Identification of Binding Sites for Myeloid Differentiation Primary Response Gene 88 (MyD88) and Toll-like Receptor 4 in MyD88 Adapter-like (Mal)*

Celia Bovijn, Anne-Sophie Desmet, Isabel Uyttendaele, Tim Van Acker, Jan Tavernier, and Frank Peelman

From the Department of Medical Protein Research, Flanders Interuniversity Institute for Biotechnology (VIB) and Ghent University, B-9000 Ghent, Belgium

Background: It is unknown how the TIR domain of the adapter Mal binds MyD88 to TLR4.

Results: We identified mutations in the Mal TIR domain that affect its interactions and signaling.

Conclusion: Four binding sites and the AB loop in the Mal TIR domain are required for binding TLR4 and MyD88.

Significance: This work provides new insights in TIR-TIR interactions.

Upon activation, Toll-like receptor 4 (TLR4) binds adapter proteins, including MyD88 (myeloid differentiation primary response gene 88) and Mal (MyD88 adapter-like) for its signal transduction. TLR4 and the adapter proteins each contain a Toll/IL-1 receptor domain (TIR domain). In this study we used random mutagenesis and the mammalian two-hybrid method MAPPIT (mammalian protein-protein interaction trap) to identify mutations in Mal that disrupt its interaction with TLR4 and/or MyD88. Our study shows that four potential binding sites and the AB-loop in the Mal TIR domain all contribute to the formation of the TLR4-Mal-MyD88 complex. Mutations in the symmetrical back-to-back Mal homodimer interface affect Mal homodimerization and interaction with MyD88 and TLR4. Our data suggest that Mal dimerization may lead to formation of potential binding platforms on the top and the side of the Mal dimer that bind MyD88 or TLR4. Mutations that affect the interaction of Mal with MyD88 also affect NF-kB activation induced by Mal overexpression. In MAPPIT, co-expression of the MyD88 TIR domain enhances Mal dimerization and binding to TLR4. Similarly, co-expression of Mal and the MyD88 TIR domain strongly promotes dimerization of the TLR4 intracellular domain in MAPPIT. The different types of TIR-TIR interactions in the TLR4-Mal-MyD88 complex thus show cooperative binding in MAPPIT. We present plausible models for the TIR-TIR interactions in the TLR4-Mal-MyD88 complex.

Toll-like receptors are type I transmembrane receptors that recognize pathogen-associated molecular patterns (1–3). TLRs oligomerize upon ligand binding, which initiates multiple signaling pathways that lead to specific inflammatory and immune responses. The intracellular C-terminal part of TLRs contains a Toll-IL1 receptor (TIR) domain, which is also found in members of the IL-1 receptor family and in TLR adapter proteins involved in the signaling pathways of TLRs and IL-1 receptor family members (4). Signaling requires multiple interactions between TIR domains (5). The TIR domains probably interact at three levels in TLR signaling: oligomerization of the receptor TIR domains (R interface), oligomerization of the adapter TIR domains (A interface), and the receptor-adapter association (S interface) (6). Five TIR adaptors have been discovered: myeloid differentiation primary response gene 88 (MyD88), MyD88 adapter-like protein (Mal), TIR domain-containing adaptor protein inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo-motif-containing protein (SARM) (7). TLR1, -2, -4, -5, -6, -7, -8, -9, and -10 use MyD88 as a signaling adapter to activate the nuclear factor κB (NF-κB) and MAP kinases (2, 3, 7). Signaling via MyD88 leads to the assembly of a death domain (DD) complex called the Myddosome (8, 9). The Myddosome crystal structure comprises six MyD88, four IRAK4, and four IRAK2 death domains, which form a helical complex (9). Four MyD88 molecules are minimally required to form one layer in the helical assembly, leading to the suggestion that higher order TLR oligomerization may be required for signaling via MyD88 (9). TLR3 and -4 use TRIF as a signaling adapter to activate interferon regulatory factor 3 (10, 11). TLR4 uniquely uses both MyD88 and TRIF as a signaling adapter. Binding of TRIF to TLR4 requires endocytosis of TLR4 and uses TRAM as a bridging adapter to recruit TRIF (11–13). TLR4 signaling via the MyD88-dependent pathway requires Mal as a bridging adapter (14, 15). Mal contains an N-terminal phosphatidylinositol 4,5-bisphosphate binding domain, directing it to the membrane, and

The abbreviations used are: TLR, Toll-like receptor; TIR domain, Toll/IL-1 receptor domain; TLR4c, intracellular domain of Toll-like receptor 4; MyD88, myeloid differentiation primary response gene 88; MAL, Mal, MyD88 adapter-like; TRIF, TIR domain-containing adapter inducing interferon-β; TRAM, TRIF-related adapter molecule; MAPPIT, mammalian protein-protein interaction trap; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl) glycine; CDTA, 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid.
a C-terminal TIR domain (16). Binding of Mal to phosphatidylinositol 4,5-biphosphate helps to recruit MyD88 to TLR4 at the cell surface (16). MyD88-dependent signaling of TLR2 is promoted by Mal, but Mal may be dispensable at higher TLR2 ligand concentrations (17, 18). Mal can play an inhibitory role in TLR3 signaling via JNK and IRF7 (17, 19). Bruton’s tyrosine kinase can interact with Mal, and this kinase is required for signaling via TLR4 (20–22). Phosphorylation of Mal by IRAK1 and -4 promotes Mal ubiquitination and degradation (23).

The crystal structures of the TIR domains of TLR1, TLR2, TLR10, IL-1RAPL, and Mal have been determined (6, 24–28). The structure of the MyD88 TIR domain was determined by NMR (29). TIR domains typically consist of a central five-stranded parallel β-sheet surrounded by helices. The BB loop between β-sheet B and α-helix B is important for the functionality of the TIR domain. A P712H mutation in this loop in mouse TLR4 abolishes LPS signaling (30). Similar mutations in the BB loop of other TLR receptor and adapter TIR domains were shown to disrupt their interactions or signaling (6, 11, 31–33).

The recent crystal structures of the Mal TIR domain provide new insights in the possible architecture of the TLR4-Mal-MyD88 complex (27, 28). Both crystal structures present a symmetrical “back-to-back” Mal-Mal interface and an asymmetrical Mal-Mal interface. The importance of the back-to-back interface for the function of Mal was demonstrated via site-directed mutagenesis (27, 28). Mutations designed to disrupt the back-to-back interaction affect the NF-κB activation induced by Mal overexpression and the formation of Mal homodimers in solution (27, 28). Unexpectedly, the predicted BB loop motif of Mal is actually part of an extended AB loop in the Mal structures. Mutations in this AB loop disrupt the interaction with MyD88 and TLR4 and affect NF-κB activation (27, 28).

Different interaction sites for TLR4 and MyD88 were proposed based on the Mal crystal structures. Valkov et al. (27) proposed a MyD88 binding site around residues Asp-96 and Ser-180 in Mal, which would provide a rationale for the phenotype of the naturally occurring D96N and S180L mutations. However, the study of Lin et al. (28) showed that the D96N and S180L Mal mutants can interact normally with MyD88 and TLR4 in GST pulldown assays and proposed the AB loop as the prime interaction site for both TLR4 and MyD88. The interaction sites of Mal for MyD88 and TLR4 thus remain a matter of debate.

Mammalian protein-protein interaction trap (MAPPIT) is a two-hybrid method based on the JAK-STAT signaling pathway of cytokine receptors (34, 35). MAPPIT allows the detection of protein interactions in intact mammalian cells and can be used to study TIR-TIR interactions (33, 36, 37). We previously used MAPPIT to test the role of a putative caspase-1 cleavage site at Asp-198 in Mal and to map mutations in the TLR4 TIR domain that affect its dimerization and its interactions with MyD88 and Tram (38, 39). In this article we combined MAPPIT with random mutagenesis and NF-κB activation assays to identify the interaction sites of Mal involved in its homodimerization and its binding to TLR4 and MyD88.

**EXPERIMENTAL PROCEDURES**

**Vectors**—The vectors pCLL (33, 34), pCLG-TLR4ic (39), pMG2-TLR4ic (39), pMG2-MyD88TIR, pMG2-SVT, pMG2-SH2β (33), pCAGGSE-Mal (38), and pMET7-FLAG-SVT (33) were as described before. pCLG-MyD88 was created by BamHI/NotI digest and ligation into the pCLG vector (40), whereas the pCLG-Mal construct was obtained by SacI/NotI digest and ligation into the pCLG vector. After digest of the pCLG-MyD88TIR bait (33) with BamHI/NotI, the MyD88TIR fragment was cloned in the pCLG vector (40), resulting in pCLG-MyD88TIR. The QuikChange® (Agilent technologies) site-directed mutagenesis method was applied for site-directed mutagenesis. All primers for mutagenesis, sequencing, and PCR amplification are listed in supplemental Table 1.

In the pMG2-Mal prey (33), we introduced an XhoI site (primers 1 and 2) to enable insertion of randomly mutated Mal TIR PCR fragments and a BclI site (primers 3 and 4) to allow exchange of these Mal TIR mutant fragment with the pMX-Mal-ires-gfp construct.

An MfeI site was introduced (primers 13 and 14) in pMX-Mal-ires-gfp (38) for exchange of the Mal mutant fragments from the pMG2-Mal mutant preys. The Mal DNA from the pMG2-Mal wild-type construct was inserted in the pMX-Mal-ires-gfp via BclI/MfeI. MalTIR mutants were exchanged from the pMG2-Mal vector via XhoI/MfeI digestion and ligation.

pMET7-FLAG-Mal and pMET7-FLAG-MalTIR plasmids were generated by amplification of Mal (primers 5 and 6) and the TIR domain of Mal (primers 7 and 8) from the pDC304-Mal vector (a kind gift of Dr. O’Neill), EcoRI/XbaI, or BstEII/XbaI digest and ligation in the pMET7-FLAG construct (41). A stop codon was introduced in the pMET7-FLAG-Mal construct before D85 (primers 9 and 10), resulting in the pMET7-FLAG-MaldeltaTIR construct.

The MyD88P200H fragment of the pMG2-MyD88P200H (33) MAPPIT prey was amplified by PCR (primers 11 and 12) and ligated into the pMET7-FLAG vector (41) after EcoRI/XbaI digest to generate pMET7-FLAG-MyD88P200H. The pXP2d2-rPAPI-luciferase reporter, originating from the rPAPI (rat pancreatic-associated protein 1) promoter was previously described (34). The pNF-conluc reporter was a gift from Dr. Alain Israel.

**Molecular Modeling of the AB-loop**—Residues 112–123 (AB loop) and 168–171 are unresolved in the Mal crystal structures. We built 1000 models for a Mal dimer using Modeler Version 9.10, with the dope_loopmodel procedure and symmetry restraints between both dimers (42). The Mal TIR structure with PDB code 2Y92 was used as template (27). The model with the best DOPE score (42) was used for mapping the mutations as described below.

**Random Mutagenesis of the Mal MAPPIT Prey**—The sequence corresponding to Tyr-86—Leu-235 in the pMG2-Mal prey construct was randomly mutated using the GeneMorph™II Random Mutagenesis kit (Agilent Technologies) with primers 55 and 56 (supplemental Table 1). The amount of plasmid (200 ng) and number of PCR cycles (10) were optimized so that one of three constructs carried a single point
Interaction Sites in the Mal TIR Domain

mutation. The PCR products were digested with XhoI and XbaI and ligated into the XhoI/XbaI opened pMG2-Mal prey. The DNA pool was transformed via electroporation into Escherichia coli DH10B. 1152 mutant single colonies were inoculated in 16 96-well blocks with 2× yeast Tryptone (YT) medium. 72 mutant prey colonies were grown in rows A–F of the 96-well plate, 12 wild-type pMG2-Mal prey were inoculated in row G, and row H was inoculated with 12 pMG2-MalP125H preys. DNA was prepared via an automated procedure using the Nucleospin®Robot-96 (Machery-Nagel) plasmid kit with the Freedom EVO 100 liquid handling platform (Tecan). The prey DNA concentration was determined with the Magellan UV A (Sigma), 530

DNA was prepared via an automated procedure using the Nucleospin®Robot-96 (Machery-Nagel) plasmid kit with the Freedom EVO 100 liquid handling platform (Tecan). The prey DNA concentration was determined with the Magellan UV spectrophotometer (Tecan) and subsequently diluted to 20 ng/μl. This Mal prey DNA was used in the automated 384-well transfections described below.

Automated Transfection Protocol in 384-Well Plates—We previously described an automated transfection procedure in 384-well plates to screen for the random mutations that affect the MAPPIT interaction (43). HEK 293T cells (HEK293T/17) were obtained from www.atcc.org. The day before transfection, 3000 HEK 293T cells/well were seeded into black 384-well plates. The next day the cells were transfected with 5 ng of bait, 5 ng of prey, and 2 ng of pXP2d2-rPAPI-luciferase reporter using a calcium phosphate precipitation method with the Freedom EVO 200 liquid handling platform (Tecan). Each bait/prey combination was transfected in 8 wells, implying that the DNA of one 96-well DNA plate was transfected over two 384-well plates. Each transfected 384-well plate thus contains transfections for 36 random Mal prey mutants, 6 wild-type Mal preys, and 6 Mal P125H prey mutants, each in combination with the same MAPPIT bait. The next day four of the eight wells were stimulated with 100 ng/ml leptin, and the other four wells (transfected with the same bait/prey transfection mix) were left untreated. The day after leptin stimulation, luciferase activity was measured using the EnSpire plate reader (PerkinElmer Life Sciences). The cells were lysed in 15 μl of cell culture lysis reagent buffer (25 mM Tris/phosphate, pH 7.8, 2 mM DTT, 2 mM CDTA, 10% glycerol, 1% Triton X-100). Just before measurement, 8 μl of luciferase substrate buffer was added (40 mM Tricine, 2.14 mM (MgCO3)2Mg(OH)25H2O, 5.34 mM MgSO47H2O, 66.6 mM DTT, 0.2 mM EDTA, 270 μM coenzyme A (Sigma), 530 μM ATP (Sigma), 470 μM Luciferin (Duchefa)).

Analysis of the Random Mutagenesis 384-Well MAPPIT Data—The “MAPPIT signal” was determined as -fold induction of luciferase activity upon leptin stimulation by dividing the median luciferase activity of the four leptin stimulated wells by the median luciferase activity of the four unstimulated wells. We calculated a “normalized MAPPIT signal” for each Mal prey mutant in a transfection by dividing its MAPPIT signal by the median of the MAPPIT signals of the six wild-type baits on the same 384-well plate. The 384-well transfections were repeated three times. The median of the three normalized MAPPIT signal values of a mutant in the three transfection experiments was multiplied by 100 to obtain the “relative MAPPIT signal” of the mutant, expressed as percentage of wild type. We determined a similar relative MAPPIT signal for each of the six wild types on the plate (supplemental Fig. 1). In this case the normalized MAPPIT signal of the wild type was calculated by dividing its MAPPIT signal by the median of the MAPPIT signals of the other five wild-type baits on the plate. The relative MAPPIT signal of the wild type is the median of the normalized MAPPIT signals of that wild type in the three transfections multiplied by 100. We determined the variation of the relative MAPPIT signal of the wild-type for the 192 transfected wild-type baits in our 384-well MAPPIT screens.

Sequences were determined on Applied Biosystems 3730XL DNA Analyzers at the VIB Genetic Service Facility with sequencing primers 57 and 58 (supplemental Table 1). The sequences were aligned to the pMG2-Mal prey protein sequence using NCBI BLASTx (44). The resulting protein alignments were concatenated and realigned using MAFFT (45) and visualized with Jalview (46) to identify amino acid substitutions. The position of the mutations on a model for Mal was visualized using University of California San Francisco chimera (47). The mutations were colored according to their relative MAPPIT signal using the “render by attribute” tool of University of California San Francisco chimera (47).

MAPPIT Protocol with Transfection in 96-Well Plates—10^4 HEK293T cells/well were seeded the day before transfection in black 96-well plates (Nunc, Thermo Scientific). Six replicate wells were transfected overnight with 20 ng of bait, 20 ng of prey, and 7.5 ng of pXP2d2-rPAPI-luciferase reporter per well using a standard calcium phosphate precipitation procedure. One day after transfection three wells were stimulated with 100 ng/ml leptin, whereas the other three wells were left unstimulated. The next day cells were lysed in 50 μl of cell culture lysis reagent buffer for 10 min, 35 μl of luciferase substrate buffer was added, and luciferase activity was measured in triplicate using a chemiluminescence reader (TopCount, PerkinElmer Life Sciences).

Determination of Prey Expression via Western Blot—The lysates of the 96-well luciferase assays were vacuum-dried and resolved in loading buffer (40 mM Tris-HCl, pH 6.8, 2% SDS, 8% glycerol, 0.01% bromphenol blue, 2.5% β-mercaptoethanol). The samples were electrophoresed on a 12% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. FLAG-tagged prey were revealed using monoclonal mouse anti-FLAG antibody M2 (Sigma). The loading control β-actin was revealed using monoclonal rabbit-anti-actin antibody (Sigma). Secondary goat-anti-mouse Dylight 680 antibody (Thermo Scientific) and secondary goat-anti-rabbit Dylight 800 antibody (Thermo Scientific) were used for detection. Western blot analysis was performed using the Odyssey Infrared Imaging System (Li-Cor).

NF-κB Reporter Assays—The day before transfection 10^4 HEK293T cells/well were seeded into black 96-well plates (Nunc). Cells were transfected with 50 ng of pMG2-Mal wild-type or mutant prey and 5 ng of pNFconluc reporter. After 48 h, the cells were lysed, and luciferase activity was measured as described above.

AlphaScreen Analysis—The day before transfection 3 × 10^5 HEK293T cells/well were seeded in 6-well plates. 1 μg of pCAGGSE-Mal and 1 μg of pMG2-Mal wild-type or mutant prey DNA was transfected per well. The cells were lysed 48 h post-transfection in 50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 5% glycerol, 0.2% Nonidet P-40, 1.5 mM MgCl2, 25 mM NaF, 1 mM
Na<sub>2</sub>VO<sub>4</sub> and complete Protease Inhibitor Mixture (Roche Applied Science). AlphaScreen experiments were performed according to the manufacturer's protocol (PerkinElmer Life Sciences). The lysates were incubated for 2 h at room temperature with biotinylated anti-E-tag antibody (Amersham Biosciences), subsequently for 1 h with the AlphaScreen FLAG™ (M2) detection kit (PerkinElmer Life Sciences) acceptor beads, and finally for 30 min with streptavidin donor beads. The lysates were transferred in triplicate into 384-well plates and measured using the EnVision plate reader (PerkinElmer Life Sciences). Expression of pCAGGSE-Mal and the preys was verified via Western blot analysis as described above using monoclonal mouse-anti-E-tag antibody (Amersham Biosciences) and rabbit-anti-FLAG antibody (Sigma).

**Transduction of Immortalized Mal Knock-out Macrophages**—

1.9 × 10<sup>6</sup> HEK293T cells were seeded in 75-cm<sup>2</sup> flasks (Nunc) in DMEM medium (Invitrogen) with 10% fetal bovine serum (Invitrogen). The next morning the cells were transfected with 10 μg of Moloney murine leukemia virus gag-pol expression vector, 1.25 μg of pCG VSV-G expression vector, and 10 μg of pMX-Mal(mutant)-IRE-S-GFP vectors using a calcium phosphate precipitation method; the virus-containing supernatant was harvested 48 and 72 h after transfection and pooled. 24 h before transduction 1 × 10<sup>6</sup> immortalized MalKO macrophage cells (a kind gift of Jonathan Kagan, Harvard Medical School, Boston, MA) were seeded into 24-well plates (Nunc) in 0.5 ml of DMEM + 10% FBS. The virus-containing HEK293T supernatants were centrifuged for 3 min at 720 × g to and filtered over a 0.45-μm nitrocellulose filter (Milllex). The virus was concentrated by ultracentrifugation at 90,000 × g for 130 min at 4°C in a SW28 rotor (Beckman) and resuspended in 100 μl of DMEM medium. The virus was added to the MalKO macrophages together with 8 μg/ml Polybrene (Sigma) and spun down for 1 h at 200 × g. After 1 h, 1 ml of fresh medium was added. After 10 days, GFP-positive cells were sorted using a DakoCytomation Mo-Flo fluorescence-activated cell sorter. Expression of Mal or its mutants was tested using Western blot with an anti-Mal antibody (FL-235; Santa Cruz Biotechnology) and secondary antibody (HRP labeled goat-anti-rabbit IgG (Jackson ImmunoResearch).

**Affect the Mal-MyD88 and Mal-TLR4 Interaction**—In this work we used MAPPIT to identify mutations of Mal that affect its dimerization or its interaction with the intracellular part of TLR4 or the TIR domain of MyD88. The principle of MAPPIT and the MAPPIT setup used in this article are outlined in Fig. 1A. MAPPIT was able to detect the interaction of a Mal prey with baits for Mal, the MyD88 TIR domain, and the TLR4 intracellular domain (Fig. 1B). A P125H mutation in the Mal AB loop was reported to disrupt the Mal-TIR interaction (31). To test whether the MalAB loop is involved in the Mal-MyD88 interaction, we tested the MalP125H mutant as the negative control in the MAPPIT assays (Fig. 1B). The P125H mutation in the Mal prey strongly reduces the luciferase readout, indicating that MAPPIT can be used to study the three tested interactions of Mal and to identify mutations that disrupt these interactions.

**Random Mutagenesis and MAPPIT Identify Mutations That Affect the Mal-MyD88 and Mal-TLR4 Interaction**—We recently developed a method that combines random mutagenesis and MAPPIT to screen for mutations that disrupt protein-protein interactions in the Mal TIR domain.
**Interaction Sites in the Mal TIR Domain**

Interactions (43). We use this method here to identify mutations in the TIR domain of the Mal prey that affect the interaction with TLR4 and/or MyD88. The sequence corresponding to Tyr-86-Leu-235 in the TIR domain of the Mal prey was randomly mutated. We tested the interaction of 1152 putative Mal prey mutants with the TLR4ic and MyD88TIR bait in 384-well MAPPIT assays. Each 384-well plate contained 36 random mutants and 6 wild-type Mal preys. We determined a relative MAPPIT signal for the mutants as described under “Experimental Procedures.” The relative MAPPIT signals of the wild type do not vary by more than 50% (supplemental Fig. 1). In contrast, we identified 114 unique Mal prey mutants with a single amino acid substitution that had a relative MAPPIT signal <50% that of the wild-type for one or both tested interactions, suggesting that these mutations possibly inhibit the interaction between bait and prey. These 114 unique mutations affect 86 different residues (supplemental Table 2). 48 of these mutants were selected for further analysis based on their solvent accessibility in a Mal homology model.

10 Mal preys with multiple amino acid substitutions seemed to specifically affect either the interaction with the TLR4 or with the MyD88 bait (supplemental Table 3). To determine whether a single amino acid substitution could mimic this specificity, we created 10 Mal preys with the corresponding single amino acid substitutions (supplemental Table 3). Only mutations that were not identified as single mutant in the screen were generated. We further created nine Mal preys with additional substitutions for various reasons (supplemental Table 4).

These 19 site-directed Mal prey mutants and the 48 selected mutants from our random mutagenesis screen were subjected to further analysis. We (re)tested the interaction of this subset of 67 Mal preys with the TLR4ic, Mal, and MyD88TIR baits in 96-well MAPPIT assays and tested their expression level via Western blot (supplemental Fig. 2). All 67 mutant preys were properly expressed. The C157S mutant shows a faster migration pattern on SDS-PAGE as discussed later. We further tested the effect of the 67 selected mutants on interaction with E-tagged Mal in AlphaScreen™ assays and on NF-κB activation upon overexpression of the Mal prey. The results are summarized in supplemental Table 2 and are described below.

**Three Potential Binding Sites and the AB Loop Are Important for the Mal-MyD88 Interaction**—The 114 mutations that affected one or both interactions in the MAPPIT screen were mapped on the crystal structure of Mal and colored according to the relative MAPPIT signal (Fig. 2). Mutations that affect the MyD88TIR-Mal bait-prey interaction are mainly found in three surface areas I, II, and III that may form potential interaction interfaces (Fig. 2). 96-Well MAPPIT assays of the MyD88 bait-Mal prey interaction of the selected Mal mutants confirm that mutations in these three areas affect the Mal-MyD88TIR interaction in MAPPIT (Fig. 3).

The first of these potential interaction interfaces corresponds to the symmetrical Mal-Mal dimer interface found in the crystal structure (Figs. 2A and 3A). The effect of mutations in this interface on the MyD88TIR-Mal bait-prey interaction may be due to disruption of the Mal-Mal interaction. This is in line with previous observations that Mal-MyD88 interaction may require Mal dimerization via the back-to-back interface (27).

Surface area II was found around residues Gln-135 and Thr-156, whereas surface area III was found around residues Arg-215 and Tyr-195. Residues that may be part of these binding sites are indicated in Figs. 2B and 3B. Surface areas II and III are at opposite sides of a Mal monomer. However, in the symmetrical back-to-back Mal dimer, surface areas II and III of both monomers became juxtaposed and formed a continuous potential binding interface (II + III). As the dimer is symmetrical, this potential II + III interface is found at two sides of the dimer (Figs. 2B and 3B). Trp-156 is at the center of the continuous interface. The site-directed W156A mutant profoundly affected the Mal-MyD88 interaction in support of a possible role of a continuous binding platform for MyD88 interaction. Mutations in the AB loop also strongly affect the Mal prey-MyD88TIR bait interaction. In the crystal structure of Mal, amino acids 112–123 of the AB loop are invisible in the electron density (27). We, therefore, built homology models based on the Mal crystal structure with the missing loop. Loops of this
length are particularly hard to model reliably, and these models at best give an estimate of the possible location of the loop. Nevertheless, the models suggest that the AB loop may come close to the potential binding sites II and III. The AB loop may thus be part of a more extensive binding site, consisting of the AB loop and potential binding sites II and/or III.

Four Potential Binding Sites and the AB Loop Are Important for the Mal-TLR4 Interaction—We similarly mapped the effect of the 114 mutations on the Mal prey-TLR4ic bait interaction in our screen (Fig. 4). Mal prey mutations that affect the interaction with the TLR4 bait again coincide with the three interfaces I, II, and III and the AB loop. 96-Well MAPPIT analysis of the TLR4ic bait-Mal prey interaction of the 67 selected Mal mutants confirms this clustering in three main surface areas (Fig. 5). Mutations that affect the TLR4ic bait-Mal prey interaction also cluster at the “top” of the symmetrical Mal dimer interface (Figs. 4B and 5B). Possibly, this area forms a binding site IV, with an important role in TLR4 binding (Figs. 4C and 5C). Also, mutations around Arg-184 at the border of this site IV tend to have a more pronounced effect on the TLR4ic bait-Mal prey than on the Mal prey-MyD88TIR bait interaction: R184I, A185V, Y187C, and Y187A. Binding site IV and the area around Arg-184 may be relatively more important for the interaction with TLR4 than with MyD88.

The Symmetrical Back-to-back Interface Is Essential for the Mal-Mal Interaction—The crystal structures of Mal suggest two possible Mal-Mal interaction interfaces (27, 28). Mutations in the symmetrical interface affect the Mal-MyD88TIR interaction and the Mal-TLR4ic interaction in MAPPIT (Figs. 2A, 3A, and 4A). Several mutations that affect the Mal-TLR4ic interaction in the 384-well MAPPIT screen are found in the dimer interface. A, mapping of the mutants on a Mal monomer, oriented as in Fig. 2A. Several mutations that affect the Mal-MyD88TIR interaction in the 96-well MAPPIT assays are found in the dimer interface. B, mapping of the mutants on a Mal dimer is shown. Residues that affect the Mal-MyD88TIR interaction in the 96-well assays are found at the side of the dimer in two potential binding sites II and III and in the AB-loop.

FIGURE 3. Mapping of the effect of 67 selected Mal prey mutations in the Mal-MyD88TIR 96-well MAPPIT test. Residues that correspond to the 67 selected Mal mutants are colored according to the effect on the Mal-MyD88TIR interaction in the 96-well MAPPIT tests as indicated on the color scale. Residues that were not mutated are colored black. Backbone atoms are colored gray. A DTT molecule linked to Cys-157 is colored blue. A, shown is mapping of the mutants on a Mal monomer, oriented as in Fig. 2A. Several mutations that affect the Mal-MyD88TIR interaction in the 96-well MAPPIT assays are found in the dimer interface. B, mapping of the mutants on a Mal dimer is shown. Residues that affect the Mal-MyD88TIR interaction in the 96-well assays are found at the side of the dimer in two potential binding sites II and III and in the AB-loop.

FIGURE 4. Mapping of the Mal-TLR4ic 384-well MAPPIT screen data on a model of Mal. Residues are colored according to the effect on the Mal-TLR4ic interaction in the 384-well MAPPIT screen, as in Fig. 2A. Several mutations that affect the Mal-TLR4ic interaction in the 384-well MAPPIT screen are found in the dimer interface. A, shown is mapping of the mutants on a Mal monomer, oriented as in Fig. 2A. Several mutations that affect the Mal-TLR4ic interaction in the 384-well MAPPIT screen are found in the dimer interface. B, mapping of the mutants on a Mal dimer is shown. The dimer interface is indicate by a dashed line. Residues that affect the Mal-TLR4ic interaction are found at the side of the dimer in two potential binding sites II and III, in the AB-loop, and in a potential binding site IV on the top of the dimer. C, the model in panel B is rotated 90° around the x axis, with binding site IV oriented toward the viewer. Residues that affect the Mal-TLR4ic interaction are found at binding site IV.
tested in an AlphaScreen proximity assay (supplemental Table 2). The effects of 38 mutations on the Mal-Mal AlphaScreen assay was mapped on the symmetrical Mal-Mal dimer in Fig. 6. Strikingly, mutations at residues Pro-155, Trp-156, Lys-158, and Glu-190 of the symmetrical interface all affect both Mal-Mal interaction assays, which supports a role of this area in Mal-Mal interaction. Other mutations that affect the Mal-Mal interaction assays are either only effective in one of both assays, buried in the structure, or surrounded by mutations that do not affect the Mal-Mal interaction at all. This suggests that the symmetrical Mal-Mal interface can be the major Mal-Mal interaction site.

In the asymmetrical interface in the Mal crystal structures, Trp-82 of one monomer binds into a pocket formed by Arg-184, Tyr-187, Pro-189, Arg-192, and Tyr-196 (27, 28). Potential binding site III, as defined above (Figs. 2B and 3B), seems to overlap with the asymmetrical interface. However, mutations of Trp-82, Arg-184, Tyr-187, or Pro-189 affected neither of the two Mal-Mal interaction assays. This suggests that the asymmetrical interface is not required for the Mal-Mal interaction.

The Suggested Mal-Mal and Mal-MyD88 Interaction Sites Are Important for NF-κB Activation—Overexpression of Mal leads to activation of NF-κB (14, 31). Similarly, overexpression of the Mal MAPPIT prey leads to a strong activation of an NF-κB reporter. We tested the effect of our 67 selected Mal prey mutants on this Mal prey-induced NF-κB activation. Most of these Mal prey mutants display a strongly reduced NF-κB activation compared with the wild-type Mal prey (supplemental Table 2). Mutations that affect the NF-κB activation coincide with the mutations that affect the MyD88TIR bait-Mal

FIGURE 5. Mapping of the effect of 67 selected Mal prey mutations in the Mal-TLR4ic 96-well MAPPIT test. Residues that correspond to the 67 selected Mal mutants are colored according to the effect on the Mal-TLR4ic interaction in the 96-well MAPPIT tests as in Fig. 3A. Mapping of the mutants on a Mal monomer is colored as in Fig. 2A. Several mutations that affect the Mal-TLR4ic interaction in the 96-well MAPPIT assays are found in the dimer interface. B, mapping of the mutants on a Mal dimer is shown. Residues that affect the Mal-TLR4ic interaction in the 96-well assays are found at the side of the dimer in two potential binding sites II and III and in the AB-loop. C, the model in panel B is rotated 90° around the x axis, with binding site IV oriented toward the viewer. Residues that affect the Mal-TLR4ic interaction are found at binding site IV.

FIGURE 6. Mapping of the effect of 38 selected Mal prey mutations on Mal-Mal interaction in AlphaScreen assays. Residues that correspond to 38 tested mutants are colored according to the effect on the AlphaScreen assay, as indicated in the color scale in panel A. Other residues are colored black. Backbone atoms are colored gray. A DTT molecule linked to Cys-157 is colored blue, A, shown is mapping of the mutants on a Mal monomer, oriented as in Fig. 2A. Several mutations that affect Mal-Mal interaction are found in the dimer interface. B, mapping of the mutants on a Mal dimer is shown. The dimer interface is indicated by a dashed line. Residues that affect the Mal-Mal interaction outside the symmetrical interface (red/orange) are either buried or are isolated and surrounded by mutations that do not affect the Mal-Mal AlphaScreen assay.
prey interaction in potential interfaces I to III and the AB-loop (Fig. 7). Mutations in the potential binding site IV on the top of the Mal dimer seem to have a less drastic effect on the NF-κB activation. Overall, our data are compatible with a model where Mal dimerization via the symmetrical interface recruits MyD88 via potential binding sites II and III and the AB-loop, leading to activation of NF-κB, although we cannot exclude a direct involvement of binding site I (corresponding with the back-to-back dimer interface) in interaction with MyD88 and/or TLR4. A MAPPIT Complementation Assay Shows That the TLR-Adaptor Complex Is Stabilized by Multiple TIR Interactions—Mutations in the symmetrical Mal-Mal interface affect the interaction with TLR4 and MyD88 and NF-κB activation, indicating that Mal dimerization via the symmetrical interface possibly is required for interaction with TLR4 and MyD88 and subsequent signaling steps. Cooperation of the different interactions may stabilize the TLR4-Mal-MyD88 complex. To assess this hypothesis, we co-transfected FLAG-tagged MalTIR and/or MyD88TIR in our MAPPIT assays. Co-expressions of part of the SV40 large T antigen as irrelevant control (SVT) or of the MalΔTIR and MyD88P200H mutants were used as negative controls in this assay. MAPPIT-fold inductions of luciferase activity that are specifically increased after co-transfection of the FLAG-tagged MalTIR and/or MyD88TIR are indicated with an arrow. A, co-expression of the Mal TIR domain increases the TLR4ic-Mal bait-prey MAPPIT signal. B, co-expression of the MyD88 TIR domain increases the Mal-Mal and TLR4ic-Mal bait-prey MAPPIT signals. C, co-expression of MalTIR plus MyD88TIR increases the TLR4ic-TLR4ic bait-prey MAPPIT signal.

**FIGURE 7.** Mapping of the effect of 67 selected Mal prey mutations on NF-κB activation. Residues that correspond to 67 tested mutants are colored according to the effect on NF-κB activation, as indicated in the color scale in panel A. Other residues are colored black. Backbone atoms are colored gray. A DTT molecule linked to Cys-157 is colored blue. A, shown is mapping of the mutants on a Mal monomer, oriented as in Fig. 2A. Several mutations that affect NF-κB activation are found in the dimer interface. B, shown is mapping of the mutants on a Mal dimer. The dimer interface is indicated by a dashed line. Residues that affect the NF-κB activation are found at the side of the dimer in two potential binding sites II and III and in the AB-loop.

TLR4ic bait with the Mal prey, suggesting that MyD88 promotes Mal dimerization and the binding of Mal to TLR4. Most striking is the strong promoting effect of co-transfection of the Mal TIR plus MyD88 TIR domain on the interaction between the TLR4ic bait and TLR4ic prey. This supports the strong cooperative nature of the TLR4-Mal-MyD88 interactions.

**FIGURE 8.** A MAPPIT complementation assay shows that the TLR-adaptor complex is stabilized by multiple TIR interactions. We co-transfected FLAG-tagged MalTIR and/or MyD88TIR in our MAPPIT assays. Co-expressions of part of the SV40 large T antigen as irrelevant control (SVT) or of the MalΔTIR and MyD88P200H mutants were used as negative controls in this assay. MAP-PIT-fold inductions of luciferase activity that are specifically increased after co-transfection of the FLAG-tagged MalTIR and/or MyD88TIR are indicated with an arrow. A, co-expression of the Mal TIR domain increases the TLR4ic-Mal bait-prey MAPPIT signal. B, co-expression of the MyD88 TIR domain increases the Mal-Mal and TLR4ic-Mal bait-prey MAPPIT signals. C, co-expression of MalTIR plus MyD88TIR increases the TLR4ic-TLR4ic bait-prey MAPPIT signal.
mutants were expressed at somewhat lower levels: A123V, Q163H, and A185V. Despite the lower expression levels, the Q163H and A185V mutants were able to restore TNF-α production to levels comparable with the wild-type Mal. Mal KO complementation with the A186V and Y195N could restore LPS-induced TNF-α release like wild-type Mal. Cells transduced with the A123V mutant showed a reduced LPS-induced TNF-α release, but we cannot exclude that this was due to lower expression levels. The M194T mutant also showed a reduced TNF-α release, suggesting that this mutant in the Mal-Mal interface affects the TLR4-Mal-MyD88 pathway.

Although A186V, Y195N, and Q163H mutations all affect NF-κB activation induced by overexpression of Mal, these mutations do not seem to affect TLR4-Mal-MyD88 signaling. This suggests that the effect of the mutation is overcome when the mutant takes part in a full TLR4-Mal-MyD88 complex, possibly by cooperative binding of the different interaction partners. Likewise, an M194T mutation hardly affects the NF-κB activation induced by overexpression of Mal but seems to affect the TLR4-Mal-MyD88 signaling. Possibly, the effect of the mutation on the Mal-Mal interaction can be overcome by the stronger overexpression of the Mal mutant in the NF-κB activation assays.

Models for Mal Interaction with TLR4 and MyD88—The MAPPIT data suggest that the sites of mutations that affect the binding of Mal with Mal, MyD88, and TLR4 strongly overlap. Mutations in the three potential binding sites, I, II, and III, can affect all three interactions in our assays and have a negative effect on NF-κB signaling. Two models shown in Fig. 10 provide a rational explanation for the very strong coincidence of mutations that affect the interaction of Mal with TLR4 or MyD88 in our assays. In a first model Mal dimerizes via the symmetrical binding site found in the Mal crystal structure. At both sides of the dimer, two identical extended binding platforms are formed that consist of residues of binding sites II and III and the AB-loop of both monomers. The binding platform can bind both MyD88 and TLR4, explaining why mutations in this platform and in the Mal dimerization site affect binding to TLR4, MyD88, and NF-κB activation (Fig. 10A). In a second model the binding site II + III of the symmetrical dimer binds MyD88. MyD88 binding stabilizes the dimer and promotes binding to TLR4 via binding site IV at the top of the symmetrical dimer (Fig. 10B). A conceptual model in which MyD88 dimerizes in a way similar to Mal shows the possibility that a MyD88 dimer may interact with a Mal dimer via a similar extended binding site (II plus III and their BB- or AB-loops (Fig. 10C). Interestingly, this model is in line with the inhibitory effect of an R196A and R288A mutation on the Mal-MyD88 interaction (29).

DISCUSSION

In this study we used random mutagenesis and MAPPIT to detect Mal mutations that affect its dimerization or its interac-
Interaction Sites in the Mal TIR Domain

When mapped on the symmetrical back-to-back Mal dimer structure, we find three additional surface areas (II, III, and IV) with mutations that affect the interactions of the Mal prey with the TLR4ic and MyD88 TIR baits. These three sites may be potential binding sites for TLR4 and/or MyD88. Potential binding site II and III are found at opposite sides of a Mal monomer. Mutations in binding site II and III of the Mal prey affect both the interaction with the MyD88 TIR and TLR4ic bait and have a strong effect on the NF-κB activation induced by Mal overexpression. In the back-to-back dimer binding sites II and III seem to form an extended binding platform that may be involved in binding of MyD88 and/or TLR4. Like mutations in the binding site II and III, mutations in the AB loop of the Mal prey affect the interaction with the TLR4ic and MyD88 TIR baits. This supports the reported role of the AB loop as a key element in the interaction with MyD88 and TLR4 (28, 29). Models for the back-to-back dimer with the AB loop show the possibility that the AB loop flanks either binding site II or III. The AB loop may thus be part of an extended binding site consisting of the AB loop plus binding site II and/or III.

Most of the Mal prey mutations that affect MyD88 TIR bait binding and NF-κB activation also affect TLR4ic bait binding. Possibly, the same site on Mal can bind to both TLR4 and MyD88. As Mal can form symmetrical dimers, a model is possible where the Mal dimer can bind MyD88 at binding sites II, III, or the AB loop at one side and bind TLR4 at a similar binding site at the opposite side (Fig. 10A). Alternatively, binding of TLR4 in our binding assays may be promoted by the binding of endogenous MyD88 in our assays, explaining why mutations that affect MyD88 binding also affect TLR4 binding.

Interestingly, mutations in binding site II and III at two opposite sides of a Mal monomer have a similar outcome, as they both affect the interactions with TLR4 and MyD88. This is reminiscent of the R196A and R288A mutations in opposite sides of MyD88, which both affect binding of MyD88 to Mal (29). A model was proposed where MyD88 is “sandwiched” between two Mal molecules, with one molecule of Mal interacting with Arg-196 and another with Arg-288 (29). Rather, possibly MyD88 may bind as proposed in Fig. 10C, in which MyD88 dimerizes in a symmetrical way with Arg-196 and Arg-288 pointing in the same direction as part of an extended binding site, as proposed for the Mal binding sites II+III.

Several mutations around Arg-184 appear to have a more pronounced effect on TLR4 binding than on MyD88 binding. Mutations in a potential binding site IV formed by at the top of the dimer affect the TLR4ic bait-Mal prey interaction. Possibly, the area around Arg-184 and binding site IV is more important for binding to TLR4. In Fig. 10B, a model is proposed where TLR4 binds to binding site IV in Mal. Binding site IV is a composite binding site with two symmetrical identical parts. We previously presented a model for a TLR4 dimer with a symmetrical binding platform for interaction with Mal and TRAM (39). Possibly, the symmetrical interaction platform in TLR4 interacts with the symmetrical binding site IV in Mal.

The Mal crystal structures display two intramolecular disulfide bridges and a dithiothreitol molecule is disulfide-linked to Cys-157 and Cys-91 (27, 28). The dithiothreitol molecule is found close to or in the potential binding site II. It is likely that this affects the local structure of binding site II. The C157S mutation affected all tested interactions with TLR4, MyD88, and Mal (supplemental Table 2). A C157S mutant Mal prey and a FLAG-tagged Mal C157S mutant (data not shown) both show a faster electrophoretic mobility on SDS-PAGE than their wild-type equivalent when expressed in HEK293T cells. It is tempting to speculate that this can be a consequence of altered disulfide bridge formation or posttranslational modification at Cys-157.

Valkov et al. (27) suggested that homo- and heterotypic associations of TLR adapter TIR domains are probably intertwined events with functional cross-talk. Our data indeed support cooperative binding between the different TIR domains. Mal is required for MyD88 binding to TLR4. Co-expression of the MyD88 TIR domain also promotes TLR4ic-Mal bait-prey interaction and the Mal-Mal bait-prey interaction in our MAPPIT assays. Co-expression of Mal plus the MyD88 TIR domain strongly promotes the TLR4-TLR4 bait prey interaction in MAPPIT. This is very similar to the strong promoting effect of TRAM co-expression on the TLR4ic bait-prey dimerization in MAPPIT (39). Co-expression of Mal alone only modestly increases the TLR4ic bait-prey dimerization, suggesting that MyD88 and Mal cooperate in promoting or stabilizing TLR4 dimerization.

The rare D96N Mal SNP was found to be inactive in NF-κB and IRF-5 signaling (48). Co-immunoprecipitation studies demonstrated that the D96N mutant was still able to interact with TLR2 and TLR4 but not with MyD88 (48). Other studies found that Asp-96 mutations do not interfere with the interaction with MyD88 but may strongly influence posttranslational modification of Mal and recruitment of MyD88 to the plasma membrane (28, 49). A Mal S180L variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria, and tuberculosis (50). The S180L attenuates TLR2 signaling (50). We tested the S180L and D96N mutations in MAPPIT, Mal-Mal AlphaScreen, and NF-κB activation assays. The S180L does not inhibit any of the assays. The D96N mutation affects all tested assays, but the D96N prey shows a slightly lower expression level (supplemental Table 2 and Fig. 2). In the
Interaction Sites in the Mal TIR Domain

Mal structures, Asp-96 and Ser-180 are close to each other, and this area was proposed as a possible MyD88 binding site (27). Ser-180 and Asp-96 are not part of one of the possible binding sites proposed in this study. Nevertheless, E95G, D96V, L146F, and G199C mutations in the area around Ser-180 and Asp-96 affect the Mal-MyD88TIR and Mal-TLR4ic interaction in our MAPPIT assays. This supports the findings that mutations in this area can affect the interactions of Mal with MyD88 and/or TLR4. However, drastic T148M, L97M, S180L, and V98M mutations in this area have no or only a limited effect on MyD88 binding or NF-κB activation, arguing against an important role of this area as MyD88 binding site (supplemental Table 2).

Mal can be cleaved by caspase-1 at residue Asp-198 (51). Based on homology modeling, Núñez et al. (52) suggested that this cleavage and release of the C-terminal fragment of Mal may expose a binding site of Mal for downstream interactions. We showed that Mal cleavage by caspase-1 is not absolutely required and suggested that Asp-198 may be part of a binding site of Mal (38). We here demonstrated that Asp-198 is probably part of binding site III. Several mutations in the C-terminal part of Mal affect interaction with MyD88 and TLR4ic and for NF-κB activation, suggesting that the C-terminal part of Mal is indispensable for its interactions.

A putative TRAF6 binding motif was identified in the TIR domain of Mal involving residues PPELRF 188–193 (53, 54). Mutation of residue E190 abolished NF-κB activation by TLR2 and TLR4 (53, 54). Our data confirm a strong effect of several mutations in this motif, including an E190G mutation, on NF-κB activation (supplemental Table 2). However, in the Mal crystal structure residues 188–193 adopt a helical conformation, which seems incompatible with the extended conformation of a peptide with a TRAF6 binding motif in the TRAF6 crystal structure (55). Mutations in the motif strongly affected the interaction with the TLR4ic bait and the MyD88TIR bait in our MAPPIT screen. As residues PPELRF 188–193 are part of the symmetrical Mal-Mal interface, it is possible that mutations at this position interfere with Mal-Mal interaction and consequently with interaction with MyD88 and TLR4 rather than with interaction with TRAF6.

Bruton’s tyrosine kinase is activated upon LPS stimulation, and its activity is required for TLR4 signaling via Mal and MyD88 (20–22). Bruton’s tyrosine kinase can interact with and phosphorylate Mal (20–22). Mutation of Tyr-86, Tyr-106, Tyr-159, and Tyr-187 affected the interaction with or phosphorylation by Bruton’s tyrosine kinase (21, 22). Tyr-86 is found at the edge of binding site II, and a Y86H mutation negatively affects all tested assays. Tyr-159 is found at the Mal-Mal symmetrical interface, whereas Tyr-187 is largely buried below Arg-184 at the border between potential binding sites III and IV. Like mutations of Arg-184, Y187A and Y187C mutations have a specific effect on the interaction with the TLR4ic bait. It is thus conceivable that mutations or phosphorylation at Tyr-86, Tyr-159, or Tyr-187 can affect the interactions via binding sites I, II, and IV.

In conclusion, our study identifies several new potential interaction sites in Mal for TIR-TIR interactions. Although mapping of our mutagenesis data to discrete binding sites remains difficult, the study shows that the potential binding sites in Mal probably extend beyond the previously described sites such as the back-to-back interface, the area around Asp-96/Ser-180, and the AB loop. Our MAPPIT assays suggest that the different types of TIR-TIR interactions cooperate in the formation of the TLR4-Mal-MyD88 complex. We propose several possible models for interaction of Mal with TLR4 and MyD88. These models can provide a basis for further experimental validation.

Acknowledgments—We thank Dr. Alain Israel and Dr. Luke O’Neill for the kind gift of plasmids. We thank Dr. Jonathan Kagan for the kind gift of immortalized Mal knock-out macrophages. We thank Dr. Sam Lievens and Dieter Defever for assistance with cell sorting.

REFERENCES

1. Gay, N. J., and Gangloff, M. (2007) Structure and function of Toll receptors and their ligands. Annu. Rev. Biochem. 76, 141–165
2. Hennessey, E. J., Parker, A. E., and O’Neill, L. A. (2010) Targeting Toll-like receptors. Emerging therapeutics? Nat. Rev. Drug Discov. 9, 293–307
3. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity. update on Toll-like receptors. Nat Immunol 11, 373–384
4. Gay, N. J., and Keith, F. J. (1991) Drosophila Toll and IL-1 receptor. Nature 351, 355–356
5. Ye, T., Gay, N. J., Mansell, A., Kobe, B., and Kellie, S. (2012) Adaptors in toll-like receptor signaling and their potential as therapeutic targets. Curr. Drug Targets 13, 1360–1374
6. Xu, Y., Tao, X., Shen, B., Horng, T., Medzhitov, R., Manley, J. L., and Tong, L. (2000) Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature 408, 111–115
7. O’Neill, L. A., and Bowie, A. G. (2007) The family of five. TIR domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 7, 353–364
8. Motshwene, P. G., Moncrieffe, M. C., Grossmann, J. G., Kao, C., Ayaluru, M., Sandercock, A. M., Robinson, C. V., Latz, E., and Gay, N. J. (2009) An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4. J. Biol. Chem. 284, 25404–25411
9. Lin, S. C., Lo, Y. C., and Wu, H. (2010) Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR1/2-1R signalling. Nature 465, 885–890
10. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003) TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. Nat. Immunol. 4, 161–167
11. Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Visintin, A., Latz, E., Monks, B., Pitka, P. M., and Golenbock, D. T. (2003) LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the toll-like receptor signaling adaptors and their ligands. Nat Rev Immunol 3, 353–364
12. Oshiumi, H., Sasai, M., Shida, K., Fujita, T., Matsumoto, M., and Seya, T. (2003) TLR-containing adaptor molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-β. J. Biol. Chem. 278, 49751–49762
13. Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-β. Nat. Immunol. 9, 361–368
14. Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A., and O’Neill, L. A. (2001) Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature 413, 78–83
15. Horng, T., Barton, G. M., Flavell, R. A., and Medzhitov, R. (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature 420, 329–333
16. Kagan, J. C., and Medzhitov, R. (2006) Phosphoinositide-mediated adapt-
tor recruitment controls Toll-like receptor signaling. Cell 125, 943–955
17. Kenny, E. F., Talbot, S., Gong, M., Golenbock, D. T., Bryant, C. E., and O’Neill, L. A. (2009) MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3. J. Immunol. 183, 3642–3651
18. Cole, L. E., Laird, M. H., Seekatz, A., Santiago, A., Jiang, Z., Barry, E., Shirey, K. A., Fitzgerald, K. A., and Vogel, S. N. (2010) Phagocytic reten-

dition of Francisella tularensis results in TIRAP/Mal-independent TLR2 signaling. J. Leukoc. Biol. 87, 275–281
19. Siednienko, J., Halle, A., Nagkall, G., Golenbock, D. T., and Miggin, S. M. (2010) TLR3-mediated IFN-β gene induction is negatively regulated by the TLR adaptor MyD88 adaptor-like. Eur. J. Immunol. 40, 3150–3160
20. Jaffeurs, C. A., Doyle, S., Brunner, C., Dunne, A., Brint, E., Wietek, C., Walch, E., Wirth, T., and O’Neill, L. A. (2003) Bruton’s tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factorκB activation by Toll-like receptor 4. J. Biol. Chem. 278, 26258–26264
21. Gray, P., Dunne, A., Brikos, C., Jaffeurs, C. A., Doyle, S. L., and O’Neill, L. A. (2006) MyD88 adaptor-like (Mal) is phosphorylated by Bruton’s tyro-
sine kinase during TLR2 and TLR4 signal transduction. J. Biol. Chem. 281, 10489–10495
22. Piao, W., Song, C., Chen, H., Wabl, L. M., Fitzgerald, K. A., O’Neill, L. A., and Medvedev, A. E. (2008) Tyrosine phosphorylation of MyD88 adaptor-like (Mal) is critical for signal transduction and blocked in endotoxin tox-
tolerance. J. Biol. Chem. 283, 3109–3119
23. Dunne, A., Carpenter, S., Brikos, C., Gray, P., Strelow, A., Wescott, H., Morrice, N., and O’Neill, L. A. (2010) IRAK1 and IRAK4 promote phos-
phorylation, ubiquitination, and degradation of MyD88 adaptor-like (Mal). J. Biol. Chem. 285, 18276–18282
24. Tao, X., Xu, Y., Zheng, Y., Beg, A. A., and Tong, L. (2002) An extensively associated dimer in the structure of the C713S mutant of the TIR domain of human TLR2. Biochim. Biophys. Res. Commun. 299, 216–221
25. Nyman, T., Stenmark, P., Flodin, S., Johansson, I., Hammarström, M., and Nordlund, P. (2008) The crystal structure of the human Toll-like receptor 10 cytoplasmic domain reveals a putative signaling dimer. J. Biol. Chem. 283, 11861–11865
26. Khan, J. A., Brint, E. K., O’Neill, L. A., and Tong, L. (2004) Crystal structure of the Toll/interleukin-1 receptor domain of human IL-1R. J. Biol. Chem. 279, 31664–31670
27. Valkov, E., Stamp, A., Dimao, F., Baker, D., Verstak, B., Roversi, P., Kellie, S., Sweet, M. J., Mansell, A., Gay, N. J., Martin, J. L., and Kobe, B. (2011) Crystal structure of Toll-like receptor adaptor MAL/TIRAP reveals the molecular basis for signal transduction and disease protection. Proc. Natl. Acad. Sci. U.S.A. 108, 14879–14884
28. Lin, Z., Lu, J., Zhou, W., and Shen, Y. (2012) Structural insights into TIR domain specificity of the bridging adaptor Mal in TLR4 signaling. PLoS ONE 7, e43202
29. Ohnishi, H., Tochio, H., Kato, Z., Orii, K. E., Li, A., Kimura, T., Hiroaki, H., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freundenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Butcher, B. (1998) DEFECTIVE LPS signaling in C3H/HeJ and C57BL/10ScCr mice. mutations in Tlr4 gene. Science 282, 2085–2088
30. Horng, T., Barton, G. M., and Medzhitov, R. (2001) TIRAP. An adapter molecule-1-mediated NF-κB and interferon regulatory factor-3 activation. J. Biol. Chem. 283, 18283–18291
31. Ulrichs, P., Peelman, F., Beyaert, R., and Tavernier, J. (2007) MAPPIT analysis of TLR3 adaptor complex. FEBS Lett. 581, 629–636
32. Eyckerman, S., Verhee, A., de Heyden, J. V., Lemmens, I., Ostade, X. V., Vandekerckhove, J., and Tavernier, J. (2001) Design and application of a cytokine receptor-based interaction trap. Nat. Cell Biol. 3, 1114–1119
33. Eyckerman, S., Lemmens, I., Lievens, S., Van der Heyden, J., Verhee, A., Vandekerckhove, J., and Tavernier, J. (2002) Design and use of a mamma-
53. Mansell, A., Brint, E., Gould, J. A., O’Neill, L. A., and Hertzog, P. J. (2004) Mal interacts with tumor necrosis factor receptor-associated factor (TRAF)-6 to mediate NF-κB activation by toll-like receptor (TLR)-2 and TLR4. *J. Biol. Chem.* **279**, 37227–37230

54. Verstak, B., Nagpal, K., Bottomley, S. P., Golenbock, D. T., Hertzog, P. J., and Mansell, A. (2009) MyD88 adapter-like (Mal)/TIRAP interaction with TRAF6 is critical for TLR2- and TLR4-mediated NF-κB proinflammatory responses. *J. Biol. Chem.* **284**, 24192–24203

55. Ye, H., Arron, J. R., Lamothe, B., Cirilli, M., Kobayashi, T., Shevde, N. K., Segal, D., Dzivenu, O. K., Vologodskia, M., Yim, M., Du, K., Singh, S., Pike, J. W., Darnay, B. G., Choi, Y., and Wu, H. (2002) Distinct molecular mechanism for initiating TRAF6 signalling. *Nature* **418**, 443–447