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A Tale of Two SNPS: Polymorphism Analysis of Toll-like Receptor (TLR) Adapter Proteins: A Dissertation

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A TALE OF TWO SNPS: POLYMORPHISM ANALYSIS OF TOLL-LIKE RECEPTOR (TLR) ADAPTER PROTEINS

A Dissertation Presented

By

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A TALE OF TWO SNPs:
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" I almost wish I hadn't gone down that rabbit-hole – and yet – and yet – it's rather curious, you know, this sort of life! "

---Alice

(From Alice's Adventures in Wonderland by Lewis Carroll)

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LIST OF ABBREVIATIONS

APCs	antigen presenting cells
BMDM	bone marrow derived macrophages
CARDs	caspase recruitment and activation domains
CD14	cluster of differentiation-14
CLRs	C-type lectin receptors
CRD	carbohydrate recognition domain
DCs	dendritic cells
DC-SIGN	dendritic cell-specific ICAM3-grabbing non-integrin
DC-SIGNR	DC-SIGN related
DD	death domain
dsDNA (or ssDNA)	double stranded deoxyribonucleic acid (or single stranded)
ELISA	enzyme-linked immunosorbent assay
FRET	Förster (or fluorescence) resonance energy transfer
FRET SE	FRET sensitized emission
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
HPRT1	hypoxanthine phosphoribosyltransferase 1
IB	immunoblot
IFN	interferon
IκB	inhibitor of κB
IKK	IκB kinase
IL-4 (IL-10, IL-13)	interleukin-4 (-10, -13)
IP	immunoprecipitation
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
IRES	internal ribosome entry site
ISRE	interferon-stimulated response element

ITAM	immunoreceptor tyrosine-based activation motif
LGP2	laboratory of genetics and physiology 2
LBP	LPS binding protein
LPS	lipopolysaccharide
LRR	leucine rich repeats
Mal	MyD88 adapter-like
MBL	mannose binding lectin
MD-2	myeloid differentiation-2
MDA-5	melanoma-differentiation-associated gene 5
mDCs	myeloid dendritic cells
MR	Mannose receptor
MyD88	myeloid differentiation protein 88
NEMO	NF-κB essential modulator
NDV	Newcastle disease virus
NF-κB	nuclear factor kappa B
NK (cells)	natural killer (cells)
NLRs	NOD-like receptors
PAMPs	pattern associated molecular patterns
PBMC	peripheral-blood mononuclear-cells
pDCs	plasmacytoid dendritic cells
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol-4, 5-bisphosphate
PRR	pattern recognition receptor
RHIM	RIP homotypic interaction motif
RIG-I	retinoic acid-inducible gene
RIP	receptor-interacting protein
RLRs	(RIG)-I-like-receptors
RLU	relative luciferase units
RSV	respiratory syncytial virus

SARM	Sterile-alpha and Armadillo motif-containing protein
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
TAB1	TAK1 binding protein
TAK1	transforming growth factor β-activated-kinase 1
TIR (domain)	Toll/interleukin-1 receptor (domain)
TIRAP	TIR domain-containing adapter protein
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	TIR domain containing adapter inducing IFN-β
TLR	Toll-like receptor
TNF	tumor necrosis factor
VSV	vesicular stomatitis virus
WT	wild type
YFP	yellow fluorescent protein

ABSTRACT

The innate immune system is the first line of defense against invading pathogens. Recognition of microbial ligands by the innate immune system relies on germ-line encoded, evolutionarily conserved receptors called pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are one such family of PRRs and are involved in innate defenses to a variety of microbes. At the core of TLR signaling pathways are Toll interleukin-1 receptor (TIR) domain containing adapter proteins. Much of the specificity of TLR pathways arise from the differential use of these adapter proteins.

The TLR signaling cascade that ensues upon ligand recognition is marked by finely orchestrated molecular interactions between the receptor and the TIR domain containing adapter proteins, as well as various downstream kinases and effector molecules. Conserving the structural integrity of the TLR components is thus essential for maintaining a robust host defense system. Sometimes, changes in a protein can be brought about by single nucleotide polymorphisms (SNPs). Studies carried out in this thesis focus on polymorphisms in MyD88 adapter-like (Mal) and myeloid differentiation protein 88 (MyD88), two TIR domain-containing adapter proteins, which incidentally are also highly polymorphic.

Mal is a 235 amino acid protein that is involved in TLR2 and TLR4 signaling. The known polymorphisms in the coding region of Mal were screened with an aim to identify SNPs with altered signaling potential. A TIR domain polymorphism, D96N, was found to be completely defective in TLR2 and TLR4 signaling. Immortalized macrophage-like cell lines expressing D96N have impaired cytokine production as well

as NF-κB activation. The reason for this loss-of-function phenotype is the inability of Mal D96N to bind the downstream adapter MyD88, an event necessary for signaling to occur. Genotyping studies reveal a very low frequency of this polymorphism in the population.

Similar SNP analysis was carried out in myeloid differentiation protein 88 (MyD88). MyD88 is a key signaling adapter in TLR signaling; critical for all TLR pathways except TLR3. In reporter assays, a death domain variant, S34Y, was found to be inactive. Importantly, in reconstituted macrophage-like cell lines derived from knockout mice, MyD88 S34Y was severely compromised in its ability to respond to all MyD88-dependent TLR ligands. S34Y mutant has a dramatically different localization pattern as compared to wild type MyD88. Unlike wild type MyD88, S34Y is unable to form distinct foci in the cells but is present diffused in the cytoplasm. IRAK4, a downstream kinase, colocalizes with MyD88 in these aggregates or "Myddosomes". S34Y MyD88, however, is unable to assemble into Myddosomes, thus demonstrating that proper cellular localization of MyD88 is a feature required for MyD88 function.

This thesis thus describes two loss-of-function polymorphisms in TLR adapter proteins Mal and MyD88. It sheds light not only on the structural aspects of signaling by these two proteins, but also has implications for the development of novel pharmaceutical agents.

CHAPTER I

INTRODUCTION

Introduction to Innate Immunity

The immune system is a network of specialized cells, tissues and organs that protect an organism against invasion by various pathogenic microorganisms. Innate and adaptive immunity are two components of the immune system with distinct properties. Innate immune responses occur rapidly and are the first line of defense that the pathogen encounters. These responses are non-specific and lack immunologic memory. An important characteristic of innate immunity is the ability to distinguish “non-self” from “self” and this is achieved by the use of pattern recognition receptors. Adaptive immune responses on the other hand have much slower kinetics and are highly specific. They have the unique feature of immunologic memory that allows them to mount a stronger and faster response to a subsequent infection by the same microorganism.

While B and T lymphocytes are the key players in an adaptive immune response, the innate response employs a variety of cells of both myeloid and lymphoid lineage. These include mast cells, natural killer cells, eosinophils, basophils as well as the phagocytic cells like dendritic cells (DCs), macrophages and neutrophils.

Mast cells: Mast cells are a type of innate immune cells that are closely associated with connective tissue and mucous membranes. They are best known for mediating allergic responses, but are also involved in wound healing and in protecting the internal surfaces of the body against pathogens (1, 2). They have large coarse granules in their cytoplasm that store a number of different chemical mediators like histamine, interleukins, heparin and various proteolytic enzymes (3). Upon stimulation, the contents of these granules are

released into the surrounding tissues, producing local responses characteristic of an inflammatory reaction. Histamine dilates blood vessels and helps in recruitment of macrophages and neutrophils (4).

Neutrophils: Neutrophils are a type of granulocyte (along with basophils and eosinophils) and are also called polymorphonuclear cells because of the distinctive appearance of their nuclei. In comparison with macrophages, neutrophils are relatively short-lived and survive for only a few days. However, they are the most abundant of all phagocytes and are highly motile. Thus, during infection neutrophils are the first cells to reach the site and defend against invading pathogens. Similar to macrophages, neutrophils attack pathogens by activating a respiratory burst. The main products of the neutrophil respiratory burst are strong oxidizing agents including hydrogen peroxide, free oxygen radicals and hypochlorite.

Dendritic cells (DCs): Dendritic cells are potent antigen presenting cells (APCs) and function to stimulate naïve T-cells (5). They thus act as a link between innate and adaptive immunity. DCs are present in a number of tissues, especially those that are in contact with the external environment like the skin (where they are called Langerhans cells) and the inner lining of lungs, nose, stomach and intestines. Numerous projections or “dendrites” that extend from the main cell body, give the cells a unique appearance and are the basis for the term “dendritic cells”. DCs are not a single population, but comprise a heterogeneous collection of cells derived from distinct hematopoietic lineages

(6). Broadly, DCs can be categorized into plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs, also known as classical dendritic cells). pDCs comprise only 0.2-0.8% of human blood cells and are so named because of their morphological similarity with plasma cells (7). They can produce type I IFN and were known as IFN producing cells (IPC) before their dendritic nature was revealed (7). Both pDCs and mDCs express pattern recognition receptors, especially TLRs. However, they both have a distinct expression profile in relation to the TLRs they express. mDCs express TLR1, TLR2, TLR3, TLR4 and TLR8, while pDCs express high levels of TLR 7 and TLR9. pDCs thus, predominantly respond to microbial nucleic acids, whereas mDCs can recognize a broader range of ligands.

Natural killer cells (NK cells): NK cells are a type of cytotoxic lymphocyte that are a part of the innate immune system (8). They are generated from the same lymphoid precursors as the B and T cells. They are also known as large granular lymphocytes and play an important role in the rejection of tumors and host defense against virally infected cells (9). NK cells are cytotoxic. They have small granules in their cytoplasm containing proteins known as perforins and proteases called granzymes (10). Upon coming close to a cell slated for death, perforins form channels in the membrane of the cell, through which granzymes and other proteins can enter, thus inducing apoptosis (11).

Macrophages: Macrophages are resident phagocytic cells that differentiate from circulating peripheral-blood mononuclear-cells (PBMCs) (12). PBMCs develop from

myeloid progenitor cells in the bone marrow, which are also the precursors to other cell types like dendritic cells. In response to macrophage colony-stimulating factor, cells of the monocyte lineage (which differentiate from myeloid progenitors) divide and differentiate into monoblasts and then pro-monocytes. They then become monocytes which are released from the bone marrow and enter the blood stream. Monocytes migrate from the blood into tissues to replenish tissue-resident macrophages (13). Macrophages in different tissues are markedly heterogeneous and reflect specialization of function in different microenvironments. In additon to lymphoid organs, macrophages are found in brain (microglial cells), liver (Kupffer cells), lung (alveolar macrophages), reproductive organs and the interstitium of organs like the heart and kidney, lamina propria of the gut etc. They are prodigious phagocytic cells and are involved in the clearing of cellular debris and cells that have undergone apoptosis. Trauma or stress induced necrosis also results in cellular debris that can be cleared by macrophages. They are equipped with a variety of receptors for phagocytosis like complement receptors and Fc receptors (14, 15). Additionally, they are equipped with a broad range of pattern recognition receptors that make them one of the primary sensors of danger in the host (16). These receptors are involved in the recognition of different pathogen associated molecules, triggering various signaling pathways to mount an immune response. Upon encountering a pathogen, a macrophage engulfs the pathogen and forms an internal membrane bound compartment called the phagosome. The phagosome fuses with the lysosome to form a phagolysosome. Various metabolic enzymes, nitric oxide, oxygen radicals and other chemical mediators in the phagolysosome digest and kill the pathogen by a process known as “respiratory

burst". In addition, macrophages release cytokines and chemokines that are involved in the inflammatory response and help recruit other cells like DCs.

Depending upon the type of cytokines they are exposed to, macrophages can be classified as either classically activated macrophages or alternative activated macrophages. The former category is used to describe effector macrophages produced during cell-mediated immune responses (17, 18). Exposure of macrophages to IFN-γ or TNF results in a population of cells that have high microbicidal activity and secrete high levels of pro-inflammatory cytokines and mediators (19). In the latter case, exposure to IL-4, IL-10 or IL-13, produces a macrophage population that can induce cellular proliferation and the production of polyamines and collagen, thereby contributing to wound healing (17, 20).

Pattern recognition receptors

In 1989, Charles Janeway Jr., proposed the pattern recognition theory, which laid the foundation for our current understanding of innate immunity (21). The innate immune system relies on germ-line encoded, evolutionarily conserved receptors called pattern recognition receptors (PRRs) to sense microbes (22). These receptors recognize conserved structures in microbes called pathogen associated molecular patterns (PAMPs). Since PAMPs are broadly expressed in pathogens and not in host cells, PRRs are able to differentiate between self and non-self. Depending on their location, these PRRs can be classified into transmembrane proteins, which include Toll-like receptors (TLRs) and C-

type lectin receptors (CLRs) as well as cytosolic proteins such as Retinoic acid-inducible gene (RIG)-I-like-receptors (RLRs) and NOD-like receptors (NLRs).

C-type lectin receptors (CLRs): C-type lectin receptors (CLRs) are a family of receptors that bind carbohydrates in a calcium dependent manner (23, 24). The sugar binding property of CLRs can be attributed to the presence of highly conserved carbohydrate recognition domains (CRDs) (24). CLRs include dectin-1, the dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), DC-SIGN related (DC-SIGNR), Mannose receptor (MR) and the circulating mannose binding lectin (MBL). They are expressed on most cell types, including macrophages and dendritic cells (DCs) and are mostly involved in defense against fungi. **Mannose receptor (MR)** was one of the first fungal PRRs to be described. It recognizes the mannose moieties in the cell walls of bacteria, viruses and various species of fungi (25, 26). **Dectin-1**, an immunoreceptor tyrosine-based activation motif (ITAM)-containing CLR is expressed on phagocytes and is a specific receptor for β-glucans which are sugar polymers found in the cell walls of fungi, including the yeasts *Saccharomyces cerevisiae* and *Candida albicans* (27-29). **Dectin-2** recognizes hyphal forms of fungi (30, 31). **DC-SIGN** has been implicated in the recognition of various viruses and other microbes. It is of particular importance because of its role in HIV recognition (32, 33).

RIG-I-like receptors (RLRs): RLR family of cytoplasmic receptors consists of retinoic-acid-inducible protein 1 (RIG-I), melanoma-differentiation-associated gene 5 (MDA-5)

and laboratory of genetics and physiology 2 (LGP2) (34, 35). RIG-I and MDA-5 recognize genomic RNA from dsRNA viruses as well as the dsRNA generated as the replication intermediate in ssRNA viruses and lead to type 1 IFN production and pro-inflammatory cytokines. Both RIG-I and MDA-5 have two caspase recruitment domains (CARDs) and a DEAD box helicase/ATPase domain (36). LGP2 lacks the CARD domain and was initially considered a negative regulator of RIG-I and MDA-5 pathways (37). Recently, however, it has been demonstrated that LGP2 acts upstream of RIG-I and MDA-5 and facilitates viral RNA recognition by these two proteins via its ATPase domain (38).

Despite a similar domain structure, RIG-I and MDA-5 detect distinctly different viral species. While RIG-I is implicated in the recognition of Paramyxoviruses [Newcastle disease virus (NDV), Sendai virus (SeV)], Orthomyxoviruses (Influenza), vesicular stomatitis virus (VSV), Japanese encephalitis virus (JEV) and Hepatitis C virus (HCV), MDA-5 is essential for recognition of picornaviruses such as encephalo-myocarditis virus (EMCV) and the synthetic dsRNA analog poly (I:C) (39, 40). Dengue virus and West Nile virus are recognized by both RIG-I and MDA-5. Short dsRNA with a 5’ triphosphate end is the preferred ligand for RIG-I, whereas MDA-5 detects long dsRNA (more than 2kb) (41-43). However, the signaling pathway downstream of both RIG-I and MDA-5 are similar. Upon recognition of dsRNA, they are recruited to the mitochondrial outer membrane via the adapter MAVS (also called IPS-1, CARDIF or VISA), ultimately leading to the activation of several transcription factors like IRF3, IRF7 and NF-κB (44).

NOD-like receptors (NLRs): NLRs are a family of cytosolic receptors containing more than 20 members (45). They are characterized by a tripartite domain structure comprising an N-terminal protein interaction domain, a central nucleotide binding oligomerization domain (NOD, also called NACHT domain) and a C-terminal region consisting of leucine-rich repeats (LRRs) (46). The N-terminal domain that mediates protein-protein interactions, also serves as the criteria for categorizing the NLR proteins (45). To date, at least 23 human and 34 mouse NLR genes have been identified (47). The function of most NLRs however remains as yet uncharacterized.

NOD1 and NOD2 were the first NLRs to be implicated in sensing of PAMPs having one and two CARD domains respectively at their N-terminus. Both NOD1 and NOD2 recognize the structures of peptidoglycan, a component of the bacterial cell wall (48). NOD1 recognizes g-D-gultamyl-mesodiaminopimelic acid (iE-DAP), while NOD2 binds muramyl dipeptide (MDP) (49, 50). They both induce NF-κB activation by engaging the adapter RIP2/RICK and lead to pro-inflammatory cytokine production.

Certain NLRs have a pyrin domain or a baculovirus inhibitor of apoptosis protein repeat (BIR) domain and are involved in the release of IL-1 family of cytokines including IL-1β, IL-18 and IL-33 (51). These proteins are components of large complexes called the “inflammasomes”, which regulate the activity of caspase-1. Caspase-1 processes the pro-form of these cytokines into mature bio-active mediators. Three NLR proteins NLR family PYD-containing 1 (NLRP1), NLRP3 and NLR family CARD-containing 4 (NLRC4) {also called ICE (caspase-1 converting enzyme) protease activating factor [IPAF]} have been identified to form inflammasomes (52). Inflammasome complexes

involve one or more NLR proteins, caspase-1 and the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC) (51).

Of all the inflammasomes, NLRP3 is the best characterized. A wide range of stimuli have been reported to activate the NLRP3 inflammasome. These include crystalline substances like uric acid crystals, silica, asbestos, β-amyloid fibrils and cholesterol crystals as well as bacteria, fungi and viruses (53-60). NLRC4 inflammasome is activated by the flagellin protein of bacteria (61).

Cytosolic DNA receptors: Although the recognition of CpG DNA motifs in the lysosomal compartment is attributed to TLR9, when dsDNA is released free into the cytoplasm, it promotes type I IFN responses through TLR9-independent pathways. These responses are chiefly mediated via the activation of TBK1 [TNFR-associated factor (TRAF) family member-associated nuclear factor κB (NF-κB) activator (TANK)-binding kinase-1], a kinase that phosphorylates and activates the transcription factor IRF3 (62).

DNA-dependent activator of IFN-regulatory factors (DAI), also known as ZBP1 or DLM-1 was the first DNA sensor implicated in the recognition of dsDNA in the cytoplasm (63). However, DAI deficient mice are still capable of producing IFN in response to cytoplasmic DNA, suggesting that DAI may play a redundant role in sensing cytosolic DNA (64). Two studies have shown that poly (dA.dT) can be transcribed into dsRNA by RNA polymerase III, which can then be detected by the RIG-I pathway (65, 66). However, DCs from RIG-I deficient mice have no defect in IFN production, signifying that cytosolic DNA detection can be cell type dependent (67). Type I IFN

production by both DAI and RIG-I converge on TBK1 and stimulator of IFN genes (STING), an endoplasmic reticulum (ER)-resident protein that functions as an adapter upstream of TBK1 (68, 69).

Recently, a member of the PYHIN protein family (pyrin and HIN200 domain–containing proteins), IFI16 and its closest mouse homolog p204 was reported to detect cytosolic viral DNA and induce type I IFN (70). Another report characterizes LRRFIP1 as a protein that signals via β-catenin and detects nucleic acids leading to activation of IFN-β by a coactivator pathway (71). Another PYHIN protein, AIM2 is involved in recognition of cytosolic DNA (72). AIM2 binds dsDNA through its HIN domain, promoting the assembly of an inflammasome with the adapter protein ASC, leading to caspase-1 activation (73, 74). AIM2 however has no role in the induction of IFN α/β.

Toll-like receptors (TLRs)

TLRs are the most well characterized and studied pattern recognition receptors. They are named for their similarity to Toll, a receptor first discovered in the fruit fly *Drosophila*. Toll was originally known for its role in embryonic development and establishing dorsal-ventral polarity of the embryo (75-77). In 1996, Lemaitre *et al.* demonstrated that the adult flies with the mutation in Toll were more susceptible to fungal infection and that the activation of Toll resulted in the production of anti-fungal peptides (78). The *Drosophila* Toll has a structure homologous to the interleukin-1 receptor (IL-1R) and it activates a transcription factor called Dorsal, which is a homolog of the mammalian NF-κB. Receptors and signaling pathways that are similar to the

Drosophila Toll have since been called Toll-like receptor (TLR) pathways. To date, thirteen TLRs have been identified in mice and ten in humans, each defined by its specificity for a particular PAMP. TLR1-TLR9 are conserved between humans and mice. TLR10 is non-functional in mice because of a retroviral insertion whereas TLR11, TLR12 and TLR13 have been lost from the human genomes.

Location of TLRs and their specificities for ligands

TLRs can be categorized into two subfamilies on the basis of their location in the cell. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface and respond mainly to bacterial surface associated PAMPs. TLR3, TLR7, TLR8 and TLR9 on the other hand, are expressed in the intracellular compartments like endosome, lysosome or endoplasmic reticulum (ER) and sense viral and bacterial nucleic acids (FIG I.1). The activation of these TLRs leads to type I IFN production in addition to pro-inflammatory cytokines.

A human homolog of Toll (hToll), now known as **TLR4,** was the first TLR to be discovered and was demonstrated to be the signal transducing component involved in responses to lipopolysaccharide (LPS) (79, 80). LPS is a component of the outer membrane of Gram-negative bacteria. C3H/HeJ mice are unable to mount an innate immune response against bacterial LPS. This defect was mapped to a point mutation (proline to histidine at residue 712) in the gene encoding for TLR4 (81, 82). In addition to being the receptor for LPS, TLR4 recognizes envelope proteins of some viruses, such as respiratory syncytial virus (RSV) and mouse mammary tumor virus (MMTV) (83-86). TLR4 is discussed in greater detail in later sections.

FIGURE I.1: Human Toll-like receptors and their ligands

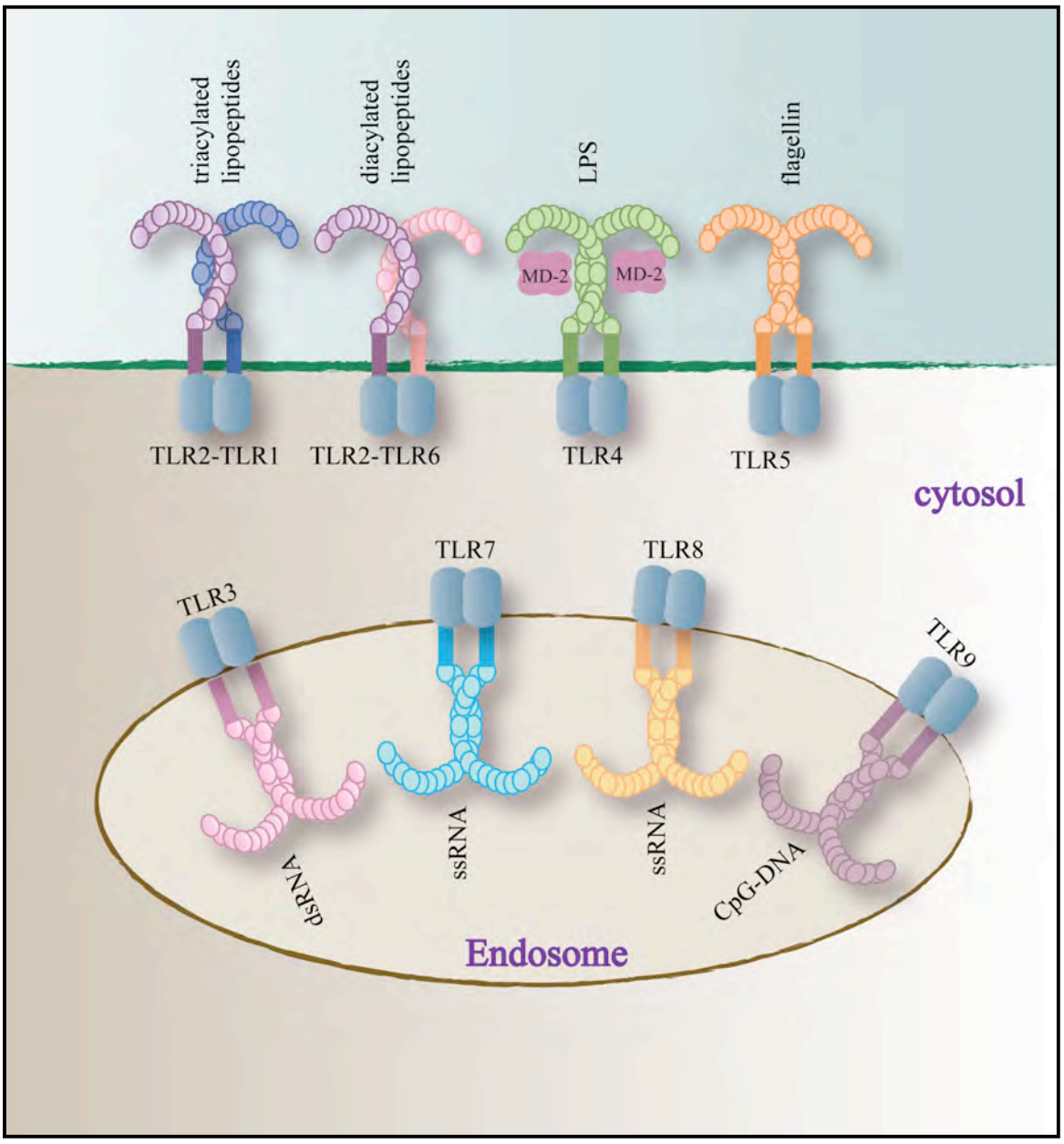


FIGURE I.1: Human Toll-like receptors at their respective locations in the cell. The best known ligands for each TLR are indicated.

TLR2 recognizes a variety of PAMPs from a range of bacteria, fungi, viruses and mycoplasma. TLR2 forms a heterodimer with either **TLR1** or **TLR6**, and does not signal effectively as a homodimer (87). The resulting TLR2/TLR1 and TLR2/TLR6 heterodimers recognize triacyl and diacyl lipoproteins respectively (88). Other ligands for TLR2 include peptidoglycan and lipoteichoic acid (45) from Gram-positive bacteria, porin from *Neisseria*, zymosan from fungi, Trypanosoma GPI-mucin and hemagglutinin protein from measles virus. TLR2 also collaborates with the CLR Dectin-1 to mount an inflammatory response against zymosan.

TLR5 recognizes flagellin, a protein found in bacterial flagella (89). It is expressed mostly on the surface of monocytes and epithelial cells (90). It has been implicated in protection against flagellated bacteria in the gut as well as in the urinary tract (91). Mouse **TLR11** is related to TLR5 and is expressed mostly in the kidney and bladder. TLR11 deficient mice are highly susceptible to infection by uropathogenic *E.coli* (92). TLR11 also recognizes a profilin like molecule from *Toxoplasma gondi* that is implicated in parasite motility and invasion (93, 94).

TLR3 recognizes polyinosinic-polycytidylic acid (poly I:C), a synthetic dsRNA analog (95). It is also involved in innate defenses against genomic RNA from dsRNA viruses, as well as dsRNA produced as a replication intermediate of ssRNA viruses such as RSV and West Nile virus (WNV) (96, 97). Mouse **TLR7** and human **TLR7/TLR8** recognize ssRNA from RNA viruses as well as imidazoqinoline derivatives like imiquimod (Human and mouse TLR7) and resiquimod (R848) (human, mouse TLR7 &

human TLR8) (98-101). TLR7 also detects RNA from bacteria such as Group B Streptococcus in endolysosomes of dendritic cells (DCs) (102).

TLR9 is the receptor for bacterial as well as viral DNA (103). It recognizes unmethylated 2' deoxyribo cytidine-phosphate-guanosine (CpG) DNA motifs, commonly present in the genomes of bacteria and viruses (104, 105). In vertebrates however, CpG motifs are present at a much lower frequency and the cytosine residues of the CpG motifs are heavily methylated, thus preventing immunostimulatory responses against host DNA (106, 107). TLR9 has also been shown to recognize malarial parasite DNA that is bound to hemozoin, a crystalline metabolite of hemoglobin, produced by the malaria parasite (108). Hemozoin acts as a carrier for DNA and transports it to the endosome, where TLR9 is present.

Structure of TLRs

All TLRs are type I transmembrane proteins (the N-terminus is outside the membrane) composed of three domains. The ectodomain is characterized by leucine-rich repeats (LRRs) and is responsible for recognizing the ligand. There is also a transmembrane domain followed by a C-terminal Toll/IL-1R (TIR) domain that is homologous to that of IL-1R. The TIR domain is the functional domain that is required for recruiting the downstream effectors and initiating the signaling pathway. The LRR domain consists of 19-25 tandem copies of the LRR motif, each 20-30 amino acids in length and comprises a β strand and an α helix, connected by a loop (109). This forms a horseshoe shaped structure that is involved in ligand binding (110).

TIR domain: The crystal structures of the TIR domains of TLR2 and TLR1 have been resolved, providing insight into the molecular aspects of the TIR domain (111). Both the structures reveal a central five-stranded parallel β-sheet surrounded by five α-helices. β-sheets and α-helices alternate with structured loops of varying length connecting them. The loop between the second β-sheet and the second α-helix is called the BB loop and is conserved in many TLRs. The BB loop is also the site for P681H mutation in TLR2, which has been shown to be homologous to the TLR4 P712H mutation, a single base change that ablates LPS signaling through TLR4 and renders C3H/HeJ mice resistant to endotoxin shock (81, 112). The conserved residues in the TIR domain are located mostly in the hydrophobic core of the structure. The start and end of the core TIR domain have a conserved (F/Y)DA and FW amino acid motif respectively with the alanine residue of the (F/Y)DA motif being the first amino acid. TIR domain may be considered as a cassette with the amino and carboxy terminals of the core domain located within 14 Å of each other. The loop regions of different TIR domains have large insertions or deletions. The size of the core TIR domain thus varies between 135 to 160 residues among the different proteins.

Ligand binding and TLR activation mechanism: insights from TLR-ligand crystal structures

Crystallographic structures of the extracellular domains of TLRs in complex with their ligands have been resolved for four different TLR-ligand complexes. Three of them employ agonistic ligands, while the fourth is a complex of the extracellular domain with an antagonist. These structures not only provide insights into the mode of recognition of

microbial patterns by TLRs; they suggest an activation mechanism that may be employed by TLR family members.

The crystal structure of the extracellular domains of TLR2 and TLR1 has been determined in association with a synthetic triacylated lipopeptide, Pam_3CSK_4 (113). The extracellular domains of TLRs comprise of a variable number of LRR motifs forming a typical horseshoe shaped structure. Both TLR2 and TLR1 extracellular domains exist as monomers in solution. However, addition of the agonist, Pam_3CSK_4 leads to the formation of a heterodimer with the lipid chains of the ligand bridging the TLRs. Two lipid chains of the lipopeptide are inserted into a pocket in TLR2, whereas the third chain inserts into a hydrophobic channel in TLR1. Additional hydrophobic interactions between TLR1 and TLR2 help to stabilize the heterodimer.

The crystal structure of two TLR3 ectodomains with the ligand, dsRNA, reveals that each RNA molecule binds to the ectodomain at two sites located at the opposite ends of the TLR3 horseshoe (95). The sugar-phosphate backbone of dsRNA interacts with the positively charged residues of TLR3, explaining why TLR3 lacks specificity for any particular sequence. In the TLR3 ectomdomain:dsRNA complex, the C-termini of the two monomeric TLR3 ectodomains are brought into close proximity, mediating protein-protein interactions. Thus, ligand-protein interaction is the main driving force behind TLR3 dimerization (95, 114).

TLR4 in association with MD-2 is responsible for the physiological response to LPS. Crystal structures of TLR4:MD-2 heterodimer in complex with LPS (agonist) as well as eritoran (antagonist) have been resolved (115, 116). The TLR4:MD-2:LPS

structure is a “m”-shaped multimer comprised of two TLR4:MD-2:LPS complexes or “units” (116). On the other hand, TLR4:MD-2:eritoran complex crystallized as a single unit (115). Both LPS as well as Eritoran bind to MD-2. The differential ability of LPS and Eritoran to dimerize two single units of TLR4:MD-2 is due to their different chemical structure. The lipid A moiety of LPS has two phosphorylated glucosamines and 6 acylated lipid chains. Eritoran however has only 4 lipid chains. LPS binds in a large hydrophobic pocket in MD-2. The two extra lipid chains in LPS displace the glucosamine backbone upwards, repositioning the phosphates which are now able to interact with positively charged residues in the second TLR4 molecule (of the second TLR4:MD-2:LPS unit) (116).

The structures of all TLR-ligand complexes determined to date reveal an “m”-shaped dimeric architecture. This suggests that dimerization of extracellular domains brings about TIR domain dimerization, thus initiating signaling (117). According to these structures, the C-termini of the extracellular domains converge to bring about close juxtaposition of the TIR domains, creating a scaffold for the intracellular adapter proteins (117). In the case of TLR9, it has been demonstrated that in a resting state, TLR9 exists as inactive dimers (118). However, binding to stimulatory DNA induces conformational changes that lead to close apposition of the TIR domains, enabling the recruitment of the downstream adapter MyD88. Thus, irrespective of whether the TLRs exist as preformed dimers or not, ligand binding induces changes in the conformation of the TIR domain, creating a scaffold for initiation of signaling.

Adapters in TLR signaling

Binding of various PAMPs to TLRs leads to activation of distinct genes, depending on the particular TLR and the cell type involved. This specificity of signaling can be attributed mostly to the differential usage of TIR domain containing adapter proteins. There are five TIR domain containing adapters: myeloid differentiation protein 88 (MyD88), MyD88 adapter-like (Mal) or TIR domain-containing adapter protein (TIRAP), TIR domain containing adapter inducing IFN-β (TRIF; also known as TICAM-1), TRIF-related adapter molecule (TRAM) and Sterile-alpha and Armadillo motif-containing protein (SARM) (FIG I.2).

MyD88 (myeloid differentiation protein 88): MyD88 was discovered in 1990 as a protein that was induced during terminal differentiation of $M1D^{+}$ myeloid precursors in response to stimulation by IL-6. “88” refers to the gene number in the list of induced genes (119). Later, MyD88 was shown to have a role in IL-1R1 signaling as well as TLR signaling (120-122). In 1997, S. Akira’s group generated MyD88 deficient mice and they were found to be largely defective in their responses to TLR2, TLR4, TLR5, TLR7 and TLR9 signaling (123). Moreover, these mice are resistant to the toxic effects of LPS and are severely compromised in their ability to clear infections by various pathogens ranging from bacterial species like *Staphylococcus aureus* and *Listeria monocytogenes* to the protozoan parasite *Leishmania major* (124, 125). Macrophages isolated from these mice have impaired cytokine production (like TNF-α and IL-6) in response to LPS. However, NF-κB and MAPK activation is not abrogated, though it occurs with delayed kinetics.

FIGURE I.2: TIR domain containing adapters

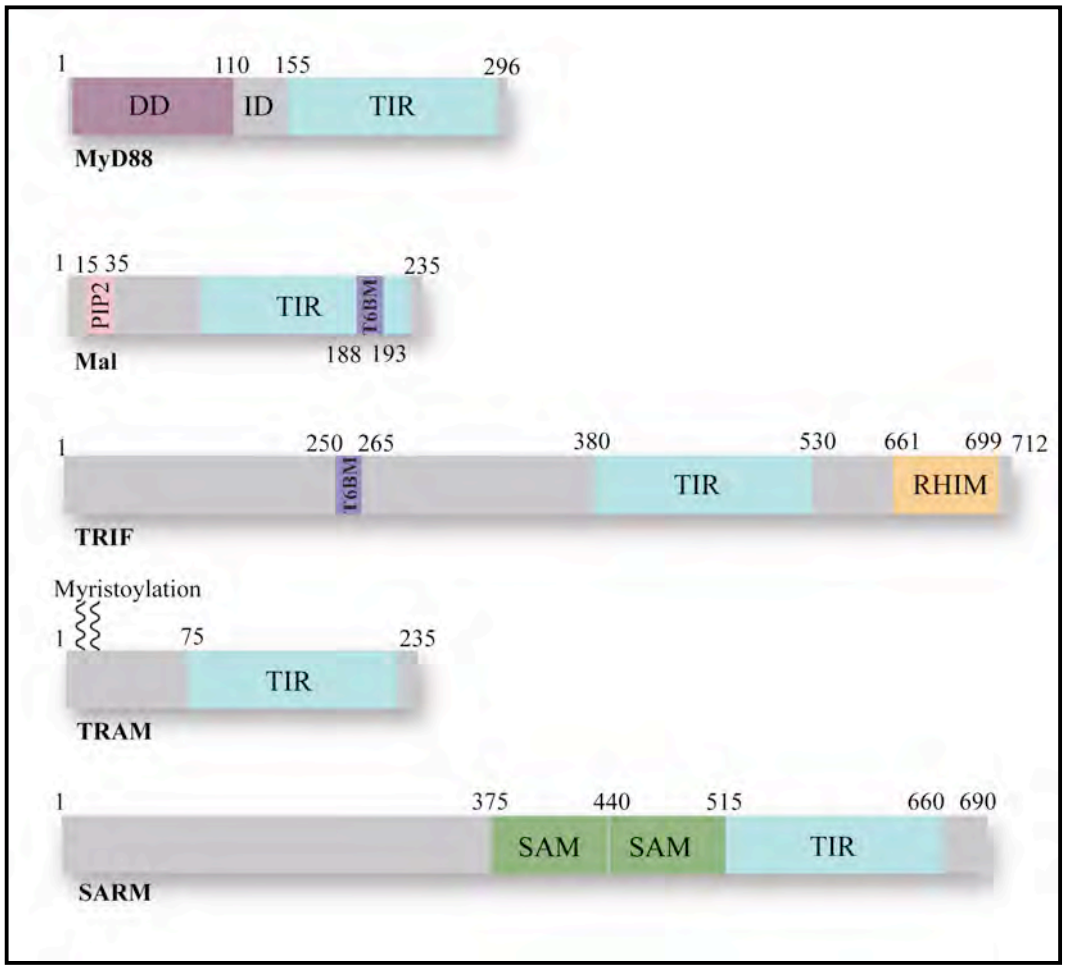


FIGURE I.2: A schematic depiction of the domain structure of the TIR domain containing adapters in TLR signaling. MyD88 has a death domain, an intermediary domain and a TIR domain. Mal/TIRAP has a phosphatidylinositol-4,5-bisphosphate (PIP2) binding domain at the N-terminus and a TIR domain at the C-terminus. TRIF has a RHIM domain and a TRAF6-binding domain (T6BM) in addition to a TIR domain. TRAM is myristoylated at its amino terminus. **Although SARM has a TIR domain, it is not an adapter protein. It also has two sterile α-motif domains**.

It was later discovered that MyD88-independent pathway mediated by the adapters TRIF and TRAM are responsible for this delayed response.

MyD88 is considered to be a universal adapter required for signaling of all TLRs (except TLR3) as well as IL-1R and IL-18R. TLR4 is unique as both a MyD88-dependent and a MyD88-independent pathway are initiated upon recognizing LPS. MyD88 is a 296 amino acid protein that has a death domain at its N-terminus and a TIR domain at its C-terminus. The death domain (in conjugation with the intermediate region) of MyD88 is important for recruiting the downstream kinases IRAK-1 and IRAK-4, while the TIR domain is responsible for TIR-TIR interactions with TLRs as well as IL1R and IL-18R (126). In the case of TLR2 and TLR4 signaling however, the bridging adapter Mal is needed for signaling. MyD88 is mostly involved in NF-κB activation and pro-inflammatory cytokine production. However, it also leads to type I IFN production upon activation of TLR7, TLR8 and TLR9. It interacts with IRF7 and IRF5 and is necessary for their activation (127, 128).

Recently, the crystal structure of a complex consisting of the death domains of MyD88, IRAK4 and IRAK2 has been resolved (129). These three proteins are present in a ternary complex consisting of 6 MyD88, 4 IRAK4 and 4 IRAK2 molecules. These oligomeric structures have been named “Myddosomes” by the authors (129). According to Lin *et al.*, the assembly of the Myddosome is a sequential process. While the MyD88 death domain by itself is able to form oligomers at high concentration, IRAK4 and IRAK2 death domains are monomeric in solution. However, in the presence of MyD88, IRAK4 is oligomerized and forms a complex with oligomeric MyD88. This then acts as a

platform for recruitment of IRAK2 (or IRAK1). The authors believe that this hierarchical assembly is essential for bringing the IRAKs into a proper conformation for their phosphorylation and activation

A splice variant of MyD88 has been described that acts as a negative regulator of MyD88-dependent signaling (130). It lacks the intermediate region and thus fails to recruit IRAK-4, thereby inhibiting NF-κB activation. In addition, a member of the IL-1R family, ST2, can sequester MyD88 and interfere with TLR4 signaling (131). IRAK-M is another death domain containing protein that acts as a negative regulator of MyD88 signaling by preventing the dissociation of IRAK1 and IRAK4 from MyD88 (132). MyD88 thus acts as an important point of control for signaling by various receptors.

Mal (MyD88 adapter-like) [also called TIR domain-containing adapter protein (TIRAP)]: Mal was the second TIR domain-containing adapter to be discovered. Two groups simultaneously identified Mal in database searches for TIR domain containing proteins (133, 134). The gene *TIRAP* is mapped to Chromosome 11. Alternative splicing of Mal generates two predominant isoforms of Mal in humans. Variant 2 has an additional segment within the 5' UTR and differs in the 3' coding region and 3' UTR, compared to variant 3. This results in a longer isoform (b) with a distinct C-terminus, compared to isoform a. Variant 3 represents the longer transcript but encodes the shorter isoform (a). Mal was discovered based on its importance for TLR4 signaling but not IL-1 signaling. Reports by Fitzgerald *et al.* and Horng *et al.* demonstrated that a proline to histidine mutation in the TIR domain of Mal results in loss

of TLR4 signaling, but does not affect IL-1 signaling (133, 134). Additionally, Mal-deficient mice were found to be defective in TLR4 and TLR2 signaling, but TLR9 and IL-1 signaling were normal (135, 136). This led to the conclusion that Mal is required for TLR2 and TLR4 signaling only. Mal deficient mice exhibit complete impairment of TLR2 mediated responses. In the case of TLR4 stimulation, these mice have a similar phenotype to MyD88-deficient mice/cells displaying delayed NF-κB and MAPK activation, thus placing Mal in the MyD88 pathway. Disease models in Mal-deficient mice reveal a role for Mal in early immune responses to *E.coli* and LPS in the lung (137). Mal is also important for the induction of anti-microbial peptides in the lung, in response to *Klebsiella pneumoniae* as well as LPS-induced airway hyper-reactivity (138).

Mal is a 235 amino acid protein with a TIR domain at its C-terminus. It lacks the death domain present in MyD88. It interacts with the receptors TLR2 and TLR4 as well as the adapter MyD88 via TIR-TIR interactions and is also capable of homodimerization with itself (133). Mal has a phosphatidylinositol-4, 5-bisphosphate (PIP2) binding domain at its N-terminus, through which it is recruited to the cell membrane (139). MyD88 is unable to bind TLR4 or TLR2 and thus Mal brings MyD88 to the receptor complex, thus acting as a bridge between the receptor and the downstream adapter MyD88. It has been suggested that the sole function of Mal is to recruit MyD88. Recently, however new properties of Mal have been revealed, with functions other than merely the recruitment of MyD88. Mal has a TRAF6 binding domain, via which it may help to recruit TRAF6 to the signaling complex (140, 141). It undergoes tyrosine phosphorylation by Bruton's tyrosine kinase (Btk), which leads to increased activation of

p65 subunit of NF-κB (142). Moreover, at higher concentrations of TLR2 ligands, PAM_2CSK_4 or PAM_3CSK_4, Mal has been shown to be dispensible for TLR2 signaling (143). This might be due to the ability of TLR1 and TLR6 to recruit MyD88 independently of Mal or due to a conducive surface charge potential of the TIR domain of TLR2 to allow for direct interactions with MyD88 TIR domain. Though Mal has no effect on pro-inflammatory cytokine production by TLR3, it has been shown to negatively influence TLR3 mediated IFN-β induction, via its ability to interact with IRF7 (144). It also interacts with caspase-1 and cleavage by caspase-1 is an important event for Mal to signal (145, 146).

TRIF (TIR domain-containing adapter inducing IFN): TRIF is the sole adapter involved in TLR3 signaling. It also mediates the MyD88-independent arm of TLR4 signaling (along with TRAM), leading to type I IFN induction. TRIF was identified independently by two groups, on the basis of its role in TLR3 signaling and in a database search for TIR domain containing proteins (147, 148). Like Mal and MyD88, overexpression of TRIF results in NF-KB activation; however only overexpressed TRIF, but neither MyD88 nor Mal, leads to strong induction of IRF3-dependent reporters such as the IFN-β gene reporter (149). The role of TRIF in TLR signaling was further confirmed by studies in TRIF-deficient mice. These mice were impaired in TLR3 and TLR4 mediated IFN-β production; while pro-inflammatory cytokine production was partially impaired in TLR4 but not TLR2, TLR7 or TLR9 (149). Further evidence that TRIF is involved in the MyD88-independent arm of TLR4 signaling came from studies in

mice deficient for both MyD88 and TRIF. Cells from these mice exhibit complete abrogation of LPS induced NF-κB activation (150). Moreover, random germline mutagenesis led to the identification of the mutation Lps2 (151). The mice carrying this mutation were unresponsive to poly I:C and response to LPS was impaired (152, 153). Mice homozygous for this mutation were resistant to LPS induced shock and were more susceptible to cytomegalovirus (CMV) infection (153). The molecular basis for this mutation was a frameshift in the gene *Trif*. TRIF was thus established as the adapter controlling TLR3 as well as MyD88-independent TLR4 signaling.

TRIF is the largest of all the TIR adapter proteins. In addition to a central TIR domain, it has a receptor-interacting protein (RIP) homotypic interaction motif (RHIM) domain at the C-terminus. Through its RHIM domain, TRIF associates with other RHIM containing proteins RIP1 and RIP3 and activates Fas-associated death domain protein (FADD)/caspase-8 axis (154). TRIF is the only TLR adapter that can induce apoptosis (155). Through its TIR domain TRIF interacts with TLR3 and with TRAM in the case of TLR4 signaling. The N-terminal domain of TRIF is responsible for its interaction with TBK1, the kinase involved in IRF3 activation (156). TRIF is also important for LPS induced upregulation of co-stimulatory molecules as well as MHC class II on dendritic cells (157).

TRAM: TRAM was the fourth TLR adapter to be discovered. Like Mal and TRIF, a search for TIR domain containing proteins led to the identification of TRAM (158-160). TRAM shares sequence similarity with other TIR domain containing proteins, especially

with TRIF. It is a 235 amino acid protein with a TIR domain at its C-terminus. TRAM is the most restricted of all adapters, involved only in the MyD88-independent axis of TLR4 signaling. TLR4 thus involves all four adapter proteins, with MyD88/Mal signaling being initiated first, followed by a delayed TRIF/TRAM response. TRAM acts as a bridging adapter between TLR4 and TRIF, playing a role similar to Mal in this regard (160).

A bipartite localization motif has been identified in TRAM. The N-terminus of TRAM has a myristoylation motif, which is required for its recruitment to the membrane (161). Mutations of the myristoylation motif, lead to mis-localization of TRAM and loss of its signaling ability. The myristoylation motif is followed by a stretch of polybasic residues. Other proteins that harbor this bipartite motif are known to shuttle between the plasma membrane and endosomes. Indeed, it was demonstrated that TRAM exists both at the plasma membrane and in the endosomal compartment (162). Currently, it is thought that TLR4 first induces MyD88/Mal signaling at the membrane and is then endocytosed and activates TRIF/TRAM signaling from endosomes (162).

SARM: SARM is a TIR domain-containing protein that also contains sterile α- (56) and HEAT/armadillo motifs (ARM) (163). However, it is not considered an adapter protein in the same sense as the other four adapters. The first studies on SARM revealed that in contrast to the other four TLR adapters, SARM was unable to induce NF-κB activation. It is now considered a negative regulator of NF-κB and IRF activation (164). It specifically inhibits TRIF-mediated gene induction, but has no effect on MyD88-dependent pathway or non-TLR signaling (164). LPS stimulation leads to an increase in SARM protein

levels, suggesting a negative feedback of the TRIF mediated pathway. Both the TIR and the SAM domains are necessary for this effect. SARM interacts directly with TRIF and this may be a mechanism by which SARM exerts its inhibitory effects. The interaction between TRIF and SARM may prevent the recruitment of the downstream signaling components like TBK1 and TRAF6. Another mechanism of inhibition might be through the recruitment of an inhibitory protein via its SAM motifs.

Interleukin-1 receptor-associated kinases (IRAKs)

Interleukin-1 receptor-associated kinases (IRAKs) are a family of intracellular kinases with an important role in the innate immune pathways (165). There are four IRAK members: IRAK1, IRAK2, IRAK-M and IRAK4. Human IRAK1, IRAK2 and IRAK4 are ubiquitously expressed, whereas IRAK-M is expressed in an inducible manner in monocytes and macrophages only. All IRAKs have a similar domain structure. At their N-terminus is a death domain (DD), followed by a proST region that is rich in prolines, serines and threonines, a central conserved kinase domain and a C-terminal domain that is absent in IRAK4. The death domain is responsible for interactions with other death domain containing proteins like MyD88 (166). The central kinase domain contains an activation loop that is essential for kinase activity. The C-terminal domain is responsible for interaction with TRAF6; IRAK1, IRAK2 and IRAK-M have three, two and one TRAF6 interaction motifs respectively (167).

IRAK1: IRAK1, the first IRAK family member to be discovered is a 712 amino acid protein, initially shown to have a role in IL-1 signaling (165, 168). It undergoes phosphorylation on critical threonine residues in the kinase domain as well as hyperphosphorylation in the proST region (169). Phosphorylation and activation of IRAK1 are followed by K-48 linked ubiquitination, leading to the degradation of IRAK1 (170). Cells from IRAK1 deficient mice exhibit reduced but not completely ablated TLR/IL-1R dependent NF-κB, MAPK and cytokine induction (171, 172). The kinase activity of IRAK1 is not essential for NF-κB activation but is implicated in TLR7 and TLR9 induced IFN-α production (173). It has also been shown to interact with TRAF3, a vital player in TLR induced type I IFN production (174).

IRAK2: The second IRAK to be identified was IRAK2, a 590 amino acid protein that shares sequence similarity to IRAK1 (126, 175). The role of IRAK2 in TLR signaling was examined using macrophages from IRAK2 deficient mice (176). These studies demonstrated that IRAK2 functions redundantly with IRAK1 in signaling and only when IRAK2 deficient mice were crossed with IRAK1 deficient mice, a dramatic impairment in TLR signaling could be observed (177).

IRAK-M: The third family member to be identified was IRAK-M, a 596 amino acid protein with unique features (178, 179). It is the only member of the IRAK family to lack kinase activity and has been reported to function as a negative regulator (132, 180). Macrophages from IRAK-M deficient mice, exhibited increased TLR/IL-1R response, suggesting an inhibitory role for IRAK-M (180).

IRAK4: IRAK4 is the most recently discovered member of the IRAK family (181-183). It is the closest homolog of the *Drosophila* Pelle protein, the only known IRAK in the fly. Unlike IRAK-1, mice deficient in IRAK4 are unable to mount a cytokine response to various TLR ligands (184). In addition, they are completely resistant to LPS-induced septic shock (184). IRAK4 interacts with MyD88 via its death domain and this interaction is necessary for NF-κB activation (130). It is also responsible for phosphorylating IRAK1, which leads to the autophosphorylation and activation of IRAK1 (185). IRAK4 has also been shown to have an important role in type I IFN production by TLR7/8/9 (186). However, it is dispensable for MyD88-independent or TRIF mediated TLR responses.

TLR4 signaling pathway

Amongst all the TLRs, TLR4 is unique in utilizing all four TIR domain-containing adapters. TLR4 recognizes lipopolysaccharide or LPS from the cell walls of bacteria.

Lipopolysaccharide (LPS): LPS is a major component of the outer membrane of Gram-negative bacteria and plays an important role in maintaining the structural and functional integrity of the bacterial membrane. LPS has three components: lipid-A, core-oligosaccharide and O-antigen (or O-polysachharide) (187). The highly hydrophobic lipid-A moiety is chiefly responsible for the endotoxic activity of LPS. Core oligosaccharide is a short chain of sugar residues that attaches directly to lipid-A and is highly variable among bacterial species. O-antigen is a highly variable region composed

of saccharidic units repeated a number of times (188). It is attached to core-oligosaccharide and is the outermost domain of LPS molecule. Since O-polysaccharide can also be targeted by antibodies, it is also known as O-antigen. Depending on the presence or absence of O-polysaccharide, LPS can be classified as “smooth” or “rough” LPS respectively.

Initiation of signaling: Even though TLR4 is the signal transducing component of the LPS recognition complex, a 25 kDa co-receptor known as Myeloid Differentiation-2 (MD-2), is also absolutely required for LPS signalling. Two additional extracellular accessory proteins are needed for full responses to LPS. They are a 60 kDa serum LPS-binding protein (LBP) and a 55 kDa LRR protein designated as CD14. The chief function of LBP is to monomerize LPS from bacterial membranes and make it readily available to CD14 (189, 190). CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein that accepts LPS monomers from LBP and transfers them to TLR4:MD-2 complex, inducing its dimerization (191, 192). TLR4 does not directly bind LPS. Instead MD-2, which exists in a complex with TLR4 is responsible for binding the lipid-A moiety of LPS (193). Ligand binding induces conformational changes to bring about dimerization of the TLR4:MD-2 complex.

MyD88-dependent and independent signaling pathways: Upon binding LPS, two pathways are initiated: MyD88-dependent and MyD88-independent or the TRIF-dependent pathway (FIG I.3).

MyD88-dependent pathway

LPS binding to the MD-2-TLR4 complex initiates a signaling cascade. Upon activation, TLR4 is thought to relocate and cluster to lipid rafts (194). TLR4 dimerization induces conformational changes in its TIR domain creating sites for the binding of Mal/TIRAP, which is normally localized to the PIP2 rich regions of the membrane. Mal, then engages MyD88 through TIR-TIR interactions. In light of the recent finding describing the Myddososme, MyD88:IRAK4:IRAK1 complex might be preformed in the cytosol and recruited to the receptor complex upon activation. Conversely, MyD88 might bind to Mal first and then assemble the Myddosome at the receptor complex. Once MyD88, IRAK4 and IRAK2 are at the receptor complex, IRAKI undergoes phosphorylation by IRAK4 on threonine residues. This activates autophosphorylation of IRAK1 that leads to its activation and subsequent dissociation from the complex. IRAK1 then interacts with TNF receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase that catalyses the addition of Lys63 (K63) linked chains on target proteins, including TRAF6 itself and IRAK1. The TRAF6-IRAK1 complex then interacts with a preformed TAK1 (transforming growth factor β-activated-kinase 1)-TAB1 (TAK1-binding protein)-TAB2 complex. The polyubiquitin chains bind to TAB2 and TAB3 and activate the kinase TAK1 (195). The K63 chains also bind to NEMO, a regulatory component of the IKK complex, thus bringing the IKK complex in proximity to TAK1 (196). Phosphorylation and activation of IKK (IκB kinase) proteins (possibly by TAK1), is followed by phosphorylation of inhibitor of κB (IκB). IκB then undergoes K48-linked polyubiquitination and finally degradation, allowing NF-κB to translocate to the nucleus

and induce gene transcription.

TRAM-TRIF dependent pathway

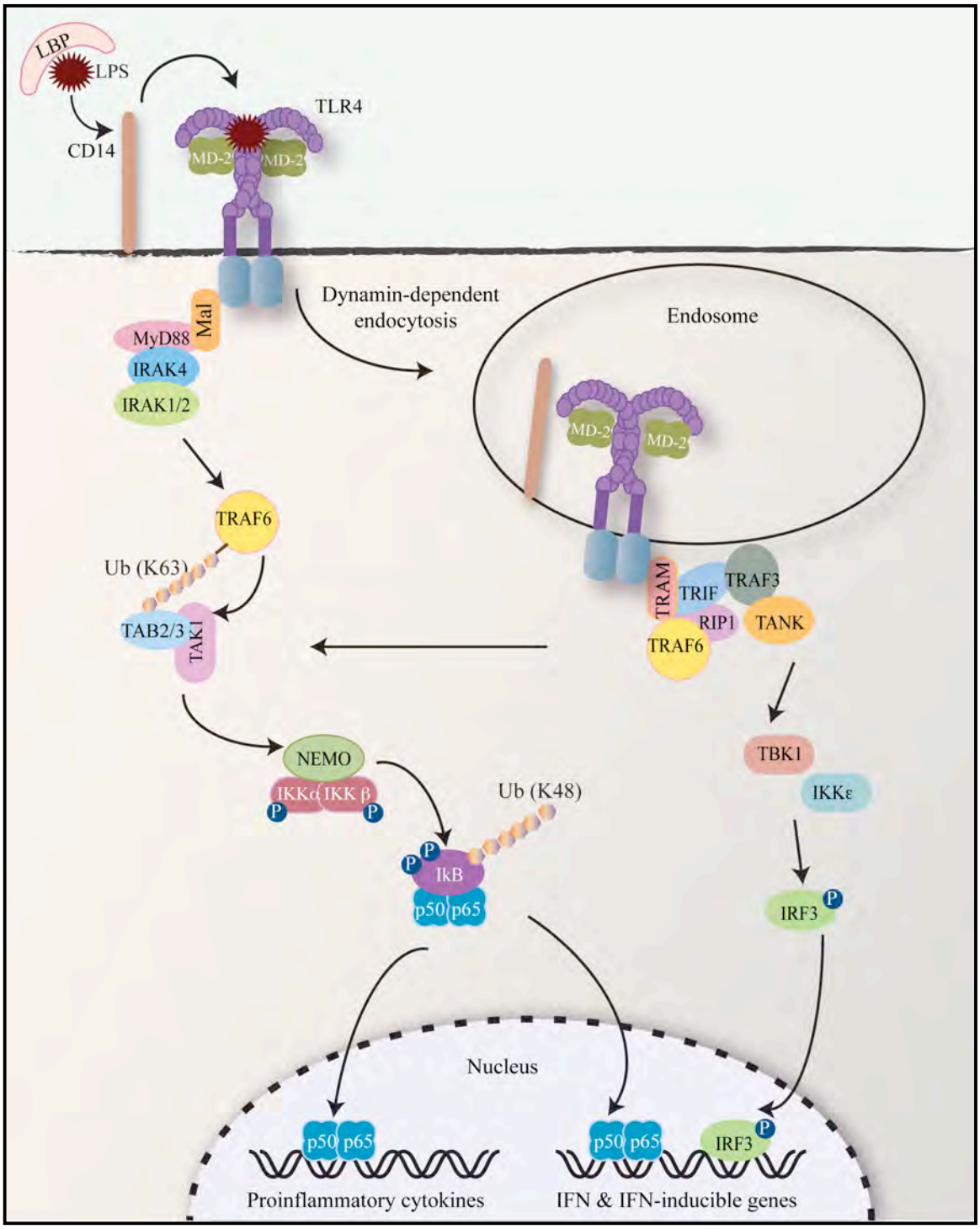
The MyD88-independent or the TRIF mediated pathway downstream of TLR4 is responsible for the delayed NF-κB activation as well as type I IFN production via IRF3 activation. MyD88 dependent and independent pathways are not triggered simultaneously upon LPS activation, but rather in a sequential manner. Upon binding of LPS to the TLR4-MD-2 complex, the Mal-MyD88 pathway is first initiated leading to pro-inflammatory cytokine production described above. The receptor complex is then endocytosed and delivered to the endolysosomes where TRAM and TRIF are recruited and signaling is initiated (158). TRIF then interacts with RIP1 via its RHIM domain. RIP1 then binds E3 ubiquitin ligase TRAF6 that catalyzes K63-linked polyubiquitination of RIP1. K63 chains of RIP1 help to recruit NEMO to the complex as well as TAK1. TAK1 activates the IKK complex leading to NF-κB and MAPK activation resulting in pro-inflammatory cytokine induction. On the other hand, IFN production by TRIF depends largely on its interaction with TRAF3 and subsequent K63-linked ubiquitination of TRAF3. This helps in recruiting TANK (TRAF family associated NF-κB activator) (197). This then allows the IKK related kinases TBK1 and IKKε to phosphorylate IRF3 and induce the type I IFN response (198).

The discovery of sequential initiation of the Mal-MyD88 and TRAM-TRIF signal transduction pathways presents a new model for initiation of TLR4 signaling. Initial LPS recognition by TLR4 leads to recruitment of the receptor to the PIP2-rich lipid rafts where the TIR domain of TLR4 engages the TIR domain of Mal, ultimately leading to the

early wave of NF-κB activation. LPS stimulation is shortly followed by dynamin-dependent TLR4 and CD14 endocytosis. The endocytosis of the receptor complex leads to dissociation of Mal from the TIR domain of TLR4, leaving the TIR domain of TLR4 available for interaction with TRAM to cause type I IFN induction. This two step activation pathway enables the categorization of the TRIF mediated TLR4 signaling with the other known endosomal TLRs that lead to IFN production. The functional separation of TLRs into pro-inflammatory cytokine or IFN inducers thus correlates with the spatial separation of cell surface or endosomal TLRs respectively.

FIGURE I.3: TLR4 signaling pathway: LPS binding to the TLR4-MD2 complex induces two pathways. MyD88-dependent pathway is triggered first, leading to NF-kB activation and pro-inflammatory cytokine production. The internalized receptor complex induces the TRIF-TRAM dependent pathway from the endosomes leading to TRAF6 mediated NF-κB activation as well as TBK1 and IKKε dependent IRF3 activation.

FIGURE I.3 TLR4 signaling pathway



Single Nucleotide Polymorphisms (SNPs)

Two randomly selected individuals share 99.9% similarity in their DNA sequence (199). The remaining 0.1% sequence that is variable or polymorphic between individuals arises because of mutation. The difference between a mutation and a polymorphism lies in its frequency in the population. When a mutation is prevalent in the population at a frequency higher than 1 %, it is called a polymorphism (200). The simplest and most basic of all polymorphisms is a single base change in the DNA called single nucleotide polymorphisms or SNP. SNPs are the most ubiquitous and the most frequently occurring of all polymorphisms. It is estimated that they occur at a frequency of 1 in about 1,000 base pairs. SNPs can be found in exons, introns, promoter regions, intergenic regions, etc. A SNP within the coding region may or may not change the corresponding amino acid due to the degeneracy of the codon usage. When a SNP does not change the amino acid of the protein, it is called a synonymous SNP, in contrast to a non-synonymous SNP that leads to a change in the amino acid sequence. Instead of a base change, SNPs can sometimes insert or delete a base, thus leading to a frame shift in the protein sequence. Moreover, a non-synonymous SNP may generate a STOP codon, causing premature termination and truncated or inactive proteins. Such polymorphisms are called nonsense SNPs.

SNPs in the non-coding regions usually have no effect on the phenotype. Sometimes however, SNPs in the introns may be present in close proximity to the splice junctions, affecting appropriate splicing of the encoded protein (201). Also, base changes in the promoter region may affect the expression of the gene and those in the untranslated

regions of the gene may influence mRNA stability (202-204). On the other hand, not all non-synonymous SNPs lead to appreciable protein changes. An amino acid may be replaced by another amino acid with very similar structure and chemical properties, in which case, the protein may still function normally; this is termed a neutral, "quiet", or conservative mutation. Alternatively, the amino acid substitution could occur in a region of the protein that does not significantly affect the protein secondary structure or function.

Genetic variation affects predisposition to disease

Variation in the DNA sequence can affect how an individual develops disease, responds to pathogens or other environmental factors (205, 206). Perhaps the most well characterized example of a SNP leading to a profound change in the phenotype of an organism is sickle cell anemia. A base change from A to T encodes a Valine instead of a Glutamate residue in the β-globin gene . This single residue change affects the shape of the β subunit of hemoglobin making the red blood cells carrying the aberrant hemoglobin sickle shape in appearance instead of the characteristic disc shaped.

TLR signaling leads to the induction of a large number of genes through multiple, finely coordinated steps in order to appropriately respond to the pathogen. Preserving structural integrity of the TLR signaling components is thus essential to maintain immunologic protection. Any changes in the structure of the many signaling molecules involved in the pathway, could affect the final outcome of the cascade. Changes in the structure can sometimes be due to SNPs in the coding region.

Many instances of molecular changes in the TLR signaling pathway have been described, often with dramatic outcomes. The two most well-studied SNPs in TLR signaling are Asp299Gly and Thr399Ile in TLR4 (207). These two co-segregating polymorphisms in the extracellular domain of TLR4 are present at a frequency of 10% in Caucasians and have been associated with hyporesponsiveness to LPS (207-209). Since they are present in the extracellular domain of TLR4, they are predicted to affect the binding of the ligand. Asp299Gly has been demonstrated to have a greater functional impact than Thr399Ile (207). These two SNPs have been associated with severe respiratory syncytial virus (RSV) bronchiolitis in infants (210). Another study also saw a correlation between the Thr399Ile SNP and increased colonization of pregnant women with *Gardnerella sp.* and other Gram-negative commensals (211). On the other hand, two studies failed to detect a significant correlation between these two SNPs and the incidence of sepsis after surgery as well as severity of severe inflammatory response syndrome (212, 213).

Other studies have highlighted the impact of SNPs on protein function and the predisposition to disease. A common STOP codon polymorphism in the signaling domain of TLR5 completely ablates signaling through TLR5 and increases the susceptibility of the individuals carrying this polymorphism to Legionnaire's disease (214). The same polymorphism has been associated with increased resistance to systemic lupus erythematosus (SLE) (215). More than one mutation in the IRAK-4 gene has been reported to lead to a truncated form of the gene and individuals harboring these SNPs are inflicted with recurrent pyogenic bacterial infections (216, 217). Thus, variation in innate

immune genes may not only affect the function of the protein, but might have a bearing on the outcome of various diseases too.

Thesis Objective and Summary

Toll-like receptors (TLRs) are the key sensors of microbial infection in mammals. At the core of TLR signaling are the TIR domain-containing adapters: signaling proteins that not only provide specificity to the TLR pathways, but are imperative for the signaling to ensue. The structural integrity of the TLR signaling components, especially the TIR adapters, is thus essential to maintain immunologic protection. The work done in this thesis addresses the molecular perturbations that can be brought about by single nucleotide polymorphisms (SNPs) in the coding region of adapter proteins. The main focus is on the adapters Mal and MyD88, which are the most polymorphic amongst all the adapter proteins and thus are good candidates for this study. I test the hypothesis that coding SNPs in the adapters Mal and MyD88 can change the signaling capability of these proteins using diverse biochemical approaches and attempt to provide a structure-function relationship for the loss-of-function phenotype observed in each case. In addition to providing unique insights into the structure of the adapter proteins Mal and MyD88, the work presented here characterizes two naturally occurring loss-of-function mutations that, though rare in the population, might still affect predisposition to various life-threatening diseases in some individuals. Moreover, by delineating the amino acids absolutely essential for signaling, this thesis brings to light potential novel drug targets for the treatment of a number of inflammatory and autoimmune diseases.

Chapter II: A TIR domain variant of Mal/TIRAP results in loss of MyD88 binding and reduced TLR2/TLR4 signaling. This chapter focuses on D96N, a TIR domain polymorphism in the adapter Mal that is completely inactive in TLR2 and TLR4

signaling. The reason for this defect is the inability of Mal D96N to bind the downstream adapter MyD88, an event that is essential for signaling to occur.

Chapter III: A natural loss-of-function mutation of MyD88 disrupts its ability to form Myddosomes: This chapter describes a death domain mutant of MyD88, S34Y, which is severely comprised in its ability to respond to all MyD88 dependent ligands. MyD88 S34Y has a noticeably different localization pattern than wild type MyD88 and is unable to form signaling complexes called Myddosomes with IRAK4 and IRAK2.

PREFACE TO CHAPTER II

This chapter of the thesis has been published in Journal of Biological Chemistry:

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FIG II.9 was contributed by J. Wong and N.J. Gay

TABLE II.1 was contributed by T.S. Plantinga and M.G. Netea

CHAPTER II

A TIR DOMAIN VARIANT OF MAL/TIRAP RESULTS IN LOSS OF MYD88 BINDING AND REDUCED TLR2/TLR4 SIGNALING

Abstract

The adapter protein MyD88 adapter-like (Mal), encoded by TIR-domain containing adapter protein (*Tirap)* (MIM 606252), is the most polymorphic of the five adapter proteins involved in Toll-like receptor (TLR) signaling, harboring eight non-synonymous single nucleotide polymorphisms (SNPs) in its coding region. We screened reported mutations of Mal for activity in reporter assays in order to test the hypothesis that variants of Mal existed with altered signaling potential. A TIR domain variant, Mal D96N (rs8177400), was found to be inactive. In reconstituted cell lines, Mal D96N acted as a hypomorphic mutation, with impaired cytokine production and NF-kB activation upon LPS or PAM_2CSK_4 stimulation. Moreover, co-immunoprecipitation studies revealed that Mal D96N is unable to interact with MyD88, a prerequisite for downstream signaling to occur. Computer modeling data suggested that residue 96 resides in the MyD88 binding site, further supporting these findings. Genotyping of Mal D96N in three different cohorts suggested that it is a rare mutation. We thus describe a rare variant in Mal that exerts its effect via its inability to bind MyD88.

Introduction

The adapter protein Mal/TIRAP (hereafter referred to as Mal) is involved in the MyD88-dependent pathway downstream of TLR2 and TLR4 (133, 134). It acts as a bridging adapter between the receptor and the sorting adapter MyD88 (139). The receptors TLR2 and TLR4 as well as MyD88, all have a predominantly electropositive surface, preventing MyD88 to bind to the receptor (218). Mal, on the other hand has a net electronegative surface charge, thus being able to interact with the receptor (TLR2 or TLR4) and recruiting MyD88 to the complex. Upon activation of TLR2 or TLR4, a signaling cascade is initiated, which leads to the activation of transcription factors such as Nuclear Factor-kappa B (NF-κB), Interferon Regulatory Factor (IRF)-5 and Activator Protein (AP-1), ultimately culminating in the production of pro-inflammatory cytokines (219). However, an uncontrolled inflammatory process can be detrimental to the host and thus in mammals a number of mechanisms have evolved to attenuate and regulate the TLR pathways. TIR domain containing adapters are also subjected to such regulation. During TLR2 and TLR4 signaling, Bruton's tyrosine kinase (Btk) phosphorylates Mal at three residues (142). Phosphorylated Mal can then interact with suppressor of cytokine signaling-1 (SOCS-1), resulting in Mal polyubiquitination and subsequent degradation (220). A recent report by Dunne *et al.* demonstrates that Mal can be phosphorylated by both IRAK-1 and IRAK-4 and this promotes its ubiquitination and degradation (221). Cleavage of Mal by caspase-1 is another event necessary for NF-κB activation by Mal (145). Mal is thus subjected to multiple regulatory mechanisms and as such serves as another level of control for TLR2 and TLR4 signaling.

The importance of Single Nucleotide Polymorphisms (SNPs) in TLR related proteins and their association with various infectious and inflammatory diseases have recently emerged (222, 223). One of the earliest studies on polymorphisms in adapter proteins demonstrates that a SNP in Mal, A186A is associated with increased susceptibility to meningeal tuberculosis (222). A study by Hill *et al.* reported that individuals heterozygous for the polymorphism S180L in Mal are protected against pneumococcal disease, bacteremia, malaria and tuberculosis (224). They further show that S180L leads to reduced TLR2 signaling and the reason for this defect is the inability of Mal S180L to bind TLR2. Within the coding region of a gene, SNPs can alter the characteristics and affect the ability of a protein to function (225). Interestingly, Mal is the most polymorphic of all adapter proteins, harboring at least 8 non-synonymous variants in its coding region. It thus appears to be an important candidate for studying genetic variation in relation to TLR2 and TLR4 signaling.

In the present study, we characterized the known polymorphisms in the coding region of Mal for effects on the function of Mal (FIG II.1). We report that one of the mutations in the TIR domain of Mal, D96N, is broadly defective in TLR2- and the MyD88 branch of the TLR4 signaling pathway. We show by biochemical and computer modeling data that the basis of this abnormality is the inability of Mal D96N to bind MyD88. While the incidence of this lesion in humans is not known, we screened three small populations for the defect and found a single heterozygous individual, suggesting that D96N is a rare mutation. We thus identify a mutation in Mal that causes a structural change, affecting the ability of the protein to bind the downstream effector MyD88.

FIGURE II.1: Domain structure of Mal

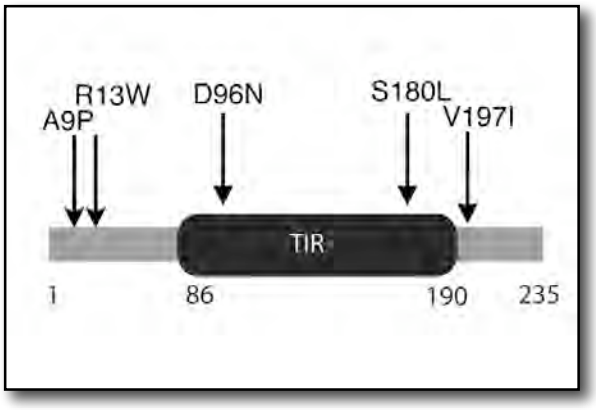


FIGURE II.1: Domain structure of Mal: A schematic showing the positions of the SNPs in the coding region of Mal.

Experimental procedures

Plasmids and site directed mutagenesis: Most of the constructs described in this work have been described elsewhere. These include: pEF-BOS-Mal-FLAG (198); pCDNA3-MyD88CFP (106); pCMV-IRF5-FLAG (226), NF-κB-luciferase, ISRE-luciferase, and Renilla-luciferase (158); and the retroviral vector pMSCV2.2-IRES-GFP (FIG II.2) (227). pEF-BOS-Mal-FLAG and pMSCV2.2-IRES-GFP-human-Mal with the different mutations in the *TIRAP* gene were generated by using a site-directed mutagenesis kit Quickchange (Stratagene), per the manufacturer's instructions.

Generation of wild type and Mal-deficient immortalized macrophage-like cell lines: Bone marrow cells were isolated from the femurs of wild type or Mal deficient mice. After red blood cell (RBC) lysis, cells were resuspended in 2.5 ml of filtered supernatant of ΨCRE-J2 cells (as the source of the recombinant retrovirus J2 with the oncogenes v-raf and v-myc) supplemented with 1.25 ml of DMEM and 1.25 ml of L929 conditioned supernatant (as the source of M-CSF) (228). Next day, supernatants were removed and cells were cultured in fresh DMEM supplemented with 20% L929 supernatants. After eight days, supernatants were removed and cells were cultured in medium without growth factors. The polyclonal population of immortalized cells were then cloned by limiting dilution method and the clones were tested based on a number of characteristics.

Transduction of immortalized macrophage-like cell line with recombinant retroviruses: FLAG-tagged human Mal (WT) or the different variants of Mal were cloned into the retroviral vector pMSCV2.2-IRES-GFP (FIG II.2) (227). HEK293T were plated in a 6-well plate at a density of $5x10^5$ cells per well. Next day the cells were tranfected with the

retroviral construct (300 ng/well), and the packaging vectors encoding gag-pol (300 ng/well) and VSV-G (30 ng/well) using genejuice. While “gag” encodes the capsid proteins, “pol” is responsible for encoding the reverse transcriptase and integrase proteins. VSV-G protein enables viral entry, by promoting attachment of the virus to the cell, allowing it to be endocytosed. It is also responsible for the broad tropism exhibited by VSV-G pseudotyped viruses. Two wells were used for each Mal variant transfection. Sixteen hours later, transfection reagent containing media was changed with fresh media. After 48 hours, supernatants containing viral particles were collected, passed through 0.45 μ syringe filter and added to previously plated Mal-deficient immortalized macrophages in a T25 flask. A week later the bulk population was subjected to FACS (fluorescence associated cell sorting) to select positively transduced cells based on the expression of GFP from the IRES element. The cells were allowed to recover for a few days and then cloned by limiting dilution to give rise to single cell clones. Equal expression of Mal or the Mal variants in selected clones was confirmed by western blotting with anti-FLAG antibody.

Luciferase reporter assay: HEK293T cells were seeded into 96-well plates at a density of 20,000 cells per well and transfected 16 h later with 40 ng of the indicated luciferase reporter genes and the indicated amounts of Mal (wt or polymorphic) with IRF-5 (10ng) construct using GeneJuice (EMD Biosciences, San Diego). The thymidine kinase Renilla-luciferase reporter was co transfected (40 ng) to normalize the data for transfection efficiency. Two days later reporter gene activity was measured using the Dual Luciferase Assay System (Promega, CA, USA).

FIGURE II.2: Retroviral vector pMSCV2.2

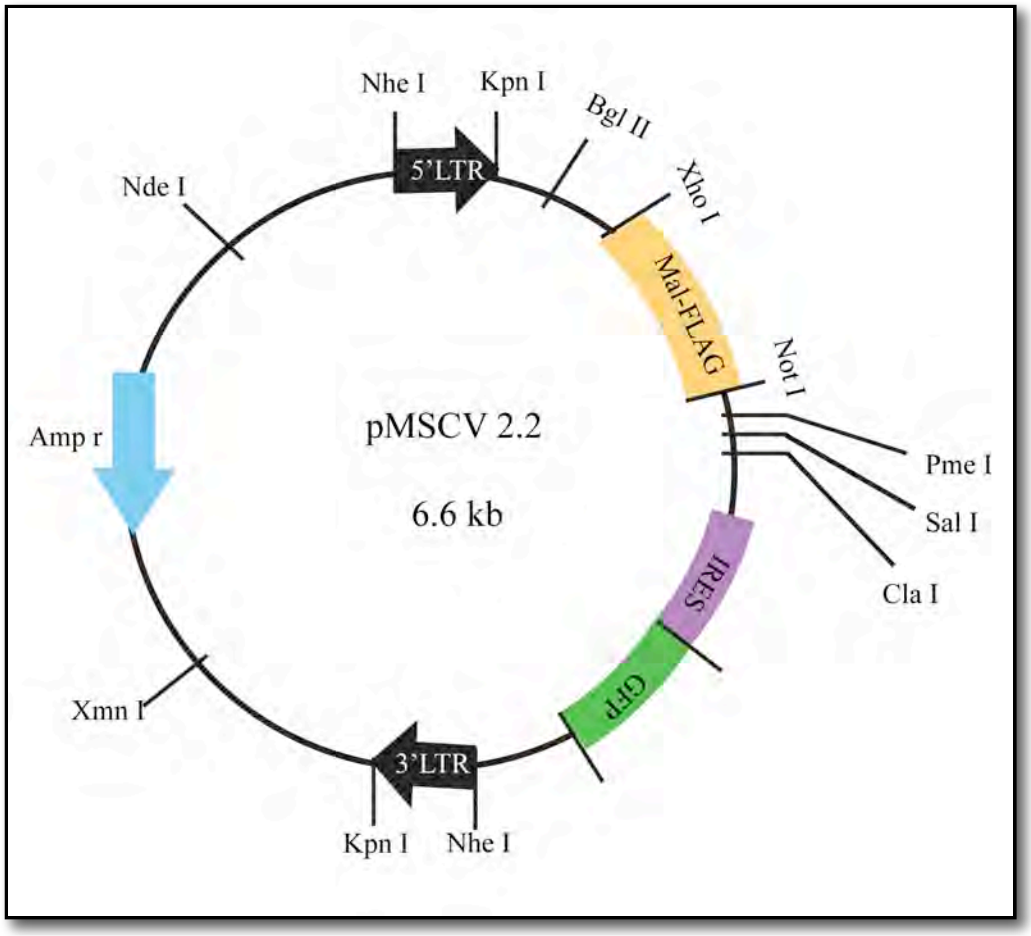


FIGURE II.2: Retroviral vector pMSCV2.2: Vector map of the retroviral construct used for reconstitution of the Mal deficient immortalized cell line. FLAG-tagged Mal (WT) or the different variants of Mal were cloned into the vector using Xho I and Not I restriction sites. GFP translated from the IRES site was used for selecting the positively transduced cells.

ELISA: Cells were seeded into 96-well plates at a density of 20,000 cells per well and stimulated overnight with the indicated concentrations of the TLR ligands. Cell culture supernatants were assayed for TNFα with ELISA kits from R&D systems, according to the manufacturer's instructions.

Quantitative Real-Time PCR: Wild type, Mal deficient or D96N expressing macrophages were plated at a density of $2x10^6$ cells per well in a 6-well plate. They were stimulated with 100 ng of LPS/ml for 2 hrs. RNA extraction and IFN-β mRNA induction analysis by quantitative Real-Time PCR was carried out as described elsewhere (229).

IkB degradation assay: $2x10^6$ Cells were seeded per well into a 6-well plate. After 24 hrs, the cells were treated with 10 nM PAM_2CSK_4 or 100 ng/ml LPS for the indicated time intervals. Cell lysates were made and run on an 8-16% SDS gel (NuSep, GA, USA), transferred to nitrocellulose membrane and probed with anti-IkB. (Cell Signaling Technology, MA, USA).

Immunoprecipitations and immunoblots: HEK293T cells were transfected with TLR2-YFP, TLR4-CFP or MyD88-CFP and FLAG-tagged Mal (WT or D96N) using genejuice transfection reagent. Two days post-transfection, cells were harvested and the lysates were incubated overnight with polyclonal anti-GFP antibody (Invitrogen, CA, USA) and Poly-A Sepharose beads. Rabbit IgG was used as a control. The processed samples were run on an SDS gel, transferred to nitrocellulose membrane and probed with an HRP conjugated anti-FLAG antibody (SIGMA, MO, USA). The immunoprecipitation of TLR2-YFP, TLR4-CFP and MyD88-CFP was checked by immunoblotting the same

membrane with monoclonal anti-GFP antibody (Invitrogen, CA, USA). Expression level of the proteins in whole cell lysates was checked by immunoblotting with HRP conjugated anti-FLAG antibody and monoclonal anti-GFP antibody.

Molecular modelling of Mal D96N: The amino acid sequences and three-dimensional structures of the TIR domains of TLR1 and TLR2, homologous proteins used as templates for comparative modelling, were obtained from the Protein Data Bank (http://www.rcsb.org/pdb). Initial alignments between Mal and its templates were obtained using the program FUGUE (230). Models were produced using the program MODELLER (231) as previously described (232). Site Directed Mutator (233) was used to calculate a stability score for a specific point mutation (96N) in the Mal TIR model. 3D structure visualization and image generation was carried out using Pymol (DeLano, W. (2002) DeLano Scientific, Palo Alto, CA, USA).

Confocal Microscopy Imaging: HEK293T cells were seeded in 35-mm glass bottom tissue culture dishes (Mat Tek Corporation, MA, USA) at a density of 250,000 cells per dish. Next day, the cells were transiently transfected with the indicated plasmids. Twenty-four hours later, live cells were imaged on a Leica SP2 AOBS confocal laser-scanning microscope.

Genotyping of TIRAP D96N in human subjects: Genetic studies were approved by the Ethical Committee of the Radboud University Nijmegen, The Netherlands. Genomic DNA from 91 African Tanzanians, 97 Chinese Han and 188 Caucasian Dutch individuals (234) were screened for the presence of *TIRAP D96N* (which encodes for Mal D96N). Screening was performed by PCR amplification and sequencing. For amplification, the

forward primer 5'-AGTGAGAGGGCACCTGGTAA-3' and the reverse primer 5'-CACAGCTCGGACACTATAGCGCC-3' were used. Sequencing was performed with the same primers on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) by the DNA Sequencing Facility, Radboud University.

Results

Transient expression of D96N does not activate NF-κB and ISRE reporters: The non-synonymous SNPs occurring in the coding region of human Mal were selected from the NCBI SNP database. These were examined for their ability to drive two signaling pathways known to function downstream of Mal. Transient expression of WT Mal in HEK293T cells triggers activation of NF-κB and IRF-5-dependent interferon stimulated response element (ISRE) luciferase reporters (145, 235). Notably, IRF5 is activated downstream of Mal and MyD88, but not TRIF or TRAM (235). As expected, WT Mal could drive both reporters. Moreover, four of the five polymorphisms screened were comparable to wild-type Mal in their ability to drive both reporters (FIG II.3). However, Mal D96N was severely compromised in driving either the NF-κB (FIG II.3A) or the IRF5-ISRE (FIG II.3B) reporter. IRF5 by itself can weakly activate the ISRE reporter, but the co-expression of wild type Mal leads to a much greater activation above this background value (FIG II.3B). In the case of Mal D96N, this increase in activity over the background level was not seen. These results suggest that Mal D96N does not drive signal transduction upon over expression.

FIGURE II.3: D96N is unable to drive either NF-κB or IRF5-dependent ISRE reporter

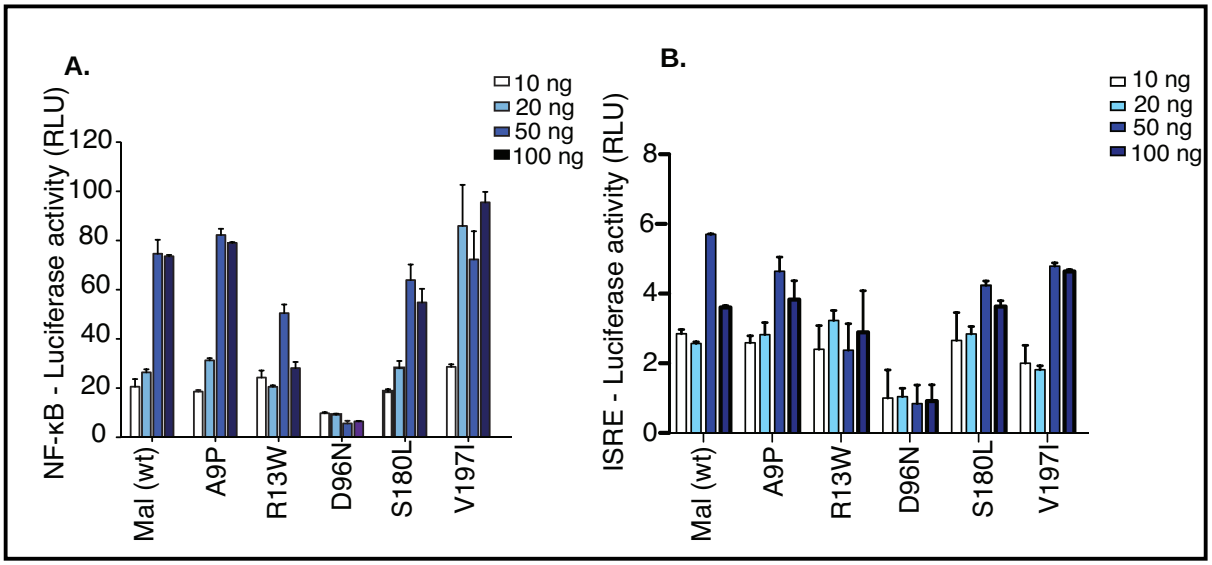


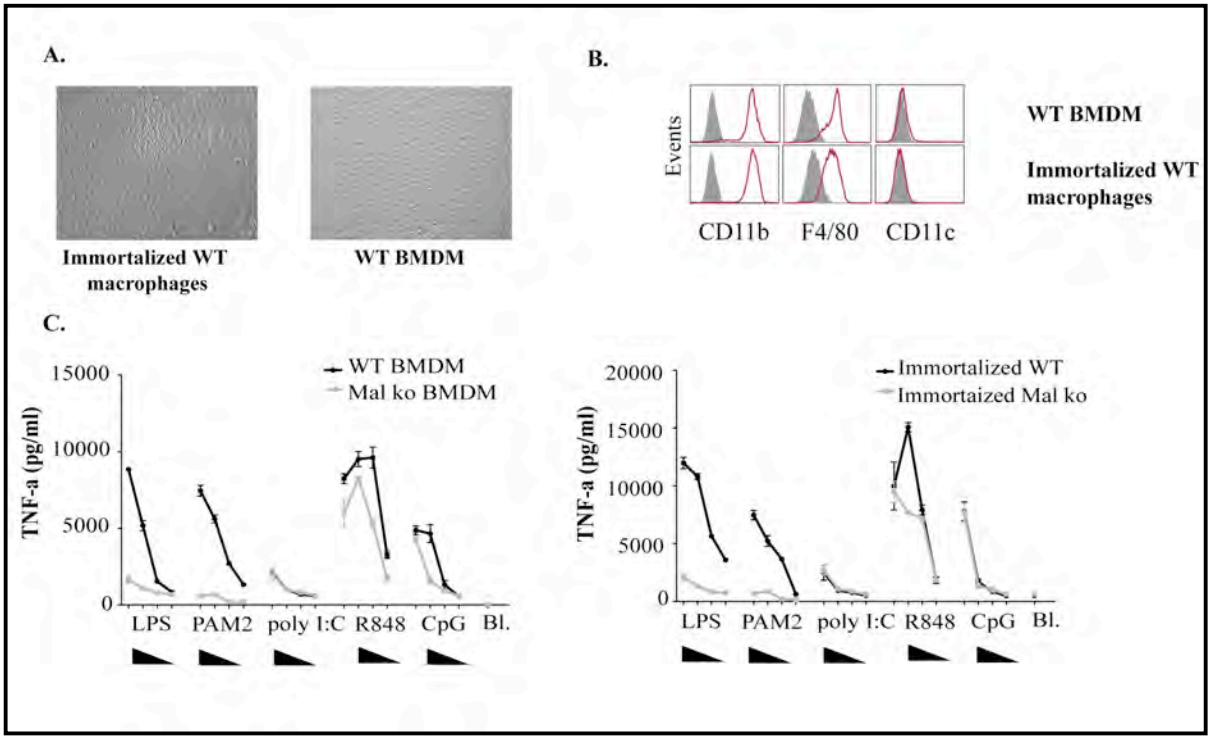
FIGURE II.3: Human Mal (hMal) carrying the D96N mutation is unable to activate either NF-κB or IRF-5. HEK293T cells were transfected with different variants of Mal and either NF- κB luciferase (A) or IRF-5 and ISRE-luciferase reporters (B). 48 hrs post-transfection, lysates were analyzed for luciferase activity. Renilla-luciferase activity was used to normalize for transfection efficiency. Results are reported as the mean of triplicate determinations ± SD. Each graph is representative of three (A) or two (B) independent experiments. RLU: Relative Luciferase Units.

The immortalized macrophage-like cell lines retain the characteristics of primary macrophages: In order to study these polymorphisms in phagocytes with a clean genetic background, we immortalized bone marrow derived macrophages (BMDM) from wild-type and Mal-deficient mice using a retrovirus encoding several oncogenes (228). The single cell clones obtained were characterized based on three criteria: morphology, surface marker expression and functional response to TLR ligands. The immortalized macrophages had a similar morphology as compared to primary macrophage culture. FIG II.4A shows wild type immortalized macrophages (left panel) with the primary macrophages (right panel). CD11b and F4/80 are the pan macrophage markers. Expression of these two surface markers was analyzed for both primary as well as immortalized wild type macrophages (FIG II.4B). Like the primary cells, immortalized macrophages expressed high levels of both CD11b and F4/80. Importantly, they were negative for CD11c, which is a pan dendritic cell marker. The cell lines were stimulated with the indicated TLR ligands and the levels of TNF-α in the supernatants was assessed. Immortalized wild type and Mal-deficient macrophage-like cell lines had a similar response pattern as their primary cell counterparts (FIG II.4C).

The expression of WT Mal functionally complements Mal-deficient macrophages: We generated retroviruses expressing FLAG-tagged wild type Mal in tandem with an internal ribosomal entry site (IRES)-encoded green fluorescent protein (GFP) (FIG II.2). This construct expresses a bicistronic mRNA that allows the translation of both Mal and GFP in the Mal-deficient immortalized cell line. The transduced cells were sorted for GFP and

FIGURE II.4: Characterization of immortalized macrophage-like cell lines. Immortalized macrophage-like cell lines from wild-type and Mal knockout mice retain the characteristics of bone marrow derived macrophages. J2 recombinant retrovirus was used for generating immortalized macrophage cell lines. Macrophage phenotype was verified by morphology (A), surface expression of the markers CD11b and F4/80 (B) as well as functional responsiveness to a panel of TLR ligands (C). The cells were treated overnight with the indicated ligands. Supernatants were subsequently analyzed for the presence of TNF-α by ELISA. Results are reported as the mean of triplicate determinations ± SD.

FIGURE II.4: Characterization of immortalized macrophage-like cell lines



then cloned by limiting dilution. In Mal-deficient macrophages, TNF-α production in response to LPS was significantly reduced whereas in response to PAM_2CSK_4, it was completely abrogated. We examined TNF-α production in the clone obtained from transducing Mal-deficient cell line with retrovirus expressing WT Mal. The single cell clone could completely restore responsiveness to LPS and PAM_2CSK_4 stimulation (FIG. 2.5A). Similarly, cells lines expressing each of the variant forms of Mal were generated. Clones with similar levels of expression of Mal, as assessed by Western blot, were selected for further study (FIG. 2.5B).

Mal D96N has impaired cytokine production: Reconstituted cell lines were stimulated with LPS and PAM_2CSK_4 and the pro-inflammatory cytokine TNFα was measured in cell supernatants by ELISA. The variants A9P, R13W, S180L and V197I were fully functional and produced comparable levels of TNF-α as WT cells. However, in agreement with our earlier experiments, D96N was significantly compromised in its ability to produce pro-inflammatory cytokines upon stimulation (FIG II.6A). The production of another pro-inflammatory cytokine, IL-6 was similar to TNFα (data not shown). As a control, TNFα production by the double stranded RNA mimetic poly I:C stimulation was also measured. Poly I:C is a TLR3 ligand, whose signaling is independent of Mal (236). In this case, TNFα production in cells expressing the D96N mutation was comparable to WT macrophages as well as to cells expressing the different variants of Mal. To test whether the MyD88-independent TLR4 signaling is intact in D96N expressing cells, we measured induction of IFN-β mRNA upon LPS stimulation,

FIGURE II.5: huMal is able to fully complement function in Mal-deficient macrophages

(A) Functional restoration of Mal-deficient immortalized macrophage cell line. Immortalized Mal knockout cells were transduced with a retrovirus carrying FLAG-tagged Mal. Single cell clones were obtained by limiting dilution and the selected clone was then tested for responsiveness to LPS and PAM_2CSK_4. WT immortalized macrophages, Mal-deficient macrophages as well as the cells expressing wt Mal were plated in a 96-well plate and stimulated overnight with LPS and and PAM_2CSK_4. TNF-α levels in the supernatants was assessed using ELISA. (B) The same strategy was used to generate cell lines expressing the different variants of Mal. For each cell line, clones were chosen for further study based on similar levels of expression of Mal or the Mal variant. The western blot shows the expression levels of the FLAG-tagged versions of different clones that were selected.

FIGURE II.5: huMal is able to fully complement function in Mal-deficient macrophages

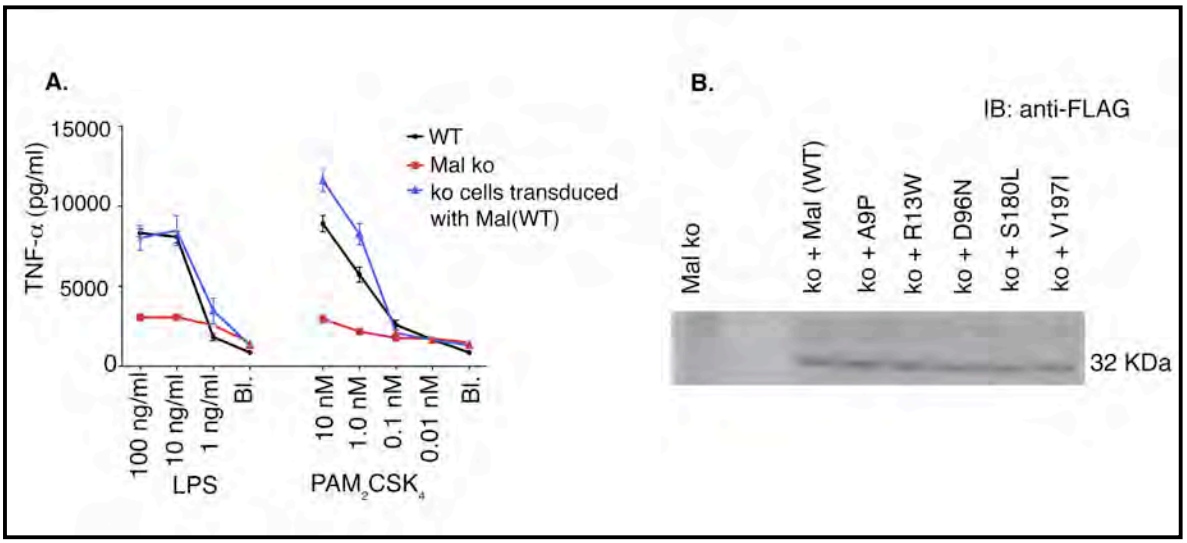
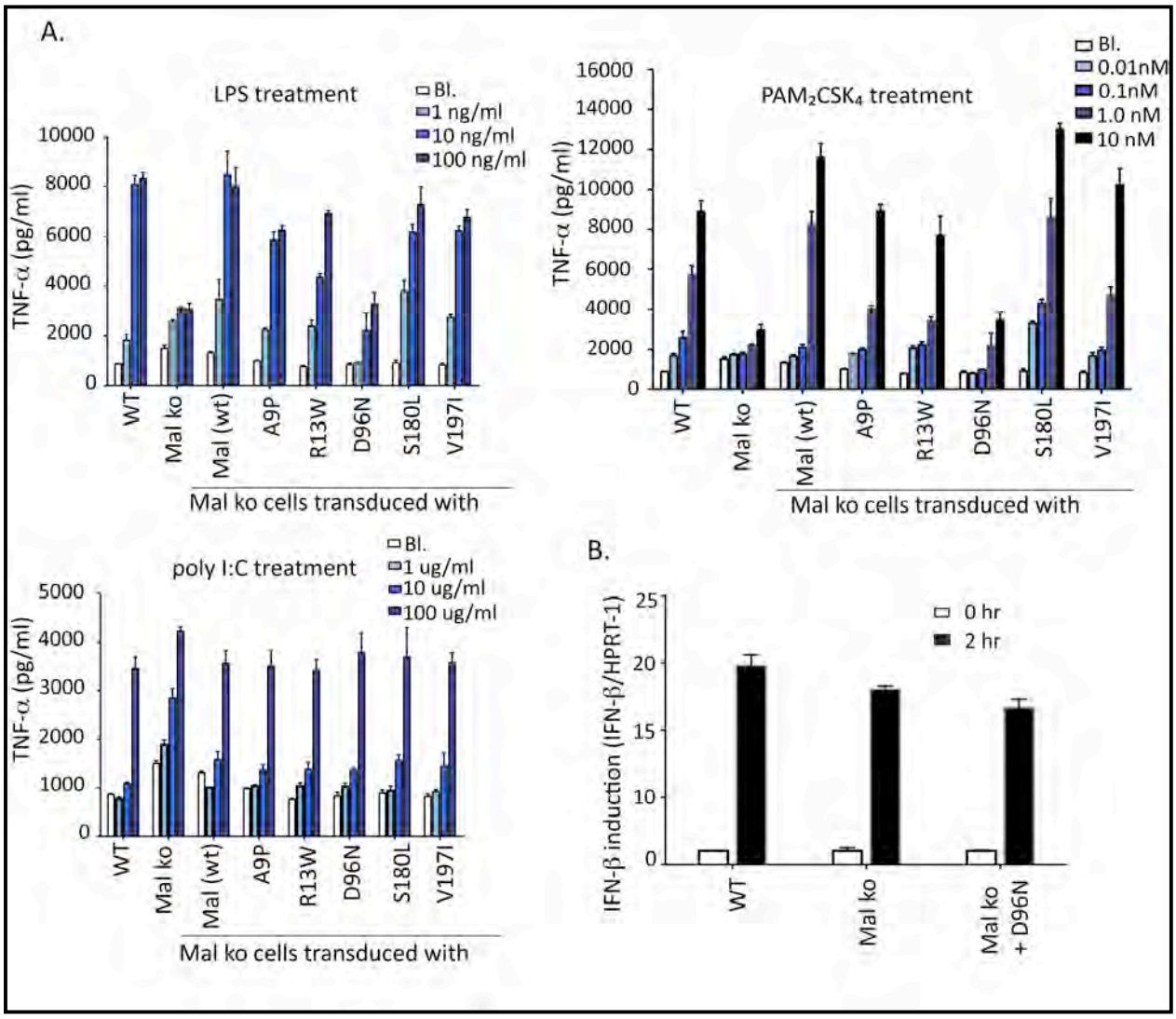


FIGURE II.6: Cells expressing D96N have impaired cytokine production: (A) Immortalized Mal-deficient macrophage cell lines expressing either WT Mal or one of the variants were stimulated with LPS, PAM_2CSK_4 and poly I:C overnight. The supernatants were then analyzed for TNF-α levels. Poly I:C, a TLR3/TRIF ligand, was used as a control for Mal-independent signaling. Results are reported as the mean of triplicate determinations ± SD. Each graph is representative of three independent experiments. (B) Immortalized WT, Mal-deficient and Mal-deficient macrophages expressing D96N were stimulated for 2 hours with LPS (100 ng/ml). Total RNA was extracted. Levels of mRNA for IFN-β were determined by Q-PCR and normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT1) level of expression. Results are reported as the mean of duplicate determinations ± SD. This graph is representative of two independent experiments.

FIGURE II.6: Cells expressing D96N have impaired cytokine production



using Real-Time PCR (FIG II.6B). D96N expressing macrophages induced comparable amounts of IFN-β mRNA as WT and Mal deficient cells. Thus, macrophages expressing the D96N variant of Mal are defective in signaling through TLR2 and the MyD88 branch of TLR4, while signaling initiated through other TLRs occurs normally.

IκB degradation assays: NF- κB is the predominant transcription factor responsible for pro-inflammatory cytokine production downstream of TLR2 and MyD88-dependent TLR4 signaling pathways. We therefore examined the activation of NF-κB (via IκB degradation) after LPS and PAM_2CSK_4 treatment in the reconstituted cell lines mentioned above. In Mal-deficient macrophages, IκB degradation is abrogated upon PAM_2CSK_4 treatment, whereas, for LPS stimulation, Mal-deficient macrophages undergo delayed degradation (237). In WT BMDMs, IκB was completely degraded at 10 min after LPS or PAM_2CSK_4 treatment (FIG II.7, row A). As expected, Mal-deficient BMDMs had a delayed degradation profile in the case of LPS (FIG II.7, left panel, row B) and exhibited no degradation in the case of PAM_2CSK_4 treatment (FIG II.7, right panel, row B). Immortalized wild type and Mal-deficient cell lines mimicked their primary cell types (FIG II.7, rows C, D). Mal-deficient cell lines expressing wild type Mal, A9P, R13W, S180L or V197I degraded IκB at 10 minutes, like the wild type immortalized cells (FIG II.7, rows E-G, I, J). In contrast however, and in agreement with the cytokine data, cells expressing the D96N mutant behaved like the Mal-deficient cells (FIG II.7, row H), with no degradation in the case of PAM_2CSK_4 and a delayed degradation profile following LPS treatment. Thus, Mal D96N is a hypomorphic, if not a null variant of Mal, fully defective in NF-κB activation.

FIGURE II.7: Mal D96N fails to degrade IκB-α effectively

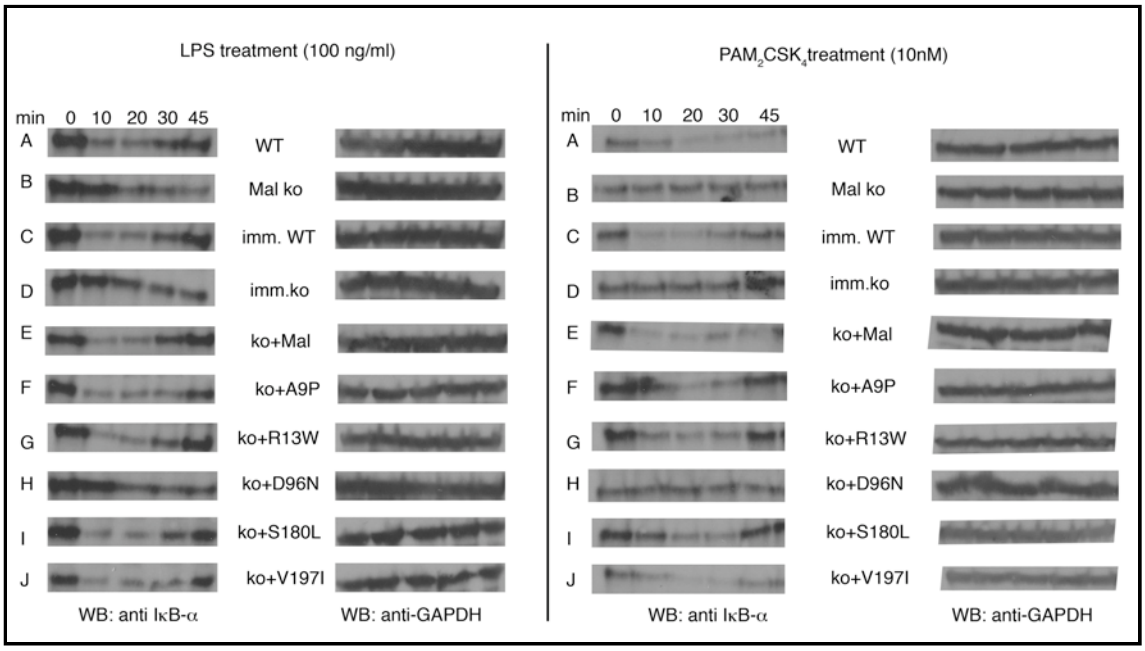


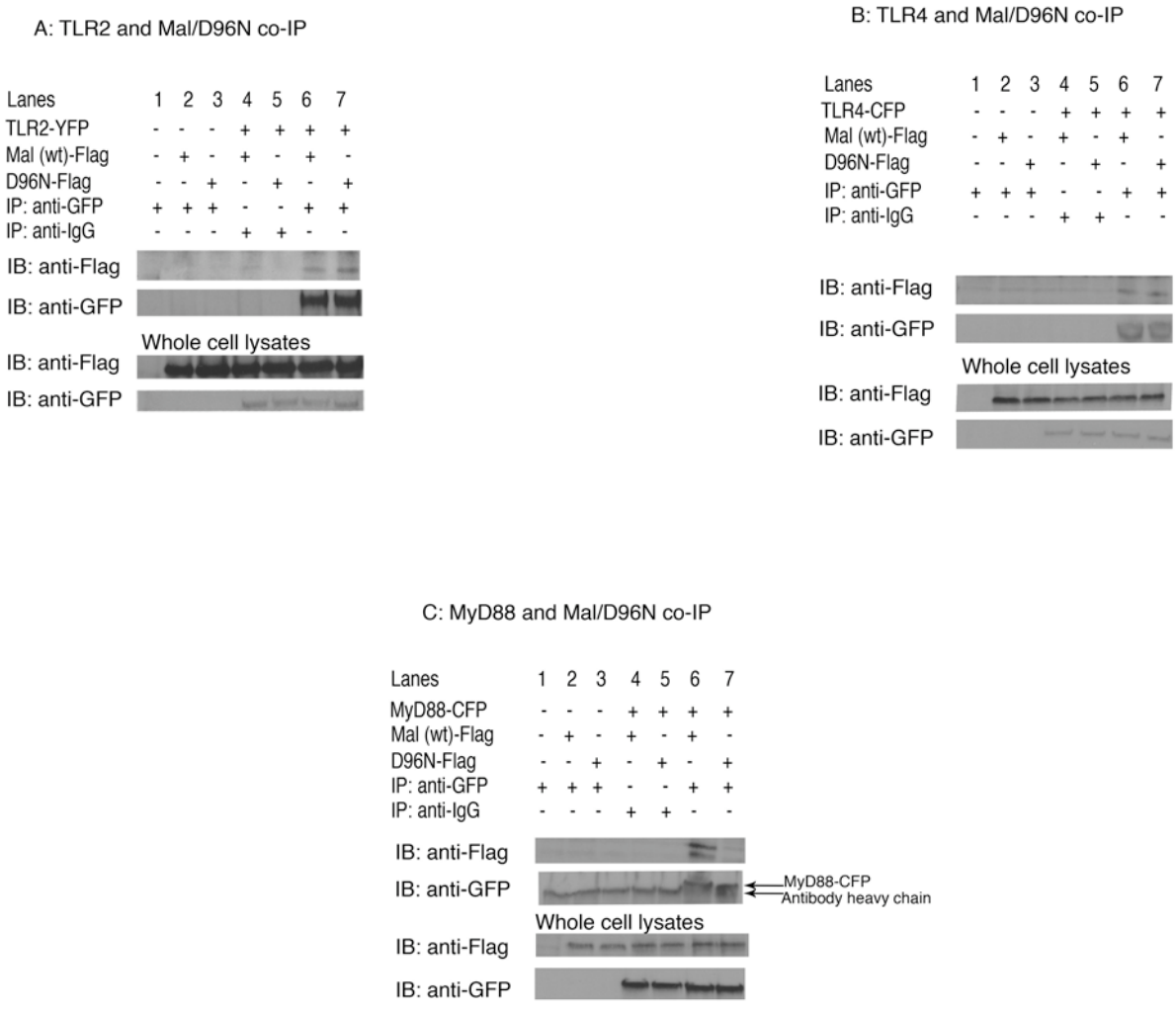
FIGURE II.7: Mal D96N fails to degrade IκB-α effectively. NF- κB activation in the reconstituted cell lines was examined via IκB-α degradation assay. The cells were stimulated with LPS (left panel) or PAM_2CSK_4 (right panel) for the indicated time points and the whole cell lysates loaded on a denaturing gel, transferred to nitrocellulose membrane and blotted with IκB-α antibody. Levels of GAPDH act as an internal loading control.

Interaction studies with TLR4, TLR2 and MyD88: Having established the failure of Mal D96N to signal, we next sought an explanation for the inability of D96N to transduce the signal downstream of TLR2 and TLR4. It is thought that Mal acts as a bridging adapter between the receptor (TLR2 or TLR4) and MyD88, and thus physically interacts with both (238). We therefore examined whether the D96N mutation affected the ability of Mal to interact with TLR2, TLR4 or MyD88. Transient transfections in HEK293T cells, followed by immunoprecipitation assays were carried out to address this question (FIG II.8). As expected, wild type Mal interacted with the receptors TLR2 (FIG II.8A) and TLR4 (FIG II.8B) and with MyD88 (FIG II.8C, lane 6). Like wild type Mal, D96N could be co-immunoprecipitated with both TLR2 and TLR4, indicating that this mutation does not affect the ability of Mal to interact with either of these receptors. However, D96N was severely compromised in its ability to interact with MyD88. While wild type Mal could co-immunoprecipitate with MyD88, D96N could not (FIG II.8C, lane 7). Thus, the loss-of-function phenotype of D96N appeared to result from its inability to interact with MyD88.

Modeling studies for Mal suggest that a change in surface charge accounts for the D96N phenotype: We generated high quality models of the TIR domains of both WT and D96N Mal, using the resolved crystal structures of TLR1 and 2 as templates (111). The Mal TIR domain was predicted to have good geometry, with none of its phi-psi dihedral angles within disallowed regions of a Ramachandran plot, and 90.6% in geometrically favored regions. JOY and Verify3D outputs (232) revealed that the environment of residues in the model of Mal are similar to those of the TLR1 and TLR2 TIR domains used as

FIGURE II.8: D96N fails to interact with MyD88. HEK293T cells were plated to half confluence in 6 well plates. After attachment, cells were transiently transfected with either TLR2-YFP (A), TLR4-CFP (B) or MyD88-CFP (C) and FLAG tagged Mal WT or Mal D96N. Two days after transfection, supernatants were removed, and cells were lysed. Anti-GFP polyclonal antibody was used for immunoprecipitation (IP). Immunoprecipitates were loaded on a denaturing gel, transferred to nitrocellulose membrane, and visualized with a monoclonal anti-FLAG-HRP antibody**.** The immunoprecipitation of TLR2-YFP, TLR4-CFP and MyD88-CFP was confirmed by immunoblotting the membrane with anti-GFP antibody. The same antibodies were used to check the level of expression of the proteins in whole cell lysates. IB: immunoblot

FIGURE II.8: D96N fails to interact with MyD88



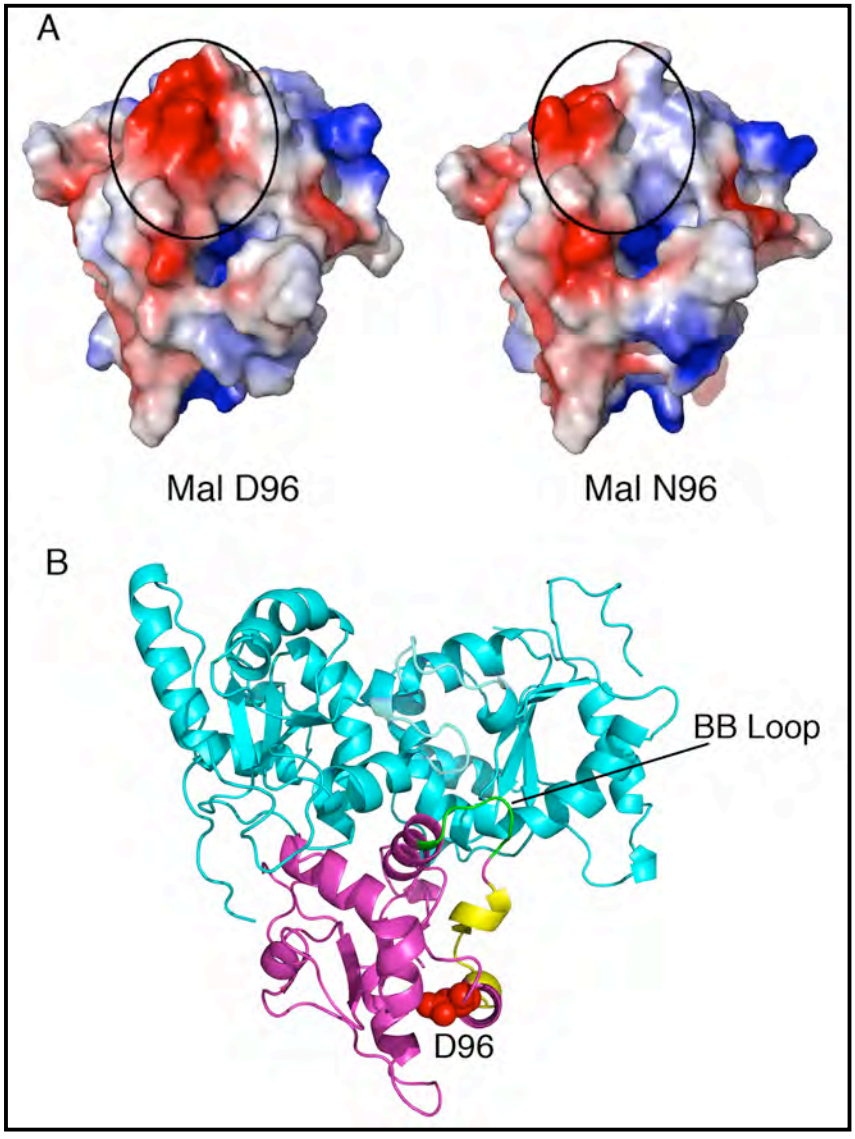
templates, and were not energetically unfavorable. Residue D96 was predicted to lie on the surface of the TIR domain in a highly negatively charged region.

Mal D96N was modeled using the same approach as the wild-type protein, and exhibited no significant changes in structure or geometry compared to wild-type Mal, as might have been expected from a change in a surface residue. This prediction was further supported using software capable of estimating the level of structural destabilization encoded by point mutations (SDM) (233). This program uses a statistical potential energy function that predicts the effect that a specific amino-acid substitution may have on the stability of that protein and produces the pseudo free energy difference between a wild-type and mutant protein (233, 239). The D96N substitution was shown to induce structural alterations with corresponding pseudo $\Delta\Delta G$ values of 0.729 kcal/mol, implying that there is no significant change to the overall stability of the Mal TIR domain caused by this mutation.

The loss of a surface-exposed negatively charged side-chain resulting from an aspartic acid to asparagine substitution (D->N) (FIG II.9A) would be predicted to be responsible for the experimentally observed disruption of the Mal/MyD88 protein interaction. FIG II.9B shows a model of the TLR4- TIR homodimer (cyan) /Mal TIR domain (magenta) complex. The change in electrostatic charge resides near a surface region (Pattern 108-122; shown in yellow) that is predicted to interact with MyD88 [(FIG II.9B) and (232)]. Moreover, residue 96 lies distal to the BB loop region of Mal (92) that has previously been proposed to interact with TLR4 [(FIG II.9B) and (232)], consistent

FIGURE II.9. The D96N mutation results in a loss of negative charge on the surface of the Mal-TIR domain in a region predicted to be involved in MyD88, but not TLR interactions. (A) Comparison of the electrostatic surfaces of wild-type (left) and N96 (right) Mal-TIR models indicating a loss of negative charge in the region of residue 96 (circled). (B) Model of the TLR4-TIR homodimer (cyan) /Mal TIR domain (magenta) complex indicating the location of Mal-D96 (red, spacefill), its proximity to the predicted MyD88 interaction surface (pattern 108-122, yellow) and its distance from the BB-loop region that is vital for Mal's interaction with TLR4 (cyan).

FIGURE II.9: The D96N mutation results in a loss of negative charge on the surface of the Mal-TIR domain in a region predicted to be involved in MyD88, but not TLR interactions.



with the immunoprecipitation studies in FIG II.8 indicating that Mal D96N retains its ability to bind to TLRs 2 and 4 even though it is no longer capable of binding MyD88.

Mal D96N fails to recruit MyD88 to the cell membrane: MyD88 is recruited to the cell membrane via TIR-TIR interactions with Mal. Exogenous MyD88 when expressed in cells, is present in condensed aggregates in the cytoplasm. Co-expression of Mal (wt) however, leads to expression of MyD88 at the cell membrane where it colocalizes with Mal (139). We expressed citrine-tagged MyD88 either by itself or in conjunction with FLAG-tagged wt Mal or Mal D96N in HEK293T cells. As expected, MyD88 when expressed by itself, could be seen as aggregate structures in the cell (FIG II.10, uppermost panel). Co-expression with wt Mal, led to the expression of MyD88 at the cell membrane (FIG II.10, middle panel). However, when we expressed MyD88 with Mal D96N, MyD88 retained its aggregate form and was not recruited to the cell membrane (FIG II.10, bottom panel). This is in accordance with our interaction data since Mal D96N is unable to interact with MyD88 and therefore fails to recruit MyD88 to the cell membrane.

Murine Mal D96N is able to drive NF-κB reporter: Murine Mal has an additional 20 amino acids at the N-terminus as compared to Human Mal. The 96th residue in human Mal is thus at position 116th in murine Mal. However, aspartic acid is conserved at this position in both human and murine Mal. We tested the activity of Mal D116N in NF-κB reporter assay. Like human Mal, murine wild-type Mal was able to drive NF-κB reporter (FIG II.11). However, unexpectedly, Mal D116N was also sufficient in its ability to drive

FIGURE II.10. Mal D96N fails to recruit MyD88 to the cell membrane

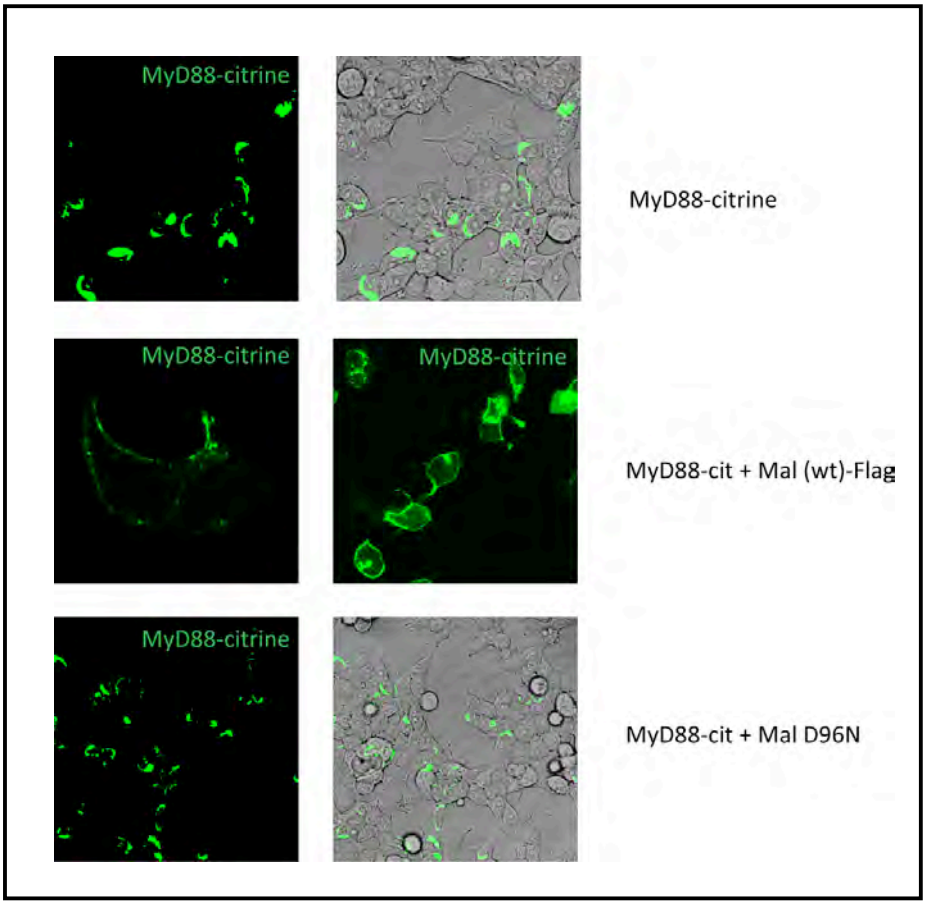


FIGURE II.10: Mal D96N fails to recruit MyD88 to the cell membrane: HEK293T cells were plated in glass-bottom confocal dishes at a density of 250,000 cells per dish. Next day, the cells were transfected with either MyD88 by itself or in conjunction with wt Mal or Mal D96N. The cells were imaged using standard confocal procedures.

FIGURE II.11: Murine Mal D96N is able to drive NF-κB reporter activity

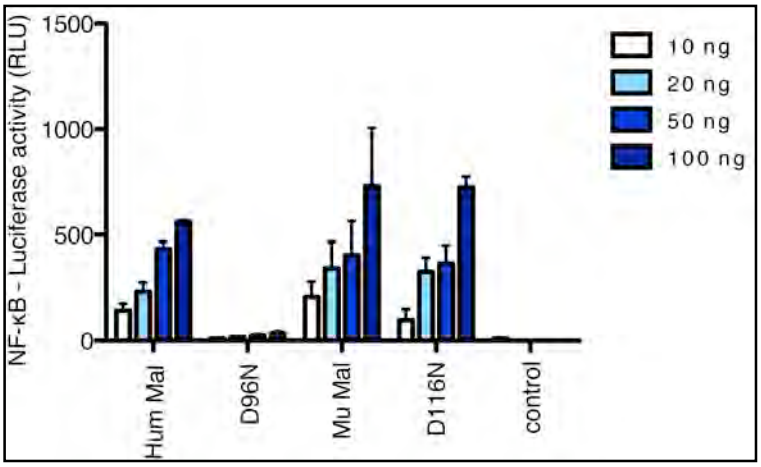


FIGURE II.11: Murine Mal D96N is able to drive NF-κB reporter activity: HEK293T cells were transfected with FLAG tagged versions of the indicated Mal constructs along with NF-κB-luciferase. Twenty-four hours later, luciferase activity was measured in cell lysates. Renilla-luciferase values were used to normalize for transfection efficiency. Results are reported as a mean of triplicate determinations ± S.D. The graphs are a representative of at-least two independent experiments.

the reporter. Thus, unlike human Mal D96N, murine Mal D116N is capable of activating NF-κB. This difference in the signaling capabilities between human and murine D96N (or D116N) might be due to the additional 20 amino acids at the N-terminus of murine Mal, which may change the conformation of the protein.

Genotyping of TIRAP in human populations: Mal D96N is an inactivating mutation, which is unable to transduce signaling downstream of TLR2 and TLR4. We next addressed the question of what the frequency of this polymorphism was in the general population. We sequenced *TIRAP* in African Tanzanian (n=91), Chinese Han (n=97) and Caucasian Dutch (n=188) individuals. The African Tanzanian and the Chinese individuals all apparently expressed wild-type Mal. However, one individual in the Caucasian Dutch cohort was heterozygous for the D96N mutation (TABLE II.1). Although this experiment is not an exhaustive assessment of the frequency of Mal D96N in all populations, we can conclude that this variation of Mal has a very low prevalence in the populations studied. This is understandable, given the strong phenotype in innate signaling that we observed in our experiments. Indeed, individuals homozygous for MalD96N would be expected to have a phenotype similar to what has been reported for MyD88 deficiency, *i.e.*, hypersusceptible to common life-threatening pathogens such as *S. pneumoniae* (240).

TABLE II.1: Genotyping of Mal in three different populations

population	N	96th residue
African Tanzanian	**91**	**All D/D**
Chinese Han	**97**	**All D/D**
Caucasian Dutch	**188**	**187:D/D; 1:D/N**

TABLE II.1 Genotyping of Mal: Genomic DNA from 91 African Tanzanians, 97 Chinese Han and 188 Caucasian Dutch individuals was screened for the presence of *TIRAP D96N* (which encodes for Mal D96N).

Discussion

TLRs are the key sensors of microbial infection in mammals. Upon encountering a pathogen, they elicit an inflammatory response involving an intricate network of signaling molecules. Changes in the structure of the TLR signaling components can have dramatic effects on the outcome of various infectious diseases. Some of these changes may be caused by the presence of a SNP in the coding region of the protein. The effects of SNPs on the structure of proteins and the consequent effect on TLR signaling have been described in many studies (241-244).

TIR domain interactions between the TLR components are a necessary event for downstream signaling to occur. SNPs in adapter proteins could thus be influential in determining the progression of infection. Incidentally, Mal is the most polymorphic of all the adapter proteins. Of the eight known coding polymorphisms in Mal, only one, S180L, has been previously described (224). Khor *et al.* found that the heterozygotes carrying this polymorphism were protected from malaria, tuberculosis and bacteremia. In view of these observations we hypothesized that SNPs in Mal could affect TLR2 and TLR4 signaling.

The data presented herein makes a strong case that a variant of Mal, D96N, is essentially non-functional. Our model would indicate that Mal D96N does not bind MyD88 due to a critically located change in charge on its surface, and hence fails to function as a bridging adapter between the receptors TLR2 and TLR4 and the sorting adapter MyD88. Its inability to engage MyD88 prevents activation of NF-kB, IRF-5 and

most likely AP-1, all of which are required to trigger pro-inflammatory cytokine production.

A noteworthy point is that Mal-deficient macrophages from mice were all reconstituted with human Mal. The TIR domains of murine and human Mal are 71% identical and indeed, human Mal was capable of fully restoring function in these cells. As expected, D96N was defective in pro-inflammatory cytokine production upon stimulation. A surprising finding was the absence of a phenotype for Mal S180L in our studies, an outcome that conceivably could be due to difference in species of the reconstituted protein and cell types, although this explanation would not hold for the ability of transfected Mal S180L to drive reporter constructs in HEK293T cells. In our experiments Mal S180L was fully active and its effect upon stimulation comparable to that of wild-type Mal. It is true that we have no truly satisfactory explanation for the discrepancy between our findings and that of Khor *et al* (224). One explanation for our findings, at least in Mal knockout macrophages complemented with the various mutant Mal constructs, is that Mal S180L may be a hypomorphic lesion, rather than a null mutation. Hence, sufficient overexpression may overcome its relative inability to signal. Indeed, Western Blots of the transduced Mal, as well as the variants, using an established anti-Mal Ab, suggested that the recombinant proteins were at least 10-100 fold more abundant in the immortalized cell lines than endogenous wild-type Mal (data not shown). Clearly, this discrepancy from previously published results need to be better analysed. To this effect, a knock-in mouse expressing the S180L lesion has been engineered (data not shown), and will need to be characterized to determine the nature of this mutation *in vivo*.

Residue 96 and the surrounding 10-12 amino acid region is fairly well conserved in the known orthologs of *TIRAP*. The presence of an aspartic acid (D) at position 96 creates a negatively charged surface that is conducive to protein-protein interactions. Conversion to uncharged asparagine would thus be expected to change the binding potential of the protein. Modeling of the TIR domains of Mal and Mal D96N suggested that the residue 96 lies away from the TLR4 or TLR2 binding site but is in very close proximity to the MyD88 binding site (Fig 5). These predictions were confirmed in co-immunoprecipitation assays and show that Mal D96N retains the ability to bind TLR2 and TL4 but is unable to bind MyD88. Hence, this characterization of a natural Mal mutant identifies, with apparent pinpoint precision, a single residue in the TIR domain of Mal that is critical for its interaction with MyD88.

The frequency of D96N in the populations we analyzed was very low. In the initial screen, we genotyped individuals from three different populations. However, we found just one individual (out of 376) who was heterozygous for D96N. Considering the deleterious phenotype we observe, the low frequency of this polymorphism is not unexpected. As the mutation exists, it is nearly certain that there are sporadic individuals born who are homozygous for D96N. Since the data suggest the failure to bind MyD88 is the reason for the phenotype of D96N, we believe that individuals carrying this mutation in a homozygous state would be extremely susceptible to both gram-negative and gram-positive infections. In view of the reported serious clinical significance of MyD88 and IRAK4 deficiencies, two gene products that are downstream of Mal and which should be similar to Mal D96N expression, we believe that Mal D96N represents a rare, potentially

life-threatening mutation.(216, 217) Indeed, individuals occasionally present for medical case with sepsis in the absence of any known risk factors; we would suggest that Mal D96N should be considered under such circumstances as a potential cause of such a clinical presentation.

In contrast to what one might expect for the rare individual with two mutant alleles, the fact that this amino acid variant exists in the population, albeit at a very low frequency, suggests that heterozygous individuals have a survival advantage under some, but still undefined, circumstances. The key to determining the nature of this hypothetical heterozygous advantage is to analyze enough individuals from enough different populations to define clinical correlates.

PREFACE TO CHAPTER III

This chapter of the thesis has been published in the Journal of Biological Chemistry:

Nagpal K, Plantinga T.S., Sirois C.M., Monks B.G., Latz E, Netea M.G., Golenbock D.T. 2011. **A Natural Loss-of-function Mutation of Myeloid Differentiation Protein 88 Disrupts Its Ability to Form Myddosomes.** *J Biol Chem* 286(13):11875-82

FIG III.15 A was contributed by Shivender Shandilya (Schiffer Lab, University of Massachusetts Medical School, Worcester, MA)

CHAPTER III

A NATURAL LOSS-OF-FUNCTION MUTATION OF MYD88 DISRUPTS ITS ABILITY TO FORM MYDDOSOMES

Abstract

Myeloid differentiation protein 88 (MyD88) is a key signaling adapter in Toll-like receptor (TLR) signaling. MyD88 is also one of the most polymorphic adapter proteins. We screened the reported non-synonymous coding mutations in MyD88 to identify variants with altered function. In reporter assays, a death domain variant, S34Y, was found to be inactive. Importantly, in reconstituted macrophage-like cell lines derived from knockout mice, MyD88 S34Y was severely compromised in its ability to respond to all MyD88-dependent TLR ligands. Unlike wild type MyD88, S34Y is unable to form distinct foci in the cells but is present diffused in the cytoplasm. We observed that IRAK4 colocalizes with MyD88 in these aggregates and thus these foci appear to be "Myddosomes". The MyD88 S34Y loss of function mutant demonstrates how proper cellular localization of MyD88 to the Myddosome is a feature required for MyD88 function.

Introduction

TLRs are a part of the innate immune system and play a critical role in host defense against a variety of pathogens. Ten functional TLRs in humans and 12 in mice have been identified to date (245). Each TLR recognizes different categories of evolutionarily conserved patterns called pathogen associated molecular patterns (PAMPs) in microbes. For example, TLR4 recognizes lipopolysaccharide (LPS) from the cell walls of bacteria while CpG-rich DNA from bacteria and viruses acts as the ligand for TLR9.

Much of the diversity of TLR pathways can be attributed to differential use of the TIR domain containing adapter proteins. There are 4 adapter proteins: MyD88, MyD88 adapter-like (Mal), also called TIR domain containing adapter protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM). MyD88 is a key adapter protein involved in signaling from all of the TLRs except TLR3 and the TRIF mediated arm of TLR4 signaling. Thus, depending on its use, TLR pathways can be divided into MyD88-dependent and MyD88-independent pathways. The engagement of a particular TLR by its ligand triggers a signaling cascade, which culminates in the secretion of various pro-inflammatory cytokines and interferons.

MyD88 is a 296 amino acid protein with a modular domain structure. In addition to the N-terminal TIR domain, it has a death domain at its C-terminus. The crystal structure of the MyD88 death domain has recently been resolved and it reveals that MyD88 exists in oligomeric form in solution (246). These oligomers of MyD88 can recruit the death domains of IRAK4 and IRAK2 to form a ternary complex called the Myddosome.

The importance of single nucleotide polymorphisms (SNPs) in TLR-related proteins has recently been brought to the forefront, with many studies implicating SNPs in disease susceptibility and outcome (247-251). Mal and MyD88 are the most polymorphic of the four adapter proteins and Mal has received the most scrutiny in this regard (222, 224, 252). However, other than a study by Von Bernuth *et al*., where children with MyD88 deficiencies were shown to have recurrent pyogenic bacterial infections (240), studies on MyD88 variants have been scarce. The central role of MyD88 in TLR signaling thus warrants a deeper understanding of the effects of SNPs on the activity/function of MyD88.

In this study we have screened the 6 non-synonymous polymorphisms (FIG III.1) in the coding region of MyD88 with the aim of identifying polymorphisms that alter the function of MyD88. We identify and characterize a death domain variant, S34Y that is completely inactive in all MyD88-dependent TLR signaling. We further show that this mutant has a localization pattern different from that of wild type MyD88 and is unable to form Myddosomes with IRAK4 and IRAK2.

Förster (or fluorescence) resonance energy transfer (FRET)

This chapter employs the technique of FRET rather extensively. Here, I have explained the basic concepts of this technique.

FRET is a valuable tool to detect dynamic molecular interactions between two fluorescently tagged proteins. Traditionally, imaging techniques rely on co-localization of proteins to demonstrate interactions. If two proteins are co-localized, this indicates that

FIGURE III.1: A schematic showing the positions of coding SNPs in MyD88

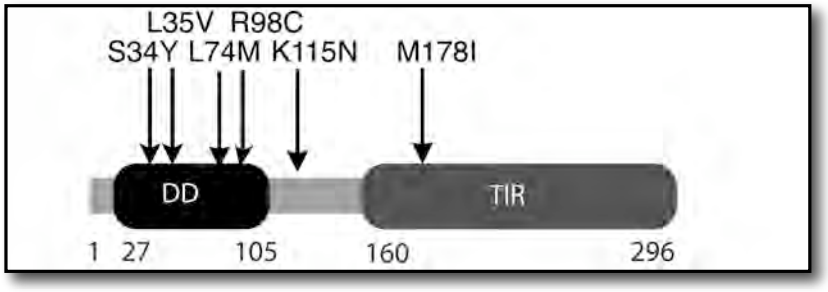


FIGURE III.1: A schematic showing the location of non-synonymous coding polymorphisms in MyD88. There are 6 SNPs in the coding region of human MyD88 (depicted here by arrows). DD (death domain) ; TIR (Toll/interleukin-1 receptor domain)

their spatial proximity is beyond optical resolution (*i.e.*, 100 nm lateral). It does not however have any bearing on the interaction between the two participating proteins. FRET can detect and quantitate interactions between two fluorescently tagged proteins. FRET occurs between two fluorophores: donor and acceptor. A prerequisite for FRET to occur, is that the donor emission spectra must overlap with the excitation spectra of the acceptor. When the two fluorphores are more than 10 nm apart, excitation of the donor leads to the characteristic donor emission. If however the donor and the acceptor are within 10 nm of each other, some of the energy from the donor emission is transferred to the acceptor fluorophore and because of the spectral overlap this energy is able to excite the acceptor leading to emission at the acceptor's emission wavelength. Thus if two proteins tagged with FRET compatible fluorophores interact, an emission at the acceptor's emission wavelength can be observed upon excitation at the donor's absorption wavelength (FIG III.2).

Sensitized emission (SE) is one of the most used methods for evaluation of FRET efficiencies. This method requires reference samples (donor only as well as acceptor only) in addition to the FRET or the experimental specimen. For each sample, the donor and the FRET signal (donor excitation only) is measured in sequence with the detection of the acceptor (acceptor excitation only). The reference samples are used to obtain calibration coefficients to correct for excitation and emission cross talk.

The equation used to calculate FRET efficiency is:

$$E_A = \frac{B - A * b - C * c}{C}$$

A, B, C correspond to the intensities of the 3 signals (donor, FRET, acceptor) and b & c are calibration coefficients calculated from the reference samples.

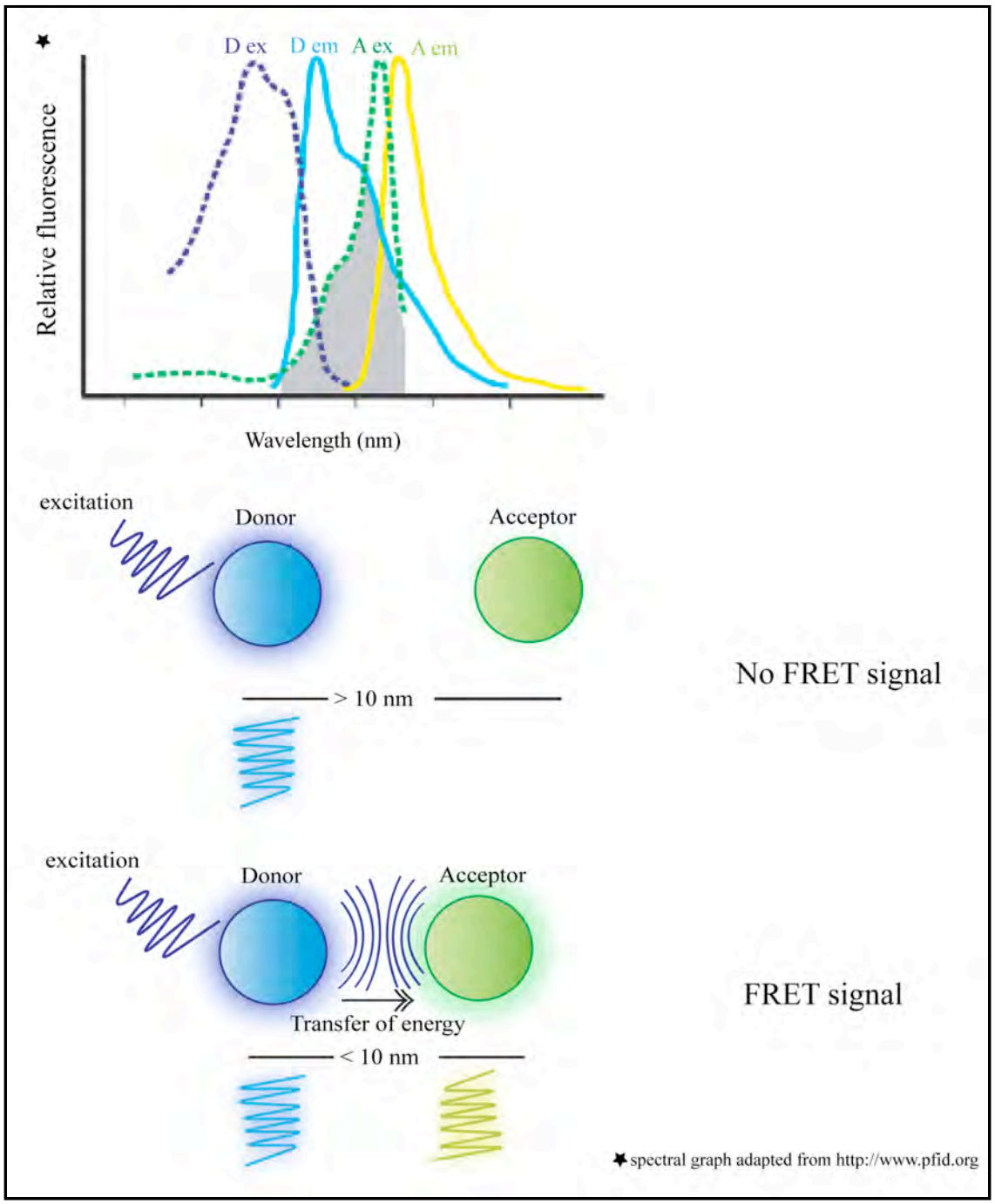
b = donor emission + crosstalk ratio

c = acceptor excitation crosstalk ratio

FRET efficiency is inversely proportional to the distance separating the FRET pair.

FIGURE III.2: Schematic representation of FRET (Förster resonance energy transfer). A typical spectral graph depicting overlap between the donor emission and the acceptor excitation spectra. The lower panels define the criteria for FRET to occur between two fluorophores.

FIGURE III.2: Schematic representation of FRET



Experimental procedures

Plasmids and site directed mutagenesis: The plasmids pEFBOS-MyD88-HA (253), pCMV-IRF5-FLAG (226), NF-κB-luciferase, ISRE-luciferase, and Renilla-luciferase (158); and the retroviral vector pMSCV2.2-IRES-GFP (227) have been described elsewhere. The different variants of MyD88 (in pEFBOS-MyD88-HA and pMSCV2.2-ISRE-GFP background) were generated by using the QuickChange site directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Cerulean-tagged IRAK-4 and citrine-tagged MyD88 were cloned into a vector that was modified from the original pCLXSN backbone from Imgenex (254). It has been named pRP by us. Subcloning was carried out using unique restriction sites in the multiple cloning site (MCS). All of the expression constructs were verified by sequencing.

Luciferase Reporter Assay: HEK293T cells were seeded in a 96 well plate at a density of 20,000 cells/well and transfected the next day with either NF-κB or interferon stimulated response element (ISRE) luciferase reporter (40 ng), renilla reporter (40 ng) and the indicated amounts of either pEFBOS-MyD88 (WT) or the different variants using Genejuice (EMD Biosciences, San Diego, USA). In the case of the ISRE reporter assay, IRF5 (5 ng) was cotransfected. Twenty-four hours post transfection, reporter gene activity was measured using the Dual Luciferase Assay System (Promega). The thymidine-kinase Renilla luciferase reporter values were used to normalize the data for transfection efficiency.

Retroviral Transduction of Immortalized Macrophage-like Cell Lines: The immortalized MyD88 deficient macrophage-like cell line has been described elsewhere (255). The

retroviral vector MSCV2.2 (227) carrying either FLAG tagged WT MyD88 or MyD88 S34Y was used to transduce MyD88-deficient cells. This retrovirus transcribes GFP from an internal ribosomal entry sequence (IRES) element. The positive cells were thus sorted based on GFP expression and the expression of the MyD88 proteins was compared by immunoblotting with anti-FLAG antibody.

Enzyme-linked Immunosorbent Assay: Wild type, MyD88-deficient and MyD88-deficient cells expressing WT MyD88 or MyD88 S34Y were seeded into 96-well plates at a density of 25,000 cells per well and stimulated overnight with TLR ligands at the indicated concentrations. TNF-α in the supernatants was measured using ELISA kits from R&D Systems as per the manufacturer's instructions. The same supernatants were also assessed for IFN-β levels as described (256).

IκB-α degradation and MAPK activation assays: Wild type, MyD88-deficient and MyD88-deficient cells expressing WT MyD88 or MyD88 S34Y were seeded into a 6-well plate at a density of $2X10^6$ cells per well. The next day, the cells were stimulated with either PAM_2CSK_4 (10nM) or R848 (20μM) for the indicated time intervals. Cell lysates were run on an SDS denaturing gel, transferred to a nitrocellulose membrane and immunoblotted with either anti-IκB-α antibody or anti-phospho-p38 antibody (Cell Signaling Technology, MA, USA). Anti-glyceride-3 phosphate dehydrogenase (GAPDH) (Advanced Immunochemicals) or anti-total p38 antibodies respectively were used to reprobe the membranes to check for equal loading of the gels.

IRAK-1 degradation assays: Cells were plated in a 6-well plate at a density of $2X10^6$ cells per well and stimulated the next day with either PAM_2CSK_4 (10nM) or R848

(20μM) for the indicated time intervals. Cell lysates were run on a denaturing gel and western blotted with anti-IRAK1 antibody (Cell Signaling Technology). GAPDH was used as an internal loading control.

Confocal Microscopy Imaging: HEK293T cells were seeded in 35-mm glass bottom tissue culture dishes (Mat Tek Corporation, MA, USA) at a density of 250,000 cells per dish. Next day, the cells were transiently transfected with the indicated plasmids. Twenty-four hours later, live cells were imaged on a Leica SP2 AOBS confocal laser-scanning microscope. A sequential scan was conducted for co-localization studies to rule out any possibility of cross talk between the different fluorophores.

Immunoprecipitations and immunoblots: HEK293T cells were transfected with MyD88-HA/S34Y-HA and either IRAK4-FLAG, IRAKI-FLAG or TRAF6-FLAG. Two days post-transfection, cells were harvested and the lysates were incubated overnight with polyclonal anti-HA antibody (SIGMA, MO, USA) and Poly-A Sepharose beads. Rabbit IgG was used as a control. The processed samples were run on an SDS gel, transferred to nitrocellulose membrane and probed with an HRP conjugated anti-FLAG antibody (SIGMA, MO, USA). The immunoprecipitation of MyD88-HA or S34Y -HA was checked by immunoblotting the same membrane with monoclonal HA antibody conjugated with HRP (SIGMA, MO, USA). Expression level of the proteins in whole cell lysates was checked by immunoblotting with HRP conjugated anti-FLAG antibody and HRP-conjugated anti-HA antibody.

Recruitment of MyD88 to the cell membrane: MyD88-deficient macrophage-like cell line was infected with retrovirus carrying either MyD88(WT)-citrine or S34Y-citrine. The

positive cells were sorted by flow cytometry and then allowed to recover for 2-3 days. The cells were seeded in 35-mm glass bottom tissue culture dishes at a density of 250,000 cells per dish. Next day, they were stimulated with either LPS (100 ng/ml) or poly I:C (20 μg/ml).

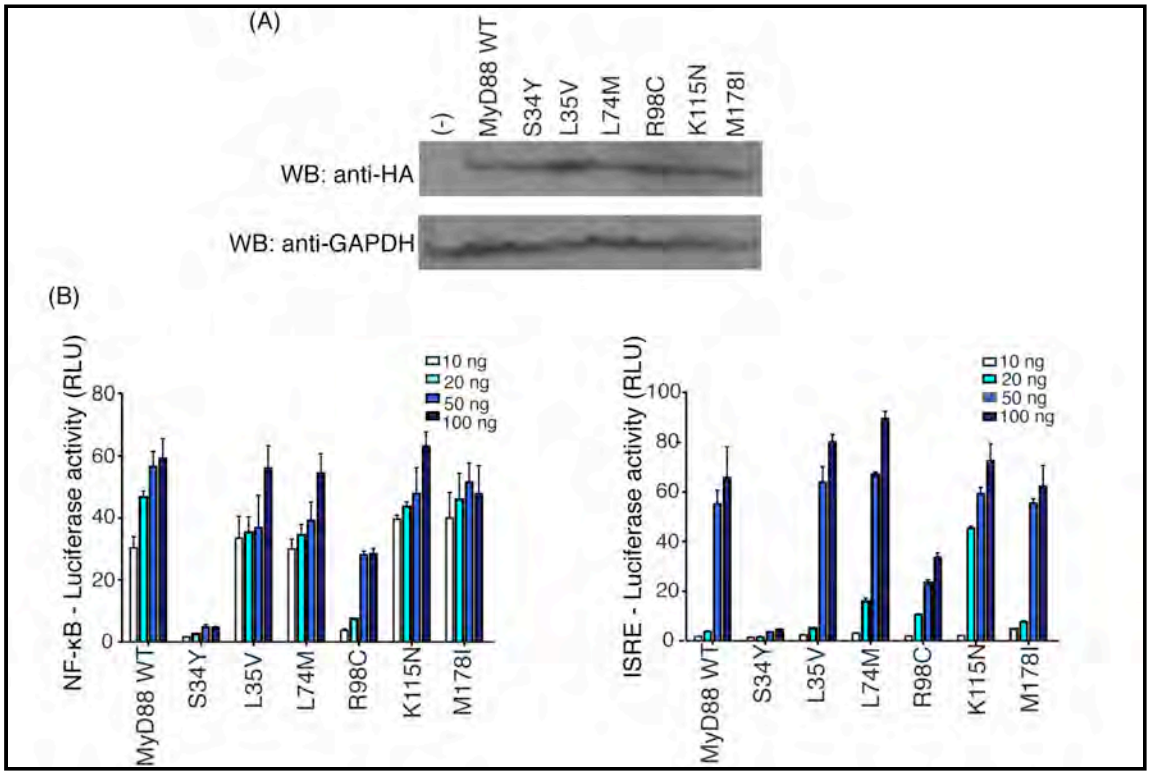
Fluorescence Resonance Energy Transfer (FRET): HEK293T cells were seeded in 35-mm glass bottom tissue culture dishes (Mat Tek Corporation, MA, USA) at a density of 150,000 cells per dish. Next day, the cells were transiently transfected with the indicated plasmids. FRET between the respective proteins was calculated by measuring sensitized emission (SE) fluorescence using the FRET SE wizard on the Leica SP2 confocal laser-scanning microscope. In each case, cerulean-tagged protein acted as the donor fluorophore while the citrine-tagged protein functioned as the acceptor fluorophore. Excitation wavelengths for the donor and acceptor were 405 nM and 514 nM respectively. The FRET efficiency is shown as a color-coded scale of values between 0 to 100%. The average of three selected regions of interest (ROIs) was used to quantitate the FRET efficiency for each sample.

Results

S34Y over-expression fails to activate both NF-κB and IRF5-dependent ISRE reporters: Human MyD88 has six non-synonymous single nucleotide polymorphisms (SNPs) in its coding region (FIG III.1). When expressed in HEK293T cells, all mutants are expressed at levels similar to wild type MyD88 (FIG III.3A), indicating no intrinsic protein stability

FIGURE III.3: MyD88 S34Y is unable to activate NF-κB and IRF-5 dependent ISRE reporters: (A) HEK293T cells were transfected with HA tagged versions of either wild type MyD88 or the different variants of MyD88 and expression levels were checked using anti-HA antibody. (B) HEK293T cells were transfected with the indicated MyD88 construct and either NF-κB-luciferase (left panel) or ISRE-luciferase + IRF5 (right panel). 24 hours later, luciferase activity was measured in cell lysates. Renilla-luciferase values were used to normalize for transfection efficiency. Results are reported as a mean of triplicate determinations ± S.D. The graphs are a representative of at-least two independent experiments.

FIGURE III.3: MyD88 S34Y is unable to activate NF-κB and IRF-5 dependent ISRE reporters:

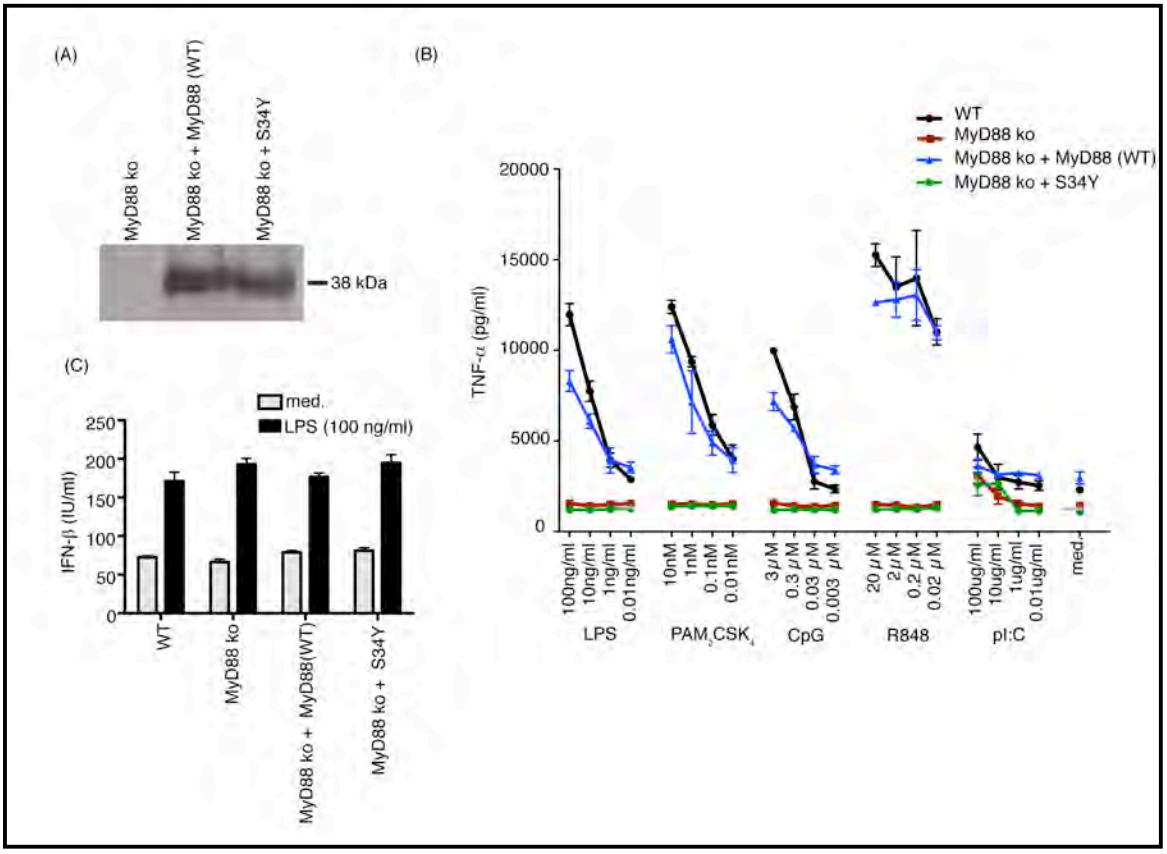


defect. To identify variants with altered function, we initially screened all the known SNPs in NF-κB reporter assays in HEK293T cells. Wild type MyD88, when overexpressed, can drive NF-κB reporter by itself. As evident in FIG III.3B (left panel), most MyD88 variants exhibited comparable (L35V, L74M, K115N, V178I) or slightly reduced (R98C) activity to wild type MyD88. One of the variants, S34Y, however, was completely inactive in driving the NF-κB reporter. Additionally, we tested the activity of the MyD88 variants in IRF5-mediated ISRE luciferase reporter assays. Interferon response factor 5 (IRF5) is a transcription factor whose activation is dependent on MyD88. Similar to the NF-κB luciferase assay, wild type MyD88 and all the variants except for S34Y were able to activate the ISRE reporter (FIG III.3B, right panel). S34Y was completely compromised in its ability to drive the ISRE luciferase reporter.

S34Y is unable to restore function in immortalized MyD88-deficient macrophage like cell line: To study these polymorphisms in a MyD88 deficient background, we transduced immortalized MyD88-deficient macrophage cell line (255) with a retrovirus MSCV2.2 (227) carrying an HA-tagged version of either wild type MyD88 or S34Y. This retrovirus transcribes GFP from an IRES element, thus making the positively transduced cells fluorescent. Western blotting with an anti-HA antibody confirmed comparable levels of expression of wild type MyD88 and S34Y (FIG III.4A). When stimulated with the different TLR ligands, immortalized wild type cells responded to all the tested ligands (FIG III.4B) and secreted appreciable amounts of the cytokine TNF-α. On the other hand, MyD88 knockout cells could not mount a response to any ligand (LPS, PAM_2CSK_4, CPG, R848) except poly I:C, which is a TLR3 ligand and signals independently of

FIGURE III.4: MyD88 deficient macrophages expressing S34Y have impaired cytokine production: (A) MyD88 deficient macrophages were infected with an IRES-GFP expressing retrovirus MSCV2.2, carrying HA tagged versions of either wild type MyD88 or the mutant S34Y. The positive cells were sorted based on GFP expression and the expression levels of the proteins were checked using an anti-HA antibody. (B) The cells were stimulated overnight with the indicated TLR ligands and the levels of TNF-α in the culture supernatants were analyzed by ELISA. Results are reported as a mean of triplicate determinations ± S.D. The graph is a representative of three independent experiments. (C) The same supernatants (only LPS stimulation) were analyzed for IFN-β levels by ELISA. Results are reported as a mean of triplicate determinations ± S.D. The graph is a representative of three independent experiments.

FIGURE III.4: MyD88 deficient macrophages expressing S34Y have impaired cytokine production but IFN-β production by the MyD88-independent pathway is intact.



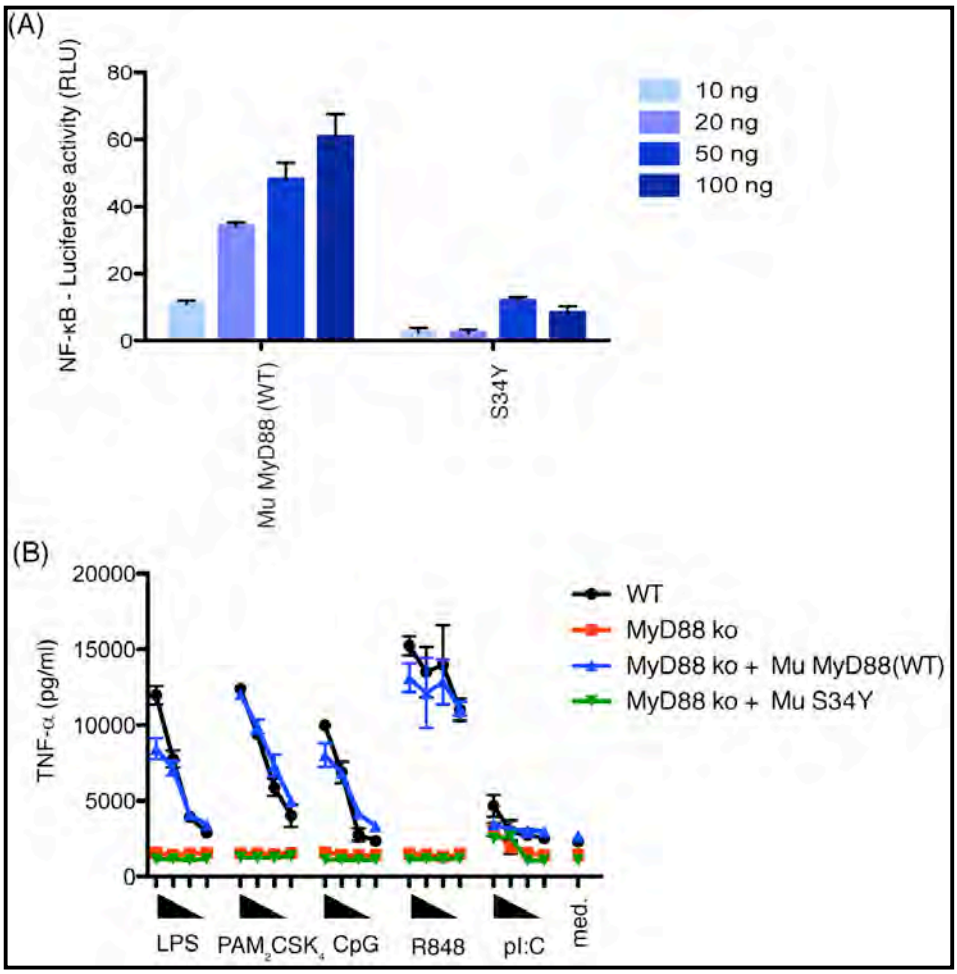
MyD88. As expected, MyD88 knockout cells expressing wild-type MyD88 behaved just like the wild-type cell line and responded to all the ligands (FIG III.4B). In contrast, cytokine production in cells expressing S34Y was completely ablated when stimulated with any of the MyD88 dependent ligands. They could, however respond just as well as the other cell lines to the TLR3 ligand poly I:C. A similar pattern was seen in the case of another pro-inflammatory cytokine, IL-6 (data not shown). To test whether MyD88-independent pathways are functional in S34Y expressing cells, we measured IFN-β production upon LPS stimulation, which is primarily mediated by the adapter TRIF. The cells expressing the mutant S34Y were able to produce IFN-β at levels similar to wild type cells (FIG III.4 C). Hence, S34Y impairs only MyD88-dependent TLR signaling, while cytokine production by MyD88-independent pathways remains unaffected.

Murine MyD88 S34Y is also a loss-of-function mutation: Serine at residue 34 is conserved between mouse and human MyD88. We thus wanted to test whether the same mutation in murine MyD88 affects MyD88 signaling. Like human MyD88, wild-type murine MyD88 can also activate NF-κB reporter when exogenously expressed in HEK293T cells. However, murine S34Y was unable to drive the NF-κB reporter (FIG III.5 A).

We also generated immortalized MyD88-deficient macrophage-like cell lines expressing either wild-type murine MyD88 or murine S34Y. These cell lines were then tested for their ability to produce pro-inflammatory cytokines in response to various TLR ligands. MyD88-deficient cells expressing wild-type murine MyD88 behaved like the wild-type immortalized cells and responded to all the tested ligands. Cells expressing

FIGURE III.5: Murine MyD88 S34Y is a loss-of-function mutation: (A) HEK293T cells were transfected with HA tagged versions of either wild type murine MyD88 or murine S34Y along with NF-κB-luciferase. Twenty-four hours later, luciferase activity was measured in cell lysates. Renilla-luciferase values were used to normalize for transfection efficiency. Results are reported as a mean of triplicate determinations ± S.D. The graphs are a representative of at-least two independent experiments. (B) MyD88 deficient macrophages were infected with an IRES-GFP expressing retrovirus MSCV2.2, carrying HA tagged versions of either wild type murine MyD88 or murine S34Y. The positive cells were sorted based on GFP expression. The cells were stimulated overnight with the indicated TLR ligands and the levels of TNF-α in the culture supernatants were analyzed by ELISA. Results are reported as a mean of triplicate determinations ± S.D. The graph is a representative of two independent experiments.

FIGURE III.5: Murine MyD88 S34Y is a loss-of-function mutation

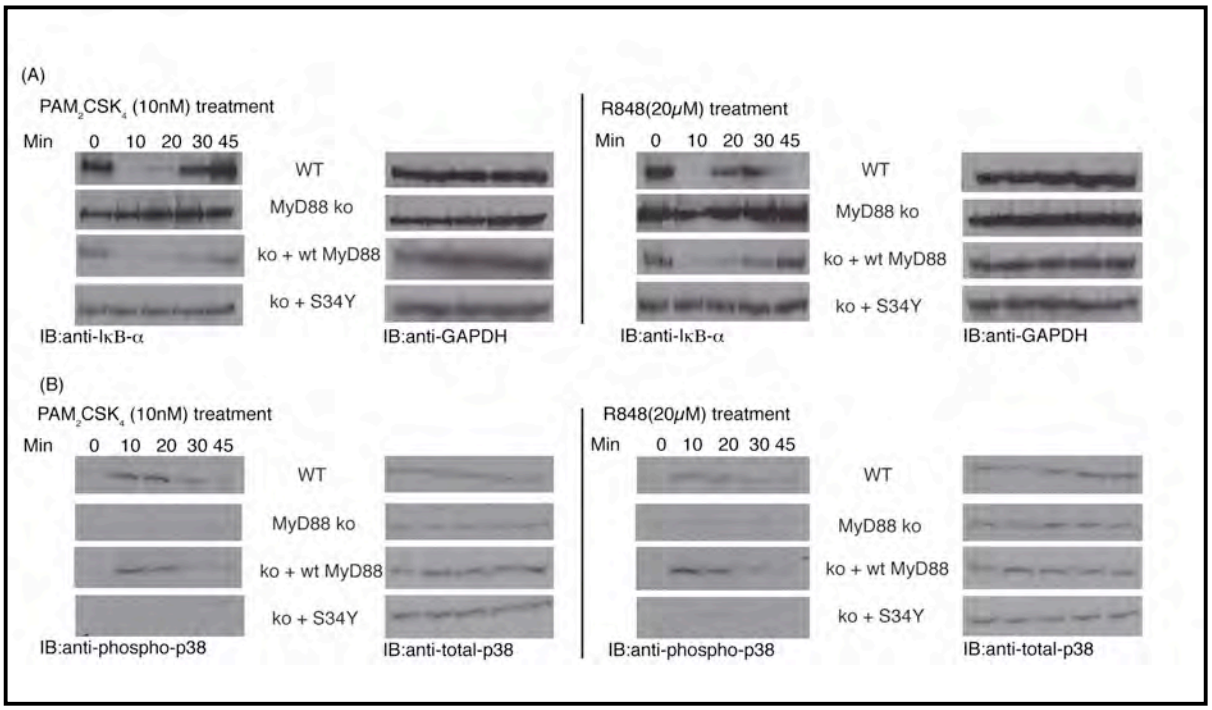


murine S34Y however, were unable to respond to any of the MyD88-dependent ligands (FIG III.5 B). Nevertheless, they could make TNF-α in response to poly I:C, which is a TLR3 ligand. Thus, like human MyD88, the mutation S34Y in mouse MyD88 also affects the signaling potential of MyD88.

NF-κB and MAPK (p38) activation is impaired in macrophages that express MyD88 S34Y: TLR engagement leads to the activation of a variety of transcription factors like NF-κB, AP-1 and interferon response factors (IRFs) (257), as well as mitogen activated protein kinases (MAPKs) (258), though NF-κB is the predominant transcription factor involved in MyD88 dependent signaling. IκB-α is the inhibitor of NF-κB and its degradation via ubiquitination implies activation of NF-κB. We thus tested the activation status of NF-κB in the cell lines expressing wild type human MyD88 or S34Y. Immortalized wild type, MyD88-deficient, MyD88-deficient cells expressing wild type MyD88 or S34Y were stimulated with either PAM_2CSK_4 (a TLR2 ligand) or R848 (a TLR7 ligand). These two ligands were selected based on the localization pattern of their receptors. TLR2 is a cell membrane receptor, while TLR7 is an endosomal TLR. Thus, using these ligands gives a complete picture of MyD88 dependent signaling from both sites-cell surface as well as the endosome. The immortalized wild type cells responded to both PAM_2CSK_4 and R848 and could degrade IκB-α starting as early as 10 minutes (FIG III.6A). MyD88 deficient cells, however, did not respond to either ligand and we could see no degradation of IκB-α in these cells. The cells expressing wild type MyD88 behaved like the wild type macrophages and could degrade IκB-α at 10 minutes. On the

FIGURE III.6: IκB degradation and MAPK (p38) activation is impaired in macrophages that express MyD88 S34Y: (A) NF-κB activation was examined by assessing IκB degradation. Cells were plated in a 6-well plate and stimulated with either 10nM PAM_2CSK_4 or 20μM R848 for the indicated time intervals. Cell lysates were made, run on a gel, transferred to nitrocellulose membrane and blotted with an anti-IκB antibody. GAPDH levels served as an internal loading control. (B) The same cell lysates were also analyzed for p38 MAPK activation by examining phosphorylation of p38 using anti-pp38 antibody. Total p38 MAPK levels acted as an internal loading control.

FIGURE III.6: IκB degradation and MAPK (p38) activation is impaired in macrophages that express MyD88 S34Y



other hand, the cells expressing S34Y were completely deficient in their ability to degrade IκB-α, when stimulated with either PAM_2CSK_4 or R848 (FIG III.6A).

Having demonstrated that S34Y expressing cells exhibit impaired NF-κB activation, we wondered if this defect in response included impaired MAPK activation. We selected p38 MAPK as a prototype of MAPK activation and examined its phosphorylation status as a marker for its activation. Phosphorylation of p38 MAPK followed the same pattern as NF-κB activation and S34Y expressing cells did not phosphorylate p38 upon stimulation with either PAM_2CSK_4 or R848 (FIG III.6B). Thus, S34Y is a non-functional mutation that renders MyD88 completely defective in signaling.

Signaling through MyD88 S34Y does not lead to the formation of a functional signaling complex: Once a TLR is activated by its respective ligand, MyD88 recruits interleukin-1 receptor associated kinase (IRAK)-1 and IRAK-4 to the receptor complex through death domain interactions. IRAK-4 then phosphorylates and activates IRAK-1, which can then autophosphorylate itself. This hyperphosphorylated form of IRAK1 then undergoes ubiquitination-mediated degradation (170, 259). These phosphorylation and degradation events are thus hallmarks of an active signal transduction pathway.

We initially hypothesized that the S34Y mutant MyD88 is unable to bind either IRAK-1 or IRAK-4. However, co-immunoprecipitation assays in HEK293T cells revealed that the presence of the S34Y mutation does not affect the ability of MyD88 to bind either IRAK-1 or IRAK-4 (FIG III.8). We thius tested whether the activation signal is effectively relayed to the downstream effectors by analyzing the degradation of

IRAK-1. Treatment with either PAM_2CSK_4 or R848 results in degradation of IRAK-1 as early as 5 minutes, in the wild type immortalized cells (FIG III.7). As expected, the MyD88-deficient cells are not able to form an active signalosome and hence cannot degrade IRAK-1. MyD88 deficient cells expressing wild type MyD88 behaved similarly to wild type cells and could respond to both the tested ligands. The cells expressing S34Y, however, were unable to degrade IRAK-1 upon stimulation with PAM_2CSK_4 or R848. Hence, even though MyD88 S34Y is able to interact with IRAK-4 and IRAK-1, these interactions are not functional and thus the activation signal is not conveyed to the downstream effectors.

Having shown that MyD88 S34Y is completely defective in signaling through the TLRs, we next sought an explanation for this phenotype. We hypothesized that the presence of this mutation might affect the interaction of MyD88 with its known binding partners. MyD88 is known to bind to the serine-threonine kinases IRAK4 and IRAK1 via death domain-death domain interactions. It can also bind to the E3 ubiquitin ligase TRAF6. We thus tested the binding of MyD88 S34Y with IRAK4, IRAK1 and TRAF6 by co-immunoprecipitation assays in HEK293T cells. These studies however, did not reveal a defect in the ability of MyD88 S34Y to interact with any of the known binding partners of MyD88 (FIG III.8). Like wild-type MyD88, S34Y was able to interact with IRAK4, IRAK1 and TRAF6.

FIGURE III.7: Macrophages expressing S34Y are unable to degrade IRAK1 effectively

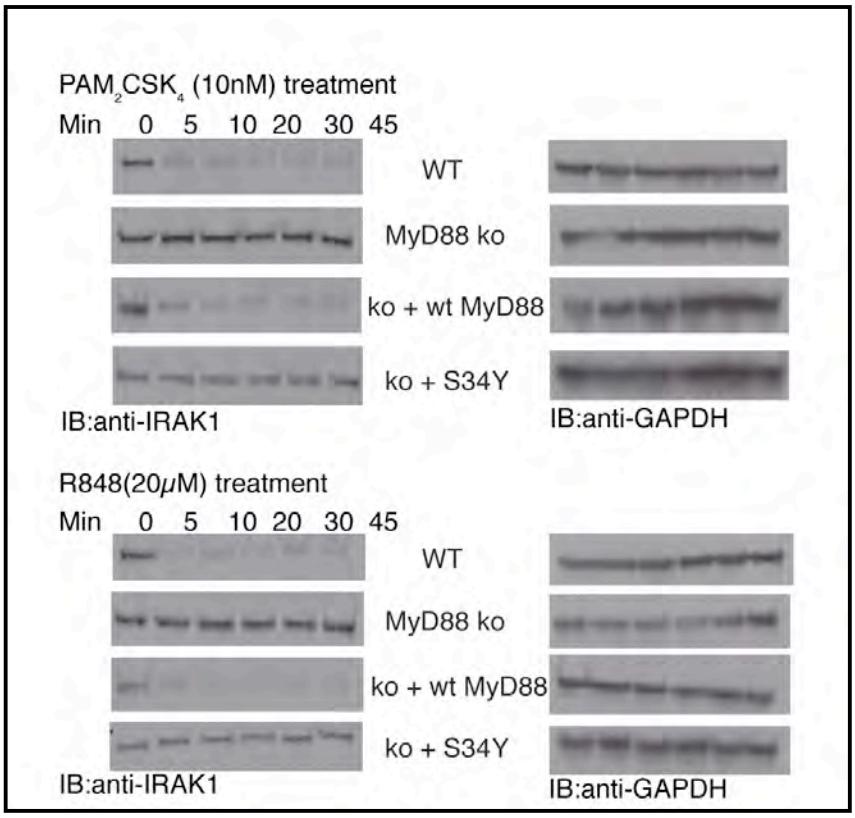


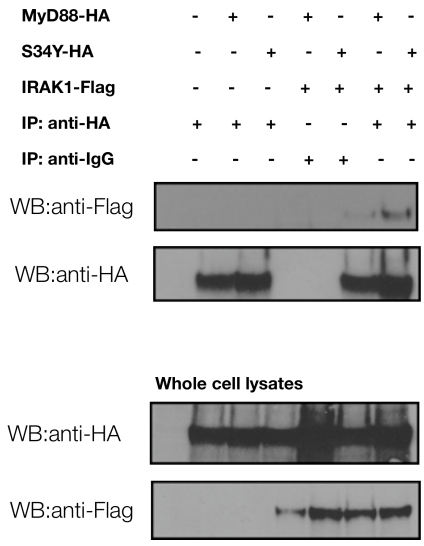
FIGURE III.7: Macrophages expressing S34Y are unable to degrade IRAK1 effectively: IRAK1 degradation was examined in the reconstituted cell lines as a marker of an active signal complex formation. Cells were plated in a 6-well plate and stimulated with either 10nM PAM_2CSK_4 or 20μM R848 for the indicated time intervals. Cell lysates were made, run on a gel, transferred to nitrocellulose membrane and blotted with an anti-IRAK1 antibody. GAPDH levels served as an internal loading control.

FIGURE III.8: MyD88 S34Y retains the ability to bind IRAK4, IRAK1 and TRAF6. HEK293T cells were transfected with the indicated plasmids. Two days after transfection, supernatants were removed, and cells were lysed. Anti-HA polyclonal antibody was used for immunoprecipitation (IP). Immunoprecipitates were loaded on a denaturing gel, transferred to nitrocellulose membrane, and visualized with a monoclonal anti-FLAG-HRP antibody. The immunoprecipitation of MyD88-HA or S34Y-HA was confirmed by immunoblotting the membrane with anti-HA antibody. The same antibodies were used to check the level of expression of the proteins in whole cell lysates. IB: immunoblot

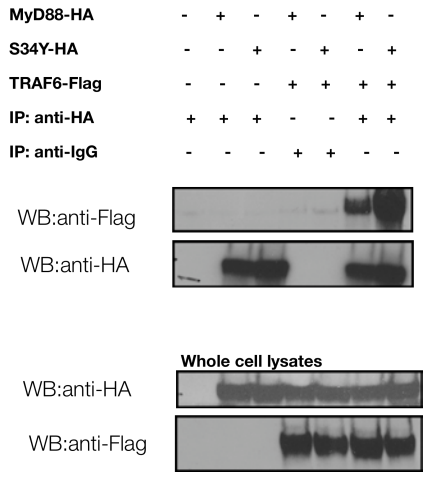
FIGURE III.8: MyD88 S34Y retains the ability to bind IRAK4, IRAK1 and TRAF6

(A) IRAK4-MyD88/S34Y interaction

(B) IRAK1-MyD88/S34Y interaction



(C) TRAF6-MyD88/S34Y interaction

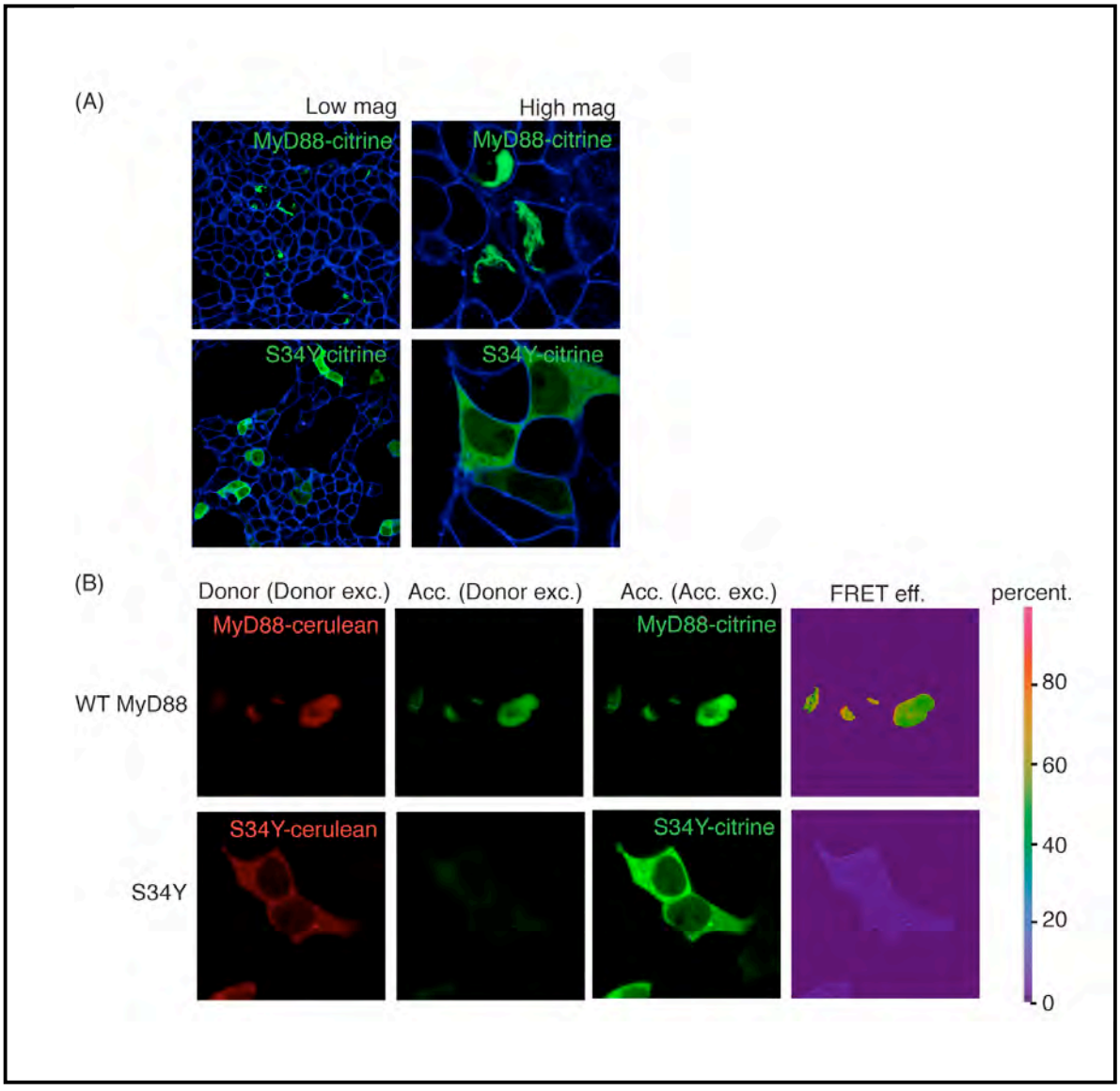


S34Y has a different localization pattern than wild type MyD88: Since the S34Y mutation did not seem to affect any interactions, we next tested whether MyD88 S34Y has a different localization pattern than wild type MyD88. In 1998, Jaunin *et al.* demonstrated that wild type MyD88, when expressed in Hela cells, is localized in fibrillar aggregates (260). A number of other groups reported similar findings, including our own group (139, 261). Indeed, we found that when citrine-tagged wild type MyD88 was expressed in HEK293T cells, it localized to aggregates or distinct foci in the cytoplasm, with almost no diffuse cytoplasmic expression (FIG III.9A, upper panel). S34Y however, was completely diffuse in the cytoplasm, with no aggregate structures (FIG III.9A, lower panel). To confirm that the aggregates we see in the case of WT MyD88 are indeed oligomeric complexes of MyD88, we carried out FRET analysis with cerulean and citrine-tagged MyD88 (WT). Both cerulean and citrine-tagged MyD88 localized to the condensed structures in the cell and a very strong FRET signal could be observed between the two differentially tagged WT MyD88s (FIG III.9B). In contrast, cerulean and citrine-tagged S34Y co-localized in the cytoplasm with a complete absence of aggregates. In this case, the FRET efficiency was significantly lower than the WT experimental set, thus demonstrating that the S34Y mutation affects the ability of MyD88 to form its own oligomeric complexes.

Some recent studies suggest that the activity/function of MyD88 might be related to its correct subcellular targeting or the ability to aggregate in the cytoplasm. A study by Nishiya *et al.* demonstrated that the signaling function of MyD88 is dependent on its correct cellular localization and the entire non-TIR region of MyD88 is responsible for its

FIGURE III.9: Unlike WT MyD88, S34Y does not form oligomeric aggregates (A) Citrine-tagged wild type MyD88 or S34Y were transfected into HEK293T cells. Next day, live cells were imaged using standard confocal procedures. Cellmask^{TM} (Invitrogen) plasma membrane stain is used to mark the cell membranes (pseudocolored blue). (B) HEK293T cells were transiently transfected with the indicated plasmids. FRET between cerulean and citrine tagged WT MyD88 or cerulean and citrine tagged S34Y was calculated by measuring sensitized emission (SE) fluorescence using FRET SE wizard on the Leica SP2 confocal laser-scanning microscope. The FRET efficiency is shown as a color-coded scale of values between 0 to 100%.

FIGURE III.9: Unlike WT MyD88, S34Y does not form oligomeric aggregates

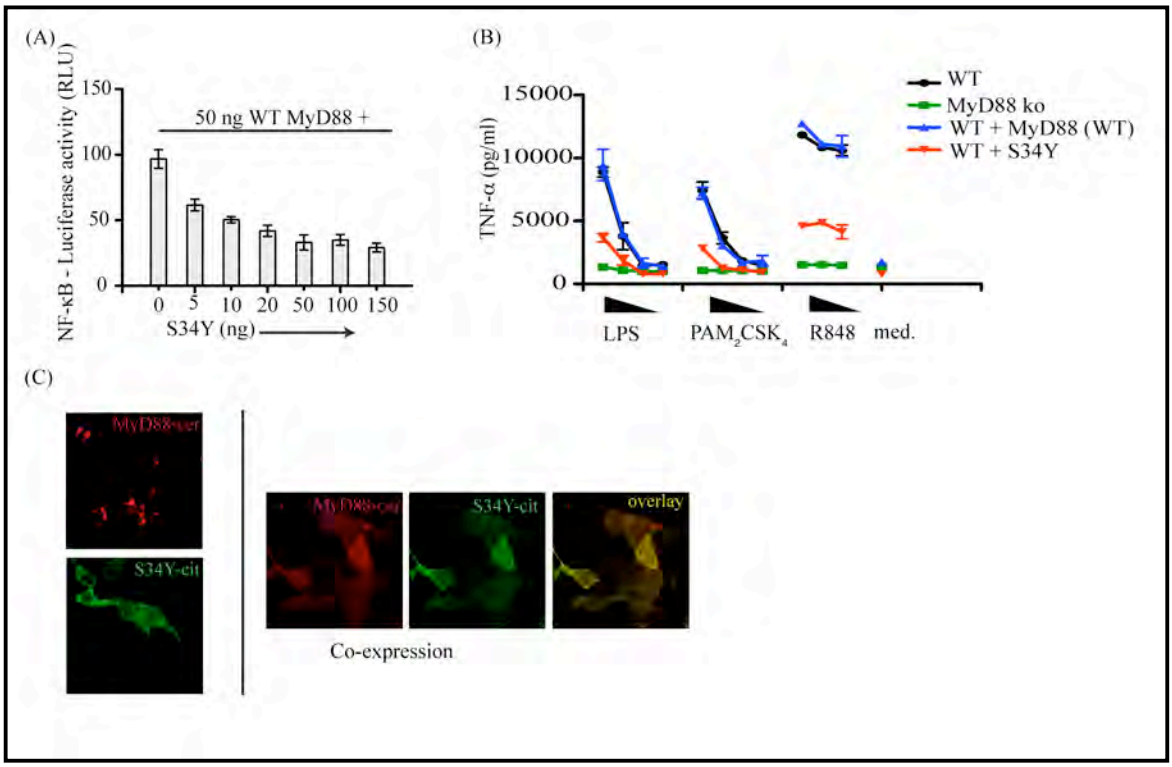


correct localization (262). They further show that the deletions or truncations in this region that change the localization of MyD88 making it diffusely expressed in the cytoplasm, also render MyD88 inactive in NF-κB reporter assays. Hence, in accordance with previously published reports, we show that proper localization of MyD88 is indispensible for its function. In addition, we show that a naturally occurring loss-of-function mutation of MyD88 changes the subcellular localization of MyD88.

MyD88 S34Y is a dominant negative mutation: Although MyD88 S34Y retains its ability to interact with the known binding partners of MyD88, these interactions are not functional and the activating signal is not relayed to the downstream effectors. We tested whether the expression of S34Y mutant interferes with the function of wild type MyD88 by sequestering the signaling proteins, thus blocking signal transduction. We co-transfected increasing amounts of DNA encoding for MyD88 S34Y, keeping the DNA amount of wild-type MyD88 constant and assessed NF-κB reporter activity. S34Y mutant reduced the ability of wild type MyD88 to drive the reporter in a dose-dependent fashion (FIG III.10A). Using retroviral infections we expressed either wild type MyD88 or S34Y in wild type immortalized cells. These cells were then tested for their responsiveness to various TLR ligands by measuring TNF-α in the supernatants of the stimulated cells. Cells expressing wild type MyD88 (in addition to the endogenous MyD88 allele) behaved just like the wild type cells and no change could be observed in terms of cytokine production (FIG III.10B). However, cells expressing S34Y (in addition to the

FIGURE III.10: MyD88 S34Y is a dominant negative mutation: (A) HEK293T cells were transfected with the 50 ng of wild type MyD88 construct and the indicated amounts of MyD88-S34Y construct, along with NF-κB-luciferase. Twenty-four hours later, luciferase activity was measured in cell lysates. Renilla-luciferase values were used to normalize for transfection efficiency. Results are reported as a mean of triplicate determinations ± S.D. (B) Wild type immortalized macrophages were infected with MSCV2.2, carrying HA tagged versions of either wild type MyD88 or the mutant S34Y. The positive cells were sorted based on GFP expression. The cells were stimulated overnight with the indicated TLR ligands and the levels of TNF-α in the culture supernatants were analyzed by ELISA. Results are reported as a mean of triplicate determinations ± S.D. (C) HEK293T cells were transfected with MyD88-cerulean or S34Y-citrine, either by themselves (left panel) or co-transfected together (right panel). The cells were imaged using standard confocal procedures.

FIGURE III.10: MyD88 S34Y is a dominant negative mutation



endogenous MyD88 allele) had a phenotype intermediate to wild type and MyD88-deficient cells. They exhibit significantly reduced cytokine production in response to all tested ligands. Thus, we demonstrate by two different assays, that S34Y mutant can reduce the signaling activity of wild type MyD88.

We wondered whether co-expression of S34Y would affect the localization of wild type MyD88. MyD88 presents as aggregates in the cytoplasm, whereas S34Y is diffuse cytoplasmic. We co-expressed wild type MyD88 tagged with cerulean and S34Y tagged with citrine in HEK293T cells. In the presence of S34Y, wild type MyD88 is unable to form condensed foci in the cytoplasm. Instead, like the mutant, wild type MyD88 is also diffused (FIG III.10C). The ability of MyD88 to form aggregates is linked to its function and by disrupting this capacity of MyD88, S34Y renders wild type MyD88 non-functional. MyD88 S34Y thus acts as a dominant negative mutation.

S34Y does not traffic to the cell membrane: When citrine-tagged MyD88 (WT) or S34Y are expressed in MyD88-deficient macrophages, they both retain their characteristic localization pattern. WT MyD88 is present in condensed aggregates whereas S34Y is diffused cytoplasmic (FIG III.11A, left panel). To investigate ligand-dependent MyD88 recruitment to the site of activation, we stimulated the cells with the TLR4 ligand, LPS. Forty-five minutes post stimulation, MyD88 could be observed at the cell membrane (FIG III.11A, right panel). This mobilization of MyD88 requires activation by a MyD88 dependent ligand, as poly I:C, a TLR3 ligand, failed to induce mobilization (data not shown). However, no change could be observed in S34Y expressing cells. LPS stimulation did not lead to S34Y trafficking to the membrane (FIG III.11, right panel).

FIGURE III.11: LPS stimulation of MyD88-deficient macrophages expressing either MyD88-citrine or S34Y-citrine.

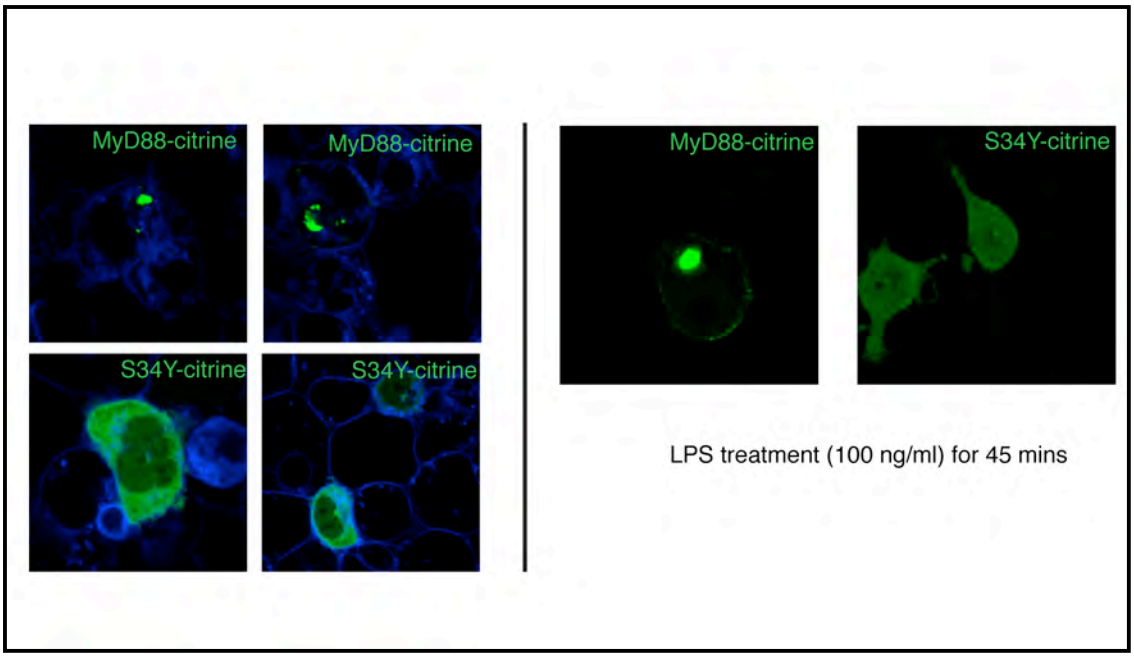


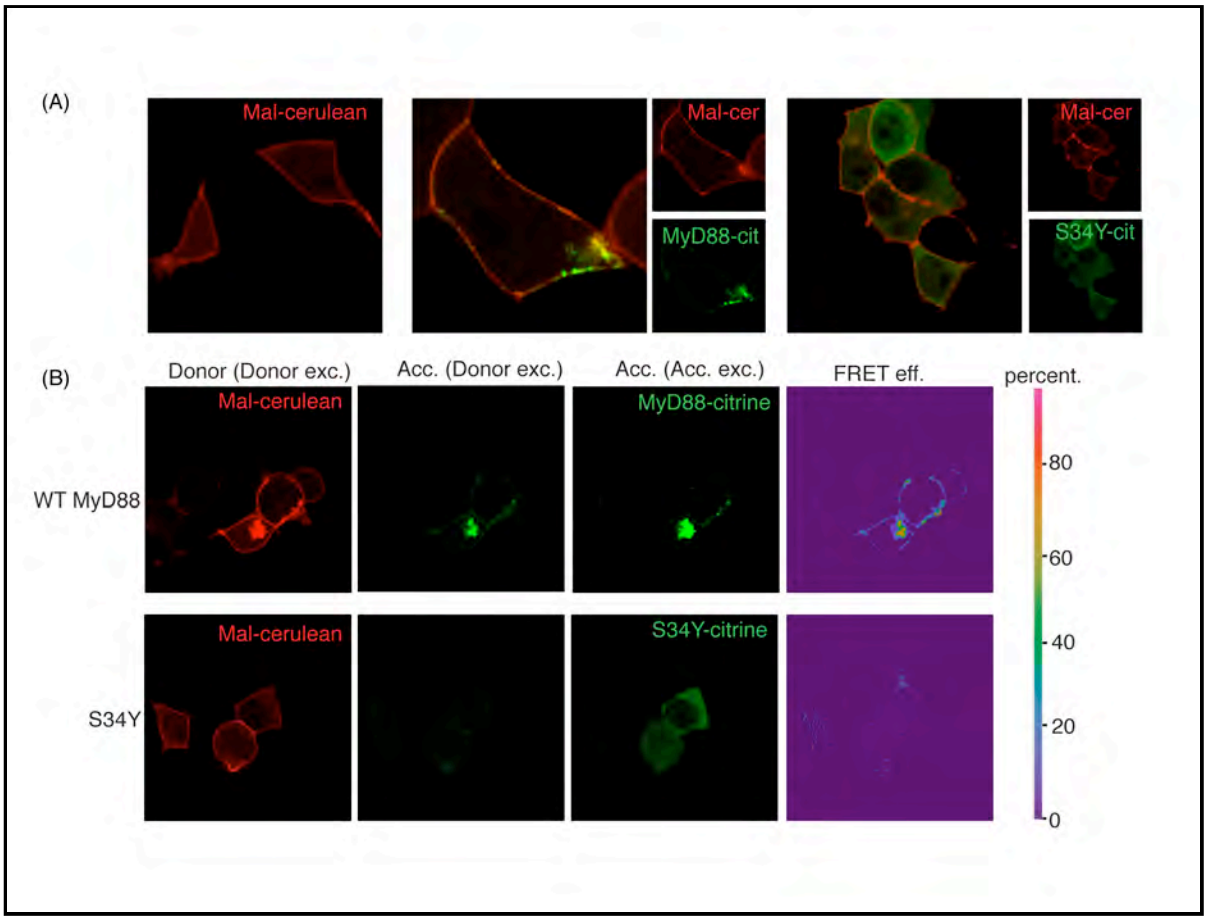
FIGURE III.11: LPS stimulation of MyD88-deficient macrophages expressing either MyD88-citrine or S34Y-citrine. Macrophages expressing either citrine-tagged MyD88(WT) or S34Y were imaged using standard confocal microscopy. Cellmask^{TM} (Invitrogen) plasma membrane stain is used to mark the cell membranes (pseudocolored blue). The cells were either left untreated (left panel) or stimulated with LPS (100 ng/ml) for 45 minutes (right panel)

We also tested the co-localization of MyD88/S34Y with the adapter Mal. When wild-type MyD88 is co-expressed with Mal/TIRAP, it relocalizes from the condensed foci to the cell membrane where it co-localizes with Mal (139). We had similar observations when we transiently transfected Mal-cerulean with wild type MyD88-citrine. Instead of being localized to distinct foci in the cytoplasm, MyD88 was co-localized at the cell membrane with Mal (FIG III.12A). As a control, we co-expressed Mal D96N with wild type MyD88. Mal D96N is a naturally occurring mutation of Mal that interferes with MyD88 binding (263). As expected, Mal D96N was unable to traffic wild type MyD88 to the membrane (chapter II, FIG II.10). In the case of S34Y co-expressed with Mal, S34Y retained its diffuse cytoplasmic expression and did not get recruited to the membrane. Since co-immunoprecipitation assays did not reveal a defect in the binding of MyD88 S34Y with Mal, we employed FRET analysis to shed some light on the interactions between Mal and MyD88/S34Y. WT MyD88 and Mal co-expression resulted in a strong FRET signal limited to the cell membrane, the site of interaction (FIG III.12B). In contrast, S34Y and Mal generated a comparatively weaker FRET signal, in the cytoplasm. Thus, even though S34Y interacts with Mal in the cytoplasm, it is unable to be recruited to the membrane, the functional site for Mal and MyD88 dependent receptor activation. The recruitment of wild-type MyD88 but not S34Y to the membrane illustrates the fact that the observed aggregates of MyD88 are functional oligomers and not merely an artifact of overexpression.

MyD88 S34Y fails to assemble into Myddosomes: Recently, the crystal structure of the MyD88-IRAK4-IRAK1 death domain complex has been determined (246). These three

FIGURE III.12: MyD88 S34Y does not traffic to the cell membrane upon co-expression with Mal. (A) HEK293T cells were transfected with cerulean-tagged Mal either by itself or in conjunction with citrine-tagged wild type MyD88 or S34Y. Next day the cells were imaged using standard confocal microscopy procedures. (B) HEK293T cells were transiently transfected with the indicated plasmids. FRET between Mal and WT MyD88 or S34Y was calculated by measuring sensitized emission (SE) fluorescence using FRET SE wizard on the Leica SP2 confocal laser-scanning microscope. The FRET efficiency is shown as a color-coded scale of values between 0 to 100%.

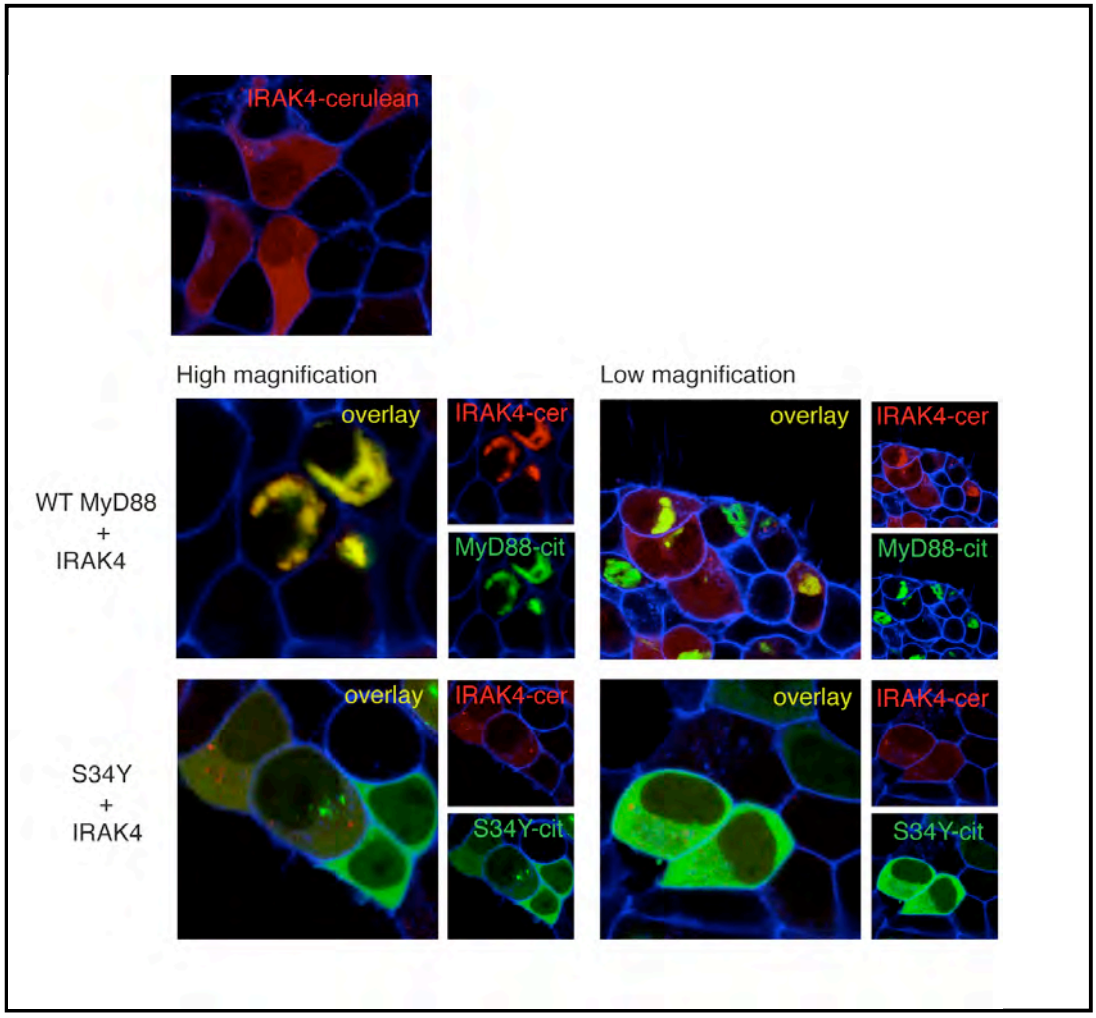
FIGURE III.12: MyD88 S34Y does not traffic to the cell membrane upon co-expression with Mal.



proteins are present in a ternary complex consisting of six MyD88, four IRAK4 and four IRAK2 death domains. These oligomeric structures have been named “Myddosomes” by the authors. We hypothesized that the foci that wild type MyD88 seems to localize to (FIG III.9A), may be Myddosomes and the S34Y mutation ablates the ability of MyD88 to form these oligomeric structures. According to Lin *et al.*, the assembly of the myddosome is a sequential process (246). While the MyD88 death domain by itself is able to form oligomers at high concentration, IRAK4 and IRAK2 death domains are monomeric in solution. However, in the presence of MyD88, IRAK4 is oligomerized and forms a complex with oligomeric MyD88. This then acts as a platform for recruitment of IRAK2 and IRAK1. The authors believe that this hierarchical assembly is essential for bringing the IRAKs into a proper conformation for their phosphorylation and activation (246). In our study, when cerulean-tagged IRAK4 was expressed in HEK293T cells, it was present diffused in the cytoplasm (FIG III.13, top panel). However, when we co-expressed citrine-tagged wild type MyD88, we observed co-localization of IRAK4 with MyD88 in distinct foci which we believe are Myddosomes (FIG III.13, middle panel). In contrast, when MyD88 S34Y was co-expressed with IRAK4, we observed both proteins to co-localize in the cytoplasm and have a diffuse expression pattern (FIG III.13, lower panel). Similar observations were made for IRAK2 and MyD88 (wild type or S34Y) co-localization (data not shown). Thus, in agreement with Lin *et al.*, we show that IRAK4 and IRAK2 colocalize with MyD88 and form “Myddosomes”. The S34Y mutation abrogates the ability of MyD88 to form these oligomeric structures.

FIGURE III.13: S34Y fails to assemble into a Myddosome: Cerulean-tagged IRAK4 (pseudocolored red) was transfected either by itself (top panel) or in conjunction with citrine-tagged wild type MyD88 (middle panel) or S34Y (bottom panel) (pseudocolored green) into HEK293T cells. Next day the cells were imaged using sequential scanning and co-localization (yellow) of the two proteins was assessed. Cellmask^{TM} (Invitrogen) plasma membrane stain is used to mark the cell membranes (pseudocolored blue). These images are representative of at least three independent experiments.

FIGURE III.13: S34Y fails to assemble into a Myddosome



To confirm the formation of a complex between MyD88 and IRAK4, we examined the occurrence of FRET between IRAK4 and WT MyD88. Cerulean-tagged IRAK4 and citrine-tagged MyD88 were used as the donor and acceptor respectively. A strong FRET signal was detected between IRAK4 and WT MyD88 in the regions of colocalization (FIG III.14A, upper panel). Three different regions of interest (ROIs) in the FRET efficiency image were selected and their average was used to denote the FRET efficiency of the sample (FIG III.14B). FRET between WT MyD88 and IRAK4 occurred at a very high efficiency of 36%. Since the efficiency of FRET is inversely proportional to the distance between the two participating proteins, such a high FRET signal implies that IRAK4 and WT MyD88 interact strongly with each other and are present in a complex. Similar FRET experiments with the mutant MyD88 S34Y, however, yielded quite a low FRET signal (FIG III.14A, lower panel) with an average efficiency of 8% (FIG III.14B), suggesting that though both IRAK4 and S34Y are present diffused in the cytoplasm they are not in close proximity and do not form a functional complex, required for the function of MyD88.

The presence of a bulky amino acid at residue 34 disrupts the formation of a Myddosome: The published crystal structure of the Myddosome provides an opportunity to investigate the effect the mutation S34Y may have on the structure of MyD88. FIG III.15 (A) shows the six death domains of MyD88 in the Myddosome. The residue 34 is colored red. The surface representation (FIG III.15 A, right panel) shows that the residue 34 is buried within the interfaces. A change in the amino acid at residue 34 might thus affect the packing of these molecules. We tested this hypothesis by investigating the

effect of mutations at this residue in an NF-κB reporter assay (FIG III.15 B). As expected, WT MyD88, with serine at the 34th position was able to drive the NF-κB reporter. S34Y, which has a big aromatic residue at this position, was unable to acivate the reporter. If serine is mutated to alanine (alanine is structurally similar to serine) at this positon, there is no effect on the ability of MyD88 to drive the NF-κB reporter i.e., S34A behaves just like WT MyD88. However, upon changing the serine to phenylalanine (S34F), an amino acid that resembles tyrosine and is a big aromatic residue, MyD88 loses its ability to drive the NF-κB reporter. This suggests that the presence of a large bulky amino acid at residue 34 might lead to steric hindrance and may affect the formation of MyD88 oligomers and subsequently the Myddosome.

FIGURE III.14: IRAK4-S34Y FRET signal is considerably reduced as compared to IRAK4-MyD88 (wt): (A) HEK293T cells were transiently transfected with the indicated plasmids. FRET between IRAK4-cerulean (pseudocolored red) and WT MyD88-citrine or S34Y-citrine (pseudocolored green) was calculated by measuring sensitized emission (SE) fluorescence using FRET SE wizard on the Leica SP2 confocal laser-scanning microscope. The FRET efficiency is shown as a color-coded scale of values between 0 to 100%. (B) FRET efficiency for both the samples (IRAK4-WT MyD88 and IRAK4-S34Y) was obtained by calculating the average efficiency of different regions in each FRET image. This experiment is a representative of two independent experiments.

FIGURE III.14: IRAK4-S34Y FRET signal is considerably reduced as compared to IRAK4-MyD88 (wt)

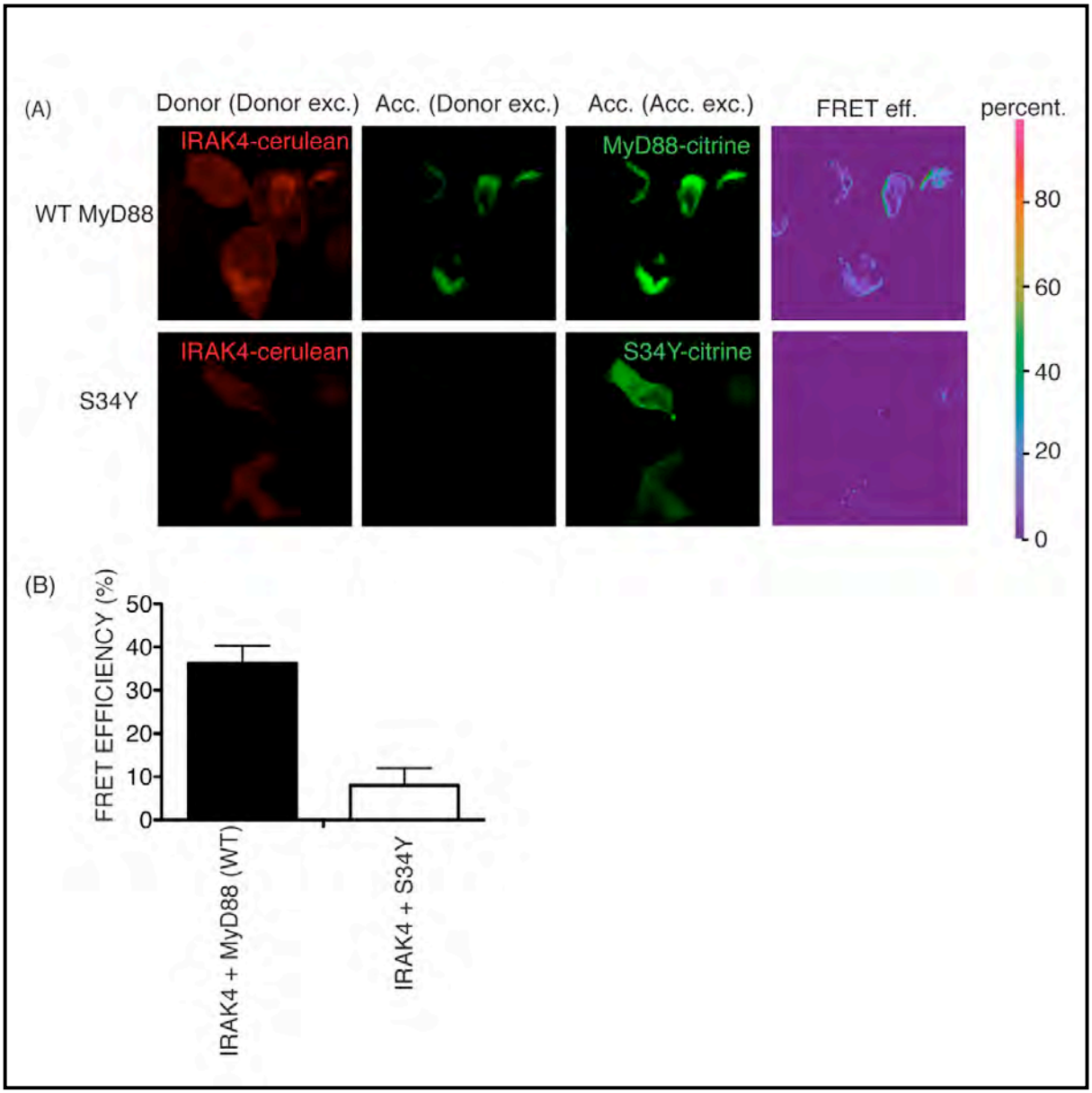
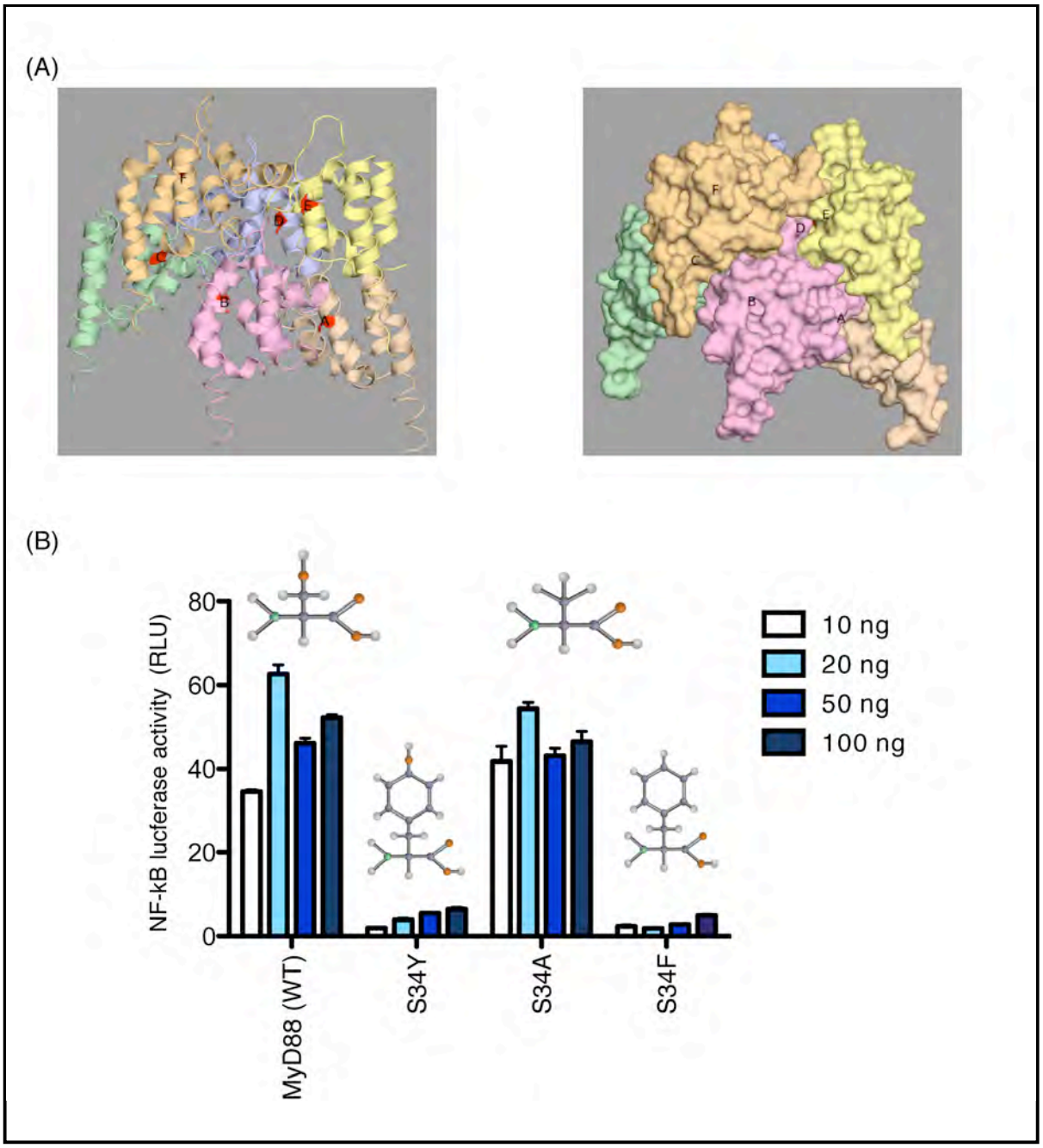


FIGURE III.15: Residue 34 is buried within the interfaces in the Myddosome structure: (A) A ribbon representation (left panel) of the six MyD88 death domains in the Myddosome structure (labeled A-F). Residue 34 is colored red. Right panel: The same six death domains shown as surface representation. Residue 34 (red) is buried within the interfaces (B) HEK293T cells were transfected with the indicated MyD88 constructs along with NF-κB-luciferase reporter. Twenty-four hours later, luciferase activity was measured in cell lysates. Renilla-luciferase values were used to normalize for transfection efficiency. The reporter only sample was used as the background and the depicted values are a ratio of the actual values to the background noise. Results are reported as a mean of triplicate determinations ± S.D.

FIGURE III.15: Residue 34 is buried within the interfaces in the Myddosome structure



Discussion

Toll-like receptors are critical for mounting an effective immune response against invading pathogens. The signaling cascade that ensues upon ligand recognition is marked by finely orchestrated molecular interactions between the receptor and the TIR domain containing adapter proteins, as well as various downstream kinases and effector molecules. SNPs in the coding regions of proteins involved in such a signaling pathway can sometimes alter the functional properties of the protein, thus affecting the outcome of the signal transduction pathway.

Human MyD88 has 6 non-synonymous SNPs in its coding region, making it one of the most polymorphic TLR adapter proteins. However, the effect of these SNPs on the function of MyD88 remains poorly understood. In our study, we have screened these SNPs with the hypothesis that some of them may alter the signaling potential of MyD88. We report that one of the polymorphisms, S34Y that lies in the death domain of MyD88, is a complete loss of function variant of MyD88. Of note, we did not observe a dramatic phenotype in MyD88 R98C, in contrast to results reported elsewhere (264).

Cellular biological studies of signaling molecules are typically done in easily transfectable cells, like HEK293, which have little in common with the innate immune cells they are thought to represent. Typically, studies in HEK293 cells are inherently compromised due to the presence of the wild-type alleles. Using immortalized macrophages from MyD88 knockout animals, we have successfully reconstituted both fully functional macrophage cell lines and mutant macrophage lines using viral expression systems. These extensive studies have shown that effector cells are

extraordinarily destabilized by both the absence of MyD88 (as would have been predicted), and by S34Y. Indeed, some of the most important effector functions, in addition to the activation of NF-κB and its associated downstream genes (such as TNFα), were clearly compromised by the expression of S34Y, including p38 activation downstream from TLRs 2 and 7. When one takes into account the variety of immune defects that we have documented in macrophages expressing S34Y, it is no surprise that S34Y appears to be a rare mutation, rather than a true "polymorphism", and is similar in incidence to MyD88 deficiency (240). Indeed, we screened 267 samples from healthy individuals, and 105 DNA samples from children with meningococcal sepsis, and failed to find a single individual in either group with even a single mutant allele (data not shown). Although knockout mice with MyD88 deficiency seem to survive well in our "clean" animal facilities, there seems to be little question that in real life, functional MyD88 is a necessity.

The death domain (and the intervening region between the death and TIR domains) of MyD88 has earlier been implicated in the proper localization of MyD88 (262). Nishiya *et al.* demonstrated that the entire non-TIR region of MyD88 is important to maintain the distinct and condensed pattern of MyD88 localization. In fact, there have been other reports that illustrate similar findings, that indeed MyD88 is present in aggregate structures in the cytoplasm (139, 260). Furthermore, this seems to be a characteristic of MyD88 that is conserved across species (265). A recent study by Liu *et al*. show that zebrafish MyD88 is also expressed as large condensed spots in the cytoplasm of cells and this observation is reproducible in different cell lines. Moreover,

there seems to be a correlation between the localization and function of MyD88. Both Nishiya *et al.* and Liu *et al.* find that deletions or mutations that change the expression pattern of MyD88 from condensed spots to diffuse cytoplasmic localization, also affect the function of MyD88, making it inactive in signaling. In view of all these studies, we compared the localization pattern of wild type MyD88 and the variant S34Y. As expected, wild type MyD88 was localized to large condensed spots, in the cytoplasm. However, S34Y had a completely different pattern of expression. It was present diffused in the cytoplasm and did not form any aggregates.

The condensed spots that MyD88 seems to localize to have as yet not been associated with any subcellular organelle or structure. In light of the report by Lin *et al.* we propose that these aggregate structures are Myddosomes; helical oligomers of MyD88, IRAK4 and IRAK2 or MyD88, IRAK4 and IRAK1. However, there is currently no method to study the exact stoichiometry of IRAK4/IRAK2/MyD88 aggregates inside the cell and hence we cannot formally demonstrate that these aggregates are Myddosomes. The presence of fluorescent oligomers is reminiscent of the ability to view other large protein assemblies devoted to innate immunity, such as the ability to view pyroptosome formation using fluorescent constructs of ASC that are expressed in the presence of an NLR and caspase 1 [the so-called “speckle” assay (60)]. Consistent with the findings by Lin *et al.* we show that MyD88 by itself is present in aggregates while IRAK4 is diffusely expressed in the cytoplasm. However, on co-expressing them together, IRAK4 co-localizes to these distinct foci along with MyD88. Most importantly, the mutant MyD88 S34Y is unable to form Myddosomes with IRAK4. The crystal

structure of the Myddosomes resolved by Lin *et al.* used just the death domains of the three proteins involved. We, on the other hand, have employed full-length proteins in all our studies and show for the first time the formation of Myddosomes in cells. More importantly, we identify a natural loss of function mutation in MyD88 that disrupts its ability to form Myddosome.

CHAPTER IV

DISCUSSION & PERSPECTIVES

As a component of the innate immune system, TLRs are at the interface of the host and the environment. Any small variation in these genes can have a profound impact on inflammatory disease pathogenesis or the outcome of the signaling pathways mediating host defense. The work described in this thesis underscores the importance of such variation in TLR adapter proteins. With the help of two naturally occurring mutations in the adapter proteins Mal/TIRAP and MyD88, I illustrate two differing accounts of molecular changes that a polymorphism can bring about in a protein and hence affect its signaling capability.

Chapter II focuses on the SNPs in the adapter Mal, the bridging protein between the receptors TLR4 or TLR2 and MyD88. We characterize a rare mutation, D96N in the TIR domain of Mal, that is completely incapable of signaling through TLR2 and TLR4. We find that the presence of this mutation affects the ability of Mal to interact with MyD88, a necessary event for the downstream signaling to occur. A closer look at the molecular changes that this mutation brings about reveals the reason behind this defect. Although protein-protein interactions are known to be mediated by a number of forces, one of the major players is electrostatic forces (266). There is a significant population of charged and polar amino acids on the surfaces of proteins, rendering them quite polar and amenable to complex formation with oppositely charged proteins (267). The surface of the TIR domain of Mal is largely electronegative, thus helping to bring the TIR domains of the receptor (TLR2/TLR4) and MyD88, both predominantly electropositive, into close proximity to each other (111). The lack of electrostatic complementarity prevents direct binding of MyD88 to TLR4 or TLR2. In the case of Mal D96N, the change from the

negatively charged aspartic acid (D) to the neutral asparagine (N) considerably reduces the net electronegative charge on the surface of the TIR domain of Mal, hence explaining the phenotype we see with respect to MyD88 binding. A study by George *et al*., report similar findings with respect to the loss-of-function phenotype of D96N (268). They however, did not see a defect in the ability of D96N to bind MyD88 in co-immunoprecipitation assays. Nevertheless, in accordance with our study, they demonstrate that Mal D96N is unable to recruit MyD88 to the cell membrane using confocal microscopy.

The extremely low frequency of Mal D96N in the population is a further testament to the dramatic changes that this single base change can bring about to the function of Mal. Although, we observed a frequency of less than 1% in the population, George *et al*., genotyped a larger cohort and found heterozygous individuals at a frequency of 1%. However, concordant with our findings, they too were unable to find individuals homozygous for *TIRAP* D96N. It is to be expected that individuals harboring this mutation, even in a heterozygous form would be particularly susceptible to both Gram-positive and Gram-negative bacterial infections. Indeed, a recent report demonstrated the association of this polymorphism with tuberculosis (TB) in a Chinese (Han) population (269). The risk of developing TB in subjects heterozygous for the N96 allele was found to be 13.37 fold higher than in individuals homozygous for the wild-type gene.

The sole purpose of Mal is thought to be the recruitment of MyD88 to the receptor complex and in this sense Mal is considered subordinate to MyD88, with no signaling

capabilities of its own. However, new functions of Mal are being brought to the forefront. For instance, Mal has a TRAF6 binding motif that is absent in MyD88 and might help to bring TRAF6 to the signaling complex (140). Mutations in this motif lead to abrogation of TLR2 and TLR4 dependent NF-κB activation (141). Mal is also implicated in inhibition of TLR3 mediated IFN-β production through its interaction with IRF7 (270, 271). One of the most interesting findings with regard to MyD88-independent Mal function is the activation of PI3K dependent Akt activation by Mal upon TLR2/6 stimulation (272). Treatment of cells with diacylated bacterial lipoproteins leads to interaction of Mal with p85α, the regulatory subunit of PI3K. This interaction drives PI3K-dependent phosphorylation of Akt, phosphatidylinositol-3,4,5-triphosphate (PIP3) generation and leading edge macrophage ruffling, the latter two events requiring the co-operation of MyD88. However, Mal or MyD88 participation is not required for Akt activation downstream of TLR2/1. The convergence of TLR2 signaling and PI3K activation at the level of Mal is quite intriguing since Mal is localized to PIP2 (substrate of PI3K) enriched regions of the membrane via its N-terminal PIP2 binding domain (139). This localization of Mal is particularly important for recruiting MyD88 to the complex and initiating signaling. An additional function of Mal might be to bring PI3K to sites rich in PIP2, its substrate, thus helping in the generation of PIP3. Localized concentrations of PIP2 and PIP3 play important roles in membrane ruffling and macrophage migration (273). PIP3 attracts a number of pleckstrin homology (PH) domain containing proteins like Akt as well as Rho family GTPases such as Rac and Cdc42. The latter two are involved in actin polymerization at the leading edge of the cell,

an event that precedes macrophage migration. Mal is thus not only involved in localization of MyD88 (and subsequent cytokine production) but also contributes to specific cellular responses, like production of second messengers and macrophage migration. Although Mal D96N is unable to recruit MyD88, it is localized to the cell membrane and hence we speculate that Akt activation in cells expressing D96N would occur normally in response to diacylated lipopeptides. However, PIP3 generation and macrophage polarization might be compromised since these responses require the involvement of MyD88. This differential response by Mal D96N could be exploited for therapeutic interventions and development of drugs targeting specific aspects of TLR2 signaling.

Given its newfound multiplicity of roles, it is not surprising that Mal is subjected to multiple levels of regulation and is an important point of control for TLR2 and TLR4 signaling (described in detail in chapter II). It is also the most polymorphic adapter protein with a number of studies demonstrating association of various SNPs in Mal with TLR2 and TLR4 mediated diseases. Our study in chapter II not only characterizes a then unknown variant of Mal, D96N, but also sheds light on the region of Mal involved in binding MyD88, using systematic biochemical approaches. The finding that a single base change in Mal can abrogate its binding to MyD88, is in itself quite significant, but the fact that this mutation naturally occurs in the population, albeit at a low frequency, lends credence to our study.

A thorough understanding of the molecular intricacies involved in TLR signaling requires a detailed structural analysis of the complex formation upon signal initiation. To

date, attempts to co-crystallize receptor and adapter complexes have been unsuccessful. Recently however, Lin *et al.* have crystallized the death domains of MyD88, IRAK4 and IRAK2 in complex with each other (129). These oligomeric complexes or "Myddosomes" are comprised of six MyD88 death domains, followed by four IRAK4 death domains and four IRAK2 death domains, arranged in a helical pattern. The structure of the Myddosome resolved by Lin *et al.* demonstrates that purified death domains of MyD88, IRAK4 and IRAK2 have a tendency to form helical oligomers in solution. However, whether this kind of assembly has any bearing in a physiological setting with full-length proteins is a question that warrants further research. Chapter III in this thesis enhances the understanding of this subject matter. We focus our studies on a death domain variant of MyD88, S34Y, that is completely defective in signaling through all MyD88-dependent TLR pathways. Wild-type MyD88 presents itself as aggregates/condensed foci in the cytoplasm. S34Y however has a dramatically different localization pattern. It fails to form aggregates and is diffused in the cytoplasm of the cell. Moreover, while IRAK4 by itself does not form aggregates/clusters, when co-expressed with wild-type MyD88 it colocalizes in distinct condensed aggregates. This is in agreement with the observations by Lin *et al.* and supports the idea that MyD88 likely acts as the platform upon which the "Myddosome" is assembled. In essence, MyD88 is the driving force behind the complex formation with IRAK4 and IRAK2. A study by George *et al.*, reported similar findings using biochemical assays like LUMIER assay (274). They show that MyD88 S34Y has reduced binding to IRAK4 and thus is unable to

form Myddosomes, although, this defect seems to be more pronounced when just the death domains of the proteins are used instead of full-length proteins.

A structural basis for the phenotype can be explained by examining the molecular characteristics of the amino acids involved. S34Y mutation leads to a change from serine, an amino acid with a small aliphatic chain, to tyrosine, a bulky aromatic amino acid, possibly causing steric hindrance in the protein and affecting its structural properties. Indeed, when serine is mutated to alanine, an amino acid with a similar side chain as serine (or a conservative amino acid change), the activity of S34A mutant is comparable to wild type MyD88 in reporter assays. On the other hand, changing serine to phenylalanine, an amino acid structurally similar to tyrosine, ablates the ability of MyD88 to signal (data not shown), suggesting that the loss-of-function effect of S34Y is not due to a requirement for a serine residue at that position, but rather due to the introduction of a bulky amino acid. The 34^{th} position of MyD88 appears to be particularly vulnerable to structural changes. Whether this intolerance towards non-conservative changes is restricted to this residue or is extended to the surrounding region would require additional mutagenesis analysis. This would help in mapping the distinct areas of MyD88 involved in cellular localization.

The few reports to date with the Myddosome as the subject have focused purely on the structural aspects of the constituents of this complex in an *in vitro* setting using purified death domains. Our studies, on the other hand, were carried out using full-length proteins expressed in cells. The concept of the Myddosome thus translates itself quite well to a cellular setting and our visualization of Myddosomes in live cells provides the

next step forward towards understanding the full significance of this structure. More importantly, we show that the S34Y mutation that ablates the ability of MyD88 to assemble into Myddosomes is completely inactive in MyD88-dependent signaling pathways. Our studies thus verify that the assembly of the proteins MyD88, IRAK4 and IRAK2 into Myddosomes is indeed a functional event required for MyD88 dependent signaling. Although not explicitly addressed in this thesis, it is likely that the S34Y mutation would also interfere with signaling downstream of receptors of the IL-1 family. MyD88 and IRAKs are essential components of the signaling cascade downstream of IL-1R and IL-18R, and thus, disruption of Myddosome formation is likely to interfere with the function of these receptors as well.

One of the questions that arise from the idea of the Myddosome is whether the assembly of this complex occurs in a ligand dependent fashion or if these signaling scaffolds are pre-formed in the cytosol and then recruited to the receptor upon initiation of signaling. While TIR-TIR interactions are considered to be unstable and transitory, those between death domains are relatively more stable. Conceivably, *in vivo* these complexes are held together by such DD-DD interactions and upon stimulation, this complex is stabilized and recruited to the receptor, which in the case of TLR4 is at the membrane. Conversely, these complexes may be assembled at the receptor when signaling is initiated; in which case MyD88 would be recruited via Mal and then oligomerize to form a platform for the sequential binding of IRAK4 followed by IRAK2/IRAK1. Our experiments support the former thought; although in the over-expression system that we employ, the molecular excess of proteins might drive the

stabilization of the Myddosome, thus allowing it to be visualized even in the absence of stimulation. Examining the ligand dependent assembly of endogenous MyD88 and IRAK4 into Myddosomes would help to better address such questions. However, determining the stoichiometry of this complex in living cells is a challenging problem. A technique that has been successfully used to understand protein scaffold formation during signal transduction is fluorescence fluctuation spectroscopy (FFS) (275-277). It is based on the principle of brightness analysis of fluorescent proteins and has been utilized to analyze protein interactions in MAPK signaling as well nuclear receptor complexes in living cells (275, 278, 279). FFS might similarly be used to comprehend the formation of Myddosomes.

The tendency to form oligomeric complexes is a characteristic of death domain (DD) containing proteins. The DD superfamily is one of the largest and most widely distributed superfamilies (166). It is evolutionarily conserved and found in many multicellular organisms, though the occurrence of proteins harboring this domain increases significantly in mammals as compared to lower organisms like *C. elegans* and *Drosophila.* One of the most important functions of these domains is to interact with other DD containing proteins to bring about the assembly of signaling complexes. DD-DD interactions are particularly important for apoptotic and inflammatory pathways where oligomeric assemblies of proteins act as scaffolds for “proximity-driven activation” of various proteins such as kinases and ubiquitin ligases (280). In the same context, the formation of the Myddosome might function to bring the IRAKs into proper conformation to allow for phosphorylation dependent activation. Indeed, we observe that

the absence of Myddosome assembly in the case of S34Y precludes degradation of IRAK1, an event dependent upon correct phosphorylation and activation of the components of the Myddosome: IRAK4 and IRAK1 (FIG III.7). IRAK2 and IRAK1 have highly redundant roles and thus a noteworthy point is that although the structure of the Myddosome was resolved using the death domains of IRAK2 in complex with those of MyD88 and IRAK4, it can be inferred that Myddosomes could be formed with the death domain of IRAK1 instead of IRAK2. In either case, it is the MyD88-IRAK4 oligomeric complex that acts as a platform for the oligomerization of either IRAK2 or IRAK1. Unlike IRAK1 or IRAK2 deficient mice, the IRAK1/IRAK2 double deficient mice are completely resistant to LPS induced septic shock and are severely compromised in their ability to respond to a variety of TLR ligands (177). This supports the existence of Myddosomes *in vivo* and provides evidence that Myddosomes are not merely an artifact of crystallography or overexpression.

A further deduction from the Myddosome is that higher order oligomeric arrangement is important for TLR signaling. The formation of such signaling platforms or “signalosomes” is being increasingly considered vital for regulation of various signaling pathways (281). For example, TLR4 and TLR2 have been reported to aggregate into lipid rich microdomains or lipid rafts upon stimulation (282). The binding of a ligand to the ectodomain of a TLR, not only induces dimerization of the receptor, but also affects the conformation of the TIR domains, establishing a foundation for the binding of the TIR domain containing adapters. In such a scenario, the recruitment of a pre-assembled Myddosome to the receptor complex would help to further multimerize the

receptor complex thus enhancing the signal. Clustering of receptors or multimerization brought about by scaffolding platforms like the Myddosome would help to explain the ability of cells to adapt their affinity/sensitivity towards a particular stimulus. For example, even though cells express a low number of IL-1Rs, they are able to mount an appreciable response upon being stimulated by a relatively low concentration of IL-1 (283, 284). This effect could be attributed to the clustering of IL-1R that the Myddosome can probably bring about.

One of the major strengths of our study is the use of immortalized macrophage-like cell lines that we generated from wild-type as well as Mal-deficient and MyD88-deficient mice. They provide an ideal system (in the absence of transgenic mice expressing mutant proteins) for studying mutations in a clean genetic background in innate immune cells. In the absence of the wild-type Mal allele, these cells can be virally infected with the complementing exogenous allele or the mutant allele, allowing the study of the respective protein/mutant without any intervention from the endogenous wild-type allele. Although retroviral vectors offer some distinct advantages in terms of stable integration and expression in the cells, their preference for integration in transcriptionally active regions of the genome might result in aberrant or modulated gene expression. Even though we used replication deficient retroviruses in our experiments, random recombination between the viral and host genome can lead to the production of transformed viruses. Results obtained from retroviral expression of genes, should thus be interpreted with caution and only in the presence of appropriate controls. Experimental approaches employed in this thesis rely heavily on exogenous expression of the

respective genes, which usually results in expression of the protein at levels significantly higher than physiological levels. Over-expression of the protein can sometimes give rise to artifacts that can obscure the results. For example, in chapter III, co-immunoprecipitation experiments with MyD88 S34Y did not reveal any defect in terms of binding with the known binding partners of MyD88. This could in part be due to the vast amount of the mutant protein being expressed in the cells, leading to non-specific interactions. This could also be due to the presence of endogenous MyD88 allele that acts as a bridge between S34Y and the interacting protein. Mere over-expression of a protein can also drive the respective signal transduction pathway in a ligand-independent manner and produce results that do not accurately reflect an activable signaling pathway. Some of the problems associated with over-expression studies can be circumvented by use of an inducible expression system.

The events occurring at the level of the receptor complex set the stage for the downstream signaling. A disruption in the pathway at that point would completely abrogate signaling. The two mutations that form the core of this thesis affect TLR signaling at the level of the receptor complex, each affecting the formation of the signaling platform by a different mechanism. Nevertheless, the consequence of either mutation is a complete block in TLR signaling pathways that are mediated by that particular adapter protein. FIG IV.1 summarizes the effect of the two SNPs, Mal D96N and MyD88 S34Y on TLR signaling pathway. Mal D96N exerts its effect by preventing the recruitment of MyD88 to the receptor complex. On the other hand, MyD88 S34Y does not allow MyD88 to form oligomers, thus inhibiting the assembly of Myddosomes.

FIGURE IV.1: A schematic showing the effect of the mutations Mal D96N and MyD88 S34Y on TLR signaling.

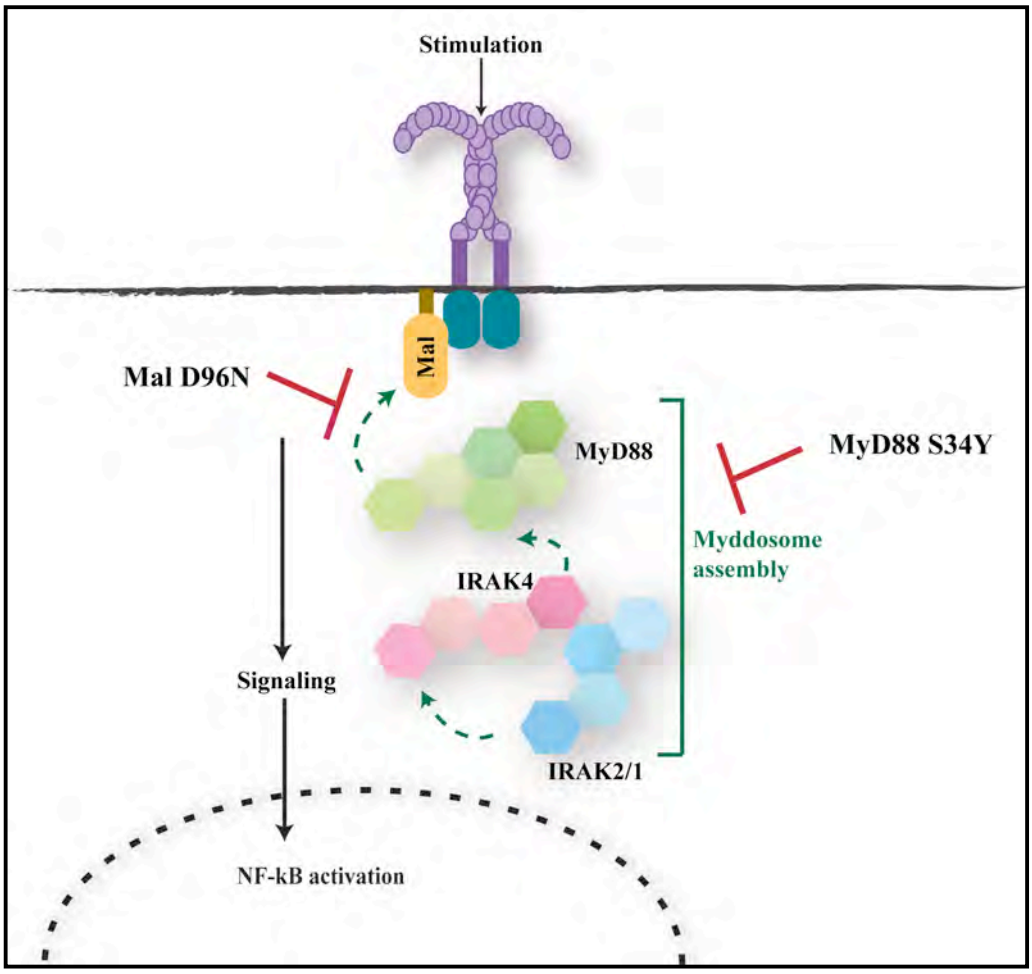


FIGURE IV.1: A schematic showing the effect of the mutations Mal D96N and MYD88 S34Y on TLR signaling. Mutation D96N, in the TIR domain of Mal, prevents the binding of Mal to MyD88. MyD88 S34Y does not allow MyD88 to form oligomers, thus inhibiting the assembly of Myddosomes.

Having studied the biochemical aspects of MyD88 S34Y, we were interested in the epidemiological significance of this polymorphism. We were unable to find a single individual carrying *MYD88* S34Y allele amongst 372 individuals we genotyped for *MYD88*. Like Mal D96N, MyD88 S34Y is an extremely rare mutant with low frequency in the population. This suggests that loss-of-function mutations in crucial adapter proteins like MyD88 and Mal encounter strong negative selection pressure. This could also emphasize the small sample size in our genotyping studies. Large sample sizes give better estimates of true population frequencies. An exhaustive attempt at genotyping *TIRAP* D96N and *MYD88* S34Y might reveal a more realistic population frequency. One of the most extensively studied polymorphisms in adapter proteins is the serine to leucine change at residue 180 in Mal (224). Individuals heterozygous for this polymorphism were found to have increased protection against a number of TLR2 mediated diseases like pneumococcal disease, bacteremia, malaria and tuberculosis. Carrying this allele in a heterozygous setting provides an advantage to the host, possibly by ensuring an optimal balanced inflammatory response. A similar example of heterozygous advantage can be seen in the case of sickle cell trait where carriers of this trait have considerable protection against malaria. The aberrant protein allele has thus been maintained in the population despite having a deleterious effect on the function of the protein. The two mutants that we characterize here, however, lead to such profound changes in the signaling pathway that they might present a distinct disadvantage to the host in terms of defense against pathogens, proving them unfavorable to be maintained in the population and hence, the low population frequency that we observe.

Not many studies have associated MyD88 variants with increased susceptibility or protection to diseases. A report by von Bernuth *et al.* however, delineates the effect of MyD88 deficiency in nine children (240). These children exhibit high mortality rate and increased susceptibility to invasive streptococcal and staphylococcal disease. Surprisingly, they have normal resistance to many common fungi, viruses, bacteria and parasites. IRAK4 deficiency in children presents with a similar narrow spectrum of invasive diseases (285). Another report by the same group describing the clinical features of 45 patients with either MyD88 or IRAK4 deficiency have similar findings (286). There might be a number of different explanations for the unremarkable clinical history of these patients. It could reflect a selection bias while recruiting patients for this study and in this respect could be a mere artifact of selection influence. Indeed, the nine children with MyD88 deficiency were all selected because of their known predisposition to invasive pneumococcal disease. When we consider the profound effect MyD88 or IRAK4 deficiency has in mice, the susceptibility of these patients to just a few pathogenic agents is quite unexpected and perhaps a more extensive study would reveal a broader spectrum of disease predisposition. Since these invasive infections are largely TLR2 and TLR4 mediated, they require Mal, in addition to MyD88 for the innate immune response. Mal and MyD88 can lead to the activation of PI3K, one of the mechanisms implicated in phagocytosis of microorganisms. Signaling through Mal and MyD88 can also lead to changes in the levels of different phosphoinositides in the cell membrane, which can affect macrophage migration (273). These infections could thus be a result of phagocytosis defects and in the absence of the cellular effects that Mal is responsible for

(which would be severely compromised in a MyD88-deficient individual, since Mal signaling for the most part is dependent on MyD88) this infection profile is predominant (287). In addition, individuals with IRAK4 and MyD88 deficiencies are highly susceptible to bacterial infections early in life. However, upon reaching adolescence, the predisposition to infections decreases considerably. This could presumably be due to the adaptive immune mechanisms having a compensatory effect. Moreover, a majority of these patients are on antibiotics and without such medical intervention, these individuals might succumb to infections. Nevertheless, these reports allude to the importance of adapter proteins in host defense and imply that variants of these proteins can influence the outcome of life-threatening disease. Since both Mal D96N and MyD88 S34Y display a null phenotype with respect to signaling, we predict that the individuals carrying these mutations would essentially have a similar phenotype to that which has been reported for MyD88 or IRAK4 deficiency.

Conservation is generally considered a structural necessity for the function of a protein. The more conserved a protein, the more critical is its role. In this essence, pattern recognition receptors or TLRs (in the context of this thesis) are highly conserved receptors that act as sentinels at the interface with the environment. The functional domains of these signaling pathways like the TIR domain and the death domain are also highly conserved domains with similar function in different organisms. Conversely, the immune system is under constant pressure to evolve in order to successfully defend the host. This can be brought about in a number of different ways. Antibodies, for example, continue to “evolve” throughout life by means of genetic recombination. HLA molecules,

on the other hand, undergo extensive gene duplication to allow for subtle differences in antigen recognition, thus increasing the repertoire of antigens that can be recognized. To a similar effect, polymorphisms in the innate immune genes are a means to provide flexibility in the host defense mechanisms. Variation in the innate immune genes suggest that genes can be highly conserved across species, yet remain highly polymorphic in individuals within the same species. Although the two mutants we describe in this thesis are extremely rare variants and may not have an effect on disease susceptibility in a population at large, they may contribute on an "individual" level to a person's immunologic competence.

TLR/IL-1R pathways have been implicated in a number of immune and inflammatory diseases like sepsis, atherosclerosis and Alzheimer's disease (209, 223, 288-292). In addition, they play a central role in autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS) and inflammatory bowel disease (IBD) (293-297) (298). Therapeutic targeting of TLR/IL-1R signaling is thus increasingly gaining attention as a potentially viable approach for many diseases of the immune system. Protein-protein interactions are a hallmark of every signaling pathway and provide an important tool for modulating the activity of TLR pathways. Intriguingly, several microbes utilize similar strategies for evading the innate immune responses. Mal and MyD88 appear to be particularly amenable to such interventions by the microbes. Viral inhibitory peptide of TLR4 (or VIPER) is a TIR domain containing-protein from Vaccinia virus (299). It binds to Mal and TRAM; masks critical binding sites on the surface of these two proteins and specifically inhibits TLR4. A number of genes encoding

TIR domain containing–proteins (Tcps) have been identified in different bacteria such as *E.coli*, *Salmonella enterica* and *Brucella melitensis* (300, 301). These proteins act as mimetics of the signaling domains, thus blocking the signaling pathway (302, 303). Taking cues from the pathogens, the development of various decoy peptides has been undertaken to target TLR function. These are short peptides that are expected to mimic the interaction surface of a particular protein and prevent the interactions between the prototype binding partners. Our study unveils two novel targets for the development of such TLR inhibitors. The BB loop region (connects the second β-sheet and the second α-helix of the TIR domain) of TIR domain has received the most attention as therapeutic targets. The work presented in this thesis highlights other regions in the TIR domain of Mal (and perhaps other adapters) that can serve as valuable therapeutic targets. We also delineate a single residue in the death domain of MyD88 that is absolutely essential for its function and thus illustrates a unique approach to TLR targeting.

By means of the studies presented in this thesis, I attempt to highlight the importance of amino acid changes in TLR adapter proteins. Such changes can have an effect on predisposition and outcome of various diseases. They also reveal invaluable information about the structure and function of the protein involved and provide novel drug targets. Systematic studies investigating the effect of natural variation in other adapter proteins, would not only help in understanding the molecular biology of these proteins, but also bring to the forefront distinct genetic variants that affect disease prognosis.

Future Prospects

Despite tremendous growth in the field of TLR signaling, the molecular workings and intricacies of adapter proteins with respect to their differential use by various TLRs, remains obscure. *In vivo* studies in transgenic mice expressing loss-of-function variants would shed light on some of these effects. These mice would not only provide clues about the spectrum of infections and outcomes that we could expect in individuals harboring these variants, but would also be an ideal experimental system to answer questions about various biochemical aspects of adapter proteins. For example, cells from these mice could be used for phagocytosis assays, interaction studies, lymphocyte proliferation assays. In the case of MyD88, an interesting facet is the apparent reliance of MyD88-dependent signaling on the formation of the Myddosomes. Mice expressing the S34Y mutant of MyD88 may help to further clarify this aspect.

Finally, some longstanding yet unanswered questions remain that are worth mentioning. First, in the absence of Mal and TRAM (for example, in the case of TLR5, TLR7 and TLR3 respectively), how are MyD88 and TRIF recruited to the receptors? Do additional adapters/factors remain undiscovered and would the discovery of such proteins, change the existing paradigms of TLR signaling? Second, Why can MyD88 signal through both surface as well as endosomal TLRs, whereas TRIF can only signal from the endosomes? Lastly, is the ability to signal from both the cell surface as well as the endosomes unique to TLR4, or do all the TLRs possess the capability to shuttle between the cell membrane and the endosomal-endoplasmic reticulum network and a plethora of signaling networks are just waiting to be revealed?

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