Mal, more than a bridge to MyD88

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Summary

The family of type 1 transmembrane proteins known as Toll–like receptors (TLRs) provide early immune system recognition and response to infection. In order to transmit their signal to the nucleus and initiate activation of pro–inflammatory and anti–microbial genes, TLRs must initiate a cytoplasmic signalling cascade, which is alternately controlled by 6 known signalling adaptors. These signaling adaptors are crucial for activating the correct immune response to any given TLR / pathogen interaction. This review will focus on one of those adaptors, MyD88 adaptor–like (Mal), also known as TIRAP.

Mal is critical for signalling by the best studied of the TLRs, the Gram negative bacterial lipopolysaccharide (LPS) sensor, TLR4. Mal's role in TLR2 signalling in response to activation of the bacterial lipopeptide receptor, TLR2, is more contentious.

Mal is a component of the so–called 'MyD88-dependent pathway' in TLR4 signalling. Recent advances in our understanding of the signalling pathways downstream of Mal highlight MyD88-indpendent roles, thus positioning Mal as multifunctional and integral for the molecular control of bacterial infections as well as inflammatory diseases.

Here we describe the sequence of molecular events involved in the signalling pathways controlled by Mal, and the importance of Mal in driving host protection against a variety of bacteria, with specific attention to the evidence for Mal's role in TLR2 signalling, recent structural findings that have altered our understanding of Mal signalling, and evidence that single nucleotide polymorphisms (SNPs) of Mal are responsible for variations in population level resistance and susceptibility to bacterial infection. © 2013 IUBMB Life, 65(9):777–786, 2013

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Toll-like receptors (TLRs) are frontline sensors of microbial infection. They detect pathogen associated molecular patterns (PAMPs) and trigger signaling

cascades that culminate in increased expression of immune and inflammatory genes. TLRs have been identified in species as distinct from humans as the earthworm Caenorhabditis elegans, the plant Arabidopsis thaliana, and the sea urchin, which is predicted to encode as many as 253 TLR sequences. These receptors are also ubiquitous within a given immune system. In humans and mice they have been identified on all known immune cells and also on cells sometimes considered to be "non-immune" barrier-type cells such as epithelial cells.

It is now understood that the most important TLRs for recognition of bacteria and their products are homodimers of TLR4, specific for LPS [1]; TLR5 which recognizes flagellin, the protein subunit component of bacterial motility known as flagella [2]; TLR9 which detects unmethylated CpG oligodeoxynucleotide DNA of bacterial and viral origin [3]; TLR1/2 heterodimers, specific for triacylated lipopeptides of Gram positive bacteria [4]; and TLR2/6 heterodimers, which detect diacylated lipopeptides, also from Gram positive bacteria [5]. In mice TLR11 can sense uropathogenic bacteria [6] and TLR13 recognizes bacterial 23S ribosomal RNA [7]. Preliminary evidence indicates that TLR12 also detects bacterial products. The other TLRs have viral specificity. TLR3 binds double stranded RNA [8], while TLR7 and TLR8 ligate variations of single stranded viral RNA [9].

Despite the array of human and murine TLRs for specific recognition of various pathogens, all TLRs maintain a base protein structure of an N-terminal extracellular leucine rich repeat (LRR) domain connected to a transmembrane domain and the key signaling C terminal cytoplasmic domain known as a Toll/IL-1R/resistance (TIR) domain.

Ligand-induced TLR dimerization allows the recruitment of six known cytosolic TIR domain-containing adaptors, namely myeloid differentiation primary response gene [88] (MyD88), which signals for all TLRs except TLR3; MyD88 adaptor-like (Mal), the subject of this review, which only signals downstream of TLR4 and TLR2; TIR domain-containing adaptor protein inducing interferon-β (TRIF), which is specific for TLR3; TRIF-related adaptor molecule (TRAM); sterile α- and armadillo-motif-containing protein (SARM), which is a negative regulator of TRIF; and B cell adaptor for PI3K (BCAP), a newly identified inhibitory adaptor [10, 11].

As it is now considered a prototypical molecule to understand the complex molecular regulation of TLR signaling, an understanding of Mal provides deep insight into the role of TLRs in infection and immunity in general. Here we review the function of Mal, an adaptor which has received much attention for its

role in bacterial infection, its complex regulation of TLR4 signaling, and genetic variation in humans.

The Role of Mal in TLR2 Signaling

Early studies identified Mal as an adaptor critical for TLR4- and TLR2-mediated activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein (MAP) kinase pathways [12-15]. The dependence of TLR4 signaling on Mal is established. More recently however, the role of Mal in TLR2 signaling has been complicated. Kenny et al. demonstrated that Mal is only necessary for interleukin (IL)-6 or NF-κB induction from TLR2 signaling at low ligand concentration [16]. At high doses of the TLR1/2 ligand Pam3Cys-Ser-(Lys4) (Pam3Cys) or the TLR2/6 ligand macrophage-activating lipopeptide-2 (Malp-2), and also with high multiplicity of infection of macrophages with Salmonella enterica, Mal deficiency had no effect, indicating that Mal might sensitize TLR2 signaling when the degree of activation is low.

Cole et al. supported these findings by demonstrating TLR2-dependent but Mal-independent signaling in response to Francisella tularaemia through retention of the bacteria within phagosomes, also highlighting the importance of localization of TLR2 PAMPs [17].

Further study in this area may yet reveal important Mal-dependent TLR2 signaling in the context of human infection in vivo, as colonization usually begins with relatively low bacterial numbers and thereby with a relatively low concentration of TLR2 ligands. The identification of the S180L Mal variant (discussed in detail later), which attenuates TLR2 signaling and protects from bacterial infection when expressed heterozygously, highlights that the "strength" of this initial Mal-mediated response to infection might be vital for subsequent control of certain pathogens. Initial exposure in vivo to low dose TLR2 bacterial ligands might be necessary to begin protective immune responses that may prevent bacterial growth and subsequent immunopathology.

In a model of gastritis and gastric cancer in gp130F/F mice that is dependent on TLR2 signaling, Kennedy et al. found that MyD88 was integral but there was no role for Mal [18]. It remains to be determined whether Mal-dependent TLR2 signaling is important for aspects of antimicrobial immunity unrelated to tumorigenesis.

It should be noted that, almost universally, published observations of the role of Mal in TLR2 signaling in vitro have experimentally used Malp-2 and/or Pam3Cys, both of which are highly specific and bioactive molecules. It is possible that

different bacteria produce lipopeptide variants that are far less bioactive. Precedence for this idea is that there are significantly different bioactivities of LPS from different bacterial species, partly attributable to the different lipid A structures displayed [19]. In this context, it is possible that TLR2-mediated detection of certain bacterial strains may be more reliant on Mal than is currently understood. Only by exposing a wider variety of bacterial lipopeptides to Mal-defective cells will a role for Mal in TLR2 signaling be ultimately determined.

The Bridge to MyD88

Mal is often described as a bridging adaptor due to its primary function of recruiting cytosolic MyD88 to interact with the activated TIR domains of TLR4 dimers at the cell membrane, thus catalyzing formation of the so-called "Myddosome" [20]. According to crystal structure analysis, the Myddosome is a hetero-complex of six MyD88 death domains (DDs) interacting with the DDs of four IL-1R associated kinase 4 (IRAK-4) and four IRAK-2 or IRAK-1 molecules [21]. Phosphorylation of IRAK-1 by IRAK-4 then propagates signaling via an interaction with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) [22], culminating in the activation of the MAP kinase pathways and NF-κB-mediated induction of pro-inflammatory and anti-microbial gene expression (including IL-6, IL-1β, and TNF-α).

The ability of Mal to recruit and catalyze the formation of the Myddosome is dependent on unique structural elements. Recent crystal structure determinations of Mal identified that unlike the BB loop-containing TIR domains of other TLR signaling molecules, including TLR1, TLR2, TLR10 [23], MyD88 [24], and the IL-1 receptor accessory protein-like 1 (IL-1RAPL) [25], Mal-TIR includes a unique extra long protruding AB loop [26]. This AB loop appears to be integral for binding of Mal with MyD88-TIR and TLR4-TIR [27]. In essence, the AB loop is a component of the "bridge."

Another vital bridging feature of Mal is the presence of an N-terminal phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain [28], which specifically binds TLR4-rich regions of the plasma membrane. Once membrane anchored via PIP2, spatial localization facilitates the above mentioned interaction between Mal-TIR and the TIR domains of MyD88, TLR2 [15], and TLR4 [12, 27].

These membrane interactions serve a vital role as important regulatory components that can skew immune responses either into MyD88/Mal-dependent surface membrane signaling pathways or TRIF/TRAM-dependent endocytic signaling pathways. Mal is now known to interact with phosphoinositol-3 kinases (PI3Ks) including the class 1A p110δ isoform, which

controls this cellular compartmentalization of TLR4 by converting PIP2 to PIP3, thus masking plasma membrane anchor sites for Mal [29], propagating the MyD88-independent endocytic TRIF pathway of TLR4 signal transduction.

MyD88 Bridging-Independent Functions of Mal

It is now accepted that Mal is more than just a bridge to MyD88. A number of studies have identified "MyD88 bridging-independent" (not to be confused with the MyD88-independent TRIF pathway) functions of Mal.

The Parallel Pathways of NF-κB Nuclear Translocation and Transactivation

TLR signaling activates NF-κB by interfering with IκB inhibition of translocation to the nucleus. However, PI3K subunit p85α-deficient B cells do not respond to LPS [30, 31], indicating that a parallel TLR PI3K-dependent pathway of NF-κB activation exists. Transactivation of the best studied NF-κB subunit p65 involves the serine phosphorylation of its transactivation 1 (TA1) domain by PI3K-Akt kinase. The combination of these parallel pathways, nuclear translocation and transactivation, are thought to be essential for optimal activation of NF-κB.

In 2000, Arbibe et al. demonstrated transactivation of the nuclear p65 subunit, identifying TLR2-Rac1 as an activator of PI3K-Akt [32]. The small guanosine-5′-triphosphate (GTP)-binding protein, Rac1, activates NF-κB activity partly through phosphorylation of IκBα [33], allowing nuclear translocation, but also through PI3K-mediated p65 transactivation [34]. For inflammatory gene expression to occur NF-κB must first be released by IκB for translocation to the nucleus where it can localize to κB-responsive elements present on relevant genes for regulation.

In the context of TLR2 signaling, Santos-Sierra et al. discovered that Mal connects TLR2/6 to PI3K activation, yet TLR1/2 mediated Akt phosphorylation is Mal-independent [35]. Diacylated lipopeptide was demonstrated to induce PIP3 formation at the leading edge of macrophages. This is a MyD88-independent Mal function, distinct from NF-κB activation.

Sanlioglu et al. also reported LPS induced Rac1-dependent NF-κB activation, thus implicating TLR4 in propagating the Rac1-PI3K-Akt pathway [36]. This evidence is supported by other data showing that LPS stimulation of human microvessel endothelial cells also induces Rac1 [37]. In that study, Equils et al. also found that dominant negative expression of Mal, but not dominant negative expression of MyD88, blocked Rac1-induced regulation of the HIV-long terminal repeat, thus

not only implicating Mal in MyD88-independent Rac1-PI3K-Akt NF-κB transactivating pathway, but also in HIV replication modulation.

Coincident with the identification of a role for Mal in the NF-κB transactivating pathway was the discovery of a putative TRAF6 binding site on Mal-TIR at amino acid position 188–193 [38]. The Pro-Pro-Glu-Leu-Arg-Phe was similar to previously characterized TRAF6 binding sequences on IRAK and TRIF, but importantly it was not present on MyD88. Mansell et al. validated the interaction by co-immunoprecipitation of TRAF6 with Mal. In this study, overexpression with Mal E190A, a mutation of the TRAF6 binding site, inhibited TLR2 or TLR4 induction of an NF-κB reporter gene relative to WT Mal. Verstak et al. further characterized this observation in 2009, confirming that Mal binds TRAF6 and recruits it to the plasma membrane [39]. Importantly this study identified that interaction between Mal and TRAF6 is required for the serine phosphorylation of p65, thereby determining a mechanism of Mal-dependent NF-κB transactivation.

Although Mal can bind TRAF6 directly, independent of MyD88, in a network sense this function is obviously integrated with MyD88 signaling, as both NF-κB translocation and transactivating p65 phosphorylation are necessary for optimal inflammatory cytokine gene expression. These two pathways might operate in parallel or sequentially.

Btk and SOCS-1

In parallel with the serine/threonine kinases that post-translationally modify TLR4 signal transduction are a group of LPS-activated tyrosine kinases with a less well defined role in TLR signaling. The initial observation by Mukhopadhyay et al. in 2002, that one of these, Bruton's tyrosine kinase (Btk), is important for antimicrobial activity including nitric oxide (NO) and pro-inflammatory cytokine production [40], in the following year led Jefferies et al. to discover that Btk is involved in TLR4 signaling and that it interacts with Mal [41]. This was further clarified in 2006 when Gray et al. demonstrated that Btk phosphorylates the TIR domain of Mal at tyrosine residues 86, 106, and 187 [42]. When these Mal tyrosine residues were replaced with phenylalanine, LPS- and Malp-2-induced signaling were inhibited, indicating that Btk phosphorylation of Mal activates and is necessary for TLR2- and TLR4-induced NF-κB activation.

Subsequently, both p65 and Mal can be polyubiquitinated for rapid proteasomal degradation, in the case of Mal as a result of binding between its PEST domain, a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T), and the SH2 domain of suppressor of cytokine signaling 1 (SOCS-1), thus forming a negative regulation on Mal-dependent p65 phosphorylation [43].

CREB and IL-10

In 2011, a further TRAF6-dependent role for Mal, distinct from NF-κB activation, was revealed. The transcription factor cAMP response element binding protein (CREB) was first described to regulate expression of the somatostatin gene [44], and has since been recognized to transactivate a variety of immune related genes including TNF-α [45], cyclooxygenase 2 (COX2) [46], and IL-10 [47]. Mellett et al. identified that in response to LPS stimulation, Mal can enhance CREB activation by signaling through TRAF6, Pellino3, p38 MAPK, and MK2 [48]. Another interesting discovery from this study was that Mal E190A mutants had no defect in their ability to activate CREB despite a known inability to activate NF-κB, thus highlighting the fact that Mal can modulate both pro- and anti-inflammatory responses via different interactive surfaces of the protein, leaving open the possibility that Mal could be therapeutically targeted to modulate this balance in a clinical setting.

Caspase-1 and the Inflammasome

In 2007, a yeast two-hybrid screen picked out a unique Mal interacting clone encoding the catalytic domain of caspase-1 [49]. The cysteine protease caspase-1 is known to cleave pro-IL-1β and pro-IL-18 into biologically active cytokines for secretion. In this context, caspase-1 is requisite for the complex known as the "inflammasome."

Because the production and secretion of mature IL-1β requires both TLR and inflammasome activation it has been postulated that TLR stimulation might directly regulate inflammasomes. However, there has been little evidence of signaling interaction. In the first piece of evidence that there is direct physical interaction, Miggin et al. found that caspase-1 does not cleave MyD88 but does cleave Mal at position D198, releasing a 4 kDa C-terminal fragment. This event is required for Mal-dependent TLR4 signaling, but not for IL-1 or TLR7, which do not signal via Mal. Subsequently, a molecular mechanism for this was hypothesized in which an E-helix is removed from Mal by caspase cleavage, thus revealing a MyD88 binding groove [50].

Another study in 2010 used a two-hybrid technique called a mammalian protein–protein interaction trap (MAPPIT) to contradict some of these findings. Ulrichts et al. confirm that caspase-1 and Mal do interact, but conclude that cleavage inhibits Mal function, and is not required for transactivation of NF-κB [51]. Inhibitory caspase cleavage of other pattern recognition receptor (PRR) pathway adaptors, TRIF and Cardif, has also been demonstrated [52], generally supporting such an inhibitory role for caspases in PRR pathways.

Equivalent amounts of the caspase-1 p10 subunit from WT and Mal-deficient macrophages indicate that Mal is not required for caspase-1 activation [49]. Nevertheless, the fact that caspase-1 does cleave Mal validates the assumption that there is an "interaction" between TLR signaling and inflammasomes by placing these two complexes in the same spatial context.

Negative Regulators of Mal Signaling

SOCS1 and caspase-1 are not the only negative regulators of Mal. Unlike the active forms, kinase inactive forms of IRAK-1 and IRAK-4 have no effect on Mal degradation, indicating that Mal degradation by phosphorylation and ubiquitination is also promoted by these IRAKs [53]. This is classic negative feedback to prevent over-stimulation, and might be a mechanism of so-called "LPS tolerance" whereby prior exposure to LPS can dampen subsequent responses to re-exposure.

TLR 3 Inhibition

All TLRs are known to signal via MyD88 with the notable exception of TLR3. TLR3 instead uses the adaptor TRIF to activate antiviral responses via IRF3. In 2007, MyD88 was shown to negatively regulate that pathway in human corneal epithelial cells [54] by inhibiting phosphorylation of JNK and thereby its activation. In 2009, the same inhibition of poly(I:C)-induced TLR3 signaling was observed in Mal-deficient macrophages [16]. One possible mechanism of this negative regulation of TLR3 signaling by Mal might be via IRAK-2. Mal may sequester that kinase such that it is less accessible for TLR3 signaling. The exact purpose of such sequestration is unknown. Hypothetically, TLR2 or TLR4 stimulation by bacteria might enhance sequestration of IRAK-2 so as to funnel more signaling resources into antibacterial pathways and away from "wasted" antiviral TLR3 signaling.

RAGE

All of the above mentioned Mal-mediated pathways are downstream of TLR2 and TLR4. MyD88 has been demonstrated to signal downstream of IL-1R, IL-18R, IFN-γ, and all TLRs except TLR3. In this regard, Mal signaling appears to be more specific than MyD88, principally responding to LPS and bacterial lipopeptides. Mal's association with the LPS receptor might explain why there is scant evidence of such promiscuity. Stimulation of Mal-deficient cells or animals with ligands that are truly LPS-free is difficult and often impossible. In most cases, heat inactivation or use of LPS-removing reagents is not sufficient to rule out LPS contamination.

To date the only evidence of a Mal/non-TLR receptor association is with the receptor for advanced glycation end products (RAGE) [55]. Despite its name, RAGE is thought to respond not only to advanced glycation end products [56], but also to an array of ligands including DNA, RNA, HMGB1 [57], amyloid fibrils [58], and S100 proteins [59]. RAGE signaling is known to induce a wide variety of responses including the typical TLR4 signaling targets: ERK1/2, p38 MAP kinase, and NF-κB. How these pathways are activated by the very short cytoplasmic tail of RAGE, which lacks obvious signaling motifs, has been a source of intrigue.

In 2011, Sakaguchi et al. published evidence that ligand activation leads to the cytoplasmic tail of RAGE being phosphorylated at Ser 391 by protein kinase C (PKC) ζ. Phosphorylated RAGE can then bind Mal or MyD88 [55]. Difficulties in understanding the role of Mal in RAGE signaling include the possibility of significant redundancy of RAGE function, the known variability of RAGE signaling in different cell types, and the fact that many RAGE ligand preparations are contaminated with LPS, which itself signals through Mal via TLR4.

Mal and Bacteria

Most of what is known about Mal signaling has been discovered in vitro using LPS or lipopeptides for specific activation of TLR2 and TLR4 pathways. Only a select few mouse pathogens have been used to gain an understanding of the function of Mal in vivo.

The general importance of Mal in protection against bacterial infection is indicated as certain bacterial species can subvert host immune responses by specifically targeting Mal or other Mal pathway molecules for negative regulation and enhancement of bacterial virulence.

Brucella is known to make a TIR domain containing protein (TcpB) with significant homology to mammalian TIR domains including Mal [60] indicating that TcpB may act as a mimic. Subsequent work identified that TcpB subverts TLR signaling [61] by degrading Mal [62] through enhancement of poly-ubiquitination [63].

Mal knockout mice are defective in their immune response to E. coli infection of the lungs, displaying a higher bacterial burden in the lungs, more bacterial dissemination to the spleen, and earlier mortality [64]. In a separate study from the same group, the same increases in virulence were also observed when Mal knockout mice were infected with Klebsiella pneumoniae, but not with the flagellated bacteria Pseudomonas aeruginosa [65]. Our own unpublished data indicates that Mal is also vital for immunity to the causative agent of whooping

cough, Bordetella pertussis. Relative to WT, infected Mal-deficient mice develop a higher B. pertussis count in the lungs, and succumb to a disseminating fatal infection.

Mal and the Barrier

Mal is entirely dispensable, and even detrimental, for host protection from intravenous administered S. enterica serovar Typhimurium [66, 67]. However, when Salmonella is administered via its natural route of infection, orally, Mal is protective [68]. Underlying this distinction between oral and intravenous infection is the function of the intestinal barrier.

Mal signaling pathways have predominantly been studied in cells of the macrophage/monocyte lineage, which function principally in inflammation and activation of the immune system. The role of Mal in other cell types, and in induction of other responses is less well documented.

The "barrier" is a generic term to describe the protective separative layer of tightly packed cells bound by tight junctions that line key mucosal sites of infection, such as the lung and intestinal epithelial layers. Bacterial breach of the barrier results in dissemination of bacteria into otherwise sterile compartments, including the blood (bacteraemia or septicaemia), which in turn can cause deadly sepsis.

In 1993, Goldblum et al. discovered that LPS perturbs pulmonary vascular endothelial barrier function by inducing the depolymerization of F-actin and consequently interfering with intercellular gap formation [69]. In 1997 he added that this effect on the barrier is mediated by tyrosine phosphorylation [70], and in 2008 his group further clarified this understanding by demonstrating that the SRC family kinases (SFK) c-SRC, FYN, and YES are activated by LPS, and that they tyrosine-phosphorylate zonula adherens proteins, thus permeabilizing the barrier via the paracellular pathway [71]. Another study identified that the barrier of pulmonary microvessel endothelial monolayers can be perturbed with lipoteichoic acid (LTA), a Gram positive bacterial cell wall component known to be sensed by TLR2 [72], providing further evidence that both TLR2 and TLR4, the two TLRs that interact with Mal, are involved in lung barrier regulation.

Goldblum's group identified a more decisive role for Mal in endothelial barrier permeability by demonstrating that LPS activation of the SFKs is mediated by TRAF6 [73], a known component of Mal signaling cascades. In this study, siRNA-

induced silencing of Mal, or overexpression of dominant negative Mal, in human lung microvascular endothelial cells (HMVEC) blocked this "LPS effect."

The "LPS effect" has also been demonstrated on epithelial cells indicating that Mal may also play a role in perturbing epithelial tight junctions. However, counter to the "LPS effect," PKC is known to induce tight junction formation, strengthening the barrier [74, 75] in vitro between cultured human nasal epithelial cells [76]. Corr et al. demonstrated that this phenomenon can be activated by Mal [68]. By using Mal bone marrow chimeras to rule out the role of Mal in macrophage/monocyte lineage cells, the susceptibility of Mal-defective mice to oral Salmonella infection was demonstrated to be due to a faulty Mal–PKC-barrier axis, thus demonstrating that Mal strengthens the intestinal epithelial barrier against bacterial infection. Counter to this are studies indicating that PKC dephosphorylates occludin and increases tight junction permeability of epithelial cells [77, 78]. The apparent conflict over the role of TLRs, MyD88, and Mal in relation to barrier permeability in some of these studies will probably be proven to be a factor of differential regulation by the different cell types and pathogens tested.

Human Mal

The Mal D96N Mutation

In 2009, Nagpal et al. published data from a screen of known Mal mutations [79]. They discovered that Mal D96N results in a loss of the bridging function of Mal. In this study, LPS-induced TNF-α production by Mal D96N macrophages was commensurate with Mal knockouts, and there was largely ablated NF-κB luciferase activity in mutant HEK293T cells. A subsequent study by George et al. supported these findings and also found that the corresponding heterozygous SNP rs8177400 was present at 0.97% of a Caucasian German population [80]. In this study, there was found to be no association between the SNP and lymphoma risk, but a more recent Chinese study found an association between Mal D96N and susceptibility to tuberculosis [81].

In combination with structural studies of Mal-TIR [26], these studies indicate that D96N is present in the MyD88 binding region of Mal and not on the TLR interactive surfaces. As such Mal D96N specifically interferes with the bridging function of Mal. Although Nagpal et al. compared Mal D96N to Mal knockout cells, in that study the only reported analysis was of MyD88 bridging function, that is NF-κB activation. Future research might compare Mal D96N mutant cells with Mal knockout cells, with special attention to the recently identified non-bridging components of Mal signaling.

The Mal S180L Polymorphism

In 2007, Khor et al. catalogued functional variants of Mal present in UK, Vietnamese, and African populations [82]. One of these SNPs encodes a serine instead of a leucine at position 180 (S180L). Data from Ferwerda et al. indicates that primary cells from LPS-primed individuals with the S180L polymorphism produce more pro-inflammatory cytokines [83]. Khor et al. found that relative to both homozygote S180 and L180, heterozygote S180/L180 Mal conveys protection from bacteremia, tuberculosis, malaria, and pneumococcal disease, indicating a "Goldilocks" model of TLR signal transduction, in which a "just right" strength of signaling is beneficial for the host. Too little cytokine signaling as in S180 homozygotes might allow unfettered bacterial colonization, and too much cytokine signaling, as in L180 homozygotes, might inhibit tolerance or negative feedback mechanisms designed to prevent systemic inflammatory response syndrome (SIRS) or related immunopathology.

There is some controversy as to the actual effect that Mal S180L has on disease incidence or severity. Nejentsev et al. provide evidence from their own data indicating no evidence of association between Mal S180L and tuberculosis [84]. They did not have data for the other three diseases associated by Khor et al. The two studies use different methods of statistical analysis and are based on different populations. A separate study of Colombian individuals found that the Mal S180L polymorphism is protective against tuberculosis [85]. But a meta-analysis by Miao et al. supports Nejentsev et al., finding no tuberculosis association [86].

Despite the ongoing controversy in relation to tuberculosis, heterozygous S180L Mal has also been associated with: Behçet's disease [87], systemic lupus erythematosus (SLE) [85] protection from serious infection in HIV-1 patients [88], and a lower risk of Trypanosoma cruzi infected individuals developing chronic Chagas cardiomyopathy [89]. Rheumatoid arthritis is not believed to be associated with the Mal S180L SNP [90] demonstrating expected disease specificity. Ladhani et al. report a fascinating association between the failure of the Haemophilus influenzae serotype b (Hib) vaccine in British patients with a Mal SNP (rs1893352) with strong linkage disequilibrium with Mal S180L [91]. This is some of the best evidence yet for a genetic explanation for general vaccine failure, with Mal playing a central role. The diverse array of infection association with Mal SNPs is not likely to be coincidental, perhaps indicating that those studies not finding tuberculosis association did so due to unique factors particular to those geographical data sets, or because of the methods used for analysis. Because of the heterogeneity of populations in Africa and the very small frequency of polymorphism in some regions, it is clear that meta-analyses and back analysis of data sets that were acquired for different purposes will not be sufficient to determine the true disease association with the S180L Mal polymorphism.

Is There Redundancy of Human Mal?

A further source of confusion as to the role of human Mal comes from work by von Bernuth et al. They discovered nine autosomal recessive MyD88-defective children, who were highly susceptible to a range of pyogenic bacteria but not to other pathogens, indicating that in humans MyD88 might only be vital for protection from pyogenic bacterial infection, and redundant in adults [92]. If true it may indicate similar redundancy in human Mal.

However, without a larger sample size and analysis of such MyD88-defective individuals from a range of locations, with and without a prior history of vaccination, it is not possible to rule out a non-redundant role for human MyD88 in protection from other non-pyogenic bacterial pathogens. It might be possible that redundancy of MyD88 may occur in those MyD88-defective children, but not necessarily in the general population. From a molecular perspective it is not clear as to why MyD88-defective individuals would be susceptible to pyogenic bacteria and not to non-suppurative bacterial species.

To date there is no equivalent published data to indicate that human Mal is similarly redundant for protection from non-suppurative bacteria. It is worth repeating that at least in mice, unlike Mal, MyD88 is known to be important not only for IL-1 signaling [93-96] but also for signaling downstream of other TIR domain-containing cytokine receptors including the IL-18R [97] and IL-33R [98] and possibly also the IFN-γ receptor [99]. Also Mal participates in signaling via the above discussed MyD88-independent pathways. These functions of Mal may prove to be vital for protection from those pathogens that von Bernuth et al. hypothesize to be resistant to MyD88-dependent immune responses. This would position Mal centrally as a target for therapeutic modulation.

Conclusion

In the complex murine and human immune systems, there is undoubtedly a degree of redundancy in the role of Mal. Other TLR pathways utilizing other adaptors, as well as other non-TLR PRR pathways, can all account for Mal-independent antibacterial immune function. But the fact that components of "Toll like" signaling pathways are so conserved between species as disparate as sea anemones, fruit flies, and humans indicates their importance.

To date the majority of knowledge regarding TLR adaptor function relates to the effect these molecules have on NF-κB activity. Although NF-κB is vital for inflammation and immunity, there are many other transcription factors that are regulated in parallel with NF-κB. It is clear that both MyD88 and Mal activate NF-κB, but as we now know, Mal is more than a bridge. The future of research into

the function of Mal in disease will certainly focus more on these other signaling pathways.

Finally, in 2012 the information flow that began with the discovery of Drosophila Toll and led to our knowledge of human TLRs came full circle. Marek et al. published the discovery of a Drosophila Mal functional homolog, dMyD88, complete with a phosphoinositide binding domain reminiscent of the Mal PIP2 binding domain [100]. This reversal of the historical flow of knowledge from mice and humans to other species represents the fact that Mal is now one of the best studied TLR signaling molecules, and highlights our recognition that Mal plays a vital conserved role at the forefront of our immunological response to microbial infection.

Figure 1.
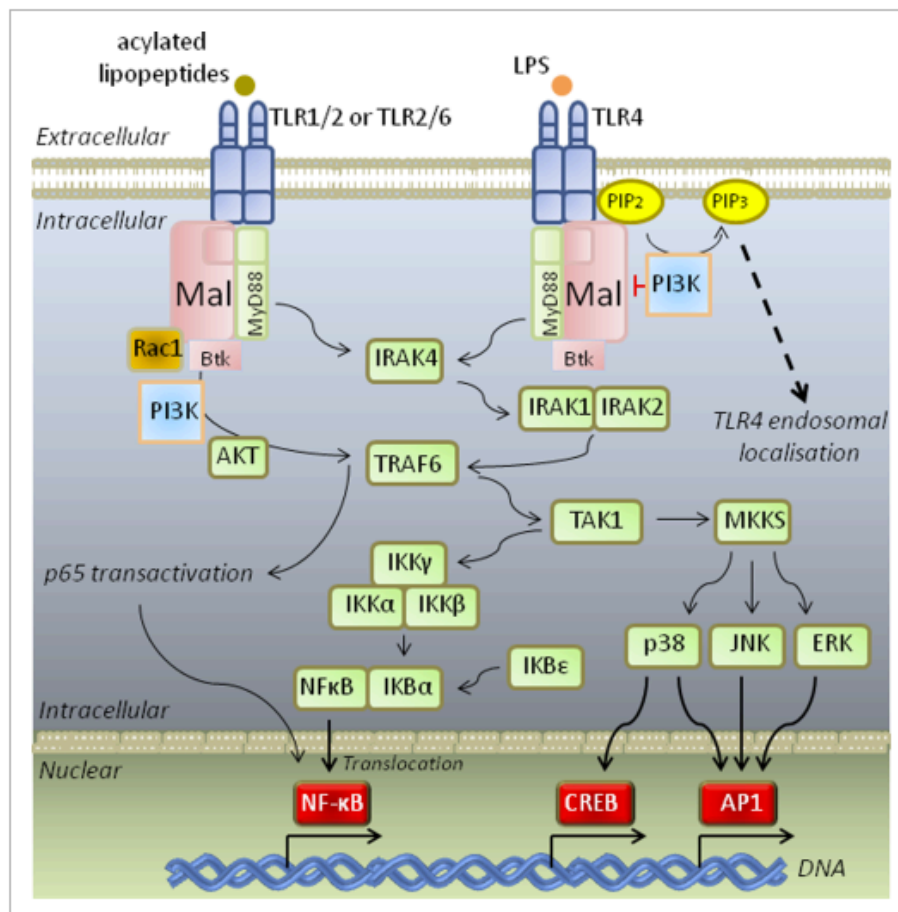
Mal in TLR signaling. Mal only signals downstream of TLR2 and TLR4. TLR4 is activated by LPS. TLR1/2 heterodimers are ligated by triacylated lipopeptides. TLR2/6 heterodimers ligate diacylated lipopeptides. Mal recruits MyD88 to the plasma membrane leading to NF-κB nuclear translocation via IRAK4, IRAK1/2, TRAF6, TAK1, and release of NF-κB from IκB. Mal also transactivates p65 NF-κB

via a MyD88-independent Rac1, PI3K, Akt, TRAF6 pathway. Mal can also activate CREB independent of MyD88 via p38 Map kinase kinase (MKK) pathways. The PI3K p110δ isoform can negatively regulate Mal signaling by converting PIP2 to PIP3 thus removing the anchor point for Mal and pushing TLR4 into endosomes for TRIF/TRAM dependent signaling.

Figure 2.
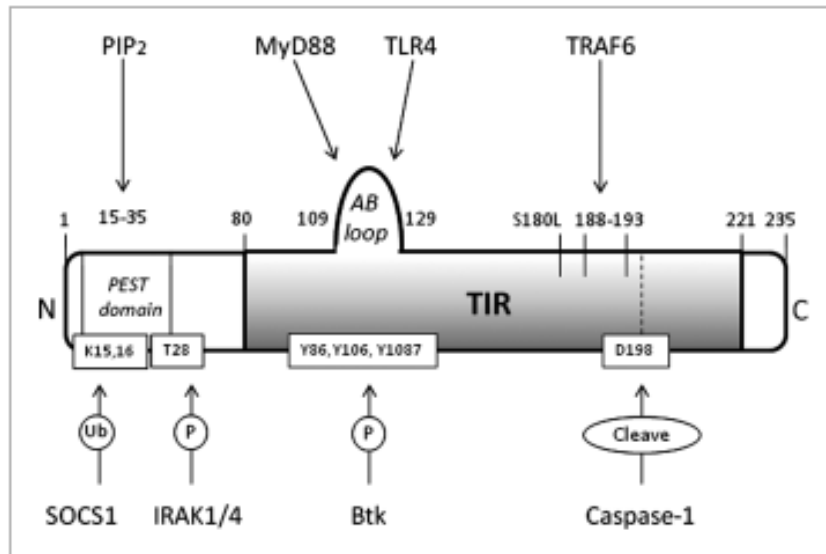
Mal binding sites (above) and post-translational modifications (below) and domains. Human and mouse Mal sites are presented together.
P = phosphorylation. Ub = polyubiquitination.

Table 1. The relationship between various pathogens, diseases and the role of Mal.

Table 1. The relationship between various pathogens, diseases and the role of Mal.

Pathogen	Disease	Effect
A. Phenotype of Mal-deficient mice		
E.coli	Lung pathology, Bacteraemia	Protective (ref: [64])
Pseudomonas aeruginosa	None	Redundant (ref: [65])
Klebsiella pneumoniae	Klebsiella pneumonia	Protective(ref: [65])
Bordetella pertussis	Whooping cough	Protective (*)
Salmonella enterica serovar Typhimurium	Salmonellosis	Redundant (ref: [66, 67]) Protective (ref: [68])
B. Human Mal S180L disease association		
Mycobacterium tuberculosis	Tuberculosis	Protective (ref: [81, 82, 85]) Redundant (ref: [84, 86])
Streptococcus pneumoniae	Invasive pneumococcal disease	Protective (ref: [82])
Trypanosoma cruzi	Chronic Chagas Cardiomyopathy	Protective (ref: [89])
Plasmodium falciparum	Malaria	Protective (ref: [82])
Haemophilus influenzae b	Vaccine failure	Protective (ref: [90])
HIV-1	AIDS	Protective (ref: [88])
N/A	Systemic Lupus Erythematosus	Protective (ref: [85])
N/A	Rheumatoid arthritis	Redundant (ref: [90])
N/A	Behçet's disease	Protective (ref: [87])

A is based on published evidence of infection models of Mal knockout mice. B is based on studies to assess disease association with human heterozygote S180L Mal SNP. (*) is our own unpublished observation. 190x254mm (96 x 96 DPI)

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