Arg753Gln Polymorphism Inhibits Toll-like Receptor (TLR) 2 Tyrosine Phosphorylation, Dimerization with TLR6 and Myeloid Differentiation Primary Response Protein 88 Recruitment*

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Running Title: R753Q polymorphism alters TLR2 signalosome assembly

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Background: TLR2 SNPs are linked to tuberculosis but the mechanisms by which they alter TLR signaling are unclear
Results: R753Q TLR2 showed impaired tyrosine phosphorylation, dimerization with TLR6, MyD88 recruitment, induction of NF-κB and cytokines upon mycobacterial challenge
Conclusion: R753Q polymorphism blocks TLR2 tyrosine phosphorylation and signalosome assembly
Significance: Deciphering how SNPs alter TLR signaling advances TLR immunobiology and facilitates design of new therapeutic strategies

SUMMARY

The Arg753Gln (R753Q) polymorphism in the Toll-IL-1 receptor domain of TLR2 has been linked to increased incidence of tuberculosis and other infectious diseases, but the mechanisms by which it affects TLR2 functions are unclear. In this paper, we studied the impact of the R753Q polymorphism on TLR2 expression, heterodimerization with TLR6, tyrosine phosphorylation, and recruitment of myeloid differentiation primary response protein (MyD) 88 and MyD88 adapter-like (Mal). Complementation of HEK293 cells with transfected WT or R753Q TLR2 revealed their comparable total levels and only minimal changes in cell surface expression of the mutant species. Notably, even a 100-fold increase in amounts of transfected R753Q TLR2 vs. WT variant did not overcome the compromised ability of the mutant TLR2 to activate nuclear factor κB (NF-κB), indicating that a minimal decrease in cell surface levels of the R753Q TLR2 cannot account for signaling deficiency. Molecular modeling studies suggested that the R753Q mutation changes the electrostatic potential of the DD loop and results in a discrete movement of the residues critical for protein-protein interactions. Confirming these predictions, biochemical assays demonstrated that R753Q TLR2 exhibits deficient agonist-induced tyrosine phosphorylation, heterodimerization with TLR6 and recruitment of Mal and MyD88. These proximal signaling deficiencies correlated with impaired capacities of the R753Q TLR2 to mediate p38 phosphorylation, NF-κB activation and induction of IL-8 mRNA in transfected HEK293 cells challenged with inactivated Mycobacterium tuberculosis or mycobacterial components. Thus, the R753Q polymorphism renders TLR2 signaling-incompetent by impairing its tyrosine phosphorylation, dimerization with TLR6 and recruitment of Mal and MyD88.

Innate immune cells, such as macrophages, dendritic cells and neutrophils, detect conserved structures of microbial
pathogens via pattern recognition receptors, including membrane-associated Toll-like receptors (TLRs)\(^2\) (1-3). TLRs are type I glycoproteins expressed on the cell surface (TLR1, TLR2, TLR4-6, TLR11) or intracellularly in the endoplasmic reticulum and endosomes (TLR3, TLR7-9) (4). They contain an ectodomain involved in ligand recognition and co-receptor interactions, a transmembrane region, and an intracellular signaling Toll-IL-1R (TIR) domain (5). TLRs expressed on the cell surface recognize bacterial lipids (e.g. LPS detection by TLR4) (2) and proteins (flagellin detection by TLR5) (6), whereas endosomal TLRs sense viral dsRNA (TLR3) (7), ssRNA (TLR7/8) (8,9), and hypomethylated CpG motifs present in microbial DNA (TLR9) (10-14). TLR2 cooperates with TLR1 or TLR6 to sense tri- or di-acylated lipoproteins, respectively, expressed by Gram positive bacteria, mycobacteria, mycoplasma and fungi (15-18).

Ligand recognition initiates TLR2 dimerization with TLR1 or TLR6 that brings together their TIR domains and triggers TLR2 tyrosine phosphorylation (15,17,19,20), forming docking platforms to enable recruitment of myeloid differentiation primary response protein (MyD) 88 (4,5). MyD88 interacts with MyD88 adapter-like (Mal) and TLR2 via TIR-TIR domain interactions (21,22), forming a scaffold to recruit interleukin-1 (IL-1) receptor-associated kinases (IRAK) 4, IRAK1, and IRAK2 (23,24) that associate with the intermediate and death domains of MyD88 (25). Clustered IRAK4 molecules undergo autophosphorylation and kinase activation, leading to IRAK4→IRAK1/2 phosphorylation and induction of kinase activity (23,26), recruitment and K63-linked ubiquitination of TNF receptor-associated factor (TRAF) 6 (4,5,27). IRAK1 also undergoes K63-linked ubiquitination via recruitment of Pellinos and TRAF6, resulting in direct recruitment of IxB kinase (IKK)\(\gamma\) to IRAK1 (28), while ubiquitinated TRAF6 recruits TGF-\(\beta\)-activated kinase (TAK) 1 by engaging ubiquitin recognition domains within inhibitor of NF-\(\kappa\)B (IxB) kinase (IKK)\(\gamma\) and TAK-interacting proteins (29). These processes activate TAK1 and the IKK complex and place them into close proximity, promoting TAK1-mediating activation of mitogen-activated protein kinases (MAPKs) and IKK-\(\beta\), resulting in nuclear translocation of transcription factors that induce transcription of inflammatory genes (28,30).

*Mycobacterium tuberculosis* (Mt) is a causative agent of tuberculosis, one of the most ancient and devastating diseases (31). TLR2 detects Mt and its structural components, and plays an important role in mounting host defense against Mt infection (15,32-35). Genome-wide association studies have identified several TIR domain-localized SNPs within TLR2, including R753Q and P631H, that have been linked to increased risk of Mt infection and incidence of tuberculosis (36-39). Peripheral blood mononuclear cells isolated from patients expressing TLR2 SNPs had impaired NF-\(\kappa\)B activation and cytokine secretion in response to Mt agonists (38,39). However, the molecular basis of compromised functions of polymorphic TLR2 variants is unclear.

To determine mechanisms by which the R753Q polymorphism affects TLR2 signaling, we used transfection-based complementation of HEK293 cells with WT or R753Q YFP-TLR2 variants to study TLR2 signalosome assembly, induction of MAPKs, transcription factors and cytokines. This paper shows that compromised signaling capacity of R753Q TLR2 is not due to lower expression of the mutant receptor species but instead results from their deficient tyrosine phosphorylation, TLR2- TLR6 assembly and impaired recruitment of Mal and MyD88 to R753Q TLR2. These findings provide a novel mechanistic insight into how the R753Q polymorphism deregulates TLR2 signaling.

**Experimental Procedures**

Reagents and cell culture – Recombinant human TNF-\(\alpha\), anti-GFP and AU1 antibodies (Abs) were from Invitrogen, Abs against TLR2, IxB-\(\alpha\), and tubulin were from Santa Cruz. Anti-phospho (p)-p38 Ab was from Promega, and anti-p-tyrosine Ab PY20 was from BD Biosciences. S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-Cys-Ser-Lys(4)-OH (Pam2Cys) and S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-Ser-Lys4-OH (Pam3Cys) and
lipoarabinomannan from *M. smegmatis* (LAM) were obtained from Invivogen, irradiated H37V Mtb, Mtb cell wall (CW) and culture filtrate (CF) preparations, and *M. smegmatis* lipoarabinomannan (LAM) were obtained from the Biodefense and Emerging Infections Research Resources Repository. HEK293T and HEK293 cells were obtained from ATCC, and maintained in DMEM medium supplemented with 10% FBS (HyClone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) (complete (c) DMEM). WT and TLR2−/− J2 retrovirus-immortalized (i) mouse bone marrow-derived macrophages (BMDMs) were kindly provided by Drs. Douglas Golenbock and Katherine Fitzgerald (University of Massachusetts Medical School, Worcester, MA) and maintained in cDMEM.

Recombinant plasmids, site-directed mutagenesis and transfection - pcDNA3-YFP-TLR2, pcDNA3-CD14, pELAM-luciferase (Luc), pTK-Renilla-Luc, pEFBOS-Flag-Mal and pcDNA3-AU1-MyD88, pcDNA3-CFP-MyD88 were described previously (40,41). pcDNA3-CFP-TLR6 was from Addgene (Addgene plasmid 13021), and pcDNA3-mCherry-Mal was constructed by restriction digestion of pEFBOS-Mal and ligation of the released Mal insert into pcDNA3-mCherry (kindly provided by Dr. Gregory Melikian (Emory University School of Medicine, Atlanta, GA) by standard molecular biological techniques. The R753Q mutation was introduced into the YFP-TLR2 expression vector by site-directed mutagenesis, and its presence was confirmed by sequencing, as described previously (42). For transient transfection, HEK293T cells were cultured and plated in 100 mm dishes (for immunoprecipitation), 6-well or 24-well plates (for gene expression studies and reporter assays, respectively), and transfected for 3 h with the respective plasmids, using SuperFect transfection reagent (Qiagen). To obtain stable transfectants, HEK293 cells transfected with the plasmids encoding WT or R753Q variants were introduced into WT and TLR2−/− iBMDMs by nucleofection, using the Nucleofector I device and the mouse macrophage nucleofection kit (Lonza), as recommended by the manufacturer. After recovery for 20 h, cells were treated for 3 h with medium or Pam3Cys (100 ng/ml), RNA was isolated, reverse transcribed, and analyzed by RT-qPCR, using gene-specific primers.

Flow cytometry - To examine TLR2 expression, untransfected HEK293 cells or HEK293 transfectants expressing WT or R753Q YFP-TLR2 variants were stained for 30 min on ice with phycoerythrin (PE)-conjugated anti-TLR2 (TL2.1, Santa Cruz), anti-HLA-A, B, C-PE (positive control), or isotype control (BD Biosciences) Abs. Cells were washed with PBS, fixed for 15 min in 2% paraformaldehyde, and analyzed by FACS on a LSRII Flow Cytometer (BD Biosciences) to measure YFP (total YFP-TLR2 expression) and PE (cell surface TLR2 expression) fluorescence. The data were analyzed using the FlowJo software (Tree Star).

Confocal Microscopy – Confocal microscopy was performed as described previously (43). In brief, cells were seeded (4 x 10⁵ cells) on poly-L-lysine-coated coverslips (Fischer Scientific) in phenol-free cDMEM, and cultured for 20 h. Transfection was performed using Lipofectamine 2000 transfection reagent, followed by gentle washing and recovery for 48 h. Cells were fixed with 2% paraformaldehyde and mounted onto glass slides using a DABCO-based anti-fade fluorescent mounting media. Images were acquired using an Olympus Fluoview 500 laser scanning confocal microscope (Olympus). Sequential scans were taken for CFP, YFP, and Cherry fluorophores, using excitation wavelengths at 458, 514, and 633 nm, respectively, as reported (44).

Isolation of RNA and real-time (RT) quantitative (q) PCR analysis - Total RNA was isolated using Trizol (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 examined by RT qPCR with primers for human IL-8, 5′-CACC
human hypoxanthine phosphoribosyltransferase (HPRT) 5'-ACCAGTCACAGGGACATAA AAG-3' (forward); 5'-GTCTGACATTGT TGCCAG TGTC-3' (reverse), mouse HPRT 5'-GCTGACCTGCTGGATTACATT-3' (forward); 5'-GTTGAGAGATCATCTCCCA-3' (reverse), and mouse TNF-α, 5'-CCCAGGCAGTCAGATCATCTTC-3' (forward); 5'-GCTTGAGGGTTTGCTACAA CATG-3' (reverse) on a MyIQ real-time PCR machine (Bio-Rad). The data were analyzed by the 2^ΔΔCT method (45).

**Co-immunoprecipitation and immunoblotting** – Cell lysates were prepared as described previously (46) and precleared with protein G-agarose beads (Roche Applied Science) for 4 h at 4°C upon rotation. Precleared cell extracts were incubated overnight at 4°C with the respective Abs 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 Applied Science). Protein G-agarose beads were added (45 μl per sample) and incubation continued for 4 h. 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). Proteins were separated by SDS-PAGE on 4-20% mini-gels (Invitrogen), electrotransferred to Immobilon-P membranes, blocked and probed with the respective Abs, as described (46,47). Densitometric quantification was performed using the Quantity One program (Bio-Rad).

**NF-κB reporter assays** – Reporter assays were performed as reported previously (46-48). In brief, cells were transfected with plasmids, using SuperFect, recovered for 24 h, and treated for 5 h with medium or stimuli. Cells were lysed in a passive lysis buffer (Promega), and firefly luciferase vs. Renilla luciferase activities were measured using dual luciferase reporter assay system (Promega) on a Berthold LB 9507 luminometer (Berthold Technologies).

**Molecular modeling studies** - Structural models of the WT and R753Q-containing TIR domains of TLR2 were built using the WT TLR2 structure (PDB accession code 1FYW) and the 3D-JIGSAW Protein Comparative Modelling Server (version 2.0, http://www.3djigsaw.org), visualized and analyzed using Cn3D 4.3 macromolecular structure viewer (NCBI, http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml) and PyMOL. Selenomethionine and dimethylarsenic cysteines modified residues from the TIR2 structure (1FYW) were stripped of modifications and replaced with Met and Cys, while retaining original conformations. Rosetta_backrub (49,50) was used to evaluate 20 models for the impact of the R753Q mutation. Resulting models were visualized using PyMOL and energy surface representations were calculated using the APBS (51,52) plug-in in PyMOL.

**Statistical analysis** - Statistical analysis was performed using the GraphPad Prism 5 program for Windows (GraphPad Software Inc.). Statistical differences were evaluated by the Student’s t-test with the level of significance set at p<0.05. Data are expressed as mean ± SD.

**RESULTS**

**R753Q polymorphism does not significantly diminish expression levels of TLR2 but drastically impairs TLR2-elicited NF-κB activation** - Since the magnitude of TLR signaling correlates with receptor expression (53,54), we examined the impact of the R753Q polymorphism on TLR2 levels. To optimize detection, we transfected HEK293 cells with vectors encoding WT or R753Q TLR2 fused at the C-terminus with YFP (42), which does not alter TLR cellular localization or functions (40,44,55). Fluorescent microscopy and FACS revealed similar YFP fluorescence in HEK293 cells transfected with WT or R753Q TLR2 fused at the C-terminus with YFP (42), which does not alter TLR cellular localization or functions (40,44,55). Fluorescent microscopy and FACS revealed similar YFP fluorescence in HEK293 cells transfected with WT or R753Q YFP-TLR2 (Fig. 1A, B), and comparable levels of WT and R753Q YFP-TLR2 were immunoblotted with anti-GFP Ab (Fig. 1C) that cross-reacts with YFP (44,55,56). FACS analyses of 293/YFP-TLR2 stable transfectants expressing WT or R753Q variants and stained with PE-conjugated anti-TLR2 Ab showed minimal changes (<20%)
in cell surface expression of WT vs. R753Q TLR2 (Fig. 1B, right). To determine whether increasing levels of R753Q TLR2 overcomes its signaling deficiencies, HEK293T cells were transfected with different input amounts of plasmids encoding WT or mutant TLR2, and Pam3Cys-induced NF-κB transactivation was determined. Figure 1D shows that ~12-fold higher input amount of R753Q TLR2-expressing plasmid was required to achieve NF-κB reporter activation similar to that caused by vector encoding WT TLR2. Importantly, the plateau of NF-κB activation elicited by WT TLR2 was ~3-fold higher compared to the maximal response caused by the R753Q variant (Fig. 1D). Thus, drastic differences in signaling between WT and the R753Q TLR2 cannot be accounted for by minor differences in expression.

Transfected R753Q TLR2 elicits diminished phosphorylation of p38, activation of NF-κB and IL-8 gene expression – To study how the R753Q polymorphism affects TLR2 signaling, we transiently transfected HEK293T cells with vectors encoding WT or mutant YFP-TLR2, and determined TLR2-driven activation of p38, NF-κB and induction of IL-8 mRNA. Treatment of 293/WT YFP-TLR2 transfectants with Pam3Cys and LAM, TLR2-TLR1 and TLR2-TLR6 agonists, respectively (34,57), led to marked phosphorylation of p38 (Fig. 2A, C) and activation of the NF-κB luciferase reporter (Fig. 1D and 3B), while cells expressing R753Q YFP-TLR2 exhibited significantly blunted responses. In contrast to WT TLR2-expressing cells, 293/R753Q YFP-TLR2 transfectants failed to phosphorylate and degrade IκB-α (Fig. 2B, C) and showed inhibited NF-κB reporter activation (Fig. 3A) when stimulated with irradiated Mtb, heat-killed S. aureus (HKSA), or M. smegmatis-derived LAM. Exposure of 293/R753Q YFP-TLR2 cells to Mtb-derived CW and CF fractions or LAM led to <2-fold NF-κB reporter induction compared to robust (4.1-4.3-fold) activation elicited by WT TLR2 (Fig. 3B).

Since MAPKs and NF-κB control expression of pro-inflammatory mediators (5,30), we next determined the impact of the R753Q mutation on TLR2-induced IL-8 gene expression. While treatment of 293/WT YFP-TLR2 stable transfectants with Pam3Cys or Mtb-derived CW resulted in 42- and 15-fold induction of IL-8 mRNA, these stimuli elicited only 4- and 2.5-fold increase in IL-8 mRNA levels in cells expressing R753Q TLR2 (Fig. 4A). TNF-α induced comparable IL-8 gene expression in 293 cells expressing WT or R753Q TLR2 (Fig. 4A), indicating that the inhibitory impact of R753Q mutation is restricted to TLR signaling.

We next sought to confirm our data in cells with the macrophage phenotype. To this end, we determined the effect of nucleofection-based complementation of TLR2−/− immortalized BMDMs (iBMDMs) human WT or R753Q YFP-TLR2 on Pam3Cys-mediated TNF-α gene expression. iBMDM cell lines replicate TLR responses of primary macrophages (58) and have been extensively used by several groups, including ours (43,58,59). As shown in Fig. 4B, Pam3Cys induced 9.2-fold induction of TNF-α mRNA in WT iBMDMs, whereas TLR2−/− iBMDMs showed no responsiveness. Nucleofection of expression vector encoding human WT YFP-TLR2 endowed Pam3Cys sensitivity, leading to ~4-fold induction of TNF-α mRNA levels, whereas only 1.9-fold increase was seen in cells nucleofected with R753Q YFP-TLR2 (Fig. 4B). Quantitative real-time PCR analyses revealed similar relative levels of human TLR2 mRNA in TLR2−/− iBMDMs complemented with WT vs. D299G TLR2 (Fig. S1), indicating comparable expression of transfected YFP-TLR2 species. Thus, the R753Q polymorphism reduces the capacity of TLR2 to activate p38, NF-κB and induce IL-8 gene expression in response to Pam3Cys, inactivated Mtb, and mycobacteria-derived CW, CF, or LAM, while not affecting TNF-α-driven expression of IL-8 mRNA.

Molecular modeling of the impact of the R753Q mutation on the structure of the TIR domain of TLR2 - The TIR2 structure (PDB code 1FYW) was used as a template structure to model the point mutation R753Q using Rosetta backrub (60). Analysis of 20 modeled Q753 TIR2 structures showed no gross conformational changes in nearby or distant main chain atoms with respect to the WT TIR2 (Fig. 5A-C). However, we noted discrete main and side chain differences, affecting residues
P746, R748, K751, and the R753Q mutation itself (Fig. 5D), thought to be involved in TIR2 interactions with TIR1 (R748 and R753) and TIR6 (R748 and K751) (61). We also observed a notable change in the electrostatic potential within the DD loop and αD region resulting from the R753Q mutation, with a relative decrease in the net positive charge (Fig. 5 E, F). Thus, the R753Q mutation is likely to change the interaction surface within the TIR domain via altered electrostatic potential of or conformational effects on the DD loop, possibly affecting TLR2 dimerization with TLR1 and/or TLR6 and recruitment of adapter proteins Mal and MyD88.

The impact of R753Q polymorphism on TLR2 tyrosine phosphorylation upon stimulation with Pam3Cys and irradiated Mtb – Previous studies from our group and other laboratories demonstrated a critical role of tyrosine phosphorylation of TLR2 for initiating signal transduction (20,40). Since the R753Q polymorphism may alter conformation and the electrostatic potential of the TIR2 domain (Fig. 5), it is possible that such changes could affect recruitment of protein tyrosine kinases (PTKs) or accessibility of tyrosine residues for PTKs. Therefore, we studied agonist-inducible tyrosine phosphorylation of WT vs. R753Q YFP-TLR2 immunoprecipitated with anti-GFP (Fig. 6A) or anti-TLR2 (Fig. 6B) Abs from 293/TLR2 stable cell lines. Immunoblotting of YFP-TLR2 immune complexes with anti-p-tyrosine Ab revealed marked induction of tyrosine phosphorylation of WT, but not R753Q, YFP-TLR2 in HEK293 transfectants stimulated with Pam3Cys or irradiated Mtb (4.8- and 7.5-folds, respectively, Fig. 6A-D). Immunoprecipitation and immunoblotting with anti-GFP Ab revealed comparable total levels of WT and R753Q YFP-TLR2 (Fig. 6A, B), indicating that different tyrosine phosphorylation was not due to lower expression of the mutant TLR2. Our data indicate that the R753Q polymorphism blocks the ability of TLR2 to undergo tyrosine phosphorylation upon stimulation with Pam3Cys or irradiated Mtb.

The R753Q polymorphism interferes with agonist-inducible TLR2 heterodimerization with TLR6 – Given that TLR2 senses Mtb and Mtb-associated lipids in cooperation with TLR6 (15,16,33), we studied whether the R753Q polymorphism affects TLR2-TLR6 assembly. To this end, 293/YFP-TLR2 stable cell lines expressing WT or R753Q species were transfected with plasmids encoding CFP-TLR6, and Pam2Cys-inducible TLR2-TLR6 hetero-dimerization was assessed by co-immunoprecipitation and Western blot analyses. Pam2Cys stimulation resulted in 5.9-8 fold increases in the accumulation of TLR2-associated TLR6 proteins in 293/WT YFP-TLR2 transfecants, whereas significantly impaired TLR2-TLR6 hetero-dimerization (0.8-0.9 fold increase) was observed in cells expressing the R753Q species (Fig. 7A, B). Comparable total levels of CFP-TLR6, WT and R753Q YFP-TLR2 were observed (Fig. 7A), indicating that differences in dimerization with TLR6 exhibited by WT vs. R753Q TLR2 variants were not due to variations in the total levels of the interacting proteins. These results suggest that changes in conformation and/or electrostatic potential of the TIR domain imposed by the R753Q polymorphism (Fig. 5) contribute to deficient agonist-inducible TLR2-TLR6 association.

R753Q TLR2 show impaired recruitment of Mal and MyD88 – Upon ligand recognition, TLR2 hetero-dimerizes with TLR1 or TLR6, resulting in docking platform assembly within their TIR domains to enable recruitment of adapter proteins Mal and MyD88 (4,5,22,30,62,63). In our next series of experiments, we examined whether compromised ability of R753Q TLR2 to hetero-dimerize with TLR6 affects the ability of TLR2 to associate with Mal and to recruit MyD88. 293/YFP-TLR2 stable cell lines expressing WT or R753Q TLR2 were co-transfected with plasmids encoding Flag-Mal or AU1-MyD88, and agonist-mediated recruitment of epitope-tagged adapters to YFP-TLR2 was determined by co-immunoprecipitation. Pam3Cys stimulation for 10 and 30 min up-regulated the amounts of Flag-Mal associated with WT YFP-TLR2, whereas it failed to increase Flag-Mal interactions with the R753Q YFP-TLR2 species over basal levels seen in medium-treated cells (Fig. 8A). Exposure to irradiated Mtb induced robust recruitment of AU1-MyD88 to WT YFP-
TLR2 evident within 15 min of stimulation, followed by its rapid dissociation (Fig. 8B). In contrast, irradiated Mtb failed to induce AU1-MyD88 recruitment to the R753Q TLR2 within the time course analyzed (Fig. 8B). Similar amounts of total transfected WT and R753Q YFP-TLR2 proteins Flag-Mal and AU1-MyD88 were seen in all samples (Fig. 8), indicating that differences in MyD88 recruitment to WT vs. R753Q TLR2 cannot be attributed to variations in total expression of the interacting transfected proteins. To confirm our results obtained by immunoprecipitation, we used confocal microscopy as an alternative approach to study co-localization of transfected YFP-TLR2, mCherry-Mal and CFP-MyD88. Pam3Cys induced increased co-localization of mCherry-Mal and CFP-MyD88 with WT YFP-TLR2, whereas the R753Q YFP-TLR2 species showed deficient co-localization (Fig. S2, S3). Taken collectively, these results demonstrate impaired capacities of R753Q TLR2 to associate with Mal and to recruit MyD88 after stimulation with Pam3Cys or irradiated Mtb.

DISCUSSION

The R753Q TLR2 polymorphism has been associated with increased prevalence of leprosy and tuberculosis (36-38,64). More than 10% of patients with atopic dermatitis express the TLR2 R753Q SNP and exhibit severe eczema (65,66). Furthermore, patients with liver transplants expressing this mutation exhibited a trend towards a higher rate of CMV infection (67), recurrence of Gram positive infections and septic shock (68). Studies with PBMCs obtained from patients with leprosy or tuberculosis expressing the R753Q TLR2 polymorphism and experiments with HEK293 cells transfected with the corresponding mutant TLR2 revealed attenuated NF-κB activation and cytokine release in response to stimulation with Mtb and defined TLR2 agonists (38,39,42,69). However, the molecular basis by which the R753Q polymorphism compromises TLR2-mediated host innate defense, promoting bacterial infections, remains poorly understood.

In this study, we employed overexpression of WT and mutant YFP-TLR2 in HEK293 cells to determine the impact of the R753Q polymorphism on TLR2 expression, tyrosine phosphorylation, and signalosome assembly, information that has not been reported previously. Since the magnitude of TLR signaling depends on relative TLR protein levels (53), we first determined the impact of the R753Q polymorphism on TLR2 expression. Using fluorescent microscopy, immunoprecipitation and immunoblot analyses, we found comparable total levels of WT and R753Q TLR2 proteins, and only slightly (<20%) reduced cell surface expression of the mutant species. Our dose-response experiments showed that even transfection of ~100-times higher amount of the R753Q TLR2-encoding vector failed to elicit the magnitude of NF-κB reporter activation comparable to the plateau of WT TLR2-inducible response. These results indicate that impaired signaling via R753Q TLR2 cannot be attributed to its slightly diminished cell surface expression. Under conditions of comparable expression of WT and mutant TLR2s, we observed significant deficiencies of the R753Q TLR2 species to activate p38, NF-κB and to induce IL-8 gene expression upon stimulation with inactivated Mtb and Mtb-derived cell wall and culture filtrate fractions, LAM and Pam3Cys. Nucleofection-based complementation of TLR2-/- iBMDMs with human WT TLR2 partially restored their responsiveness to Pam3Cys, whereas the R753Q TLR2 version failed to impart Pam3Cys sensitivity, confirming our results in cells with the macrophage background. In contrast, TNF-α induced comparable levels of IL-8 mRNA in 293/TLR2 transfectants that express WT or R753Q TLR2, demonstrating the specific impact of the R753Q mutation on TLR2 signaling.

Very little and controversial information has been reported in the literature regarding the impact of the R753Q polymorphism on TLR2 expression. One study showed no significant changes in expression levels of TLR2 in healthy volunteers and patients with familial Mediterranean fever expressing WT and R753Q TLR2 (70). In contrast, another group found lower α-CD3 Ab-inducible expression of R753Q vs. WT TLR2 in T cells obtained from healthy volunteers or patients with atopic dermatitis, with distinct modulation of WT and R753Q
TLR2 levels upon stimulation with lipoteichoic acid (69). Of note, two other loss-of-function TIR2 domain mutations, P681H and P631H, do not affect TLR2 expression levels (50,71). Our data support findings showing the lack of the impact of the TIR2 mutations on TLR2 expression levels. However, we cannot exclude that the R753Q polymorphism could affect TLR2 trafficking and localization, an important regulatory mechanism of TLR2 signaling (72), analogous to the reported effect of the P6531H mutation (71).

To gain initial insights into how the R753Q mutation could interfere with TLR2 signaling, we undertook molecular modeling of the secondary structures of WT and R753Q-containing TIR domains. These studies suggested significant changes in the electrostatic potential of the DD loop and α-D region with accumulation of the net positive charge, in conjunction with discrete main and side chain differences, affecting P746, R748, K751, and the R753Q mutation itself. Notably, one model of TLR2 hetero-dimerization postulates that the dimerization interface involves the interaction of the DD loop region of the TIR2 and the BB loops of TIR1 or TIR6 (61). Interestingly, according to our molecular modeling, changes in electrostatic potential and discrete main and side chain differences within the DD loop region imposed by the R753Q mutation affect residues involved in TIR2 interactions with TIR1 (R748 and R753) and TIR6 (R748 and K751) (61). Since TLR2 hetero-dimerization with TLR6 is the initial step required for cell activation in response to Mtb and Mtb-derived lipids (15,32-35), we sought to obtain direct biochemical evidence on whether the R753Q polymorphism affects TLR2-TLR6 assembly. Using co-immunoprecipitation and Western blot analyses, we found a significantly lower ability of the R753Q TLR2 to associate with TLR6 upon stimulation with Pam2Cys, a defined TLR2-TLR6 agonist (73), confirming the direct impact of the mutation of TLR2-TLR6 hetero-dimerization predicted by molecular modeling.

The molecular basis by which the R753Q mutation interferes with TLR2-TLR6 dimerization is unclear. TLR2 hetero-dimerization with TLR6 is initiated via ligand recognition by LRR-containing receptor ectodomains (17,74,75), and is thought to involve subsequent cooperative engagement of their respective TIR domains (17). Since the R753Q polymorphism is localized to the TIR domain and spaced at a significant distance from the ligand-recognition interface in the ectodomain, direct conformation impact of the mutation on ligand recognition is unlikely. It is tempting to speculate that the R753Q mutation affects cooperative TIR2-TIR6 interactions, due to changes in the electrostatic potential and conformation within the DD loop region imposed by the mutation. Further structural and biochemical studies are required to determine the precise molecular mechanism of R753Q-mediated interference.

Agonist-mediated dimerization of the TLR2 TIR domains is thought to form a scaffold to which TIRAP/Mal and MyD88 adapters and different kinases, including protein tyrosine kinases (PTKs) and IRAKs, are recruited (4,5,21,22,30,63,76). Previous studies by us and others demonstrated the importance of agonist-inducible tyrosine phosphorylation of TLR2, TLR3, TLR4, and TLR9 for endowing signaling competence (20,40,76-79). They revealed c-Src as a likely PTK candidate phosphorylating TLR3 (79), and members of the Src kinase and Bruton tyrosine kinase families as putative PTKs that phosphorylate TLR4 (40). However, the identity of PTKs involved in tyrosine phosphorylation of TLR2 is unknown. Since the R753Q polymorphism inhibits TLR2-TLR6 hetero-dimerization that assembles a scaffold TIR interface to which PTKs are recruited, this could translate into the failure of the mutant TLR2 to become tyrosine phosphorylated. Indeed, this paper demonstrates for the first time that in contrast to WT TLR2, the R753Q TLR2 fails to undergo tyrosine phosphorylation upon stimulation with Pam2Cys or irradiated Mtb, known TLR2-TLR6 agonists. Experiments are ongoing to determine the mechanisms of impaired agonist-inducible tyrosine phosphorylation of R753Q TLR2 and to determine PTKs involved in TLR2 tyrosine phosphorylation.
TLR2-TLR6 hetero-dimerization and tyrosine phosphorylation of TLR2 is important for assembly of TIR docking platforms to enable recruitment of downstream adapter proteins and kinases. Significant and novel findings reported herein are that the R753Q polymorphism alters agonist-inducible association of the mutant TLR2 with a sorting adapter protein Mal and blocks recruitment of the signaling adapter MyD88. Consistent with dysregulated R753Q TLR2 signalosome assembly, the mutant receptor exhibited deficient phosphorylation of p38, activation of NF-κB reporter and expression of IL-8 mRNA in 293/TLR2 transfectants stimulated with irradiated Mtb, Mtb-derived components and Pam3Cys. Because of the reported regulation of microbial phagocytosis by TLR signaling, it is plausible that deficient signal transduction of R753Q TLR2 could promote Mtb replication in infected macrophages, underlying a decreased resistance of R753Q carriers to Mtb infection and increased incidence of tuberculosis.

In summary, this paper shows for the first time that the R753Q mutation renders TLR2 signaling-deficient by impairing TLR2-TLR6 hetero-dimerization, tyrosine phosphorylation, and recruitment of Mal and MyD88, while not affecting TLR2 expression. We also provide important and novel mechanistic insights into R753Q-mediated disruption of TLR2 signaling. Furthermore, our data suggests that peptide mimics or small molecule components promoting TLR2-TLR6 hetero-dimerization may overcome signaling deficiency of the R753Q TLR2 and could serve as an important therapeutic modality for treatment of TLR2-deficient patients.

References


**FOOTNOTES**

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The abbreviations used are: TLR, Toll-like receptor; TIR, Toll-IL-1-receptor; MyD, myeloid differentiation primary response protein; Mal, MyD88 adapter-like; IL-1, interleukin-1; IRAK, IL-1 receptor-associated kinase; TRAF, TNF receptor-associated factor; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; IKK, IκB kinase; TAK, TGF-β-activated kinase; MAPKs, mitogen-activated protein kinases; Mtb, *Mycobacterium tuberculosis*; Abs, antibodies; p, phospho; LAM, lipoarabinomannan; PE, phycoerythrin; RT, real-time; q, quantitative; PTK, protein tyrosine kinase.

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mCherry, and to Dr. Adam Puche (University of Maryland School of Medicine) for guidance and advance with confocal microscopy analyses.

**FIGURE LEGENDS**

**Figure 1. Expression levels and signaling capacities of WT and R753Q YFP-TLR2 species.** HEK293 cells were transiently (A, C, D) or stably (B) transfected with expression vectors encoding WT or R753Q YFP-TLR2 variants. A: After recovery for 48 h, cells were analyzed by fluorescent and phase contrast microscopy to determine expression levels of the TLR2 variants. B: Total expression of transfected YFP-TLR2 variants was determined by FACS analysis, using the YFP fluorescence intensity (FITC channel) (top panel). TLR2 cell surface expression was determined by staining of cells with TL2.1-PE (anti-TLR2 Ab) or isotype control IgG-PE, followed by FACS analyses of PE fluorescence (bottom panel). C: Whole cell lysates were examined by immunoblotting using anti-GFP and anti-tubulin Abs. D: HEK293 cells were co-transfected with the indicated amount of plasmids expressing WT or R753Q YFP-TLR2, along with pELAM-Luc (0.2 μg per well). After recovery for 20 h, cells were treated for 5 h with medium or Pam3Cys (100 ng/ml), and firefly Luc activity was determined in cell lysates and normalized for protein levels. The data of a representative experiment (n=12, A; n=5, B; n=3, C, D) are shown.

**Figure 2. The impact of R753Q polymorphism on TLR2-mediated phosphorylation of p38, IKK-α and degradation of IkB-α.** WT or R753Q YFP-TLR2 variants were overexpressed in HEK293T cells following transient transfection of the corresponding expression vectors. After recovery, cells were stimulated for the indicated times with 100 ng/ml Pam3Cys (A), irradiated Mtb (B, killed Mtb per HEL cell ration 10:1), or 5 μg/ml LAM (C). YFP-TLR2 proteins were immunoprecipitated from whole cell lysates with anti-GFP Ab and analyzed by immunoblotting with anti-GFP Ab. Whole cell lysates were examined by Western blot analyses using Abs against GFP, p-p38, total p38, p-IκB-α, IkB-α and tubulin. The results of a representative experiment (n=3) are presented.

**Figure 3. R753Q TLR2 mediates impaired activation of NF-κB luciferase reporter in response to inactivated M. tuberculosis, S. aureus, mycobacteria-derived cell wall and culture filtrate fractions, LAM and Pam3Cys.** HEK293T cells were transiently transfected with pcDNA3-YFP plasmids encoding WT or R753Q TLR2, along with pELAM-Luc (NF-κB reporter) and pTK-Renilla-Luc (to normalize for transfection efficiency). A: After recovery for 24 h, cells were stimulated with irradiated Mtb or HKSA using inactivated bacteria/HEK cell ratios 50:1 and 10:1. B: HEK transfectants were treated for 5 h with medium or stimulated with 100 ng/ml Pam3Cys, Mtb-derived CW and CF fractions, and LAM (5 μg/ml each). Firefly vs. Renilla luciferase activities were determined in cell lysates, the numbers in bacteria- or lipid-stimulated cells were normalized to medium control values and presented as fold induction. The summary of three experiments (mean ± SD) is shown. *p<0.05, **p<0.01, ***p<0.005.

**Figure 4. The effect of R753Q polymorphism on TLR2-elicited cytokine gene expression.** (A): HEK293 cells stably transfected with plasmids encoding WT or R753Q YFP-TLR2 were treated for 3 h with medium, 1 μg/ml Pam3Cys or 5 μg/ml Mtb CW preparation. (B): Mouse WT or TLR2-/- iBMDMs were nucleofected with pcDNA3-YFP or expression vectors encoding human WT or R753Q YFP-TLR2 variants. After recovery for 24 h, cells were treated for 3 h with medium or 1 μg/ml Pam3Cys. RNA was isolated, converted into cDNA, and subjected to RT qPCR analyses with the respective gene-specific primers. Data were processed according to the 2^ΔΔCT method. Shown is the summary (mean ± SD) of three independent experiments (A) and a representative experiment (mean ± SEM, n=4). *p<0.05.

**Figure 5. Molecular modeling of the structures of the TIR domains of WT and R753Q TIR2 species.** Ribbon models of WT R753 (A) and mutant Q753 (B) TIR domains of TLR2 and their overlay (C) are shown. (D) A close-up of the DD-loop – αD region encircled in (C). The electrostatic surfaces of the WT (R753) (E) vs. mutant Q753 (F). The electrostatic potential was calculated by solving the Poisson-Boltzman equation using the APBS PyMol plugin and depict a range from – (red) / + (blue)
1k_BT/e_c, where k_B is the Boltzmann constant, T is the temperature and e_c is the charge of the electron. Surfaces of the TIR2 domains are depicted.

**Figure 6.** The impact of R753Q polymorphism on agonist-induced tyrosine phosphorylation of TLR2. HEK293 stable transfectants expressing WT or R753Q TLR2 were stimulated for the indicated time points with 100 ng/ml Pam3Cys (A) or treated for 15 min with medium or irradiated Mtb (inactivated Mtb/HEK cell ratios 50:1) (B). Cell lysates were immunoprecipitated with anti-GFP (A) or anti-TLR2 (B) Abs and subjected to immunoblot analyses with anti-GFP and anti-p-tyrosine Abs. Total YFP-TLR2 levels were examined by immunoblotting of cell lysates with anti-GFP Ab, and protein loading was controlled by immunoblotting with anti-tubulin Ab (B). Panels (C) and (D) represent quantification of Western blot data shown in (A) and (B), respectively, with the numbers in the brackets indicating fold increases in agonist-mediated tyrosine phosphorylation of TLR2 normalized to medium-treated values. Shown are the data of a representative (n=3) experiment.

**Figure 7.** The R753Q polymorphism impairs the ability of TLR2 to heterodimerize with TLR6 upon Pam2Cys stimulation. A: 293/YFP-TLR2 stable cell lines expressing WT or R753Q YFP-TLR2 variants were co-transfected with pcDNA3-CFP-TLR6. After recovery for 20 h, cells were treated for the indicated time points with medium or 100 ng/ml Pam2Cys. Whole cell lysates were prepared, TLR2 species were immunoprecipitated with anti-TLR2 Ab (TL2.1) and analyzed by immunoblotting with anti-TLR6 Ab to detect TLR2-associated TLR6. Total levels of transfected YFP-TLR2 and CFP-TLR6 proteins were assessed by Western blot analyses of whole cell lysates with anti-TLR2 and anti-TLR6Abs, respectively. Immunoblotting of cell lysates with anti-tubulin Ab was used to control for protein loading. The results of a representative (n=3) experiments are shown. B: Quantification of the results shown in (A). TLR2-TLR6 arbitrary units were calculated by measuring densitometric values for TLR2-TLR6, TLR2, TLR6 and tubulin at each time point, subtracting background values, normalizing for tubulin, and then by dividing TLR2-TLR6 values by those calculated for TLR2 and by TLR6.

**Figure 8.** R753Q TLR2 shows deficient capacity to recruit Mal and MyD88 in response to agonist stimulation. HEK293T cells were transiently transfected with pcDNA3-AU1-MyD88 in combination with pcDNA3-WT YFP-TLR2 or pcDNA3-R753Q YFP-TLR2, and control cells were transfected with pcDNA3. After recovery for 24 h, cells were treated with medium or stimulated with irradiated Mtb (inactivated Mtb/HEK cell ratio 50:1) for the indicated time points, cell lysates were prepared and subjected to immunoprecipitation with anti-TLR2 Ab. TLR2 immune complexes were examined by Western blotting with anti-GFP and anti-AU1 Abs to determine total YFP-TLR2 levels and the amount of AU1-MyD88 associated with TLR2s. Whole cell lysates were subjected to immunoblotting with anti-AU1 and anti-tubulin Abs to analyze total AU1-MyD88 expression and to control for protein loading, respectively. The results of a representative (n=2) experiment are shown.
Figure 2

A) Transfected YFP-TLR2:
- WT
- R753Q

Pam3Cys (min): 0 5 15 0 5 15

IP: α-GFP
IB: α-GFP

YFP-TLR2
p-p38
Tubulin

B) Transfected YFP-TLR2:
- WT
- R753Q

Irradiated Mtb (min): 0 5 15 30 60 0 5 15 30 60

IP: α-GFP
IB: α-GFP

YFP-TLR2
IkB-α
Tubulin

C) Transfected YFP-TLR2:
- WT
- R753Q

LAM (min): 0 5 15 30 0 5 15 30

IB: α-GFP

YFP-TLR2
p-IκB-α
IkB-α
Tubulin

p-p38
total p38
Tubulin
Figure 3

A

![Graph showing NF-κB reporter activation for YFP-TLR2 WT and R753Q in response to different conditions.](image)

B

![Graph showing NF-κB reporter activation for YFP-TLR2 WT and R753Q in response to different ligands.](image)
Figure 6

A. Transfected YFP-TLR2:
   WT     R753Q
   IP: α-GFP  |  IP: α-GFP
   IB: α-GFP  |  IB: α-GFP
   Pam3Cys (min): 0 5 15 30 0 5 15 30
   YFP-TLR2
   YFP-TLR2
   p-YFP-TLR2
   p-YFP-TLR2
   Western
   Tubulin

B. Transfected YFP-TLR2:
   WT     R753Q
   IP: α-TLR2
   IB: α-GFP
   Irradiated Mtb: - + - +
   YFP-TLR2
   p-YFP-TLR2

C. Graph showing p-TLR2/TLR2 (arbitrary units x 10^-2) vs. Pam3Cys (min):
   WT TLR2
   R753Q TLR2
   (4.8) (1.2) (2.3)

D. Bar graph showing p-TLR2/TLR2 (arbitrary units):
   WT
   R753Q
   Medium
   Irradiated Mtb
   (7.5) (1) (1) (1)
Figure 7

A

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IP: α-TLR2
IB: α-TLR6

CFP-TLR6

B

TLR2-TLR6 Arbitrary Units

Pam2Cys (min)

WT
R753Q

0.8
0.9
5.9
8.0
Figure 8

<table>
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<td>0 10 30</td>
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**A**

IP: α-Flag or IgG
IB: α-GFP

**B**

YFP-TLR2: WT R753Q pcDNA3
AU1-MyD88: + + + + + +
Irradiated Mtb (min): 0 5 15 30 0 5 15 30

IP: α-TLR2
IB: α-AU1

WESTERN

IP: α-AU1
IB: α-AU1

Flag-Mal

α-GFP

YFP-TLR2

AU1-MyD88

α-GFP

YFP-TLR2

AU1-MyD88

α-GFP

YFP-TLR2

AU1-MyD88

α-GFP
Arg753Gln Polymorphism Inhibits Toll-like Receptor (TLR) 2 Tyrosine Phosphorylation, Dimerization with TLR6 and Myeloid Differentiation Primary Response Protein 88 Recruitment
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