red) of permeabilized resting macrophages from a TLR1 602I/I (top) or TLR1 602S/S (bottom) blood donor. (B) Monocyte-derived macrophages were stimulated with auramine-O labeled *M. avium* (green) for 15 minutes. Cells were subsequently fixed, permeabilized, and stained for TLR1 (red). Primary human monocyte-derived macrophages from venous blood donors of the indicated TLR1 602 genotypes were stimulated for 12 hrs with *Mycobacterium avium* or *Mycobacterium tuberculosis* in the presence or absence of cytochalasin D (cD), followed by ELISA quantification of secreted TNFα (C) or IL-6 (D). Error bars represent the standard deviation of at least three donors (black bars; TLR1 602I/I or TLR1 602I/S donors, grey bars; TLR1 602S/S donors) (DIC; differential interference contrast, Mtb; *Mycobacterium tuberculosis*, cD; cytochalasin D).
Figure 3.3 TLR1 602S/S macrophages resist downregulation of MHCII in response to soluble, but not particulate, mycobacterial agonists.

(A) Primary human monocytes of different TLR1 602 genotypes were incubated with or without *M. tuberculosis* membrane fraction (Mtb mem) for 24 hours. Surface levels of MHCII were assessed by flow cytometry (black bars; TLR1 602I/I or I/S, gray bars; TLR1 602S/S). Error bars represent the standard deviation from at least three different blood donors. Asterisks denote significant differences versus unstimulated cells (*p<0.05, **p<0.005).

(B) Representative histograms of monocyte MHCII expression (filled histograms; isotype, black histograms; unstimulated cells, gray histograms; stimulated with Mtb membrane fraction).

(C) Primary human monocyte-derived macrophages were stimulated with soluble and particulate TLR1/2 agonists for 24 hours and surface levels of MHCII were assessed by flow cytometry (black bars; TLR1 602I/I or I/S, gray bars; TLR1 602S/S). Error bars represent the standard deviation from at least three blood donors. Asterisks denote significant differences versus unstimulated cells (*p<0.05). (D) Representative histograms from indicated genotypes (filled histograms; isotype, black histograms; unstimulated cells, gray histograms; stimulated cells). (Mtb mem; mycobacterial membrane fraction, Mav; *Mycobacterium avium*).
**Figure 3.4 TLR1 602S/S monocytes and macrophages resist mycobacterial inhibition of IFNγ-induced CD64.**

(A) Primary human monocytes of different TLR1 602 genotypes were incubated with or without *M. tuberculosis* membrane fraction (Mtb mem) in the presence or absence of IFNγ for 24 hours. Surface levels of monocyte CD64 were assessed by flow cytometry. Bars represent the fold change in CD64 expression versus unstimulated cells (black bars; TLR1 602I/I or I/S donors, gray bars; TLR1 602S/S donors). Error bars represent the standard deviation from at least three different blood donors. Asterisks denote significant differences versus unstimulated values (*p<0.05, **p<0.005). (B) Representative histograms of CD64 expression (filled histograms; isotype, black histograms; IFNγ-stimulated cells, gray histograms; co-stimulated with IFNγ and Mtb membrane fraction). (C) Primary human monocyte-derived macrophages were stimulated with soluble and particulate TLR1/2 agonists in the presence or absence of IFNγ for 24 hours. Surface levels of CD64 were assessed by flow cytometry. Bars represent the fold change in CD64 expression versus unstimulated cells (black bars; TLR1 602I/I or I/S donors, gray bars; TLR1 602S/S donors). Error bars represent the standard deviation from at least three different blood donors. Asterisks denote significant differences versus unstimulated values (*p<0.05).
Figure 3.4 continued.
(D) Representative histograms from indicated genotypes: top row (filled histograms; isotype, black histograms; IFNγ-stimulated cells, gray histograms; co-stimulated with IFNγ and Mtb membrane fraction) and bottom row (filled histograms; isotype, black histograms; IFNγ-stimulated cells, gray histograms; co-stimulated with IFNγ and *M. avium* Mav).
Figure 3.5 TLR1 602S/S monocytes resist induction of host arginase-1 when stimulated with soluble, but not particulate, mycobacterial agonists.

(A) Blood monocytes from donors of the indicated TLR1 602 genotypes were stimulated with various TLR agonists for 24 hrs followed by detection of Arginase-1 in cell lysates via Western blot. Loading controls were performed by detection of actin. (B) Primary human monocytes from the indicated donors were stimulated with *M. tuberculosis* (Mtb) membrane fraction (top), or live *M. avium* (bottom) for 24 hours. Levels of Arginase-1 were determined by Western blot. Relative protein loading is denoted by actin blots.
Figure 3.6  **TLR1 602S/S monocytes resist induction of host arginase-1 following prolonged stimulation with, soluble but not particulate, mycobacterial agonists.**

Blood monocytes from four different donors of the indicated TLR1 genotypes were stimulated with various TLR agonists for 48 hours followed by detection of Arginase-1 in cell lysates via Western blot. Relative protein loading is denoted by actin blots.
Figure 3.7 Killing of mycobacteria is marginally enhanced in TLR1 602S/S macrophages.

(A) Primary human monocyte-derived macrophages from three different individuals of the indicated TLR1 602 genotypes were stimulated with or without Mtb membrane fraction in the presence or absence of IFNγ for 24 hours. These macrophages were subsequently infected with *M. avium* (MOI 10:1) and bacterial killing was allowed to proceed for 2 days. Colony forming units plated from macrophages lysates were finally counted (gray bars; TLR1 602S/S donors, black bars; TLR1 602I/S or TLR1 602I/I donors). Error bars represent the standard deviation from three different blood donors. (B) The same experimental protocol as above was applied using two TLR1 602S/S donors, one TLR1 602I/I donor, and one TLR1 602I/S donor. However, the time allowed for killing was increased to 4 days (light gray and white bars; TLR1 602S/S donors, dark gray bar; TLR1 602I/S donor, black bar; TLR1 602I/I donor). (C) Macrophages from the same donors used in panel B were allowed to kill bacteria for 6 days using the same protocol (light gray and white bars; TLR1 602S/S donors, dark gray bar; TLR1 602I/S donor, black bar; TLR1 602I/I donor) (Mtb mem; *M. tuberculosis* membrane fraction, cfu; colony forming unit).
Figure 3.8  Pre-treatment with IFNγ enhances bacterial uptake but does not affect enhanced killing of *M. avium* by TLR1 602S/S macrophages.

Primary human monocyte-derived macrophages from three different individuals of the indicated TLR1 602 genotypes were stimulated for 24 hours with or without PAM3CSK4 or Mtb membrane fraction following IFNγ pre-treatment for 24 hours. These macrophages were subsequently infected with *M. avium* (MOI 10:1) and bacterial killing was allowed to proceed for 3 days. Colony forming units plated from macrophages lysates were finally counted (gray bars; TLR1 602S/S donors, black bars; TLR1 602I/S or TLR1 602I/S donors). Error bars represent the standard deviation from three different blood donors (PAM3; PAM3CSK4, Mtb mem; *M. tuberculosis* membrane fraction, cfu; colony forming unit).
References


Clinical Implications

Many aspects of the TLR1 I602S polymorphism are striking and unexpected. First, this polymorphism is frequently represented in the human population. Single nucleotide polymorphisms are numerous in the genome, making up 90% of the genetic variation in humans, but are rarely commonly frequent between individuals (7). In fact, for a variant to be considered a SNP, it must occur in at least 1% of the population. Depending on ancestral heritage, the TLR1 602S allele can be present anywhere from 75% in Caucasians to only 1% in individuals of East Asian descent (8). Second, SNPs rarely cause noticeable phenotypes. Many SNPs are present in non-coding DNA, such as introns or intergenic sequence, and cannot be attributed with influence on any known biological process. In addition, SNPs frequently encode silent amino acid substitutions, either changing a residue to one chemically similar to the original or leaving the amino acid unchanged due to the redundancy in the genetic code. The TLR1 602S allele converts a non-polar isoleucine to a polar serine and confers a prominent phenotype in the form of abrogated trafficking of the receptor to the plasma membrane. A single nucleotide change altering a residue proximal to the transmembrane helix in the TLR1 cytoplasmic domain completely interrupts proper display in resting cells and subsequently confers almost full unresponsiveness to soluble synthetic and bacterial agonists. While many phenotypes of this nature could easily be attributed to faulty overall expression of the deficient receptor, the TLR1 602S variant is expressed at equally stable levels as those of TLR1 602I. Interestingly, this single nucleotide change establishes its phenotype in an arguably more complex mechanism, by affecting the trafficking processes required for proper anterograde transport of the receptor to the cell surface.

The third striking aspect of this polymorphism is the presence of another phenotype associated with the homozygous TLR1 602S/S genotype: protection against mycobacterial disease. While many known SNPs confer protection to various illnesses, few loss-of-function phenotypes are associated with resistance to infectious disease. One well characterized example is the heterozygote advantage conferred to sickle cell carriers against malarial disease (11). A beta-hemoglobin SNP in homozygous form causes aggregation of the protein within red blood cells, reducing cell flexibility and altering the cell morphology. Heterozygotes are
incompletely recessive for this trait, producing both normal and altered red blood cells. Plasmodium parasites, which spend a portion of their life cycle inside these cells, reproduce inefficiently in heterozygotes, as sickled cells will lyse prematurely upon infection. Interestingly, protective allele frequencies correlate geographically with disease incidence. In this example, a loss-of-function allele in a protein unrelated to immunity protects against an infectious parasite.

Even rarer however are mutations which confer protection against infectious disease through a specific loss of immune function. A well known example of such a phenomenon is the Δ32 mutation in human CCR5 (17). This chemokine receptor typically recognizes both RANTES and macrophage inflammatory proteins, but also serves as a co-receptor for viral entry of macrophage-tropic HIV-1. A premature stop codon deletes the C-terminal end of the receptor, leading to diminished expression and reduced capacity of leukocytes to chemotax (15). Subsequently, macrophages with lowered surface levels of CCR5 are less amenable to infection by HIV virions, and individuals homozygous for this deletion are significantly less likely to be seropositive for the virus. Thus a perturbation in an immunity gene leads to protection against an infectious disease. There are few characterized examples of such concepts, as the purpose of a fully functional immune system is to protect against pathogens.

Another example of this rare occurrence is the TLR1 I602S polymorphism. Similarly to the CCR5 mutation, this polymorphism affects surface expression of an immune receptor (albeit by dissimilar means), which decreases its typical function but protects against the development of *M. tuberculosis* and *M. leprae* related diseases (8, 12, 13, 20). It is not surprising that a 32 amino acid deletion could have highly detrimental effects on CCR5 protein function, which makes the fact that a single nucleotide change in TLR1 confers significant protection against highly successful pathogens incredibly impressive.

In addition to offering protection against mycobacterial subversion of macrophage function as described in Chapter 3, differential activation of TLR1 602S may confer resistance through the enhancement of Th1 immunity. A study by Strle et al. showed that *in vitro* stimulation of PBMCs from TLR1 602S/S Lyme disease patients with live *Borrelia burgdorferi* lead to significantly higher production of IFNγ over TLR1 602I individuals (19). Interestingly, TLR1 602S/S Lyme patients also had greater serum levels of IFNγ and chemokines, which was associated with heightened Lyme arthritis. In addition, greater production of IFNγ and IL-2 has been observed in response to BCG vaccination among South African infants possessing the TLR1 602S allele (16). IFNγ has been well characterized as an essential component of anti-
mucobacterial defense and, if TLR1 602S/S individuals are able to drive greater production of this Th1-skewing cytokine, they may be poised to heightened immunity against mycobacteria and other intracellular pathogens.

There have also been clinically detrimental effects associated with the TLR1 602S polymorphism in association with vaccine efficacy, however. The triacylated lipoprotein of *Borrelia burgdorferi*, OspA, has been used as a subunit vaccine component against Lyme disease. It was shown that TLR1 and TLR2 deficient mice have impaired antibody production upon vaccination which was correlated with hyporesponsiveness to OspA stimulation (1). Interestingly, a subpopulation of human patients also exhibited lower OspA antibody titers upon vaccination, an observation which may be associated with TLR1 602S homozygosity. Indeed it has been shown that TLR1/2 heterodimers play an essential role in mediating recognition of whole Borrelia cells, whereby antibody blocking of TLR1 in vitro leads to lower cytokine production in response to spirochete stimulation f PBMCs (14). These seemingly contradictory results, of enhanced Th1 immunity to Lyme disease but reduced efficacy of Lyme vaccination, potentially reveal differential roles TLR1 602S may be playing during OspA stimulation versus actual Borrelia infection.

TLR1 602S allele frequencies exhibit differential geographic and racial distributions. Peoples of African descent possess an I:S frequency of 75:25%; East Asian, 99:1%; Caucasian, 25:75%; Hispanic 70:30%; Nepalese, 96:4%; and Turkish, 47:53%. There are presently no known selective forces which explain these observed distributions. It is interesting to note however that areas of the world endemic with the highest incidences of mycobacterial disease, such as India and Asia, also possess the lowest frequencies of the protective TLR1 602S allele. Even before the discovery of the TLR1 I602S polymorphism, it was observed that African Americans had twice the risk of developing tuberculosis as Caucasians (18). This study, which examined 25,000 nursing home patients, could find no pre-disposing environmental or social factors to explain this difference.

If TLR1 function can indeed be usurped by mycobacteria via methods described in Chapters 3, it is conceivable that TLR1 could potentially be a target for anti-mycobacterial therapies (6). Would blocking of surface TLR1 602I in lung granulomas by monoclonal antibodies protect infiltrating monocytes from inhibitory signals mediated by soluble mycobacterial agonists? Could small-molecule inhibitors of TLR1/2 activity similarly be protective or therapeutic, especially in combination with first line anti-mycobacterial drugs like
isoniazid or rifampin? Alternatively, the currently unknown signaling pathways induced by TLR1 to facilitate the immunomodulatory activities of mycobacteria may be a target as well. If these pathways differ from the normal TLR1/2 cascade, then perhaps drugs targeting those signaling proteins could inhibit mycobacterial influences without blocking protective TLR1/2 activity.

Chemical inhibition of TLR1 as a means of anti-mycobacterial therapy would only be effective if the true nature of protection conferred by TLR1 602S/S is based on the lack of surface expression and subsequent reduced function. It remains possible that intracellular TLR1 602S possesses an alternative function to that of surface displayed TLR1 602I. Activation of intracellular TLRs, such as the endosomally expressed TLR3, 7, 8, and 9, leads to signals and outputs alternative to those of surface-expressed TLRs. TLR4 has been shown to use alternative signaling components to produce interferons when internalized, as opposed to pathways utilized when the receptor is displayed on the plasma membrane (9). Could ER-bound or endosomally-recruited TLR1 602S induce distinct signals and functions to those of surface TLR1 602I? These questions among others need to be answered before any therapeutic potential through the manipulation of TLR1 can be designed.

Unanswered Questions and Future Experimentation

Although we have evidence that the TLR1 I602S polymorphism lies within a sequence necessary for TLR1 trafficking, we still don’t fully know why this sequence is important and how it contributes to targeted surface trafficking of TLR1. Many other trafficking motifs have similarly been identified in various TLRs, but no mechanisms describing how they function as such have been described. As seen in Figures 2.13 and 2.14, the ER chaperone PRAT4A behaves in a positive fashion for TLR surface trafficking, and this enhancement of surface expression (for TLR1) requires this 6 amino acid motif (5). Interestingly however, this region is not required for efficient interaction between PRAT4A and TLR1. It is hypothesized that PRAT4A is strictly expressed in the ER lumen and thus only interacts with the extracellular portion of the receptor. This idea is supported by mutagenesis experiments performed on TLR4, where co-immunoprecipitations between TLR4 and PRAT4A revealed the PRAT4A binding site to be in the N-terminal portion of TLR4 (10). The TLR1 trafficking domain resides in the cytoplasmic domain but seems to affect the trafficking enhancement by PRAT4A. How does amino acid information within the cytoplasmic domain affect the function of an ER lumen-expressed chaperone? Is
there another protein involved in the receptor/chaperone complex that spans the ER lumen’s membrane to contact the cytoplasmic domain of the TLRs? UNC93B1 is a known TLR chaperone present in the ER with multiple transmembrane-spanning domains, and has been shown to be required for trafficking of TLR3, 7, and 9. It is conceivable that an as-yet unidentified protein could mimic the function of UNC93B1 for surface-expressed TLRs.

Overexpression of PRAT4B negatively regulates TLR surface trafficking and an enhanced interaction between TLR1 602S and PRAT4B may account for this receptor variant’s phenotype (Fig. 2.15). Similarly to PRAT4A, if PRAT4B is expressed in the ER, how does a cytoplasmic polymorphism affect the interaction between the ligand binding domain of the receptor and the chaperone (10)? No studies have been performed to address the binding site for PRAT4B, but there may be yet another protein in the receptor/chaperone complex whose function could answer questions both for PRAT4A and PRAT4B. It would also be pertinent to determine if PRAT4A and PRAT4B bind simultaneously to TLRs and compete for TLR1 binding in a manner which regulates the release of TLRs from the ER to the cell surface. It would be fascinating to follow up on PRAT4B’s function especially if it is indeed a negative regulator of TLR function. As seen in Figure 2.20, stimulation of monocytes with IFNγ upregulates PRAT4A but not PRAT4B (5). If PRAT4B expression and its downregulation of TLRs acts as a damage-control response to inflammation, would anti-inflammatory cytokines upregulate PRAT4B? If so, manipulation of PRAT4B expression could be used as an anti-inflammatory therapeutic. Alternatively, enhancement of PRAT4A activity could potentially boost immune recognition of pathogens or tumors.

There are also many unanswered questions about how the TLR1 602S polymorphism protects against mycobacterial disease. The only measure for protection performed is the statistically significant absence of disease symptoms in homozygotes. However, it is not known whether this correlates to lack of infection. Does the polymorphism protect against infection establishment or does the observed hyporesponsiveness of TLR1 602S/S cells actually protect against disease by limiting collateral damage to chronically infected tissues? We hypothesize that monocytes infiltrating granulomas are pre-conditioned for immunosuppression by mycobacterial components. To further support this we would need to determine if monocytes recognize mycobacterial components before they reach whole bacteria or if mycobacterial components leak from granulomas to deactivate surrounding leukocytes before they even reach the granuloma border. TLR1 602S may also protect by possessing a unique signaling function.
which better adapts monocytes and macrophages to kill internalized mycobacteria. If TLR1 602S/S macrophages are better able to kill engulfed mycobacteria, it would be important to determine if this is linked to arginase-1/iNOS activity *in vivo*. Also we are interested in determining if there are other mechanisms by which mycobacteria use TLR1 signalling to inhibit macrophage function. Is the subversion of TLR1 by mycobacteria macrophage-specific, or are T cells and other immune cells affected?

Finally, perhaps the most interesting question to ask is also the most difficult to answer. Studies have revealed that TLR1 possesses the highest rate of missense mutation over any other TLR and that the TLR1-6-10 gene cluster is a hotspot of positive selection (2-4). It is conceivable that evolutionary selective pressures applied in the past could explain today's pattern of racial distribution for each particular allele frequency of the TLR1 I602S polymorphism. Selective pressure for specific alleles of genes related to host immunity often comes in the form of an infectious disease, where individuals with one specific allele were more likely to resist or survive exposure to a pathogen. The prevalence of such a pathogen may have been confined to certain areas of the world dominated by particular races of people, thus an allele may appear to segregate based on race simply due to spatial organization of the number of people exposed to a pathogen.

Since homozygosity for TLR1 602S protects individuals from tuberculosis and leprosy, one might hypothesize that mycobacterial infections promoted the observed pattern of allele frequencies. While these diseases can be devastating and disfiguring, especially for immunocompromised patients, slowly progressing mycobacterial infections rarely kill individuals prior to their reproductive years. Regardless of whether a person is infected with *M. tuberculosis* or *M. leprae*, they would still have the potential to reproduce and pass on any allele, deleterious or advantageous, before they might succumb to infection. If the worldwide distribution of TLR1 602S is dependent on a past epidemic, the pathogen would need to be much more virulent than mycobacteria. Infection by the causative agent of the highly virulent Black Plague, *Yersinia pestis*, would be a potential candidate for this hypothesis. Unfortunately no studies have been performed to examine whether the TLR1 602S protects against this disease. While it is probable that mycobacterial infection is not a strong enough selective pressure, it is interesting to note that areas of the world with the highest incidences of mycobacterial disease, like Asia and India, also have the lowest frequencies of the protective TLR1 602S allele. Other areas of the world, such as Europe and the Americas, have higher
frequencies of TLR1 602S and lower rates of mycobacterial infection. It is likely that these distributions have a great deal to do with prevalence of high poverty and less access to medical care, but it could be suggested that low levels of protective alleles in local populations also contribute. The sheer number of questions left unanswered about TLR1 I602S reveals how truly little we know and have yet to discover about this fascinating polymorphism.


References


