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TYROSINE PHOSPHORYLATION OF MYD88 ADAPTER-LIKE (MAL) IS CRITICAL FOR SIGNAL TRANSDUCTION AND BLOCKED IN ENDOTOXIN TOLERANCE*

Wenji Piao[†], **Chang Song**[†], **Haiyan Chen**[†], **Larry M. Wahl**[‡], **Katherine A. Fitzgerald**[§], **Luke A. O'Neill**[‡‡], and **Andrei E. Medvedev**[†]

[†]Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201, USA [‡]NIDCR/NIH, Bldg. 30, Rm. 325, MTS 4352, Bethesda, MD 20892, USA [§]Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA 01605, USA [††]School of Biochemistry and Immunology, Trinity College, Dublin, Ireland

Abstract

Toll-like receptor (TLR) 4 recognition of lipopolysaccharide (LPS) triggers signalosome assembly among TLR4, sorting (*e.g.*, MyD88-adapter-like, Mal) and signaling (*e.g.*, MyD88) adapters, initiating recruitment and activation of kinases, activation of transcription factors and production of inflammatory mediators. In this study, we examined whether tyrosine phosphorylation of Mal regulates its interactions with TLR4, MyD88, IRAK-2, TRAF-6 and is important for signaling. Overexpression of wild-type (WT) Mal in human embryonic kidney (HEK) 293T cells induced its constitutive tyrosine phosphorylation and led to activation of p38, NF-κB, and IL-8 gene expression. Mutagenesis of Y86, Y106, and Y159 tyrosine residues within the Toll-IL-1R (TIR) domain impaired Mal tyrosine phosphorylation, interactions with Bruton tyrosine kinase (Btk), phosphorylation of p38, and NF-κB activation. LPS triggered tyrosine phosphorylation of endogenous Mal and initiated Mal-Btk interactions in 293/TLR4/MD-2 cells and human monocytes that were suppressed in endotoxin-tolerant cells. Compared to WT-Mal, Y86A-, Y106A-, and Y159A-Mal variants exhibited higher interactions with TLR4 and MyD88, while associations with IL-1R-associated kinase (IRAK)-2 and TNFR-associated factor (TRAF)-6 were not affected. Overexpression of Y86A and Y106A Mal in 293/TLR4/MD-2 cells exerted dominant-negative effects on TLR4-inducible p38 phosphorylation and NF-κB reporter activation to the extent comparable to P125H Mal-mediated suppression. In contrast, tyrosine-deficient Mal species did not affect NF-κB activation when signaling was initiated at the post-receptor level by overexpression of MyD88, IRAK-2 or TRAF-6. Thus, tyrosine phosphorylation of Mal is required for adapter signaling, regulates Mal interactions with TLR4 and receptor signaling, and is inhibited in endotoxin tolerance.

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Address correspondence to: Dr. Andrei E. Medvedev, Department of Microbiology and Immunology, University of Maryland School of Medicine, 660 West Redwood Street, Howard Hall Bldg., Rm. HH 324, Baltimore, MD 21201. Phone: (410) 706-5854; Fax : (410) 706-2129; E-mail: E-mail: amedvedev@som.umaryland.edu.

Toll-like receptors (TLRs)[1] are immune sensors of pathogen-associated molecular patterns (PAMPs) that mediate early activation of host innate immune defense and are crucial for initiating and orchestrating subsequent adaptive immune responses (1–3). Eleven mammalian TLRs express an ectodomain containing leucin-rich repeats (LRR) and implicated in ligand recognition and co-receptor interactions, a transmembrane domain, and a cytoplasmic domain with an intracellular Toll-IL-1R (TIR) domain essential for signal transduction (4–7). Epithelial and endothelial cells, macrophages, neutrophils, and dendritic cells express TLRs on the cell surface (TLR2, TLR4, TLR5, TLR11) or in intracellular endosomes (TLR3, TLR7–9) (8–10). This allows for early recognition of bacterial (TLR2, TLR4, TLR5, TLR9), viral (TLR3, TLR7–9) or parasitic (TLR11) PAMPs (11–18) and mounting of effective immune defense networks.

Sensing of PAMPs induces TLR oligomerization and conformational changes that create docking platforms within the TIR domains, allowing recruitment of adapter proteins and kinases (1,2,19–21). TIR-containing adapters can be classified into “signaling” adapters, MyD88 (22,23) and TIR domain-containing adapter inducing interferon-β (TRIF) (24–26), and “sorting”, or “bridging” adapters, MyD88-adaptor like (Mal, also called TIR domain-containing adapter protein) (27,28), and TRIF-related adaptor molecule (TRAM, 29–32) (33–35). The fifth adapter, sterile α and HEAT/armadillo motifcontaining protein does not signal by its own and is involved in negative regulation of TRIF signaling (36). MyD88 is used by all TLRs except for TLR3 that solely relies on TRIF (22–26), Mal participates in TLR2 and TLR4 signaling (27,28), whereas TRIF is utilized by TLR3 and TLR4 (24–26). TRAM shows the most restricted TLR utilization pattern, acting as a “bridging” adapter in TLR4 signaling (29–32,35). The “bridging” adapters have been postulated to act by delivering cytoplasmic “signaling” adapters into a close proximity to cell surface-localized TLRs, whereas endosomal localization of TLR3 and TLR7-9 is thought to allow these TLRs to directly associate with MyD88 or TRIF (31,33–35). However, IL-1R, IL-18R, and TLR5 are expressed on the cell surface but they do not require “bridging” adapters for their interactions with MyD88 (rev. in 35). It is possible that specific utilization of “bridging” adapters by some, but not all, TLRs could be due to different structures of their TIR domains, yet, how exactly the adapters initiate or specify signaling is still poorly understood. In addition to adapter engagement, signalosome formation at TLRs involves recruitment and activation of IL-1R-associated kinase (IRAK)-4, IRAK-1 and TNFR-associated factor (TRAF)-associated NF-κB activator (TANK)-binding kinase (TBK)-1. These result in activation of downstream adapter TRAF-6 and TGF-β-activated kinase (TAK)-1, IκB kinases (IKK), and mitogen-activated protein kinases, leading to induction of transcription factors NF-κB, IFN-regulatory factor (IRF)-3, IRF-5 and IRF-7 (22–33,37–43). A complex interplay of activated transcription factors ultimately turns on expression of a plethora of inflammatory and antimicrobial genes, MHC, costimulatory and accessory molecules (rev. in 2,21,34,35).

Prior exposure to LPS induces a state of endotoxin tolerance that is characterized by suppressed induction of proinflammatory cytokines and increased or unchanged expression of anti-inflammatory and antimicrobial mediators (44–46). A subset of septic shock survivors show a tolerant-like phenotype manifested by decreased macrophage responses to LPS and increased incidence of secondary bacterial infections (47). Therefore, endotoxin tolerance has been postulated to curtail excessive inflammatory responses and prevent septic shock. While LPS tolerance was initially suggested to result from suppressed cell surface expression of TLR4/

[1]The abbreviations used are: TLR, Toll-like receptor; PAMPs, pathogen-associated molecular patterns; TIR, Toll-IL-1R; leucin-rich repeats, LRR; lipopolysaccharide, LPS; MAPK, mitogen-activated protein kinase; HEK, human embryonicnic kidney cells; Btk, Bruton's tyrosine kinase; TRIF, TIR domain containing adapter-inducing interferon; TRAM, TRIF-related adapter molecule; TANK, TRAF-associated NF-κB activator; TBK, TANK-binding kinase; IKK, IκB kinase; IRFs, interferon regulatory factors; IFNs, interferons; Ab, antibody; p, phospho; hu, human; HA, haemagglutinin; HPRT, hypoxanthine phosphoribosyltransferase; WT, wild-type; PKC, protein kinase C; PIP2, phosphatidylinositol-4,5-biphosphate; SOCS, suppressor of cytokine signaling.

MD2 (48), other studies showed unaltered TLR4 expression, but suppressed TLR4 tyrosine phosphorylation, MyD88 recruitment to TLR4, IRAK-1-MyD88 interactions, and IRAK-1 activation as molecular hallmarks of endotoxin tolerance (44–46,49–51).

Posttranslational modifications of receptors and adapters play an important role in regulating several signaling systems, including TLRs. TLR2, TLR3, TLR4, and TLR5 undergo agonist-induced tyrosine phosphorylation, and mutations of tyrosines within their TIR domains suppress TLR signaling capacities (51–54), alter recruitment of MyD88 to TLR4 and IRAK-1 activation at TLR4 (51). Tyrosine phosphorylation of Mal and serine phosphorylation and myristoyaltion of TRIF were found to regulate TLR signaling (55–57). For instance, Mal is tyrosine phosphorylated by Bruton's tyrosine kinase (Btk), and mutations of Y86 or Y106 render Mal functionally-incompetent and confer upon it the capacity to act as a dominant negative inhibitor of LPS-mediated NF-κB reporter activation (55). However, functional significance of Mal tyrosine phosphorylation in activation of endogenous signaling intermediates was not characterized. In addition, molecular mechanisms by which Mal tyrosine deficiency alters its signaling functions still remain unknown. It is also unclear to what extent “reprogramming” of LPS responsiveness in endotoxin tolerance is linked to altered posttranslational modifications of adapter proteins.

In this study, we determined tyrosine residues within the TIR domain important for Mal activation of endogenous signaling intermediates, and examined how tyrosine deficiency of Mal affects its interactions with Btk, TLR4, MyD88, IRAK-2, and TRAF-6. In addition, we studied whether endotoxin tolerance alters LPS-induced Mal tyrosine phosphorylation and Mal-Btk interactions. Our results indicate a critical role for Mal tyrosine phosphorylation in regulating associations with Btk and TLR4, as well as in TLR4-initiated IRAK-1 phosphorylation, IκB-α degradation, NF-κB reporter activation, and IL-8 gene expression.

Experimental Procedures

Reagents and cell culture

Anti- MyD88, IκB-α, tubulin, β-actin, anti-Btk, anti-haemagglutinin (HA)-horseradish peroxidase (HRP) and IRAK-1 antibodies (Abs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho (p)-p38 and anti-p-IRAK-1 Abs was from Promega (Madison, WI) and Abcam (Cambridge, MA), respectively. Anti-Flag Ab (M2), M2-HRP conjugate, and anti-HA Abs were obtained from Sigma (St. Louis, MO), anti-GFP and AU1 Abs were from Invitrogen (Carlsbad, CA), and anti-p-tyrosine Ab PY20 was from BD Biosciences (San Jose, CA). Ultrapure *E. coli* 0111:B4 LPS repurified according to Hirschfeld et al. (58) and free of lipoproteins was obtained from Invivogen (San Diego, CA). Human embryonic kidney (HEK) 293T cells were from ATCC (Manassas, VA), and maintained in DMEM medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and 10% FBS (HyClone, UT) (complete DMEM). HEK293 cells stably expressing untagged TLR4 and Flag-MD-2 (293/TLR4/MD-2), 293/YFP-TLR4/MD-2, and 293/YFP-MyD88 cell lines were kindly provided by Dr. Douglas Golenbock (University of Massachusetts Medical School, Worchester, MA). These cell lines were maintained in complete DMEM medium supplemented with 0.5 mg/ml neomycine. Human monocytes were prepared by counter flow elutriation and resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FBS.

Recombinant plasmids and transient transfection

pCDNA3-YFP-TLR4, pCDNA3-CD14, pCMV-β-galactosidase, pEFBOS-HA-MD-2 and pELAM-luciferase, were obtained from Dr. Douglas T. Golenbock. pEFBOS-His/Flag-human

(hu) MD-2 was provided by Dr. Kensuke Miyake (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). pEFBOS-Flag-Mal WT and P125H mutant, pDC304-HA-Mal, pCDNA3-YFP-MyD88, pCDNA3-Btk, pCDNA3-Myc-IRAK-2, and pRK5-Flag-TRAF-6 were described previously (27,51,55). pFlag-CMV-1 encoding WT CD4-TLR4 was kindly provided by Dr. Stephen T. Smale (Howard Hughes Medical Institute, UCLA, Los Angeles, CA), and pRL-TK-*Renilla* luciferase was from Promega. Y86A, Y106A, Y159A, Y187A, Y195A and Y196A mutations were introduced into the TIR domain of WT Flag-Mal or HA-Mal by site-directed mutagenesis, using Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). HEK293T cells were cultured overnight in 100 mm tissue culture dishes (2.5×10^6 cells per dish) and co-transfected for 3 h with expression vectors as described in Figure Legends (10 μg total plasmid DNA per dish) using Superfect transfection reagent (Qiagen, Valencia, CA). After 24 h, cells were collected and whole cellular extracts were prepared as described (51). For real-time PCR analyses, transfections were performed in 6-well plates according to the manufacturer's protocol as specified in the Figure Legends.

Isolation of RNA and real-time RT-PCR analysis

Total RNA was isolated using RNeasy kits (Invitrogen), residual genomic DNA was digested with DNase and RNA was re-purified as recommended by the manufacturer. cDNA was prepared from 1 μg total RNA using Reverse Transcription System (Promega), and subjected to real-time PCR with gene-specific primers for human IL-8 5'-CACCGGAAGGAACCATCT CACT-3' (forward); 5'-TGCACCTTCACACAG AGCTGC-3' (reverse) and hypoxanthine phosphoribosyltransferase (HPRT) 5'-ACCAGT CAACAGGGGACATAAAAG-3' (forward); 5'-GTCTGCATTGTTTTGCCAGTGTC-3' (reverse), on a MyIQ real-time PCR detection system (Bio-Rad, Hercules, CA). Real-time PCR data were analyzed using $-2^{\Delta\Delta CT}$ method as described (59).

Co-immunoprecipitation and immunoblotting

Whole cell lysates (1 mg total protein) were precleared with protein G-agarose beads (20 μl per sample, Roche Applied Science, Indianapolis, IN) for 4 h at 4°C upon rotation. Precleared cell extracts were incubated overnight at 4°C with 1 μg/ml of respective Ab in lysis buffer containing 20 mM HEPES (pH 7.4), 0.5% Triton X-100, 150 mM NaCl, 12.5 mM β-glycerophosphate, 50 mM NaF, 1 mM DTT, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, and protease inhibitor cocktail (Roche). Protein G-agarose beads were added (45 μl per sample) and incubation continued for 4h. Beads were washed 5 times with lysis buffer, and resuspended in Laemmli sample loading buffer (50 mM Tris-Cl, pH 6.8/10% glycerol/2% SDS/0.1% bromophenol blue/5% 2-mercaptoethanol). Immunoprecipitated proteins were separated on 4–20% mini-gels (Invitrogen), electrotransferred to Immobilon-P membranes, blocked and probed with the respective Abs.

NF-κB reporter assays

HEK293T or 293/TLR4/MD-2 cells were plated into 24-well tissue culture plates (10^5 cells per well) and grown for 20 h. Expression vectors pELAM-luciferase was added (0.3 μg/well each) to corresponding transfection mixtures, pCMV-β-galactosidase reporter or pRL-TK-*Renilla* luciferase (0.1 μg/well) were co-transfected for normalization, and total plasmid DNA was adjusted to 1 μg/well with pcDNA3. Following transfections, cells were recovered for 24 h, lysed in a passive lysis buffer (Promega) and firefly luciferase *vs.* β-galactosidase or *Renilla* luciferase activities were measured using Reporter Assay System (Promega) and β-galactosidase assay kit (Tropix, Galacto-Light System) on a Berthold LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Where pRL-TK-*Renilla* luciferase was used

for normalization, firefly and Renilla luciferase activities were measured with dual luciferase reporter assay system (Promega).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 program for Windows (GraphPad Software Inc., San Diego, CA). Statistical differences among experimental groups were evaluated by the Student's t-test with the level of significance set at $p<0.05$. Values are expressed as mean ± SD.

RESULTS

Mal undergoes tyrosine phosphorylation that correlates with signaling and is abolished by mutagenesis of Y86, Y106 and Y159 tyrosine residues within the TIR domain

To examine whether Mal is subject to tyrosine phosphorylation, two approaches were applied. First, 293/TLR4/CD14/MD-2 cells were transfected with pEFBOS-Flag-Mal to express constitutively-active Mal that signals cell activation in a TLR-independent manner (27,28). Immunoprecipitation of Mal with α-Flag Ab and immunoblot analysis with α-p-tyrosine-specific Ab revealed constitutive Mal tyrosine phosphorylation (Fig. 1A, top panel). LPS stimulation slightly reduced tyrosine phosphorylation of overexpressed Mal within 15–30 min, most likely due to Mal protein degradation (Fig. 1A, bottom panel). Secondly, we assessed LPS-inducible tyrosine phosphorylation of endogenous Mal. 293/TLR4/MD2 cells or primary human monocytes were stimulated with LPS, endogenous Mal proteins were immunoprecipitated and analyzed by immunoblotting with α-p-tyrosine Ab (to detect Mal phosphorylation) *vs.* α-Mal Ab (to assess total protein expression). Alternatively, proteins phosphorylated on tyrosines were immunoprecipitated with α-p-tyrosine Ab, followed by the detection of phosphorylated Mal species within this fraction by immunoblotting with α-Mal Ab. LPS induced tyrosine phosphorylation of endogenous Mal within 1–5 min that reached a plateau level by 15–30 min post-stimulation (Fig. 1B and C, Figs. 3A and B).

To identify tyrosine residues important for Mal phosphorylation and signaling, six tyrosines within the TIR domain of Mal were mutated to alanines and the effect of these mutations on Mal tyrosine phosphorylation and cell activation was analyzed. Unlike WT Mal, overexpression of Y86A, Y106A, and Y159A Mal variants in HEK293T cells led to a decrease in the intensity of the bands immunoreactive with α-p-tyrosine Ab (Fig. 2A, top panel) and disappearance of the α-Flag-Ab-reactive Mal bands with slower electrophoretic mobilities which, as reported earlier (55), correspond to phosphorylated Mal (Fig. 2A, 2nd and 3rd panels from the top). Interestingly, the mutation of a conserved proline to histidine within the TIR domain of Mal (P125H) that renders Mal signal-incompetent (27,28, and Fig. 2A) also abolished Mal tyrosine phosphorylation (Fig. 2A, top three panels). Similar to the P125H mutant, Y86A, Y106A, and Y159A Mal variants showed significantly reduced capacities to mediate IκB-α degradation, p38 phosphorylation (Fig. 2A), NF-κB reporter activation (Fig. 2B), and to up-regulate IL-8 mRNA expression (Fig. 2C), compared to robust responses elicited by WT Mal. In contrast, mutagenesis of Y187, Y195, and Y196 tyrosines did not affect Mal tyrosine phosphorylation, Mal-mediated IκB-α degradation, p38 phosphorylation, and had a minimal (Y187A and Y195A) or no effect (Y196A) on Mal ability to activate NF-κB reporter or IL-8 gene expression (Fig. 2). These data show functional significance of Mal tyrosine phosphorylation for activation of endogenous signaling intermediates and suggest Y86, Y106 and Y159 as important phospho-acceptor sites within the TIR domain of Mal.

Induction of endotoxin tolerance correlates with suppressed LPS-inducible Mal tyrosine phosphorylation

Since Mal is an essential "bridging" adapter in TLR4 signaling, we sought to determine whether reprogrammed responsiveness to LPS observed in endotoxin-tolerant cells (44–47,51) is associated with altered Mal tyrosine phosphorylation. To this end, LPS-inducible tyrosine phosphorylation of endogenous Mal was examined in control and endotoxin-tolerant 293/TLR4/MD2 cells and primary human monocytes. Tyrosine phosphorylation of Mal was observed as early as 1 min, and persisted throughout 5–30 min after LPS stimulation of medium-pretreated 293/TLR4/MD-2 cells and human monocytes (Fig. 3A and B, top panels). LPS pretreatment of 293/TLR4/MD-2 cells and human monocytes for 20 h led to the induction of endotoxin tolerance, as evidenced by suppressed LPS-induced phosphorylation of p38 and IRAK-1 (Fig. 3) and diminished IκB-α degradation (Fig. 4B). Altered LPS responsiveness correlated with reduced levels of Mal tyrosine phosphorylation and the inability of LPS to re-induce this response in endotoxin-tolerant 293/TLR4/MD-2 cells and human monocytes (Fig. 3A and B). Decreased levels of total Mal protein expression (~20–30%, Fig. 3) were not observed in other experiments that showed even higher total expression of Mal in endotoxin-tolerant cells (Fig. 4B). These data show that endotoxin tolerization correlates with the failure of LPS to induce Mal tyrosine phosphorylation, suggesting an important role of tyrosine phosphorylation of Mal in efficient TLR4 signaling.

P125H, Y86A, Y106A, and Y159A mutations within the TIR domain of Mal and induction of endotoxin tolerance inhibit associations of Mal with Btk

Btk was reported to interact with and phosphorylate Mal on tyrosines (55) but it was unknown which tyrosine residues are important for Mal associations with Btk. To elucidate this, untagged Btk was co-expressed in 293/TLR4/MD-2 cells together with Flag-tagged WT Mal, the P125H or Y86A, Y106A, Y159A mutants showing defective tyrosine phosphorylation and signaling, or the Y196A Mal variant that undergoes phosphorylation and is signaling-competent. Cells were treated with medium or incubated with LPS, and the effect of the P/H or Y/A mutations on Mal constitutive and LPS-inducible associations with Btk was examined by immunoblotting of Mal immunoprecipitates with α-Btk Ab. WT and Y196A Mal variants constitutively associated with Btk (Fig. 4A, left panel), and LPS stimulation further increased Mal-Btk interactions (Fig. 4A, right panel), showing a correlation with its tyrosine phosphorylation patterns and signaling activity (Fig. 2–Fig. 4). In contrast, the P125H, Y86A, Y106A, and Y159A mutants failed to interact with Btk constitutively and showed significantly impaired associations with Btk upon LPS stimulation (Fig. 4A, top panel). Thus, deficient Mal-Btk associations of the P125H, Y86A, Y106A, and Y159A Mal mutants are likely to account for the lack of tyrosine phosphorylation of these Mal species and their signaling-incompetent features.

Next, we used primary human monocytes to confirm LPS-inducible association of endogenous Mal and Btk, and to study whether endotoxin tolerization affects constitutive and LPS-mediated Mal-Btk interactions. Monocytes were pretreated for 20 h with medium or tolerized with LPS, washed, re-stimulated with LPS, and interactions of endogenous Mal and Btk were examined in cell lysates by co-immunoprecipitation. LPS caused an increase in the amount of Btk associated with Mal within 5–15 min in medium-pretreated cells (Fig. 4B) that correlated with enhanced tyrosine phosphorylation of Mal (Fig. 3B). In contrast, LPS failed to up-regulate Mal-Btk associations over constitutive levels in endotoxin-tolerant monocytes, correlating with the lack of LPS-inducible Mal tyrosine phosphorylation (Fig. 3B and Fig. 4B). Taken collectively, these results suggest an important role of the P125, Y86, Y106, and Y159 sites within the Mal TIR domain in mediating associations with Btk, and show impaired LPS-induced Btk recruitment to Mal in endotoxin-tolerized cells.

Tyrosine-deficient Y86A, Y106A, and Y159A Mal variants show increased association with TLR4 and exert a dominant-negative effect on TLR4-inducible signaling

In our next series of experiments, we studied whether mutations of tyrosine residues within the TIR domain of Mal alter Mal interactions with TLR4. CD4-TLR4 was co-expressed in HEK293T with WT or P125H, Y86A, Y106A, Y159A, or Y196A Flag-Mal variants, and TLR4-Mal interactions were studied by co-immunoprecipitation. TLR4 proteins expressing CD4 epitopes were immunoprecipitated with α-CD4 Ab and immune complexes were analyzed by immunoblotting with either α-Flag Ab, to detect associated Flag-Mal, or with α-CD4 Ab, to assess total TLR4 expression. These experiments revealed higher associations with TLR4 exhibited by Y86A, Y106A, and Y159A Mal variants compared to WT and Y196 Mal species (Fig. 5A, top panel). Since CD4-TLR4s express both CD4 and Flag epitopes (51), reciprocal immunoprecipitation of overexpressed Flag-Mal with α-Flag Ab was ruled out because it would have immunoprecipitated both CD4-TLR4 and Flag-Mal. For this reason, we used Ab against native, untagged Mal proteins to immunoprecipitate overexpressed Mal variants. Subsequently, Mal immune complexes were subjected to immunoblot analyses with α-Flag Ab to allow the simultaneous detection of CD4-TLR4 (~75 kDa) associated with Mal vs. total Flag-Mal expression (~35 kDa). This reciprocal immunoprecipitation confirmed increased amounts of TLR4 associated with Y86A, Y106, and Y159A Mal species compared to WT and Y196A Mal variants (Fig. 5A, the second panel from the top). Similar total proteins levels of Flag-Mal variants and CD4-TLR4 were observed in all samples (Fig. 5A), indicating that differential Mal-TLR4 associations were not due to different rates of Mal degradation or variations in the expression of total Mal or TLR4 proteins.

To delineate whether increased associations of Y86A and Y106A Mal species with TLR4 is functionally significant, we tested the ability of tyrosine-deficient Mal variants to exert a dominant-negative effect on TLR4-initiated signaling. Co-expression of Y86A, Y106A, and Y159A, but not Y196A, Mal species in 293/TLR4/MD-2 cells inhibited LPS-inducible p38 and IRAK-1 phosphorylation (Fig. 5B and data not shown) and NF-κB reporter activation (Fig. 5C). These results indicate that Y86A, Y106A, and Y159A mutations led to increased association of Mal with TLR4, conferring upon Mal the ability to act as a dominant-negative inhibitor of TLR4-induced activation of IRAK-1, p38 and NF-κB.

Y86A, Y106A, and Y159A Mal variants retain their interactions with MyD88, IRAK-2, and TRAF-6 and do not affect the ability of these signaling intermediates to mediate post-receptor signaling initiated by their overexpression

In addition to TLR4, Mal associates with MyD88, IRAK-2 and TRAF-6 and serves as a bridging adapter to initiate signalosome formation at the receptor to transduce a signal downstream (22,27,28,33–35). Therefore, it was of interest to examine the effect of tyrosine deficiencies on the ability of Mal to interact with a principal signaling adapter MyD88, a principal kinase IRAK-2, and a principal downstream adapter TRAF-6, and to link possible observed changes in interactions to signaling functions of these intermediates. As shown by co-immunoprecipitation, Y86A, Y106A, and Y159A Flag-Mal species associated with co-expressed AU-1-MyD88 to a greater extent compared to MyD88 interactions exhibited by WT and Y196A Mal variants (Fig. 6A and B). Likewise, increased amounts of Y86A Mal were found in complex with co-expressed YFP-MyD88 as compared to WT or Y196A Mal species (Fig. 6C). This phenomenon was demonstrated by two different co-immunoprecipitation approaches: immunoblot detection of Flag-Mal variants associated with immunoprecipitated epitope-tagged MyD88 proteins (Fig. 6A–C, top panels), and assessment of amounts of epitope-tagged MyD88 in complex with immunoprecipitated Flag-Mal (Fig. 6A–C, the second blots from the top). Equal total expression levels of AU-1-MyD88, YFP-MyD88, and Flag-Mal variants were observed (Fig. 6A–C), indicating that differences in protein-protein interactions could not be attributed to different expression levels of MyD88 and Mal variants.

To test functional consequences of increased interactions of tyrosine-deficient Mal species with MyD88, the effect of co-expression of Y86A, Y106A, Y159A, and Y196A Mal variants on NF-κB reporter activation initiated by overexpression of AU-1-MyD88 was measured. No inhibition of MyD88-triggered NF-κB reporter activation was observed upon co-expression of either Mal species (Fig. 6D).

Similar approaches were employed to examine the effect of Y/A mutations within the TIR domain of Mal on its ability to interact with co-expressed Myc-IRAK-2 or Flag-TRAF-6. Comparable amounts of WT, P125H, Y86A, and Y196A Flag-Mal variants were detected in complex with Myc-IRAK-2 by co-immunoprecipitation (Fig. 7A). Reverse immunoprecipitation also showed similar quantities of Myc-IRAK-2 associated with Flag-Mal species (Fig. 7A). Likewise, overexpressed WT, P125H, and Y86A HA-Mal proteins exhibited comparable interactions with co-expressed Flag-TRAF-6 (Fig. 7B). This conclusion was confirmed by two complementing co-immunoprecipitation approaches, the detection of Flag-TRAF-6 present in complex with immunoprecipitated HA-Mal variants and immunoblot analysis of Flag-TRAF-6 immunoprecipitates for the detection of interacting HA-Mal species. Co-expression of different Mal variants did not suppress NF-κB reporter activation initiated at the post-receptor levels by overexpression of Myc-IRAK-2 (Fig. 7C) or Flag-TRAF-6 (Fig. 7D). Taken collectively, these data indicate that the Y86A, Y106A, and Y159A mutations led to an increased association of Mal with MyD88 but did not affect its interactions with IRAK-2 and TRAF-6, and did not influence postreceptor signaling initiated by overexpression of MyD88, IRAK-2 or TRAF-6.

DISCUSSION

Signalosome formation at the receptor complex is thought to involve TLR oligomerization, conformational changes, and post-translational modifications of TLRs and adapters, including phosphorylation events. Agonist engagement of TLR2, TLR3, TLR4 and TLR5 triggers their tyrosine phosphorylation, and mutations of several tyrosines in the TIR domains of these receptors impair TLR signaling (51–54). In addition to TLRs, adapter proteins are subject to several posttranslational modifications. For instance, myristoylation and serine phosphorylation of TRAM is important for its membrane anchoring and signaling (56,57). LPS triggers tyrosine phosphorylation of MyD88 (60), and tyrosine phosphorylation of Mal has been reported to be important for TLR2 and TLR4 signaling (55). Non-classical K63 ubiquitination of TRAF-6, necessary for induction of its functional activities, is another example of post-translational modifications of adapters (61). However, little is known about mechanisms by which posttranslational modifications of adapters affect signalosome assembly and TLR signaling.

Several lines of evidence shown herein stress an important role for Mal tyrosine phosphorylation in TLR4 signaling. We demonstrate a correlation of signaling incompetence of the P125H Mal mutant with its failure to interact with Btk, a key kinase involved in Mal tyrosine phosphorylation (55), and to undergo tyrosine phosphorylation. These results are reminiscent of our previous findings of compromised tyrosine phosphorylation of mouse or human TLR4 as a consequence of the P712H or P714H mutations associated with signaling deficiencies of these TLR4 species (51). This paper also reveals significance of Y86, Y106, and Y159 tyrosine residues within Mal for its interactions with Btk, Mal tyrosine phosphorylation and Mal-mediated activation of p38 phosphorylation, IκB-α degradation, IL-8 mRNA expression, and NF-κB reporter induction. It is plausible that P125, along with Y86, Y106, and Y159 tyrosines, provide docking sites for candidate kinases whose recruitment initiates phosphorylation of Y86, Y106, and Y159 tyrosine residues in a co-operative manner, with each reaction depending on the other. Although previously published suppression of Mal phosphorylation by a Btk inhibitor LFM-A13 (55) and the interactions studies shown here

suggest Btk as the key kinase responsible for tyrosine phosphorylation of Mal, we cannot rule out the involvement of other kinases. Interestingly, protein kinase C (PKC)-δ was also reported to bind Mal within its TIR domain and PKC-δ depletion from RAW cells suppressed TLR2- and TLR4-induced signaling (62). Thus, the involvement of a multi-kinase complex (*e.g.*, Btk-PKC-δ) in Mal phosphorylation is possible, suggesting a cooperative role for P125 and different tyrosine residues to efficiently dock Btk and PKC-δ, allowing tyrosine phosphorylation to take place. Studies are in progress to test this hypothesis and to delineate the role of tyrosine residues within the TIR domain of Mal in Mal associations with PKC-δ.

Induction of endotoxin tolerance re-programs subsequent cellular responses to LPS, suppressing induction of pro-inflammatory cytokines, while not affecting or even enhancing expression of anti-inflammatory mediators and antimicrobial effectors (44–47). Several important changes at the level of signalosome assembly have been revealed in endotoxin tolerance, including disrupted recruitment of MyD88 to TLR4 and altered IRAK-1-MyD88 association (49,50). In addition, we recently found that LPS-inducible TLR4 tyrosine phosphorylation is necessary for signaling and is drastically compromised in endotoxin-tolerant 293/TLR4/MD-2 cells and human monocytes (51). Hence, it is possible that dysregulated post-translational modifications of “bridging” and/or “signaling” adapters may play a role in disrupted signalosome complex formation observed in tolerant cells, leading to reprogramming. More specifically, we reasoned that altered tyrosine phosphorylation of Mal may be important for endotoxin tolerization, a possibility that was not addressed previously. To the best of our knowledge, this paper shows for the first time that endotoxin tolerance is associated with compromised associations of Btk with Mal, leading to inhibited Mal tyrosine phosphorylation. Because of the suggested role for Mal as a “bridging” adapter that recruits a “signaling” adapter MyD88 to the TLR4 receptor complex, it is tempting to speculate that impaired recruitment of MyD88 to TLR4 coupled with deficient IRAK-1 activation is associated with compromised tyrosine phosphorylation of both TLR4 (51) and Mal (shown in this study).

This report demonstrates increased interactions of Y86A, Y106A, and Y159A Mal variants with TLR4 that translate into the ability of these Mal species to act as dominant-negative inhibitors of TLR4-elicited p38 phosphorylation and NF-κB reporter activation. To gain an insight into mechanisms by which Mal tyrosine phosphorylation regulates TLR4 signaling, we examined interactions of WT and signal-incompetent, tyrosine-deficient Mal variants with TLR4, MyD88, IRAK-2, and TRAF-6. The signal-incompetent Y86A, Y106A, and Y159A Mal variants incapable of undergoing tyrosine phosphorylation were found to exhibit significantly higher associations with CD4-TLR4 compared to that observed with WT Mal. These increased associations are functionally significant as the mutagenesis of the Y86, Y106, and Y159 tyrosines rendered Mal into a dominant-negative inhibitor of TLR4 signaling. In contrast, higher interactions of the Y86A and Y106A Mal species with constitutively-active, overexpressed MyD88 are not translated into their ability to inhibit MyD88-induced NF-κB reporter activation. When tested for interactions with IRAK-2 and TRAF-6, both the WT and Y86A Mal variants showed similar associations with these signaling intermediates and moderately enhanced NF-κB reporter activation induced by overexpression of IRAK-2 and TRAF-6. These data suggest that tyrosine phosphorylation of Mal regulates the process of signalosome assembly at the level of TLR4, while failing to affect signaling initiated at the post-receptor levels. Indeed, once overexpressed, activated MyD88, IRAK-2 or TRAF-6 trigger activation of downstream signaling cascades in a TLR- and ligand-independent fashion (most likely, due to their enforced oligomerization) (1,22,23,35). Under these conditions, tyrosine-deficient Mals (*e.g.*, Y86A) do not suppress cell stimulation, suggesting their failure to sequester signaling-competent downstream intermediates.

It remains unknown how tyrosine phosphorylation of Mal regulates signalosome formation and signaling at the level of TLR4. Studies with molecularly-engineered MyD88 species demonstrated the importance of the delivery of MyD88 to the plasma membrane, showing a possibility to bypass the requirement for Mal if MyD88 could be plasma-membrane redirected due to the presence of grafted membrane-anchoring domains (33). However, a key question that remains unknown is how Mal carries out MyD88 recruitment. One possible mechanism is that Mal acts exclusively at the plasma membrane, by associating with TLR4 and creating docking platforms to recruit cytoplasmic MyD88. This possibility is supported by the membrane localization of Mal and generation of phosphatidylinositol-4,5-biphosphate (PIP2) by agonist-stimulated CD11b/CD18 that could anchor Mal at the plasma membrane through the PIP2-binding domain of Mal (33). This process is thought to facilitate Mal re-localization to membrane microdomains enriched with TLR4, leading to Mal-TLR4 interactions and co-localization (33). Despite a relative distance of the conserved Y86 from the PIP2 domain (aa 15–36), Y86 tyrosine phosphorylation may initiate conformational changes affecting the PIP2 domain and, hence, influencing Mal microdomain localization/TLR4 association within the plasma membrane. This could eventually affect the assembly of docking platforms formed by TLR4/Mal that are necessary for recruitment of downstream signaling adapters and kinases.

Alternatively, Mal could recruit MyD88 to TLR4 by acting as a shuttle molecule between the plasma membrane and the cytoplasm. Interestingly, serine phosphorylation of another “bridging” adapter, TRAM initiates its translocation from the plasma membrane, supposedly leading to dissociation of TRAM-TRIF complex to the cytoplasm where it triggers signaling (57). Likewise, a similar regulation of Mal translocation from the cell membrane by its tyrosine phosphorylation could be envisioned, due to a lower interaction of tyrosine-phosphorylated, constitutively-active WT Mal with TLR4. In contrast to WT Mal, stronger associations of tyrosine-deficient Y86A, Y106A, or Y159A Mal variants with TLR4 may suggest retention of tyrosine-deficient Mal at the plasma membrane. This increased association of tyrosine-deficient, signaling-incompetent Mal species with TLR4 is different than the failure of the serine-deficient, signal-compromised TRAM variant to interact with TLR4 in either constitutive or LPS-inducible manners (57). Thus, despite Mal and TRAM are both considered as “bridging” adapters, phosphorylation of these adapters seems to differently regulate their localization and signalosome formation.

Tyrosine phosphorylation of Mal targets it for ubiquitination by suppressor of cytokine signaling (SOCS)-1 and subsequent proteosomal degradation (63), leading to down-regulation of TLR4 signaling due to unavailability of Mal. However, it is also plausible that Mal degradation at early stages of signal transduction promotes a release of activated components of the MyD88/IRAK-4/IRAK-1/TRAF-6 signalsome from the receptor. Hence, impaired tyrosine phosphorylation of Mal could suppress its degradation, preventing dissociation of signaling components MyD88, IRAK-2, and TRAF-6 from the receptor complex, and, as a result, inhibiting signaling. Mal was also found to be cleaved by caspase-1 that generates signaling-competent Mal species necessary for signal transduction (60). Although Y86 and Y106 are situated distantly from the D198 residue that is critical for caspase-1-mediated cleavage of Mal (60), tyrosine phosphorylation of Y159 could potentially be involved in changes of Mal conformation and its molecular electrostatic charge that may promote Mal-caspase-1 interactions. Thus, phosphorylation of different tyrosine residues within Mal could regulate its functions at several levels by non-redundant mechanisms.

In spite of uncompromised abilities of tyrosine-deficient Mal species to interact with constitutively-active MyD88, IRAK-2 and TRAF-6, Mal tyrosine phosphorylation could confer upon Mal the ability to shuttle from the membrane and to bind non-activated downstream signaling intermediates, delivering them to TLR4. This would initiate orchestrated signalosome assembly, leading to MyD88/IRAK-2/TRAF-6 complex formation,

posttranslational modifications, and activation of downstream signaling. In line with this hypothesis, tyrosine de-phosphorylated Mal would be incapable of translocating from the membrane to the cytoplasm and/or would not be able to bind inactive, non-oligomerized cytoplasmic intermediates and recruit them to TLR4, failing to initiate competent signalosome formation. Studies employing cell fractionation, biotinylation and confocal microscopy are in progress to further elucidate molecular mechanisms by which Mal posttranslational modifications affect TLR4 signalosome formation and activation of signaling intermediates at the receptor complex.

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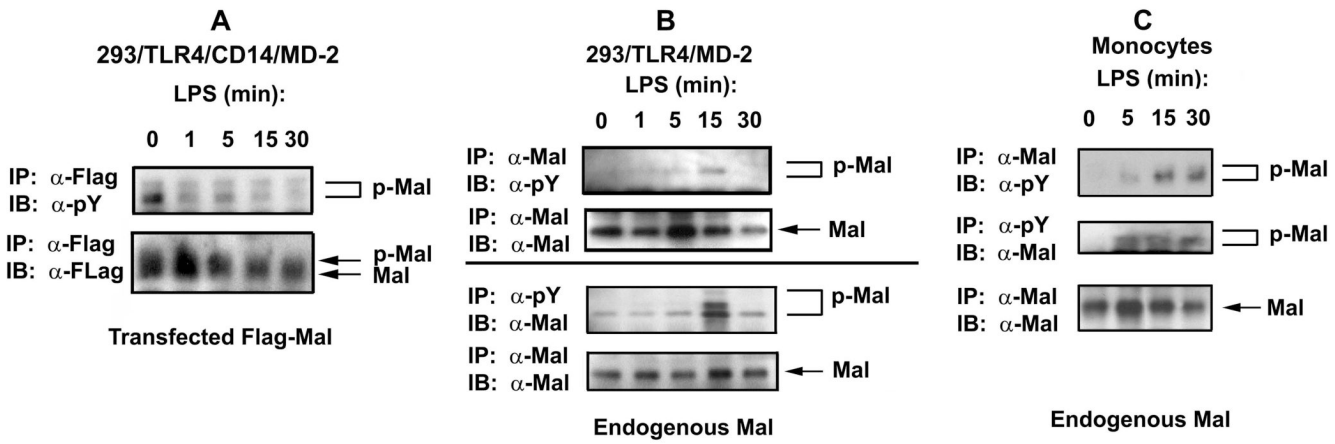

Figure 1. Mal undergoes tyrosine phosphorylation in a constitutive (upon overexpression) or a LPS-inducible (endogenous Mal) manner

(A) HEK293T cells were plated into 100-mm tissue culture dishes and transiently transfected with pCDNA3-CD14 (1 μg per dish), pCDNA3-TLR4 (4 μg per dish), pEFBOS-HA-MD-2 (1 μg per dish), and pEFBOS-Flag-Mal (WT, 4 μg per dish). To study LPS-inducible Mal tyrosine phosphorylation, the 293/TLR4/MD-2 stable cell line (B) and human monocytes (C) were used. Cells were treated with medium or stimulated with 100 ng/ml LPS as shown. Flag-Mal or endogenous Mal were immunoprecipitated with α-Flag or α-Mal Abs, respectively, and subjected to immunoblotting with α-p-tyrosine Ab PY20 (Mal phosphorylation) and α-Flag (A) or α-Mal (B and C, top panels) Abs (total protein expression of overexpressed or endogenous Mal). Alternatively, total tyrosine-phosphorylated proteins were immunoprecipitated with PY20 Ab, followed by immunoblot detection of p-Mal within this fraction, using Abs against endogenous Mal (B and C, middle panels). To examine total levels of endogenous Mal proteins, Mal proteins were immunoprecipitated with Ab against endogenous Mal and subjected to immunoblotting with α-Mal Ab (B and C, bottom panels). Results of a representative experiment (n=3) are shown.

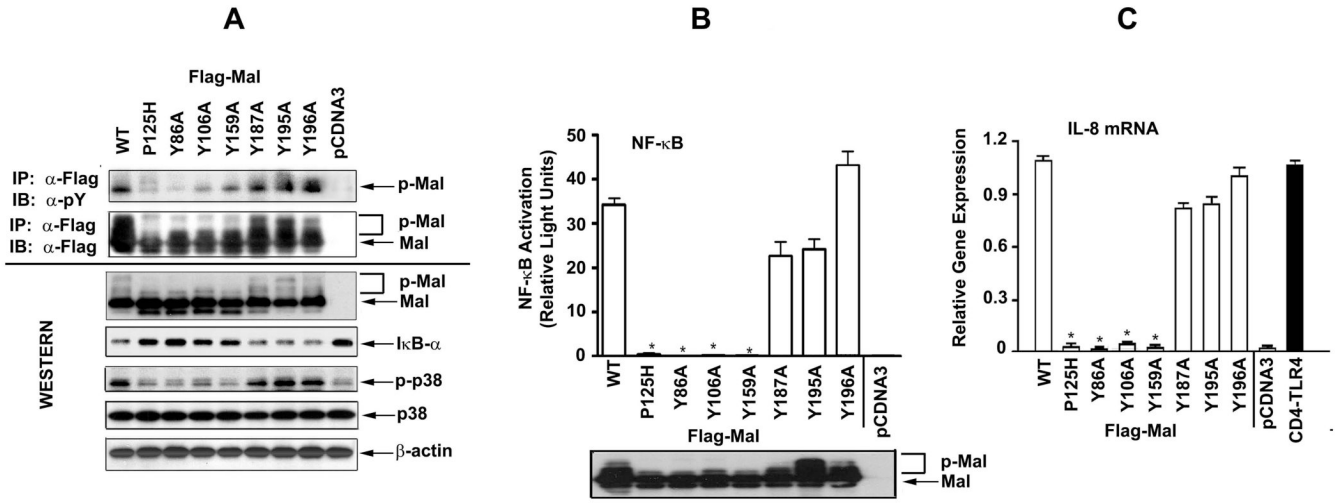

Figure 2. P125H, Y86A, Y106A, and Y159A mutations in the TIR domain of Mal impair Mal tyrosine phosphorylation and its ability to signal p38 phosphorylation, IκB-α degradation, NF-κB reporter activation, and IL-8 mRNA expression

WT or P125H, Y86A, Y106A, and Y159A mutations in the TIR domain of Mal WT or P125H, Y86A, Y106A, Y159A, Y187A, Y195A and Y196A Flag-Mal variants were overexpressed in HEK293T cells (the vector input was 10 μg per 100-mm dish), and pCDNA3 transfection was used as a negative control. Cell extracts were analyzed by immunoblotting with α-Flag Ab (A and B) or Abs against IκB-α, p-p38, and total p38. β-Actin immunoblot was used to control for protein loading (A). (A, top two panels): Mal species were immunoprecipitated with α-Flag Ab and subjected to immunoblot analyses with α-p-tyrosine Ab PY20 or α-Flag Ab to detect Mal phosphorylation and total protein expression, respectively. (B): NF-κB reporter activation was analyzed in cell extracts by measuring luciferase vs. β-galactosidase activities, and the total levels of Mal were analyzed in the same cell lysates by immunoblotting with α-Flag Ab (bottom panel). (C): RNA was isolated, cDNA was prepared and IL-8 and HPRT mRNA expression levels were analyzed by real-time RT-PCR with gene-specific primers. *p<0.005 reflect statistically significant differences in gene expression and reporter activation between Flag-Mal WT and the indicated mutants. Shown are the results of a representative (n=3) experiments.

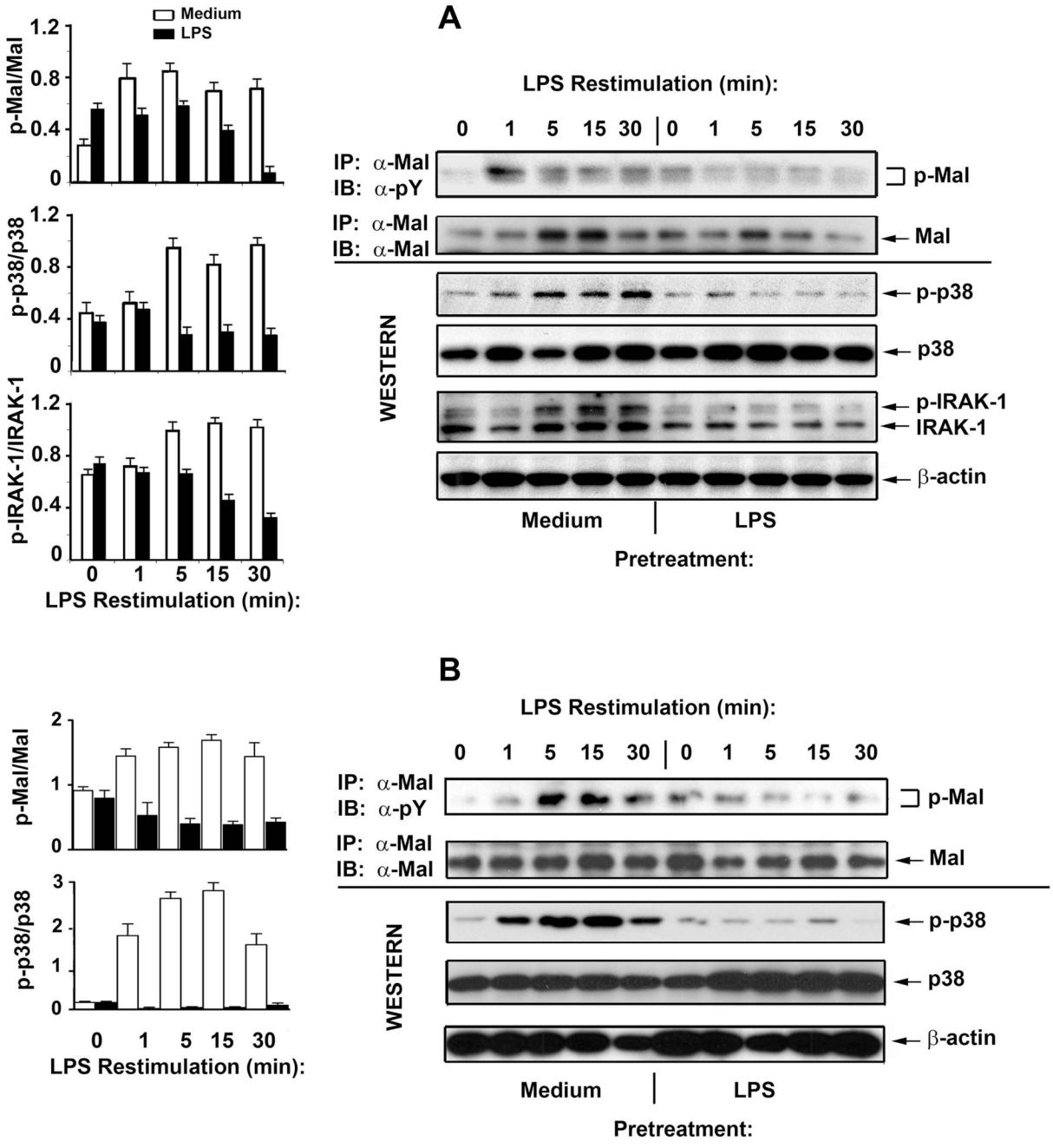

Figure 3. LPS-inducible Mal tyrosine phosphorylation is inhibited in endotoxin-tolerant cells

The HEK293/TLR4/MD-2 stable cell line (A) or human monocytes (B) were pretreated for 20 h with medium or 10 ng/ml LPS, washed and re-stimulated with 100 ng/ml LPS as indicated. Endogenous Mal proteins were immunoprecipitated from cell extracts with α-Mal Ab, followed by immunoblotting with α-p-tyrosine Ab PY20 (to measure Mal phosphorylation) and α-Mal Ab (to detect Mal total protein expression). For controlling cell activation and endotoxin tolerance induction, IRAK-1 and p38 phosphorylation were examined by immunoblotting of whole cell lysates with the corresponding Abs. The results of a representative experiment are shown, and densitometric quantification of relative protein levels of p-Mal, p-p38, and p-

IRAK-1 (normalized to total Mal, p38, unmodified IRAK-1 and β-actin, respectively) from three (A) and six (B) experiments are presented on the left.

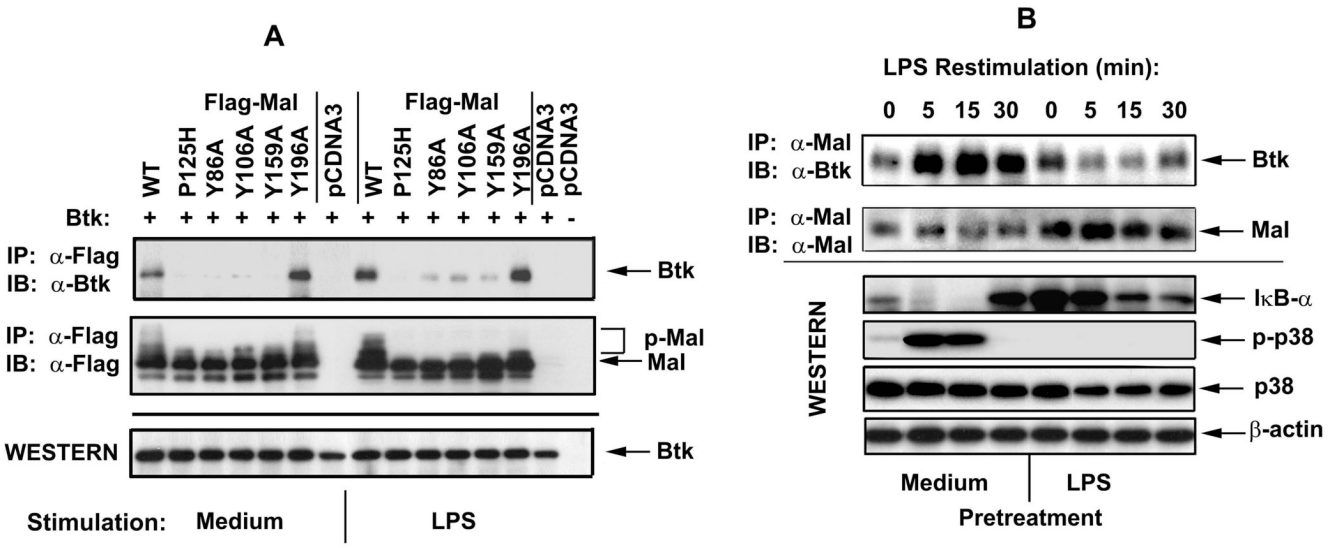

Figure 4. P125H, Y86A, Y106A, and Y159A mutations in the TIR domain of Mal and endotoxin tolerance induction suppress Mal-Btk associations

(A): WT, P125H, Y86A, Y106A, Y159A, and Y196A Flag-Mal variants were co-expressed in HEK293/TLR4/MD-2 cells along with Btk (the input vector amounts were 4 μg each per 100-mm dish). After recovery for 24 h, cells were treated with medium or stimulated for 30 min with 100 ng/ml LPS, and cell lyzates were prepared. Mal species were immunoprecipitated with α-Flag Ab, and their interactions with Btk were analyzed by immunoblotting with α-Btk Ab and compared to total protein levels of Mal, as detected by Western blot analyses of Flag-Mal immunoprecipitates with α- Flag-HRP Ab. Total protein levels of overexpressed Btk were analyzed by immunoblotting of cell lysates with α-Btk Ab. (B): Human monocytes were pretreated for 20 h with medium or tolerized with 10 ng/ml LPS, washed and re-stimulated with 100 ng/ml LPS for the indicated time intervals. Total endogenous Mal proteins were immunoprecipitated from cell extracts with α-Mal Ab, and subjected to immunoblot analyses with α-Btk and α-Mal Abs to assess Mal-Btk associations and Mal total protein levels, respectively. The results of a representative experiment (n=3) are shown.

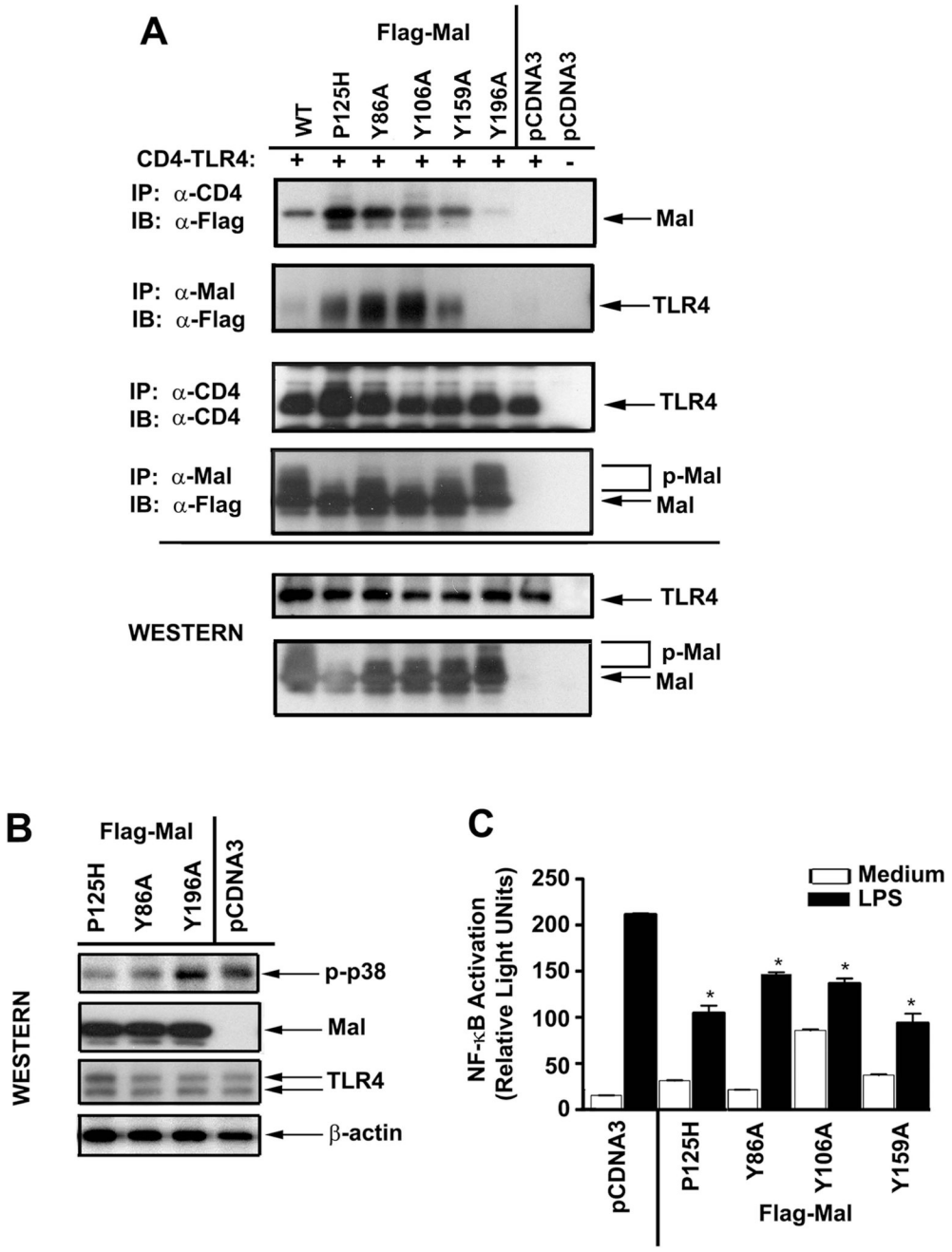

Figure 5. Y86, Y106, and Y159 tyrosine deficiencies within the TIR domain of Mal increase Mal associations with TLR4 and exert a dominant-negative effect on TLR4-mediated p38 phosphorylation and NF-κB reporter activation

(A) WT, P125, Y86A, Y106A, Y159A, and Y196A Flag-Mal variants were co-expressed in HEK293T cells along with CD4-TLR4 (5 μg of each expression vector per 100-mm dish), and pCDNA3 transient transfection was used as a negative control. CD4-TLR4 and Flag-Mal proteins were immunoprecipitated with α-CD4 and α-Mal (to avoid co-precipitation of CD4-TLR4 that also expresses Flag tag) Abs, respectively. Flag-Mal proteins (~35 kDa) associated with TLR4 were detected by immunoblotting of CD4-TLR4 immunoprecipitates with α-Flag Ab, and compared to total expression of CD4-TLR4, as detected by the immunoblotting with

α-CD4 Ab. Reciprocally, Mal proteins were immunoprecipitated with α-Mal Ab, and Mal interactions with CD4-TLR4 vs. total expression of transfected Flag-Mal were analyzed by immunoblotting with α-Flag Ab to detect CD4-TLR4 (~75 kDa) and Flag-Mal (~35 kDa), respectively. Total expression levels of transfected Flag-Mal and CD4-TLR4 were also examined by immunoblotting of whole cell lysates with α-Flag and α-CD4 Abs, respectively. (B): P125H, Y86A, and Y196A Mal species or pCDNA3 were expressed in 293/YFP-TLR4/MD-2 cells, cells were recovered for 20 h and stimulated for 5 min with 100 ng/ml LPS. Thereafter, p38 phosphorylation was examined by immunoblotting of cell lysates with α-p-p38 Ab, and YFP-TLR4 and Flag-Mal protein levels were assessed by immunoblotting with α-GFP and α-Flag Abs, respectively. β-actin immunoblot was run to control for equal protein loading. (C). pEFBOS-Flag-Mal expression vectors encoding P125H, Y86A, Y106A, and Y159A Mal species were co-transfected in 293/TLR4/MD-2 cells with pELAM-lucifearse and pCMV-β-galactosidase reporters. Cells were recovered for 20 h, stimulated with 1μg/ml LPS for 6 h, and luciferase vs. β-galactosidase activities were measured in cell extracts. *$p<0.05$ reflect statistically significant differences in reporter activation between pCDNA3 control and the indicated mutants. Results of a representative experiment (n=3) are depicted.

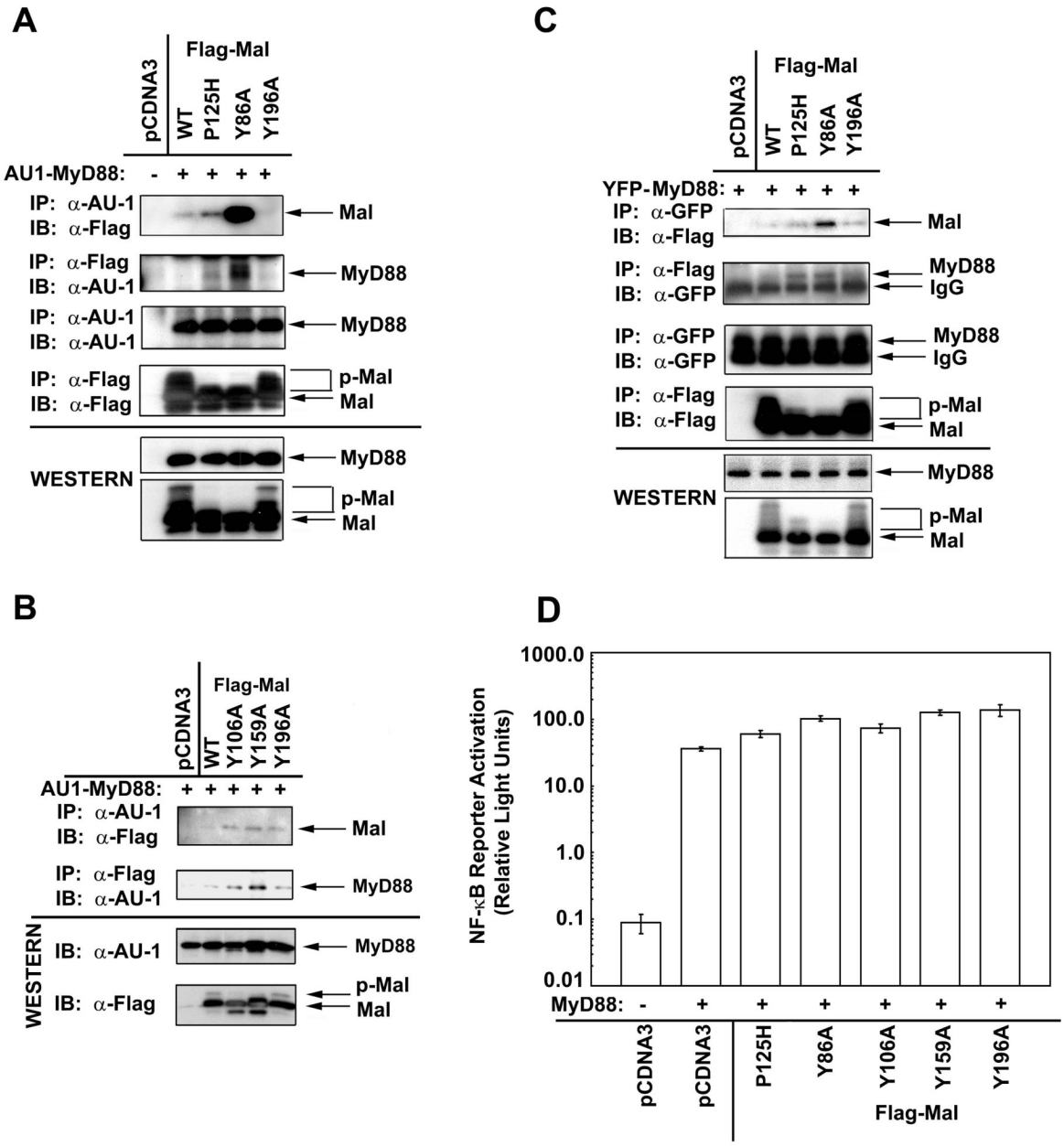

Figure 6. The effect of the P125H, Y86A, Y106, Y159A, and Y196A mutations within the TIR domain of Mal on its interactions with MyD88 and MyD88-mediated NF-κB reporter activation

pEFBOS expression vectors encoding Flag-tagged WT, P125H, Y86A, Y106A, Y159A, or Y196A Mal variants (5 μg per dish) were co-transfected into HEK293T cells along with pCDNA3-AU-1-MyD88 (A and B) or pCDNA3-YFP-MyD88 (C) (5 μg per dish each) in 100-mm tissue culture dishes. Cell extracts were prepared, Flag-Mal species or AU-1-MyD88 (A, B) and YFP-MyD88 (C) were immunoprecpitated with the corresponding α-epitope Abs. AU-1-MyD88 and YFP-MyD88 immunoprecipitates were analyzed for interactions with Flag-Mal variants by immunoblotting with α-Flag Ab, and total protein levels of overexpressed MyD88 were measured with α-AU-1 or α-GFP Abs. Flag-Mal immune complexes were immunoblotted with α-AU-1 (A and B) or α-GFP (C) Abs to examine associations with epitope-tagged MyD88 *vs.* total Mal expression analyzed by immunoblotting with α-Flag-HRP. (D)

pCDNA3-AU-1-MyD88 (0.1 μg per well) and pEFBOS-Flag-Mal expression plasmids encoding WT or mutant Mal variants (0.5 μg per well) were transfected in HEK293T cells along with pELAM-luciferase (0.3 μg per well) and pTK-*Renilla* luciferase (0.1 μg per well) reporters. NF-κB reporter activation was studied by measuring firefly vs. *Renilla* luciferase activities. The data of a representative experiment (n=4) are shown.

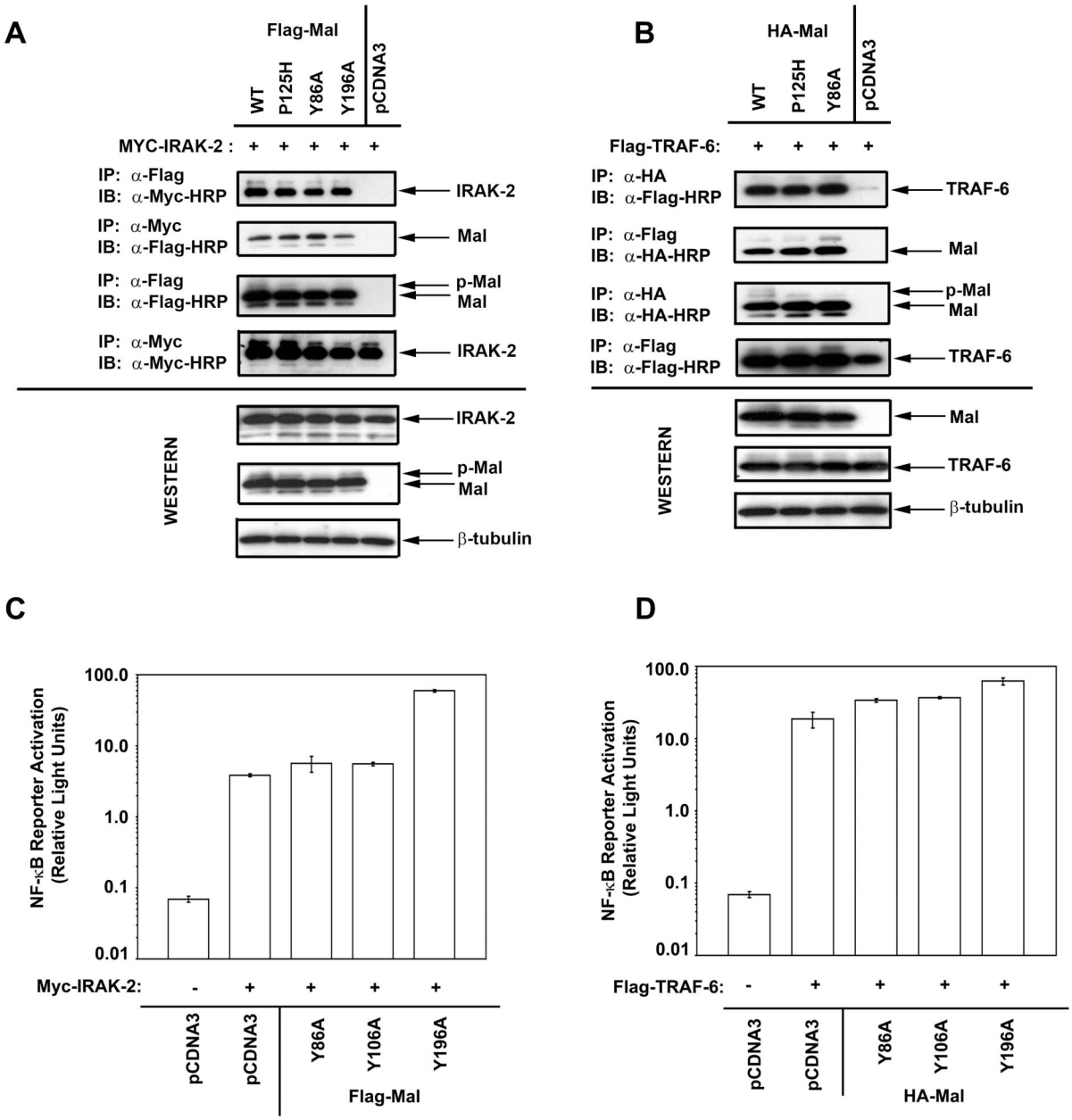

Figure 7. The P125H, Y86A, and Y196A mutations within the TIR domain of Mal do not affect Mal interactions with IRAK-2 and TRAF-6 and NF-κB reporter activation triggered by IRAK-2 and TRAF-6 overexpression

Flag-tagged (A) or HA-tagged (B) WT, P125H, Y86A or Y196A Mal variants were co-expressed in HEK293T cells along with Myc-IRAK-2 (A and C) or Flag-TRAF-6 (B and D) similar to the protocol described in Fig. 6. Mal species were immunoprecpitated with α-Flag (A) or α-HA (B) Abs, and analyzed for interactions with Myc-IRAK-2 (A) or Flag-TRAF-6 with α-Myc or α-Flag Abs. In addition, Myc-IRAK-2 (A) and Flag-TRAF-6 (B) were immunoprecipitated with α-Myc or α-Flag Abs, and immunoblotted with α-Flag (A) or α-HA Abs to examine the presence of co-immunoprecipitated Mal species. Total protein levels of all expressed protein were analyzed with the corresponding α-epitope Abs. (C and D): WT, Y86A, Y106A, and Y196A Flag-Mal (C) or HA-Mal (D) species were co-expressed together with

Myc-IRAK-2 (C) or Flag-TRAF-6 (D). For reporter assays, expression vectors encoding IRAK-2 and TRAF-6 were used at input amounts of 50 ng per well that gave ~30–40% of plateau levels of NF-κB reporter activation caused by overexpression of these intermediates. pEFBOS-Mal plasmids were used at 500 ng per well, and the NF-κB and *Renilla* luciferase reporters were used as described in Fig. 6. Firefly vs. *Renilla* luciferase activities were measured in cell lysates to assess NF-κB reporter activation. The data of a representative experiment (n=3) are presented.