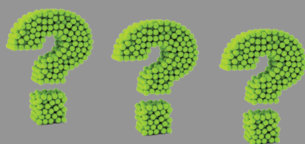

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MyD88 Adaptor-Like Is Not Essential for TLR2 Signaling and Inhibits Signaling by TLR3[1]

Elaine F. Kenny,* Suzanne Talbot,† Mei Gong,‡ Douglas T. Golenbock,‡ Clare E. Bryant,† and Luke A. J. O'Neill[2]*

Although a clear role for the adaptor protein myeloid differentiation factor-88 (MyD88) adaptor-like (Mal, or TIRAP) in TLR4 signaling has been demonstrated, there is limited information on its role in TLR2 signaling. Here we have systematically analyzed the role of Mal in signaling by TLR2, TLR4, and as a control TLR3 in murine macrophages and dendritic cells. Mal was not required for the induction of IL-6 or NFκB activation at high concentrations of the TLR1/2 ligand Pam_3Cys-Ser-$(Lys)_4$ or the TLR2/6 ligand macrophage-activating lipopeptide-2 and was required for these responses only at low ligand concentrations. Similarly, induction of IL-6 by *Salmonella typhimurium*, which is sensed by TLR2, required Mal only at low levels of bacteria. Mal was required for IL-6 induction at all concentrations of the TLR4 ligand LPS. Mal deficiency boosted IL-6 induction by the TLR3 ligand polyinosinic-polycytidylic acid. Activation of JNK, but not p38 or IκB degradation, was similarly potentiated in response to polyinosinic-polycytidylic acid in Mal-deficient macrophages. MyD88 was vital for all TLR2 and TLR4 responses and, similar to Mal, was also inhibitory for TLR3-dependent IL-6 and JNK induction. MyD88 interacted with the Toll/IL-1R domains of TLR1, TLR2, TLR4, and TLR6. Mal interacted with the Toll/Il-1R domains of TLR1, TLR2, and TLR4 but not with TLR6. Our study, therefore, reveals that Mal is dispensable in TLR2 signaling at high ligand concentrations in macrophages and dendritic cells, with MyD88 probably coupling to the TLR2 receptor complex at sufficient levels to allow activation. An inhibitory role for Mal in TLR3 signaling to JNK was also demonstrated. ***The Journal of Immunology,*** **2009, 183: 3642–3651.**

The TLRs are a family of transmembrane glycoproteins that activate the innate immune response and act as a first line of host defense (1, 2). There have been 10 human and 13 mouse TLRs characterized to date, all of which contain leucine-rich repeats (3). The leucine-rich repeats are sufficiently flexible to allow for the binding of such diverse ligands as lipopeptides which bind TLR2, viral dsRNA which binds TLR3, and LPS which with MD2 binds TLR4 (4). Signaling is initiated by the binding of various adaptor proteins to the Toll/IL-1R (TIR)[3] domain of the TLRs. Four activating adaptors have been identified: myeloid differentiation factor-88 (MyD88); MyD88 adaptor-like (Mal); TIR domain-containing adaptor-inducing IFN-β (TRIF); and TRIF-related adaptor molecule (TRAM) (5). Their binding, through their own TIR domains, activates signaling cascades resulting in the recruitment of the IL-1R-associated kinases (IRAK) and ultimately the activation of transcription factors such as NFκB and IFN-regulatory factor 3 (IRF3). The final products of TLR activation are cytokines, chemokines, and other inflammatory molecules (6, 7).

The role of these adaptors has been clarified through the generation of knockout mice. Mice lacking MyD88 demonstrate its central role in response to all TLR ligands tested, with the exception of TLR3 (8). MyD88 is also not required for the activation of IRF3 by TLR4 which proceeds via the TRIF/TRAM pathway (4, 6). Studies into the role of Mal in TLR signaling firstly revealed its function in response to TLR4 activation (9, 10). Mice lacking the TIR domain of Mal or the entire gene coding for Mal were then generated. It was established that Mal was required for TLR2 and TLR4 signaling only, and Mal was postulated to act as a bridging adaptor for MyD88 (11, 12). Subsequent to the identification of the requirement of Mal in TLR2 signaling the generation of TLR1-, TLR2-, and TLR6-deficient mice revealed that TLR2 must heterodimerize with either TLR1 or TLR6 to recognize tri- and diacylated lipopeptides, respectively (13–15).

Further work into TLR4 signaling revealed the role of TRIF and TRAM in activating the MyD88-independent signal cascade leading to IRF3 activation (16). Studies into mice lacking TRIF demonstrated that it was the sole adaptor required for TLR3 signaling (17). Recently, a novel inhibitory role for MyD88 in TLR3 signaling has been discovered in which MyD88 prevents the overactivation of the TRIF pathway upon TLR3 stimulation by inhibiting JNK phosphorylation (18).

Although a clear role for Mal in TLR4 signaling has been demonstrated, there is more limited information regarding its role in TLR2 signaling. It has been previously demonstrated that Mal can bind TLR2 (12), but whether it can bind TLR1 or TLR6 has not been examined. Further, whether Mal might be inhibitory toward TLR3, in a similar manner to MyD88, has not been determined.

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[3] Abbreviations used in this paper: TIR, Toll/IL-1R; BMDC, bone marrow-derived dendritic cell; BMDM, bone marrow-derived macrophage; HEK-293T, human embryonic kidney T cell; IRAK, IL-1R-associated kinase; Mal, MyD88 adaptor-like; MyD88, myeloid differentiation factor-88; TRIF, Toll/Il-1R domain-containing adaptor-inducing IFN-β; TRAM, TRIF-related adaptor molecule; IRF3, IFN-regulatory factor 3; Pam_3CSK_4, Pam_3Cys-Ser-$(Lys)_4$; Malp-2, macrophage-activating lipopeptide-2; MOI, multiplicity of infection; HA, hemagglutinin; poly(I:C), polyinosinic-polycytidylic acid.

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Here, we have analyzed the role of Mal in the TLR2-, TLR3-, and TLR4-signaling pathways in macrophages and dendritic cells. We demonstrate that Mal has a greater role to play in TLR4 signaling than in TLR2 signaling. Mal is not necessary for TLR2 signaling at high ligand concentrations or in response to high levels of *Salmonella typhimurium* but is required for TLR4 signaling at all ligand concentrations tested. We have shown that TLR1, TLR2, TLR4, and TLR6 all interact with MyD88 and that TLR1, TLR2, and TLR4 but not TLR6 interact with Mal. Finally, we have revealed a greater inhibitory role for Mal in TLR3 signaling to JNK and IL-6 induction when compared with MyD88. Thus, we propose that Mal is required for TLR4 signaling, is inhibitory in TLR3 signaling, and is only required to sensitize the system in TLR2 signaling, where MyD88 plays a more central role.

Materials and Methods

Plasmids and reagents

Generation of the Mal-hemagglutinin (HA) and pGEX-4T2 TLR4-TIR have been previously described (19). The TIR domains of hTLR1, TLR2 and TLR6 were subcloned from flag- or yfp-tagged plasmids containing the full length TLRs into the *Eco*RI and *Sal*I restriction sites of the pGEX-4T2 vector (Amersham Biosciences). The source of other plasmids was: pGBKT7 TLR1-TIR-Myc, TLR2-TIR-Myc, TLR4-TIR-Myc and TLR6-TIR-Myc and pACT2 empty vector, Mal-HA and MyD88-HA (provided by Dr. S. Miggin, Institute of Immunology, National University of Ireland, Maynooth, Eire); and MyD88-Myc (provided by Dr. Marta Muzio, Mario Negri Institute, Milan, Italy).

The following Abs were used: anti-Myc (Santa Cruz Biotechnology); anti-HA (Convance); phospho-p38 MAPK, phospho-JNK MAPK, and IκBα (Cell Signaling Technology). TLR agonists used were synthetic tripalmitoyl lipopeptide Pam_3Cys-Ser-$(Lys)_4$ (Pam_3CSK_4) from Invivogen, 2-kDa macrophage-activating lipopeptide (Malp-2) from Alexis, LPS from *Escherichia coli*, serotype EH100, from Alexis, and polyinosinic-polycytidylic acid (poly(I:C)) from Invivogen. The Mal inhibitor peptide and Mal control peptide were from Calbiochem. The IL-6 ELISA kit was from R&D Systems.

Cell culture

We generated immortalized MyD88-deficient, Mal-deficient, TRIF-deficient, and wild-type bone marrow-derived macrophages (BMDMs). This was done as previously described for similar cell lines using the J2 retrovirus encoding v-*raf* and v-*myc* (20–22). Primary TLR1-deficient, TLR2-deficient, TLR6-deficient, and wild-type littermate BMDMs were a gift from K. Fitzgerald (University of Massachusetts Medical School, Worchester, MA). Primary BMDMs and bone marrow-derived dendritic cells (BMDCs) were isolated from femurs and tibiae of Mal-deficient mice and their wild-type littermates and cultured in BMDM medium (RPMI 1640 supplemented with 10% (v/v) FCS, 2 mM L-glutamine and a 1% penicillin-streptomycin solution (v/v)), in petri dishes. For maintenance of the BMDMs in culture, this medium was further supplemented with 20% (v/v) of supernatant taken from L929 cells (a murine M-CSF-producing cell line). For maintenance of the BMDCs in culture, the medium was supplemented with 40 ng/ml GM-CSF. The human embryonic kidney T cell line (HEK-293T) was purchased from the European Cell Culture Collection. All cell lines (bar the primary cells) were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, and a 1% penicillin-streptomycin solution (v/v).

Cytokine analysis

The BMDMs and BMDCs (2×10^5 cells/ml in 96-well plates) were stimulated for 18 h with the ligands indicated in the legends of Figs. 1, 2, and 3. The wild-type macrophages were treated with 1 μg/ml Mal inhibitor peptide or Mal control peptide for 1 h before poly(I:C) stimulation. The supernatants were collected, and IL-6 production was measured by ELISA.

Salmonella *cytokine assay*

S. typhimurium strain SL1344 in midlog growth phase was added to the primary Mal-deficient and wild-type macrophages at multiplicities of infection (MOI) of 1, 10, or 30. After a 2-h incubation, the cells were incubated in BMDM medium containing 50 μg/ml gentamicin for 1 h to kill extracellular *Salmonella*. The cells were then further incubated in BMDM medium containing 10 μg/ml gentamicin until the end of the experiment (22 h). The supernatants were collected, and IL-6 production was measured by ELISA.

Western blot analysis

The immortalized BMDMs (8×10^5 cells/well of a 12-well plate) were stimulated the TLR ligands as outlined in the legends of Figs. 4 and 5. The cells were washed twice in 1× ice-cold PBS and lysed in 100 μl of low stringency lysis buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 10% glycerol (v/v), 0.5% Nonidet P-40 (v/v), 1 mM EDTA, 1 mM sodium orthovanadate, 0.1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The cell lysates were centrifuged at 13,000 rpm for 10 min, supernatants were collected, and the protein concentration of each was determined using Coomassie Bradford reagent (Pierce) according to manufacturer's instructions. Samples containing equal protein concentrations were generated using 5× SDS sample loading buffer (125 mM Tris-HCl (pH 6.8), 15% glycerol (v/v), 2% SDS (v/v), 10 mg/ml bromophenol blue) containing 50 mM DTT. Normalized samples were then analyzed by SDS-PAGE and immunoblotted for the phosphorylation of p38, phosphorylation of JNK, and IκBα per the manufacturer's instructions. Densitometric analysis of band intensities was determined using Multi Gauge version 2.2 software.

Yeast-two-hybrid analysis

The vectors containing pGBKT7:TIR domains of TLR1, 2, 4, and 6 were used as bait against pACT2:full-length MyD88 or Mal. The AH109 strain of yeast was used to perform the assay as described by the manufacturer (Matchmaker Gal4 Two-Hybrid System 3; Clontech). The yeast grown on histidine-leucine-tryptophan-deficient agar plates indicate protein-protein interactions.

GST-pulldown assay

HEK-293T (3×10^6cells/10-cm dish) cells were transiently transfected with 3 μg of MyD88-Myc or Mal-HA using the GeneJuice transfection reagent (Novagen) according to the manufacturer's instructions. Cells were lysed 24 h later in low stringency lysis buffer and incubated for 3 h at 4°C with recombinant GST fusion proteins (TIR-TLR1, -2, -4, and -6) coupled to glutathione-Sepharose. The complexes were washed three times in lysis buffer, separated by SDS-PAGE, and immunoblotted for the presence of MyD88 using an anti-Myc Ab or Mal using an anti-HA Ab.

Statistical analysis

Data are expressed relative to untreated cells, and are the means ± SD of triplicate determinations. For comparison between two groups, Student's *t* test was used. A p value of <0.05 was considered significant.

Results

Cytokine production in response to TLR2 activation is MyD88 dependent at high and low ligand concentrations but is Mal dependent at low ligand concentrations only in immortalized BMDM

To test the role of Mal in signaling by TLR1/2, TLR2/6, TLR3, and TLR4, we used immortalized BMDMs from MyD88-deficient and Mal-deficient mice and their wild-type littermates. These cells were stimulated with various concentrations of the TLR1/2 ligand Pam_3CSK_4 and the TLR2/6 ligand Malp-2, as well as LPS and poly(I:C).

As shown in Fig. 1*A*, 20 nM and 50 nM Pam_3CSK_4 produced an 8- to 10-fold increase in IL-6 production in wild-type macrophages (Fig. 1*A*, ■). This was impaired by 70% in the Mal-deficient macrophages (Fig. 1*A*, □), and completely abolished in the MyD88-deficient macrophages (Fig. 1*A*, ▨). However, in response to 200 nM Pam_3CSK_4 the Mal-deficient macrophages produced levels of IL-6 comparable with the wild-type macrophages, whereas MyD88-deficient macrophages remained fully impaired.

As shown in Fig. 1*B*, stimulation of the macrophages derived from MyD88-deficient and Mal-deficient mice with Malp-2 revealed a similar trend; 20 or 50 nM concentrations of Malp-2 resulted in impaired induction of IL-6 in the Mal-deficient macrophages, whereas the wild-type macrophages exhibited a robust response. At the high concentration of 200 nM of Malp-2, the Mal-deficient macrophages gave a response similar to that of the

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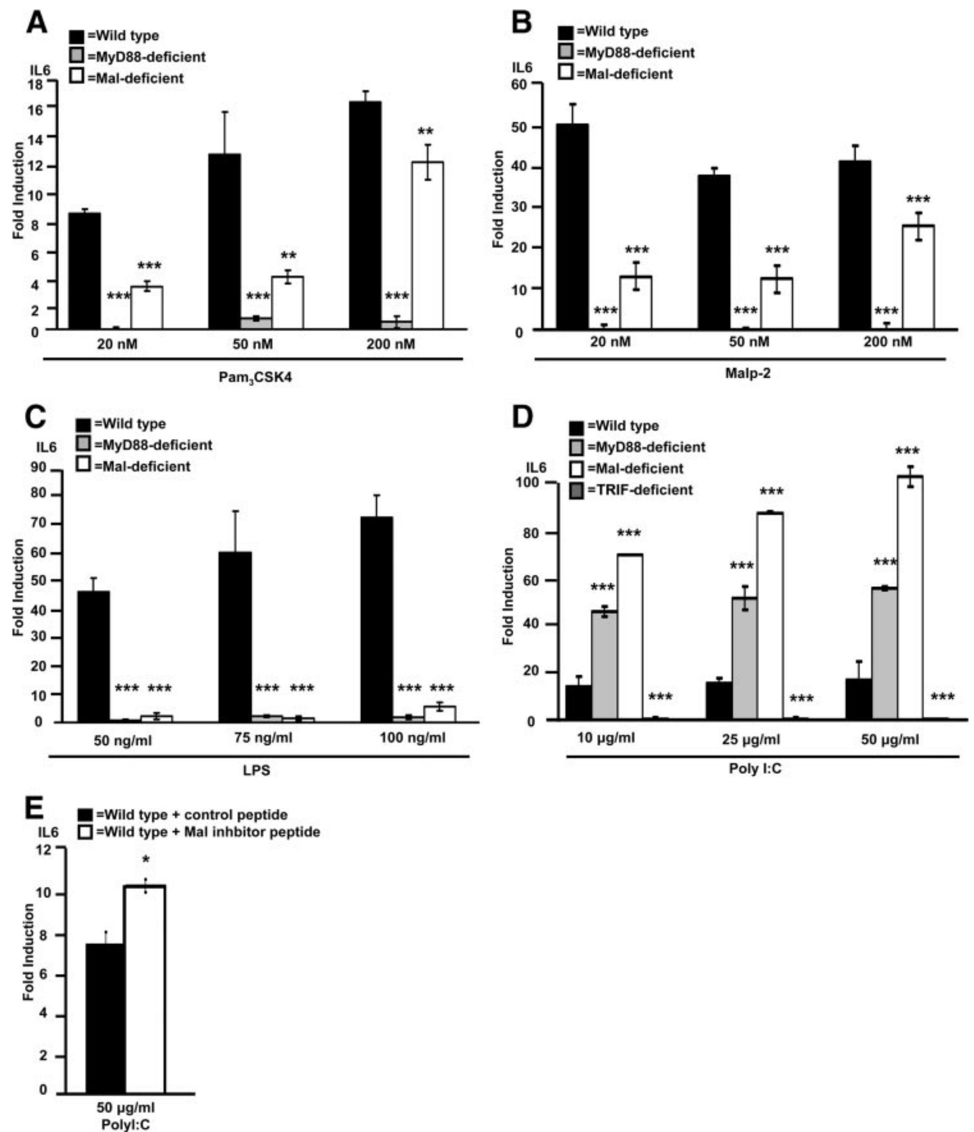

FIGURE 1. IL-6 production in response to TLR2 ligands is MyD88 dependent, is Mal dependent at low ligand concentration only, and is enhanced in response to poly(I:C) in the absence of MyD88 or Mal. Immortalized BMDMs from wild-type, MyD88-deficient, Mal-deficient (*A–C*), and TRIF-deficient (*D*) mice were treated with 20, 50, and 200 nM concentrations of either Pam_3CSK_4 (*A*) or Malp-2 (*B*), 50, 75, and 100 ng/ml LPS (*C*) or 10, 25, and 50 μg/ml poly(I:C) (*D*). Immortalized wild-type macrophages were treated for 1 h with 1 μg/ml Mal inhibitor peptide or 1 μg/ml control peptide and stimulated with 50 μg/ml poly(I:C) (*E*). After 18-h incubations, IL-6 production was measured by ELISA. The basal levels of IL-6 produced ranged from 110 to 160 pg/ml. Similar results were obtained in two additional experiments. ***, $p < 0.005$; **, $p < 0.01$; *, $p < 0.05$; NS, $p > 0.05$; significant differences between wild-type and MyD88-, Mal-, or TRIF-deficient macrophages.

wild-type macrophages. The MyD88-deficient macrophages were again unable to produce IL-6 to a level above the basal amounts. These data show that Mal is required for IL-6 production at low TLR2 ligand concentration only, with an absolute requirement for MyD88 at all TLR2 ligand concentrations.

Cytokine production in response to TLR4 activation is MyD88 and Mal dependent, but in response to TLR3 activation MyD88 and Mal play an inhibitory role

We next tested for LPS responsiveness. As shown in Fig. 1*C*, the MyD88-deficient and Mal-deficient macrophages were impaired for IL-6 production at all concentrations of LPS tested when compared with the wild-type cells, as expected.

We also tested TLR3 signaling. As shown in Fig. 1*D*, TLR3 activation at three concentrations (10, 25, and 50 μg/ml) of poly(I:C) led to enhanced IL-6 production in the MyD88-deficient and the Mal-deficient macrophages relative to wild-type cells. At all three concentrations, the wild-type macrophages produced a 12- to 15-fold induction of IL-6, while the MyD88-deficient macrophages showed 40- to 50-fold induction of IL-6. Similarly, the Mal-deficient macrophages showed 65- to 95-fold induction of IL-6. TRIF-deficient macrophages were unable to produce IL-6 in response to all three concentrations of poly(I:C) (Fig. 1*D*, ▨). The dependency of poly(I:C) on TRIF indicated the role for TLR3 in poly(I:C) signaling.

To test that the enhanced production of IL-6 in response to poly(I:C) stimulation seen in the Mal-deficient macrophages was not due to an artifact of genetic deletion, we next treated the wild-type macrophages with a Mal inhibitor peptide and a control peptide for 1 h before 50 μg/ml poly(I:C) stimulation. As shown in Fig. 1*E*, the inhibition of Mal through the use of the Mal peptide (Fig. 1*E*, □) resulted in enhanced IL-6 production when compared with the wild-type macrophages treated with the control peptide (Fig. 1*E*, ■). This revealed that Mal can indeed inhibit TLR3 signaling.

These results verify the absolute requirement of MyD88 and Mal in TLR4 signaling to activate IL-6 production and demonstrate inhibitory roles for MyD88 and Mal in TLR3 signaling. Mal deficiency consistently resulted in a more enhanced response to poly(I:C) when compared with MyD88 deficiency.

A role for Mal is evident only at low concentrations of TLR2 ligands, and Mal is inhibitory for TLR3 in primary BMDM and BMDC

To verify the results seen in the immortalized BMDMs, we next generated primary BMDMs and BMDCs from Mal-deficient mice

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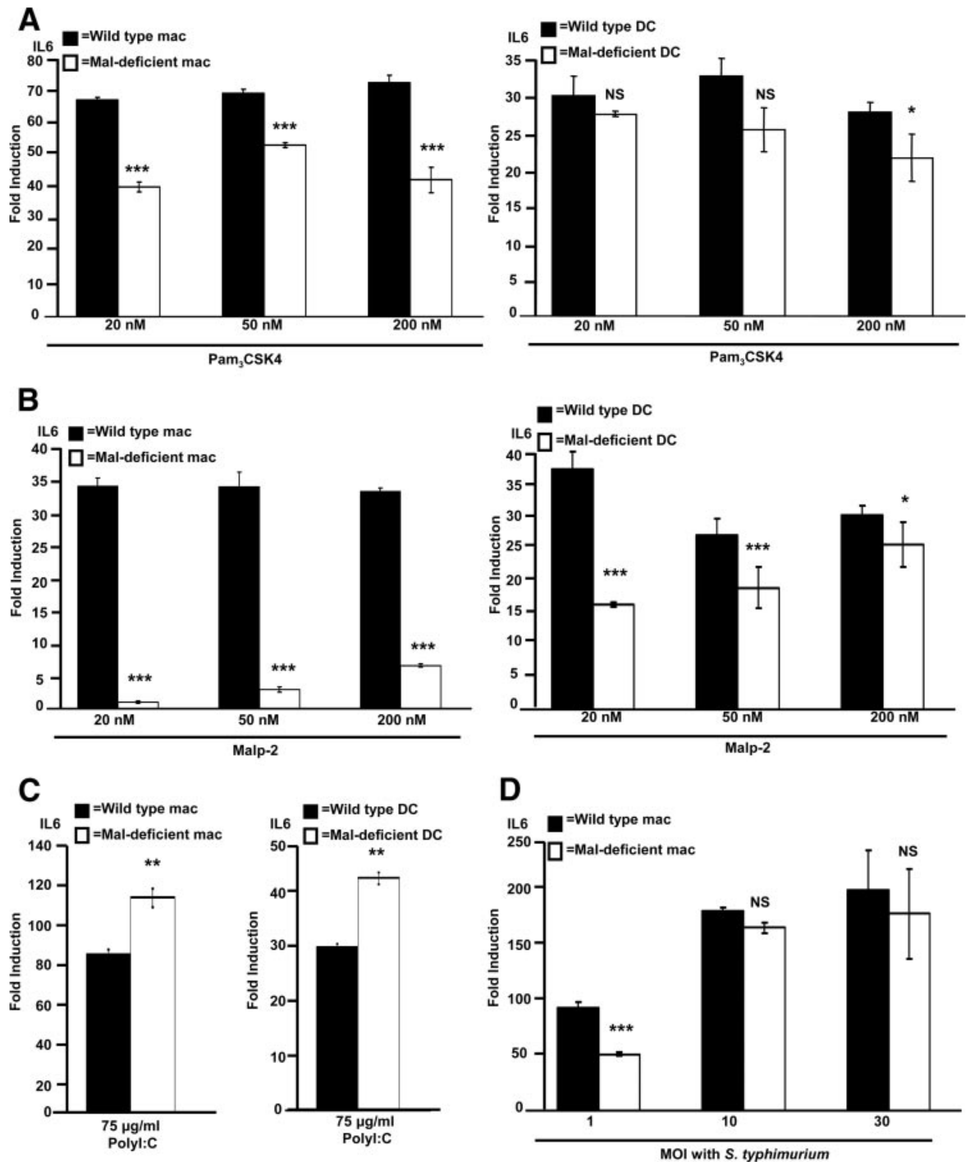

FIGURE 2. IL-6 production in response to TLR2 ligands is Mal dependent at low ligand concentration only and is enhanced in response to the TLR3 ligand poly(I:C) in the absence of Mal in primary BMDMs and BMDCs. Primary BMDMs (mac) and dendritic cells (DC) from wild-type and Mal-deficient mice were treated with 20, 50, and 200 nM concentrations of either Pam_3CSK_4 (*A*) or Malp-2 (*B*) or 75 μg/ml poly(I:C) (*C*). Primary BMDMs from wild-type and Mal-deficient mice were treated with *S. typhimurium* at MOI of 1, 10, and 30 (*D*). After 18- to 22-h incubations, IL-6 production was measured by ELISA. The basal levels of IL-6 produced by the primary macrophages and dendritic cells ranged from 110 to 130 pg/ml. Similar results were obtained in one further experiment. ***, $p < 0.005$; **, $p < 0.01$; *, $p < 0.05$; NS, $p > 0.05$; significant differences between wild-type and Mal-deficient macrophages and dendritic cells.

and their wild-type littermates. As shown in Fig. 2*A*, in response to 20, 50, and 200 nM Pam_3CSK_4, the primary Mal-deficient macrophages (Fig. 2*A*, □, *left*) produced a 40- to 50-fold induction of IL-6. Stimulation of the wild-type macrophages (Fig. 2*A*, ■, *left*) with the three concentrations of Pam_3CSK_4 resulted in a 70-fold induction of IL-6. Thus, the level of IL-6 production in the Mal-deficient macrophages was only partially inhibited. The Mal-deficient dendritic cells (Fig. 2*A*, □, *right*) produced 25- to 30-fold induction of IL-6 in response to the three Pam_3CSK_4 concentrations tested. When compared with the 30- to 35-fold induction of

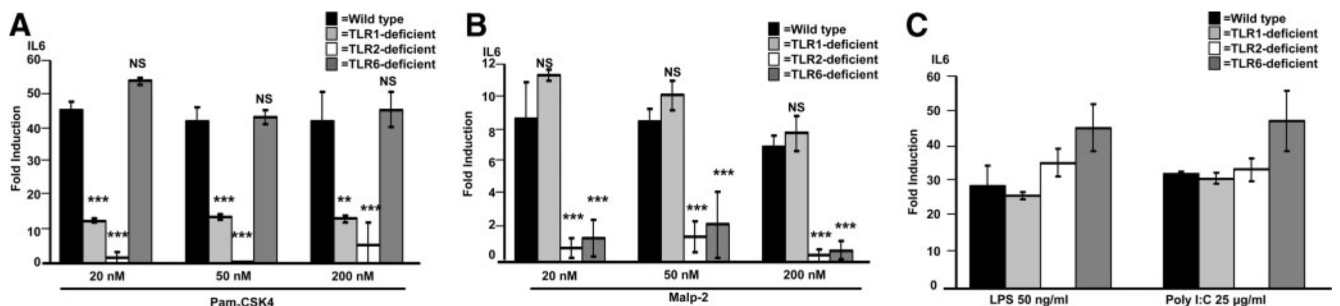

FIGURE 3. IL-6 production in response to Pam_3CSK_4 is TLR1/2 dependent and in response to Malp-2 is TLR2/6 dependent. Primary BMDMs from wild-type, TLR1-deficient, TLR2-deficient, or TLR6-deficient mice were treated with 20, 50, and 200 nM concentrations of either Pam_3CSK_4 (*A*) or Malp-2 (*B*), 50 ng/ml LPS, and 25 μg/ml poly(I:C) (*C*). After 18-h incubations, IL-6 production was measured by ELISA. The basal levels of IL-6 produced ranged from 95 to 170 pg/ml. Similar results were obtained in two additional experiments. ***, $p < 0.005$; **, $p < 0.01$; NS, $p > 0.05$; significant differences between wild-type and TLR1-, TLR2-, or TLR6-deficient macrophages.

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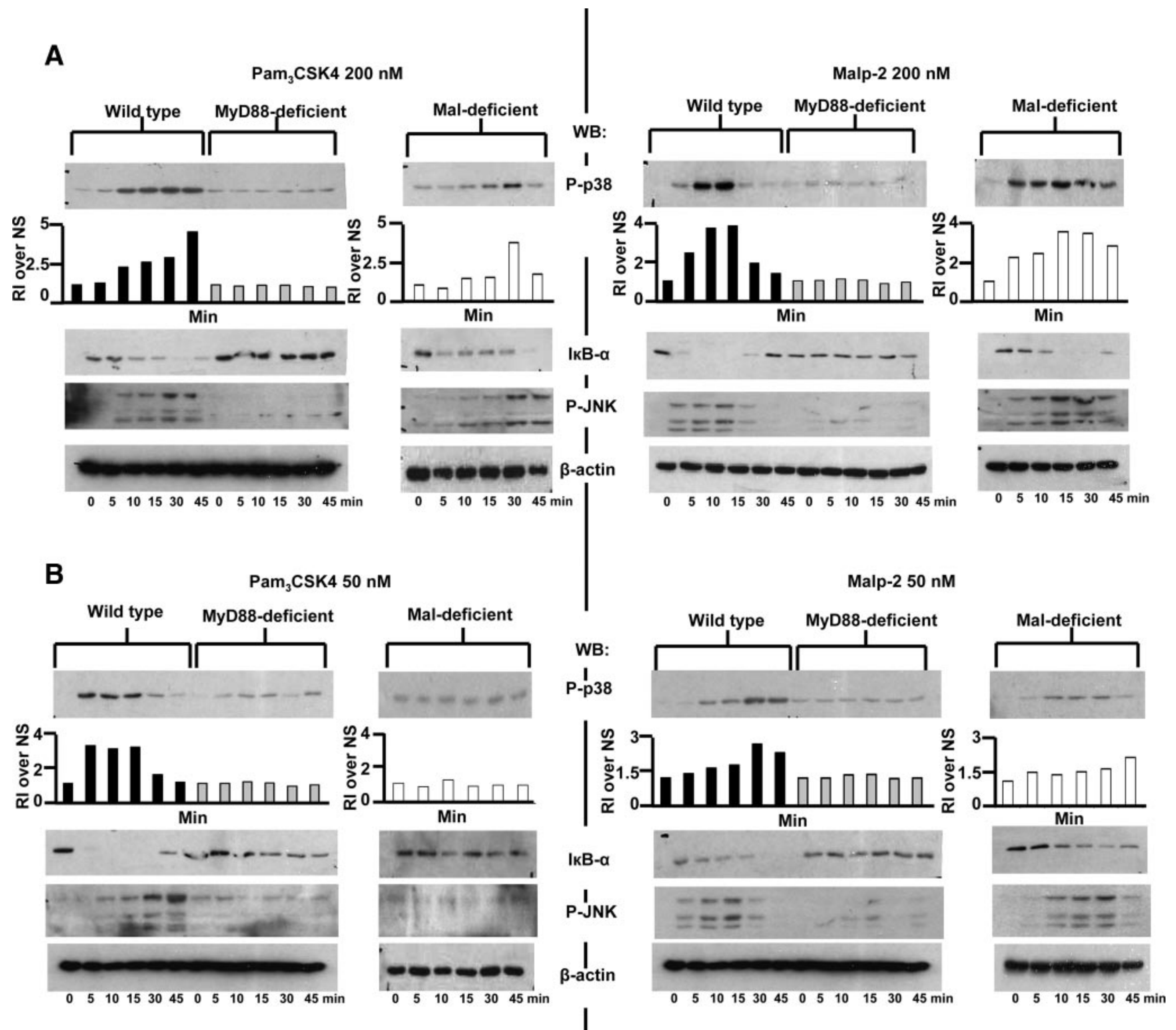

FIGURE 4. Downstream signal activation in response to TLR2 ligands are MyD88 dependent at all ligand concentrations and Mal dependent at low ligand concentrations only. Immortalized BMDMs from wild-type, MyD88-deficient, and Mal-deficient mice were treated for the indicated times with either 200 nM Pam_3CSK_4 or 200 nM Malp-2 (*A*). The macrophages were also treated with either 50 nM Pam_3CSK_4 or 50 nM Malp-2 (*B*). The cell lysates were collected, and p38 phosphorylation, IκBα degradation, and JNK phosphorylation were determined by Western blot (WB). β-Actin was included as a loading control. Densitometric analysis of band intensities was determined for the phospho-p38 blots, in which each band was normalized to its β-actin and the relative intensity (RI) of the bands over the nonstimulated (NS) control (set at 1) were calculated. Data are representative of three experiments.

IL-6 by the wild-type dendritic cells (Fig. 2*A*, ■, *right*), the lack of a requirement for Mal in response to Pam_3CSK_4 was revealed.

As shown in Fig. 2*B*, this trend was also seen in the Mal-deficient dendritic cells and somewhat in the Mal-deficient macrophages in response to Malp-2 stimulation at 20, 50, and 200 nM. The Mal-deficient macrophages (Fig. 2*B*, □, *left*) show impairment in IL-6 production at all three concentrations tested when compared with the wild-type macrophages (Fig. 2*B*, ■, *left*). However, increasing amounts of IL-6 are produced by these cells as the ligand concentration is increased to 200 nM. The Mal-deficient dendritic cells (Fig. 2*B*, □, *right*) demonstrate the concentration dependence of Mal in TLR2/6 signaling more clearly. At the low concentrations of 20 and 50 nM Malp-2, the Mal-deficient dendritic cells induce 15- to 20-fold production of IL-6, whereas stimulation of the wild-type dendritic cells (Fig. 2*B*, ■, *right*) results in 30- to 40-fold induction of IL-6. At the high concentration of 200 nM Malp-2, however, the Mal-deficient dendritic cells induce 25-fold production of IL-6, and the wild-type dendritic cell stimulation results in a 30-fold induction of IL-6, demonstrating that Mal is not required at this high ligand concentration. These results suggest a requirement for Mal in TLR2/6 signaling at low ligand concentrations only and a lack of requirement for Mal in TLR1/2 activation.

We next examined the production of IL-6 in response to 75 μg/ml poly(I:C) stimulation in the primary wild-type and Mal-deficient macrophages and dendritic cells. As shown in Fig. 2*C*, stimulation of the wild-type macrophages (Fig. 2*C*, ■, *left*) resulted in an 80-fold induction of IL-6. In comparison, stimulation of the Mal-deficient macrophages (Fig. 2*C*, □, *left*) with poly(I:C) resulted in 120-fold induction of IL-6. The inhibitory role of Mal in TLR3 signaling was also demonstrated in the primary dendritic cells. Stimulation of the wild-type dendritic cells (Fig. 2*C*, ■,

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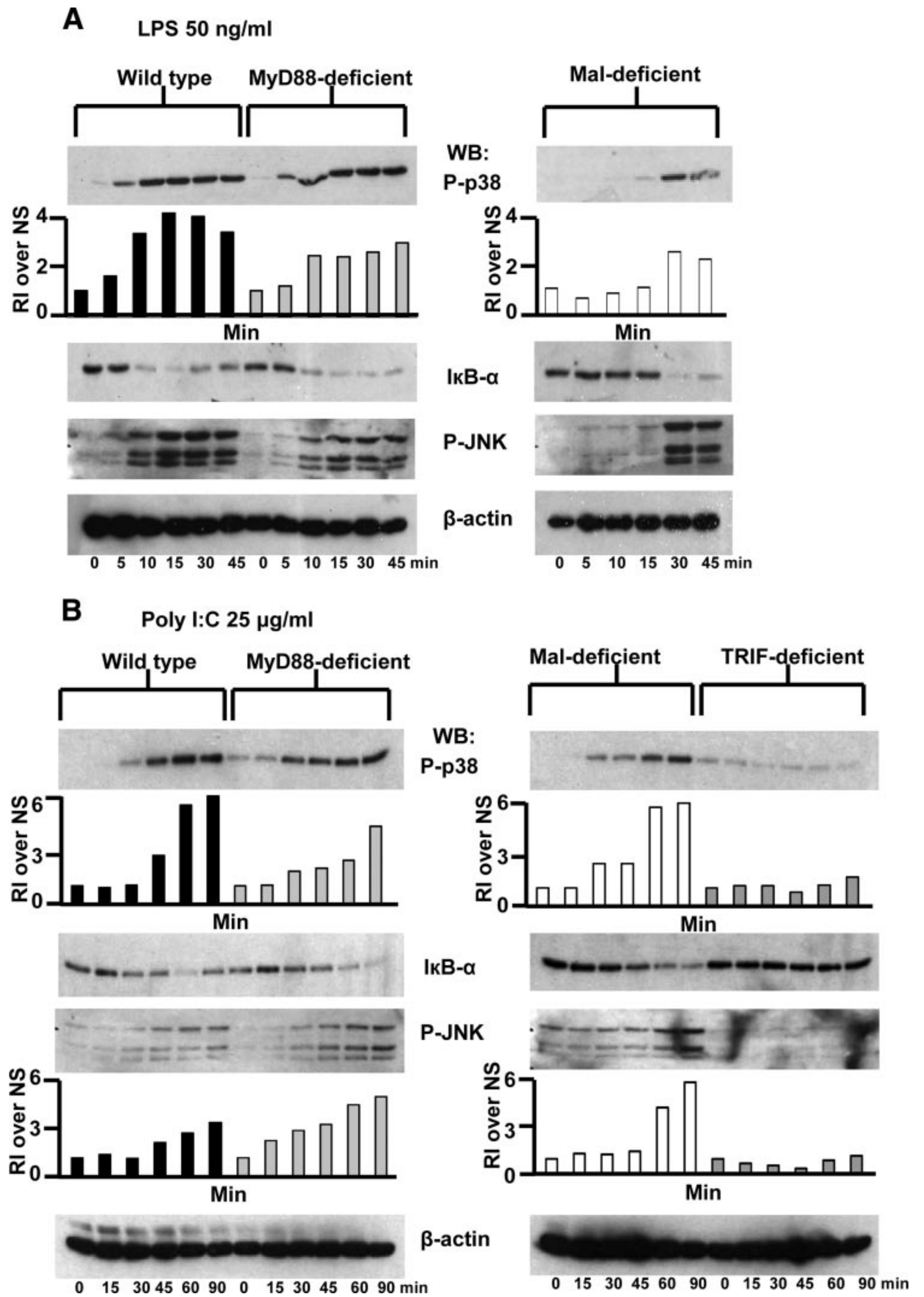

FIGURE 5. Downstream signals in response to TLR3 and TLR4 ligands are relatively normal in the absence of MyD88 and Mal with the exception of enhanced JNK phosphorylation upon TLR3 activation. Immortalized BMDMs from wild-type, MyD88-deficient, and Mal-deficient mice were treated for the indicated times with 50 ng/ml LPS (*A*). The same macrophages as well as TRIF-deficient macrophages were treated for the indicated times with 25 μg/ml poly(I:C) (*B*). The cell lysates were collected, and p38 phosphorylation, IκBα degradation, and JNK phosphorylation were determined by Western blot (WB). β-Actin was included as a loading control. Densitometric analysis of band intensities was determined for the phospho-p38 blots (and p-JNK in *B*), in which each band was normalized to its β-actin and the relative intensity (RI) of the bands over the nonstimulated (NS) control (set at 1) were calculated. Data are representative of three experiments.

right) with poly(I:C) resulted in 30-fold induction of IL-6; however, the Mal-deficient dendritic cells (Fig. 2*C*, □, *right*) resulted in a 45-fold induction of IL-6 in response to poly(I:C) stimulation. These results verified the inhibitory role of Mal in TLR3 signaling revealed with the immortalized macrophages.

We also tested the wild-type and Mal-deficient macrophages in response to *S. typhimurium* stimulation because it is known to be sensed by TLR2 (23). As shown in Fig. 2*D*, treatment of Mal-deficient primary macrophages with *S. typhimurium* also revealed concentration dependence. Stimulation of the Mal-deficient macrophages (Fig. 2*D*, □) with *S. typhimurium* at a MOI of 1 resulted in blunted production of IL-6 compared with levels produced by the wild-type macrophages (Fig. 2*D*, ■). When the MOI was increased to 10 and 30, the Mal-deficient macrophages regained their ability to produce IL-6 in levels comparable with the wild-type macrophages.

Pam_3CSK_4 is TLR1/2 dependent and Malp-2 is TLR2/6 dependent

In case there was a contaminant in the ligand preparations, revealed at the high ligand concentrations, we next tested Pam_3CSK_4 and Malp-2 responses in TLR1-deficient, TLR2-deficient or TLR6-deficient macrophages, as appropriate. As shown in Fig. 3*A*, Pam_3CSK_4 was impaired in TLR1- and TLR2-deficient macrophages (Fig. 3*A*, ▨ and □, respectively), whereas Malp-2 was impaired in the TLR2- and TLR6-deficient macrophages (Fig. 3*B*, □ and ▨, respectively), as expected. The TLR1-deficient, TLR2-deficient, and TLR6-deficient macrophages produced IL-6 in

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amounts similar to that of the wild-type macrophages in response to the TLR4 ligand LPS and the TLR3 ligand poly(I:C), as anticipated (Fig. 3*C*).

Activation of downstream signaling molecules upon TLR2 stimulation is MyD88 dependent at all ligand concentrations but Mal dependent at low concentrations only

To verify that TLR2 signaling requires Mal at low ligand concentrations only, as shown for IL-6 production, the signaling molecules that lead to NFκB and AP1 activation were next examined. The immortalized wild-type, MyD88-deficient, and Mal-deficient macrophages were stimulated with Pam_3CSK_4 and Malp-2 at a high (200 nM) and a low (50 nM) concentration over various times and examined for their ability to induce phosphorylation of p38 and JNK and degradation of IκBα. As shown in Fig. 4*A*, within 10 min of stimulation with 200 nM Pam_3CSK_4 (Fig. 4*A*, *left*) or 200 nM Malp-2 (Fig. 4*A*, *right*), the wild-type macrophages activated all three downstream molecules tested and continued to activate these proteins for all other time points to 45 min. The MyD88-deficient macrophages were unresponsive to both ligands with all signaling molecules tested. In contrast the Mal-deficient macrophages were responsive for all three signals following treatment with the high concentration of ligand. Densitometric analysis of p38 phosphorylation reconfirmed this trend, as depicted below the Western blots. As similar activation patterns were seen for phosphorylation of JNK and degradation of IκBα, their relative intensities were not graphed.

The macrophages were then tested for their ability to activate these signaling molecules at the low ligand concentration of Pam_3CSK_4 and Malp-2, both at 50 nM. As shown in Fig. 4*B*, the wild-type macrophages responded to both Pam_3CSK_4 (Fig. 4*B*, *left*) and Malp-2 (Fig. 4*B*, *right*) within 5–10 min of stimulation with induction of p38 and JNK phosphorylation and IκBα degradation. The Mal-deficient macrophages showed no activation of p38 or JNK and marginal activation of IκBα degradation in response to 50 nM Pam_3CSK_4 and delayed activation of the three in response to Malp-2 at 50 nM. The MyD88-deficient macrophages did not respond to either ligand. Phosphorylation of p38 was analyzed by densitometry to confirm the trend seen by Western blot analysis. These results reconfirm the requirement of MyD88 to activate a signaling cascade in response to TLR2 stimulation at all ligand concentrations. Mal is again shown to only be required in TLR2 signaling in response to low ligand concentration.

MyD88 or Mal deficiency does not affect TLR3 or TLR4 signaling, except in the case of JNK activation by TLR3 which is potentiated

The wild-type, MyD88-deficient, and Mal-deficient macrophages were next treated with 50 ng/ml LPS and 25 μg/ml poly(I:C) for various times and tested for their ability to induce phosphorylation of p38 and JNK and degradation of IκBα. As shown in Fig. 5*A*, the wild-type macrophages produced a response to LPS similar to the one shown previously for the TLR2 ligands. The MyD88-deficient macrophages were capable of activating all three signaling proteins tested in a manner comparable with that of the wild-type macrophages. The Mal-deficient macrophages were also responsive to LPS stimulation but showed a delay in phosphorylation of p38 and JNK and degradation of IκBα. Densitometric analysis of p38 phosphorylation again confirmed the trend revealed by Western blotting. The ability of the MyD88-deficient and the Mal-deficient macrophages to activate downstream signaling proteins is due to the ability of TLR4 to activate the TRIF-dependent pathway to NFκB (24, 25). Why these macrophages do not produce any IL-6 despite activating p38 and JNK and degrading IκBα remains unexplained.

As shown in Fig. 5*B*, treatment of the wild-type macrophages with 25 μg/ml poly(I:C) resulted in normal downstream responses as seen for all other ligands tested; however, a slower activation was seen and required long stimulation times of 60 and 90 min. Treatment of the MyD88-deficient and Mal-deficient macrophages with poly(I:C) led to the phosphorylation of p38 and degradation of IκBα to the same extent as the wild-type macrophages. An interesting result was seen in the MyD88-deficient and Mal-deficient macrophages with regard to JNK phosphorylation; however, were stimulation with poly(I:C) resulted in enhanced JNK phosphorylation in comparison with wild-type cells. This is evident in both cells at the 60- and 90-min time points. Activation of all three signals in response to poly(I:C) was abolished in the TRIF-deficient macrophages showing poly(I:C) stimulation acts through TLR3. Densitometric analysis on the phosphorylation of p38 and JNK reconfirmed the activation patterns revealed in the Western blots.

TLR1, TLR2, and TLR4 directly interact with both MyD88 and Mal, but TLR6 interacts with MyD88 only

We next tested the ability of MyD88 and Mal to directly interact with the TLRs involved using yeast-two-hybrid and GST-pulldown assays. The interactions of MyD88 and Mal with TLR4 have been widely documented so these were used as controls. In the yeast-two-hybrid assay TLR1 and TLR6 only interacted with MyD88 (Fig. 6, *A* and *D*), whereas TLR2 and TLR4 interacted with MyD88 and Mal (Fig. 6, *B* and *C*), indicated by the growth on agar plates lacking tryptophan, histidine, and leucine. Western blotting confirmed that all the TLRs and adaptors were expressed in similar levels in the yeast (Fig. 6, *E* and *F*).

A GST-pulldown assay was next conducted. For this, we constructed GST-TIR fusion proteins for TLR1, TLR2, and TLR6. As shown in Fig. 6*G*, overexpressed Myc-tagged MyD88 was pulled down with GST-TLR1, -TLR2, -TLR4, and -TLR6, thus demonstrating that MyD88 can interact with all the TLRs tested. Each sample contained similar levels of MyD88 expression. Similarly, as shown in Fig. 6*H*, overexpressed HA-Mal was pulled down with GST-TLR1, -TLR2, and -TLR4. It was not, however, pulled down with TLR6 (Fig. 6*H*, *lane 5*) and therefore interacts only with TLR1, TLR2, and TLR4. HA-Mal was expressed at similar levels in all samples.

These results indicate that TLR2 and TLR4 interact with MyD88 and Mal to initiate signaling, that TLR6 is likely to only signal through its interaction with MyD88, and interestingly that TLR1 is capable of interacting with both MyD88 and Mal.

Discussion

Previous work on the role of Mal in TLR signaling has suggested its bridging role in TLR2 and TLR4 signaling to the MyD88-dependent pathway (26). The evidence for this is that the surface of MyD88, TLR2, and TLR4 are predicted to be electropositive. The surface of Mal, on the other hand, is predicted to be electronegative and would be expected to help in the recruitment of MyD88 (19).

Much work has been undertaken to identify the important regions of Mal involved in its signaling to NFκB. This has led to the identification of several phosphorylation sites, a caspase-1 cleavage site, and a PEST domain allowing it to be degraded by suppressor of cytokine signaling-1 (27–29). A single-nucleotide polymorphism has also been reported in the gene encoding Mal, which converts a serine at position 180 to a leucine. A heterozygous state for this polymorphism has been shown to be protective against

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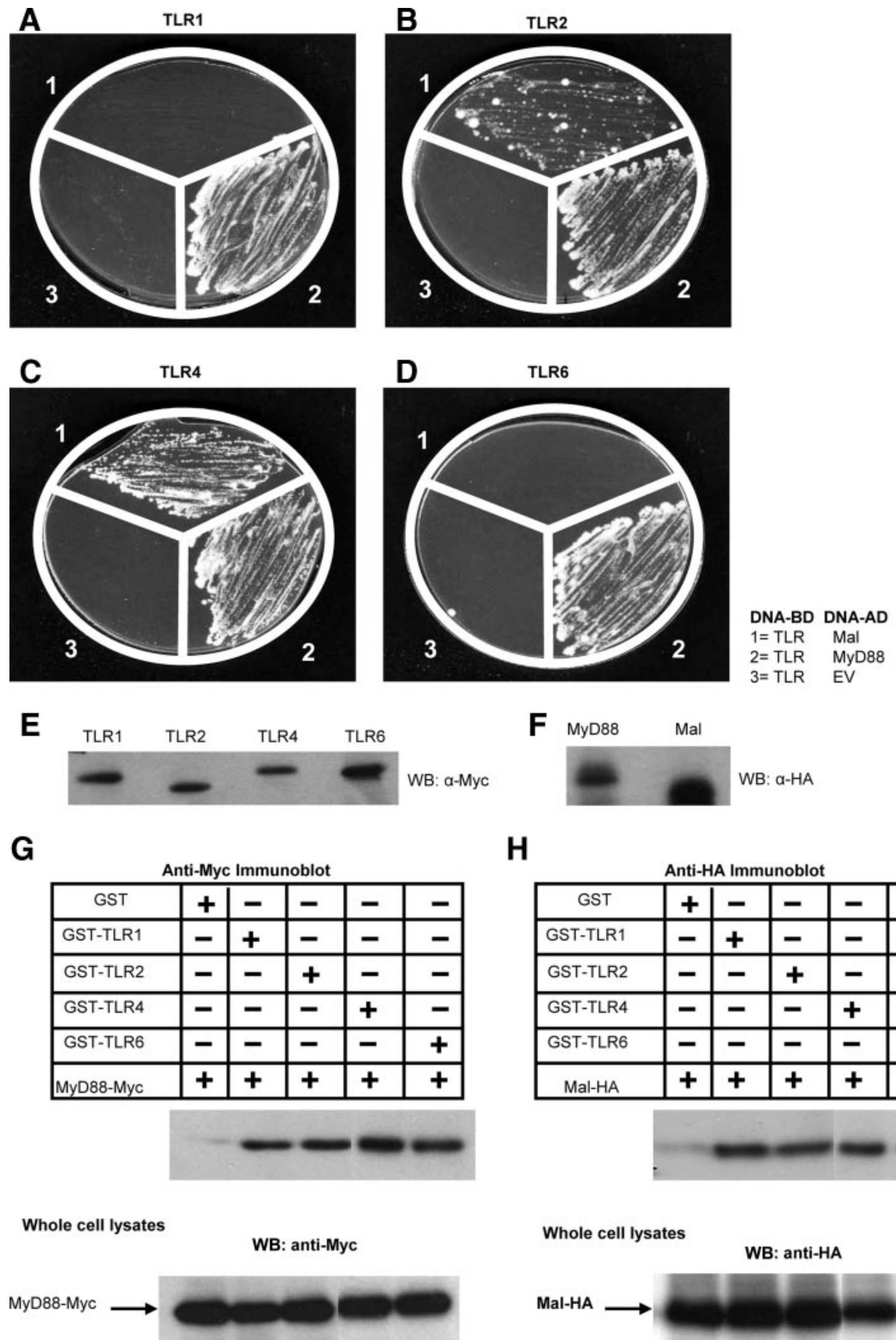

FIGURE 6. MyD88 can interact with TLR1, TLR2, TLR4, TLR6, whereas Mal interacts with TLR1, TLR2, and TLR4. The AH109 strain of yeast was transformed with the TIR domains of TLR-1, -2, -4, and -6 and grown at 30°C for 3 days on agar plates lacking tryptophan. The yeast were retransformed with Mal, MyD88, or empty vector (EV) and grown on plates lacking tryptophan, histidine, and leucine at 30°C for 1 wk (*A–D*). After both transformations, yeast extracts were tested for the presence of the TLR-Myc-tagged (*E*) and adaptor-HA-tagged (*F*) proteins by Western blot (WB). *G* and *H*, HEK-293T cells were transiently transfected with MyD88-Myc or Mal-HA for 24 h, lysed in low stringency lysis buffer, and incubated with GST-fusion proteins containing the TIR domain of TLR1, TLR2, TLR4, and TLR6 for 3 h at 4°C. The samples were washed in lysis buffer and analyzed by Western blot with Myc and HA Abs. Data are representative of three experiments.

GST	+	−	−	−	−
GST-TLR1	−	+	−	−	−
GST-TLR2	−	−	+	−	−
GST-TLR4	−	−	−	+	−
GST-TLR6	−	−	−	−	+
MyD88-Myc / Mal-HA	+	+	+	+	+

several diseases including malaria and systemic lupus erythematosus (30, 31).

Here we have taken an in-depth look at the role of Mal in TLR2, TLR3, and TLR4 signaling. As predicted, Mal is required to activate the MyD88-dependent pathway in TLR4 signaling to produce IL-6. Conversely to what is currently reported concerning the requirement of Mal for TLR2 signaling to produce IL-6, we have shown that Mal is somewhat dispensable at high concentrations of the TLR1/2 ligand Pam_3CSK_4 and the TLR2/6 ligand Malp-2.

This was also seen in primary BMDMs and BMDCs from Mal-deficient and wild-type mice, demonstrating that this is not just macrophage specific. In the primary cells, less dependence on Mal was seen in response to TLR1/2 activation as has been previously shown (32). Mal is also dispensable for IL-6 production at high levels of *S. typhimurium* infection. When the ligand concentration is dropped the requirement for Mal is more obvious. It therefore appears that the presence of Mal increases the efficacy of TLR2 signaling at lower concentrations of ligand.

The concentration-dependent requirement of Mal in TLR2 signaling was further demonstrated when the activation of downstream molecules was tested. Stimulation with a high ligand concentration in the absence of Mal allowed the activation of the downstream signaling proteins p38, JNK and the degradation of IκBα. The low ligand concentration stimulation resulted in a loss of this activation in TLR1/2 signaling and impairment in TLR2/6 signaling. For all concentrations of TLR2 ligands, MyD88 reveals its absolute requirement to allow IL-6 production and the activation of downstream signaling molecules and thus is the central activator of TLR2 signaling.

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To establish why Mal is not essential to TLR2 activity, we determined where in the complexes MyD88 and Mal bound. The first evidence came from the yeast-two-hybrid assay which suggested TLR1 and TLR6 bound MyD88 only and that TLR2 bound both MyD88 and Mal. To confirm this in a mammalian system a GST-pulldown assay was conducted and gave similar results with one exception. The ability of TLR1 to directly bind Mal was shown in this assay but not the yeast-two-hybrid. This may be due to the artificial nature of the two-hybrid assay using yeast to express mammalian proteins and expecting the proteins to interact as normal. The GST-pulldown assay involves expressing mammalian proteins in a mammalian system (HEK-293T cells), and therefore is a more reliable method for the examination of protein-protein interactions. Hence, we believe these experiments have revealed TLR1 as a binding partner of Mal as well as MyD88. This study is therefore the first demonstration of Mal interacting with TLR1.

In our investigations into the role of Mal in TLR2 signaling, we have also revealed a previously unreported inhibitory role for Mal in TLR3 activation. The absence of Mal allows for enhanced IL-6 production after poly(I:C) stimulation. Inhibition of Mal in wild-type cells by the addition of a Mal inhibitor peptide also resulted in enhanced IL-6 production upon poly(I:C) stimulation, verifying that the inhibition of TLR3 signaling is due to Mal. Examination of the downstream signaling molecules revealed normal p38 phosphorylation and IκBα degradation but enhanced phosphorylation of JNK in the absence of Mal. Similar to our study, this has been previously reported for MyD88 (18).

When taken together, these results demonstrate a more complicated method of signaling through Mal than was previously thought. We propose the following updated role for Mal in TLR2 and TLR4 signaling. TLR2 and TLR4 use Mal and MyD88 to signal. Both TLR2 and TLR4 require the presence of Mal to recruit MyD88; however, TLR2 can also use its ability to dimerize with TLR1 and TLR6 to allow for Mal-independent recruitment of MyD88. TLR1 also binds Mal, allowing for more MyD88 recruitment. At high ligand concentrations, activation of TLR2 can occur in the absence of Mal due to greater coupling of TLR1 or TLR6 to the complex, which allow for sufficient MyD88 recruitment. This coupling might be less effective at low ligand concentrations which therefore require Mal to stabilize MyD88 in the complex. The inability of the MyD88-deficient macrophages to activate any TLR2 pathways demonstrates its central role in TLR2 signaling. This is due to its ability to interact with TLR1, TLR2, and TLR6. In TLR4 signaling, on the other hand, a lack of MyD88 does not completely abolish downstream signaling due to the activation of the TRIF-dependent pathway. As TLR2 has no access to this branch of signaling MyD88 is the crucial molecule required, with Mal used to somehow sensitize the system at low ligand concentrations or low levels of bacteria. An example of a bacterium in this regard is *S. typhimurium*, which we found could induce IL-6 in Mal-deficient macrophages at high levels of infection. Low levels were, however, Mal dependent.

The mechanisms by which both MyD88 and Mal can inhibit the activation of JNK and hence dampen down TLR3 signaling is currently under investigation. Since MyD88 and Mal can interact directly with IRAK2 (9, 33), the inhibitory role may be due to their ability to sequester IRAK2 away from TLR3. The interaction of TLR3 directly with IRAK2 has previously been shown to be crucial for TLR3 signaling (34). IRAK2 has also recently been shown to be important for TLR4-mediated mRNA stabilization and translational control in macrophages (35). It was shown that IRAK2 can form complexes with several MAPK kinases and TRAF6 upon TLR4 activation. A lack of IRAK2 did not prevent phosphorylation of p38, in response to TLR4 activation, to a great extent suggesting another MAPK as the target of the complex formed with IRAK2. TLR3 may also require IRAK2 to act in this manner to allow normal activation of its signaling pathways that utilize TRAF6.

In conclusion, we propose that Mal is required in TLR2 signaling only as a method of sensitizing the host response to low levels of ligand, with Mal-independent recruitment of MyD88 to the TLR2 complex at high ligand concentration allowing for signaling without Mal. This could also apply to low level infection with pathogens sensed by TLR2, where again, Mal may have a sensitizing role. Mal is crucial for TLR4 responses as there is no alternative method for MyD88 recruitment and is inhibitory in TLR3 signaling.

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Disclosures

The authors have no financial conflict of interest.

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