



# The R753Q polymorphism in Toll-like receptor 2 (TLR2) attenuates innate immune responses to mycobacteria and impairs MyD88 adapter recruitment to TLR2

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Goutham Pattabiraman, Rahul Panchal, and Andrei E. Medvedev<sup>1</sup>

From the Department of Immunology, University of Connecticut Health Center, Farmington, Connecticut 06030

Edited by Luke O'Neill

Toll-like receptor 2 (TLR2) plays a critical role in host defenses against mycobacterial infections. The R753Q TLR2 polymorphism has been associated with increased incidence of tuberculosis and infections with non-tuberculous mycobacteria in human populations, but the mechanisms by which this polymorphism affects TLR2 signaling are unclear. In this study, we determined the impact of the R753Q TLR2 polymorphism on macrophage sensing of *Mycobacterium smegmatis*. Upon infection with *M. smegmatis*, macrophages from knock-in mice harboring R753Q TLR2 expressed lower levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 compared with cells from WT mice, but both R753Q TLR2- and WT-derived macrophages exhibited comparable bacterial burdens. The decreased cytokine responses in R753Q TLR2-expressing macrophages were accompanied by impaired phosphorylation of IL-1R-associated kinase 1 (IRAK-1), p38, ERK1/2 MAPKs, and p65 NF- $\kappa$ B, suggesting that the R753Q TLR2 polymorphism alters the functions of the myeloid differentiation primary response protein 88 (MyD88)-IRAK-dependent signaling axis. Supporting this notion, HEK293 cells stably transfected with YFP-tagged R753Q TLR2 displayed reduced recruitment of MyD88 to TLR2, decreased NF- $\kappa$ B activation, and impaired IL-8 expression upon exposure to *M. smegmatis*. Collectively, our results indicate that the R753Q polymorphism alters TLR2 signaling competence, leading to impaired MyD88-TLR2 assembly, reduced phosphorylation of IRAK-1, diminished activation of MAPKs and NF- $\kappa$ B, and deficient induction of cytokines in macrophages infected with *M. smegmatis*.

*Mycobacterium tuberculosis* (Mtb)<sup>2</sup> targets over a third of the world population, causing tuberculosis or latent infection that

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<sup>1</sup> To whom correspondence should be addressed: Dept. of Immunology, University of Connecticut Health Center, L. Bldg., Rm. 3019, 263 Farmington Ave., Farmington, CT 06030. Tel.: 860-679-7979; Fax: 860-679-2128; E-mail: medvedev@uchc.edu.

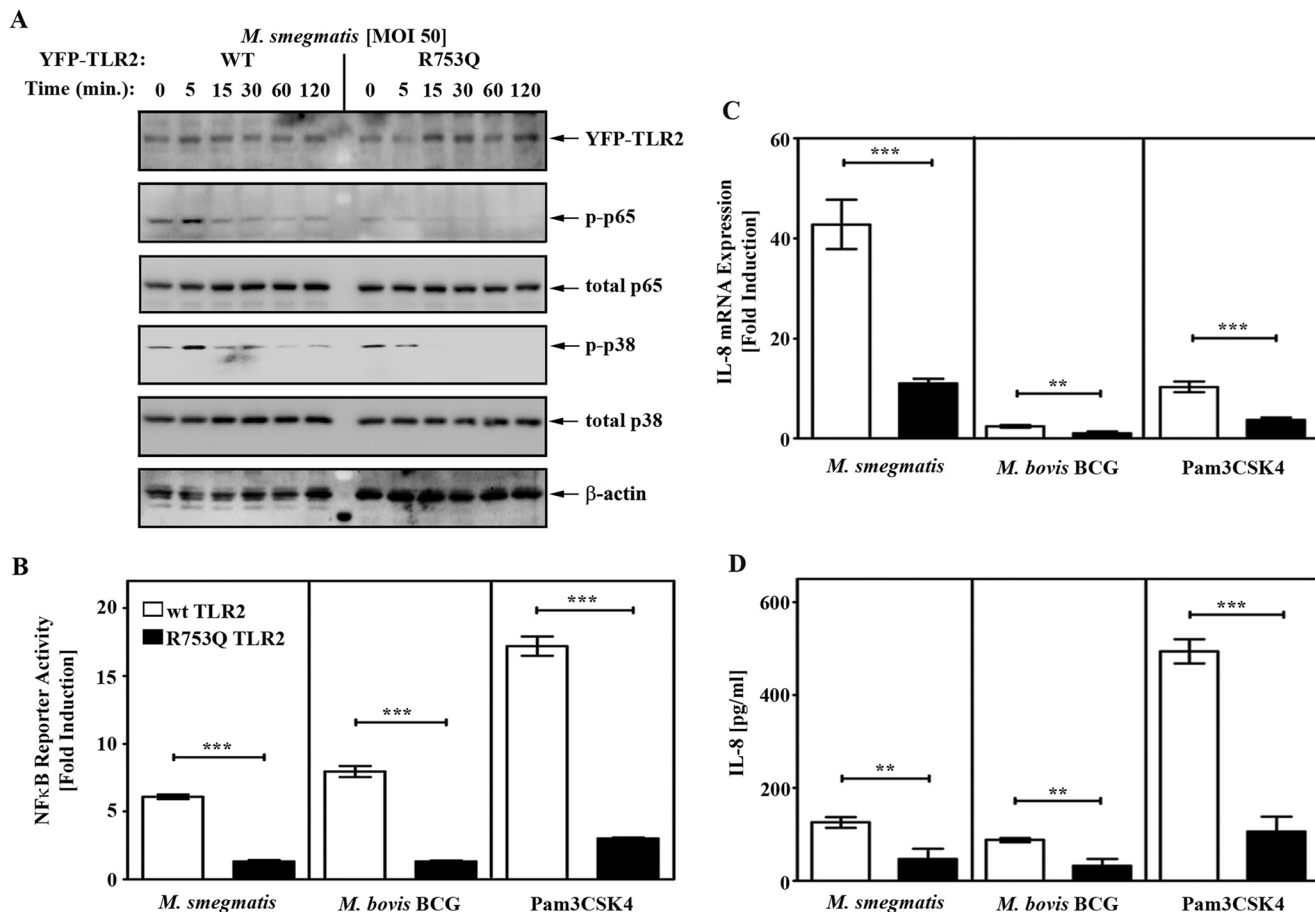
<sup>2</sup> The abbreviations used are: Mtb, *Mycobacterium tuberculosis*; TLR, Toll-like receptor; BCG, bacillus Calmette-Guérin; TIR, Toll-IL-1R; IRAK, IL-1R-associated kinase; KI, knock-in; m.o.i., multiplicity of infection; BMDM, bone marrow-derived macrophage; MFI, mean fluorescence intensity; Ab, antibody; Pam<sub>3</sub>CSK<sub>4</sub>, S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-Ser-Lys4-OH; cDMEM, complete DMEM; Luc, luciferase; TK, thymidine kinase; PE, phycoerythrin.

could be reactivated because of co-infection with HIV, diabetes, malnutrition, or immunosuppressive interventions (1–3). Mtb infects alveolar macrophages and results in the formation of granulomas where bacilli reside for the period of latency, whereas during active disease, the granuloma becomes necrotic and caseous, leading to Mtb escape and spreading (3, 4). Macrophages detect mycobacterial lipoproteins, lipoarabinomannan, or DNA via membrane-associated TLRs or by cytosolic nucleotide-binding oligomerization domain-containing protein 2, nucleotide-binding oligomerization domain-like receptor, pyrin domain-containing protein 3, absent in melanoma 2, and cyclic GMP-AMP synthase (2, 5–8).

TLRs are expressed on the cell surface (e.g. TLR5), in endosomes (TLR3 and TLR7–9), or in both compartments (e.g. TLR2 and TLR4) (9) in macrophages, neutrophils, dendritic cells, or epithelial and endothelial cells (10). TLR2 plays a critical role in the detection of and host defense against Mtb and other mycobacteria, including *Mycobacterium bovis* BCG, *Mycobacterium avium*, or *Mycobacterium smegmatis* (11–17). Upon binding of ligands to the ectodomain, TLR2 heterodimerizes with TLR1 or TLR6, leading to Toll-IL-1R (TIR) domain assembly to recruit myeloid differentiation primary response protein 88 (MyD88) adapter-like and MyD88 (18). Subsequently, several molecules of IL-1R-associated kinase 4 (IRAK-4), IRAK-1, and IRAK-2 are recruited and interact with MyD88 via their death domains, activating IRAK activities and initiating recruitment of TNFR-associated factor 6 (TRAF6) (19, 20). TRAF6 interacts with and activates TGF- $\beta$ -activating kinase 1, which propagates signals downstream, activating transcription factors and inducing inflammatory cytokines (21).

Genome-wide association studies have identified several polymorphisms within TLR2, including R753Q, R677W, and P631H, that have been associated with increased risk of infection with Mtb, *M. avium*, or *Mycobacterium abscessus* as well as higher incidences of tuberculosis and leprosy (22–29). Our previous studies in HEK293 transfectants stimulated with TLR2 agonists or irradiated Mtb have shown that R753Q TLR2 undergoes deficient tyrosine phosphorylation, compromised TLR6 assembly, and impaired recruitment of Mal and MyD88 (30). However, the impact of the R753Q polymorphism on the signaling capacities of endogenous TLR2 in macrophages during infection with live mycobacteria has not been studied. The mechanisms by which signaling deficiencies of R753Q TLR2 could underlie increased susceptibility to Mtb infection in human carriers of TLR2 SNPs are unknown.

## R753Q TLR2 polymorphism and mycobacterial infection



**Figure 1. The TLR2 R753Q polymorphism attenuates TLR2-mediated p38 MAPK and NF- $\kappa$ B activation and inhibits IL-8 expression in HEK293 transfectants incubated with live *M. bovis* BCG or *M. smegmatis*.** A, HEK293 cells stably transfected with the pcDNA3-YFP expression vectors encoding WT or R753Q TLR2s were incubated for the indicated times with *M. smegmatis* (m.o.i. 50). Cells were lysed, and whole-cell lysates were examined by Western blot analyses using the indicated Abs. The results of a representative experiment ( $n = 3$ ) are shown. B, 293/TLR2 stable cell lines expressing WT or R753Q TLR2 species were co-transfected with pELAM-Luc and pTK-*Renilla*-Luc. Cells were treated for 24 h with Pam<sub>3</sub>CSK<sub>4</sub> (1  $\mu$ g/ml), *M. smegmatis*, or *M. bovis* BCG (all at m.o.i. 50), and cell lysates were prepared to determine firefly versus *Renilla* luciferase activities. The numbers from treated cultures were normalized to medium control values and expressed as -fold induction. C, 293/TLR2 cells stably expressing WT or R753Q TLR2s were treated for 24 h with 1  $\mu$ g/ml Pam<sub>3</sub>CSK<sub>4</sub>, live *M. smegmatis*, or *M. bovis* BCG (m.o.i. 50). RNA was isolated, converted to cDNA, and subjected to real-time PCR analysis with the respective primers. Data were processed according to the  $2^{-\Delta\Delta CT}$  method (58). D, cells were stimulated for 24 h with 1  $\mu$ g/ml Pam<sub>3</sub>CSK<sub>4</sub>, *M. smegmatis*, or *M. bovis* BCG (m.o.i. 50). IL-8 levels were determined in supernatants by ELISA. The data (mean  $\pm$  S.D.) of a representative experiment ( $n = 3$ ) are shown. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

In this study, we sought to determine the impact of the R753Q TLR2 polymorphism on macrophage responses to live infection with *M. smegmatis*. Using macrophages from knock-in (KI) mice harboring endogenous R753Q TLR2, we show that the R753Q polymorphism impairs activation of IRAK-1, NF- $\kappa$ B, and MAPKs and induction of inflammatory cytokines during infection. HEK293 transfectants expressing R753Q TLR2 exhibited deficient recruitment of MyD88 to the mutant TLR2 upon exposure to live *M. smegmatis*. To the best of our knowledge, this is the first demonstration of deficient MyD88-TLR2 assembly and activation of IRAK-1 as the mechanism by which the R753Q polymorphism impairs innate responses of macrophages during infection with live mycobacteria.

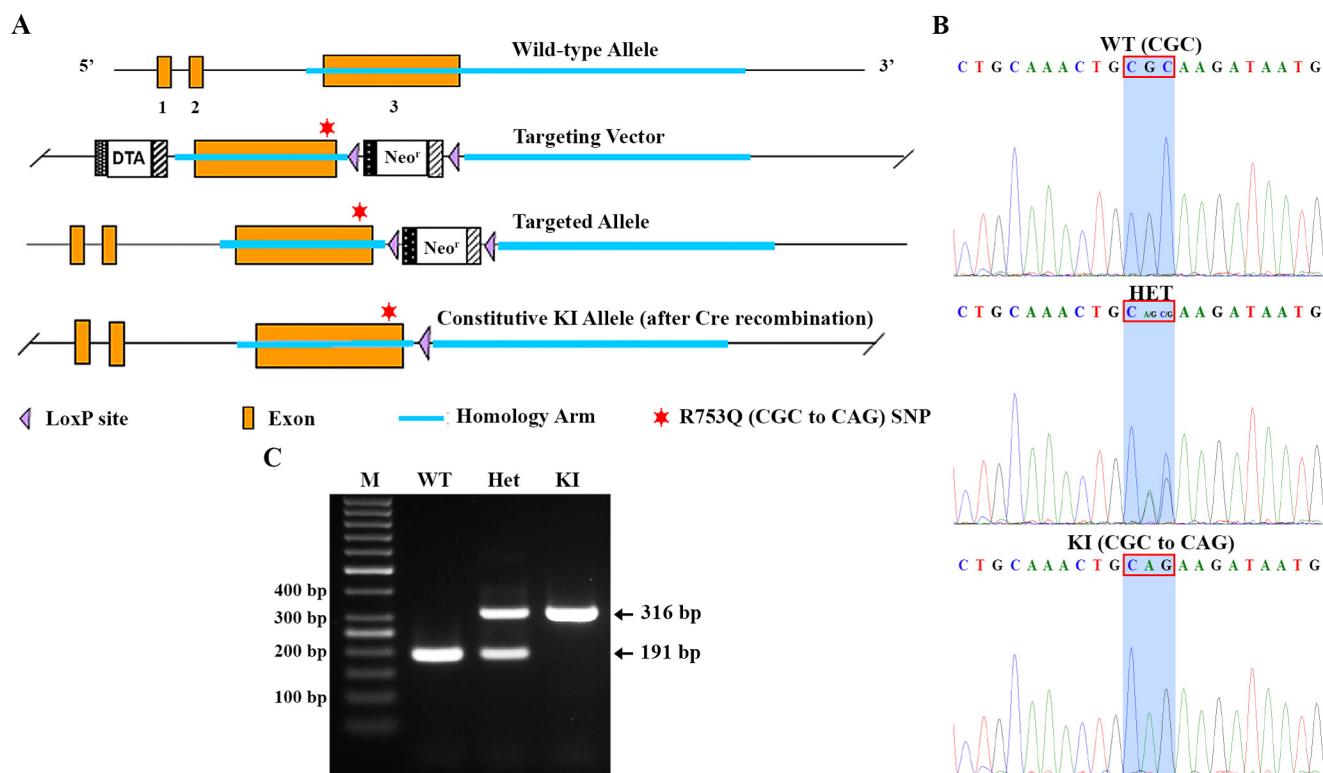
### Results

#### The R753Q TLR2 SNP impairs MAPK and NF- $\kappa$ B activation and IL-8 induction upon exposure of HEK293/TLR2 transfectants to *M. smegmatis* or *M. bovis* BCG

To examine the impact of the R753Q TLR2 SNP on cell responses to live mycobacteria, we first used HEK293 cells

stably expressing YFP-tagged human WT (293/TLR2<sup>WT</sup>) or R753Q TLR2 (293/TLR2<sup>R753Q</sup>). Cell activation was judged by phosphorylation of p38 MAPK and p65 NF- $\kappa$ B, activation of the NF- $\kappa$ B-driven luciferase reporter, and induction of IL-8 expression. Exposure of 293/TLR2<sup>WT</sup> cells to *M. smegmatis* (multiplicity of infection (m.o.i.) = 50) markedly enhanced phosphorylation of p38 MAPK and p65 NF- $\kappa$ B (Fig. 1A) and led to a 6.1-fold increase in NF- $\kappa$ B reporter activation (Fig. 1B). In contrast, 293/TLR2<sup>R753Q</sup> cells showed deficient phosphorylation of p38 MAPK and p65 NF- $\kappa$ B and impaired activation of the NF- $\kappa$ B reporter (78% decrease) in response to *M. smegmatis* and *M. bovis* BCG (Fig. 1).

Because MAPKs and NF- $\kappa$ B mediate the expression of inflammatory cytokines (21), we next examined induction of IL-8 in HEK293 cells expressing WT or R753Q TLR2 in response to mycobacteria. Stimulation of 293/TLR2<sup>WT</sup> cells with Pam<sub>3</sub>CSK<sub>4</sub>, *M. smegmatis*, or *M. bovis* BCG resulted in ~10-, ~43-, and ~2.5-fold up-regulation in the levels of IL-8 mRNA, whereas 293/TLR2<sup>R753Q</sup> transfectants showed 74%, 64%, and 58% inhibition in the activation of these responses,



**Figure 2. Generation of TLR2 R753Q KI mice.** A, the structure of the mouse TLR2 gene, the targeting vector, the targeted allele, and the constitutive allele after Cre recombination. After negative selection for identifying the C57BL/6 embryonic stem cells carrying the R753Q TLR2 mutation, the constitutive R753Q KI allele was obtained after Cre recombination. The stars indicate the sites of mutation. B, the CGC-to-CAG replacement was verified by DNA sequencing. Shown are sequencing data from WT (top panel), heterozygous (HET, center panel), and homozygous KI (bottom panel) mice. C, PCR analysis of DNA samples obtained from tail snips of WT and TLR2 R753Q KI mice. The WT and mutant alleles were identified by their sizes of 191 and 316 bp, respectively.

respectively (Fig. 1C). Similarly, in contrast to a marked up-regulation of IL-8 secretion observed in 293/TLR2<sup>WT</sup> cells exposed to Pam<sub>3</sub>CSK<sub>4</sub>, *M. smegmatis*, or *M. bovis* BCG, a significantly lower induction of IL-8 secretion was observed in 293/TLR2<sup>R753Q</sup> in response to stimulation with Pam<sub>3</sub>CSK<sub>4</sub> or mycobacteria (Fig. 1D). Immunoblot analyses revealed a similar expression of WT versus R753Q TLR2 in HEK293 stable transfectants (Fig. 1A and data not shown), indicating that the observed differences in cell responses to live *M. smegmatis* or *M. bovis* BCG were not due to altered expression of the mutant TLR2. These data indicate that the R753Q SNP attenuates the ability of TLR2 to activate p38 MAPK- and p65 NF- $\kappa$ B-driven pathways in response to *M. smegmatis* and *M. bovis* BCG infection, leading to deficient induction of IL-8.

#### Macrophages from mice expressing endogenous R753Q TLR2 exhibit impaired activation of IRAK-1, MAPKs, and NF- $\kappa$ B and deficient induction of inflammatory cytokines upon infection with *M. smegmatis*

To confirm the results obtained in overexpression systems in macrophages expressing the endogenous TLR2 mutation, we engineered KI mice harboring the R753Q TLR2 SNP (R753Q KI mice) (Fig. 2A). The presence of the R753Q SNP was confirmed by Southern blotting, sequencing of DNA samples, and PCR genotyping (Fig. 2, B and C, and data not shown). BMDMs from WT, TLR2 KO, or R753Q TLR2 KI mice were used to study the effect of the R753Q SNP on TLR signaling and expression of TNF $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10, cytokines involved in macro-

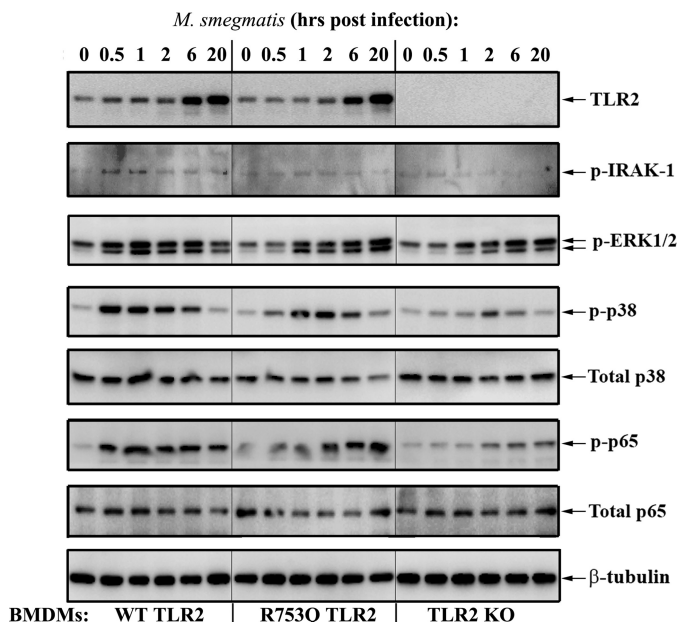
phage anti-mycobacterial defenses (31–33). Infection of WT BMDMs with *M. smegmatis* led to robust phosphorylation of IRAK-1, p38, ERK1/2 MAPK, and p65 NF- $\kappa$ B (Fig. 3, left panels). TLR2 KO BMDMs exhibited minimal activation of these signaling intermediates (Fig. 3, right panels), whereas significantly attenuated activation of IRAK-1, MAPKs, and NF- $\kappa$ B were seen in BMDMs from R753Q TLR2 KI mice (Fig. 3, center panels).

Because MAPKs and NF- $\kappa$ B govern the expression of inflammatory cytokines that regulate host immune responses against pathogens (10, 21), we next determined the impact of the R753Q TLR2 SNP on the expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10. BMDMs from WT mice responded to infection with *M. smegmatis* by 4- to 18-fold up-regulation of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 mRNA expression as well as a marked increase in the secreted levels of these cytokines (Fig. 4, A and B). In contrast, there was a lack of production of these cytokines in TLR2 KO BMDMs, whereas BMDMs from R753Q KI mice expressed significantly lower levels of cytokine mRNA and protein. These results indicate that the R753Q SNP impairs TLR2-mediated activation of IRAK1, MAPKs, NF- $\kappa$ B, and induction of inflammatory cytokines in macrophages infected with *M. smegmatis*.

#### The R753Q polymorphism does not affect TLR2 expression and mycobacterial uptake

TLR2 traffics between the plasma membrane and endosomes and has been implicated in bacterial uptake and phagocytosis

## R753Q TLR2 polymorphism and mycobacterial infection



**Figure 3. Macrophages harboring R753Q TLR2 show impaired activation of IRAK-1, MAPKs, and NF- $\kappa$ B upon mycobacterial infection.** BMDMs from WT, TLR2 KO, or R753Q TLR2 KI mice were infected for 2 h with *M. smegmatis* (m.o.i. 10), washed, incubated in gentamycin-containing medium, and lysed at the indicated times after infection. Whole-cell lysates were examined by Western blot analyses with the indicated Abs. The results of a representative experiment ( $n = 5$ ) are shown.

(34–39). Thus, the R753Q SNP could impair macrophage responses to *M. smegmatis* because of altered TLR2 expression or uptake of bacilli. To address these questions, we first compared the expression of WT and mutant TLR2 in BMDMs derived from WT *versus* R753Q TLR2 KI mice. Real-time PCR and Western blot analyses of BMDM samples obtained from the respective mice revealed comparable mRNA and protein levels of total WT and R753Q TLR2 variants (Fig. 5, A and B). Furthermore, FACS analyses showed similar levels of cell surface TLR2 species in BMDMs derived from WT or R753Q TLR2 KI mice (Fig. 5C).

Second, we assessed whether the presence of the R753Q SNP affects bacterial uptake by macrophages. Real-time PCR analyses showed that BMDMs from WT, R753Q TLR2 KI, or TLR2 KO mice exhibit comparable levels of mycobacterial 16S rRNA (40) 2 h after infection with *M. smegmatis* (m.o.i. 10) (Fig. 6A). Similarly, FACS demonstrated similar percentages and mean fluorescent intensity (MFI) of GFP-positive WT, R753Q TLR2 KI, and TLR2 KO bone marrow-derived macrophages (BMDMs) after infection with GFP-expressing *M. smegmatis* (Fig. 6, B and C). Furthermore, we also found similar bacterial cfu in cell lysates from *M. smegmatis*-infected BMDMs isolated from WT *versus* R753Q TLR2 mice or mice lacking TLR2 (Fig. 6D). Taken together, these results indicate that the R753Q SNP does not affect TLR2 expression or uptake and survival of *M. smegmatis* in macrophages.

### R753Q TLR2 exhibits impaired recruitment of MyD88 upon *M. smegmatis* infection

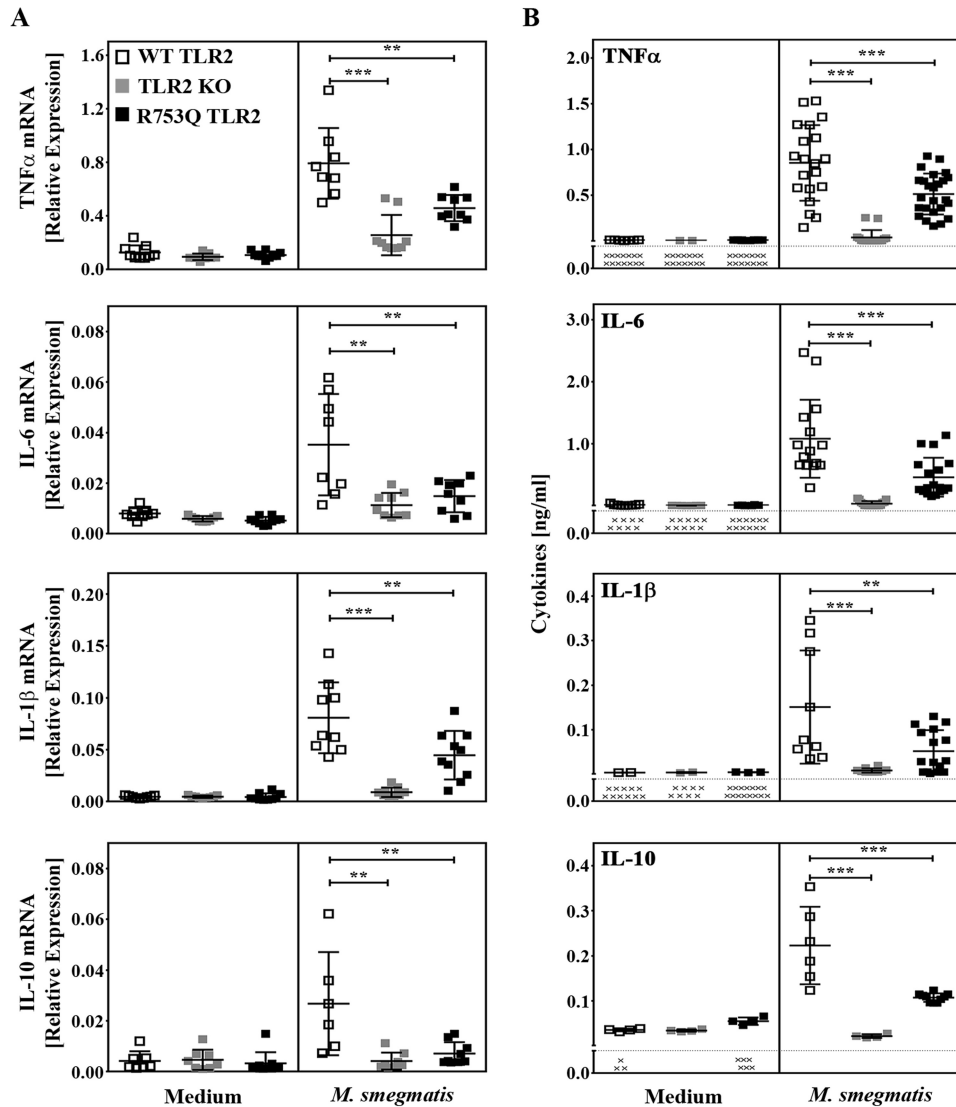
To gain insights into the mechanisms by which the R753Q SNP alters TLR2 signaling, we analyzed the recruitment of endogenous MyD88 to transfected YFP-tagged WT or mutant

TLR2 species in HEK293 cell transfectants exposed to live *M. smegmatis*. Co-immunoprecipitation analyses revealed that exposure of 293/TLR2<sup>WT</sup> to *M. smegmatis* resulted in association of endogenous MyD88 with WT TLR2 as early as 5 min, with a steady accumulation of TLR2-associated MyD88 up to 60 min after infection, followed by a decline at 120 min (Fig. 7). In contrast, we noted drastically impaired MyD88 recruitment to the mutant TLR2 species in 293/TLR2<sup>R753Q</sup> cells incubated with *M. smegmatis* (Fig. 7). Comparable levels of total WT, R753Q TLR2, and MyD88 proteins were observed in whole cell lysates, indicating that differences in *M. smegmatis*-inducible MyD88-TLR2 associations cannot be accounted for by variations in total levels of the interacting proteins. Thus, the R753Q polymorphism impairs the ability of TLR2 to recruit MyD88 after infection with live *M. smegmatis*, leading to deficient activation of IRAK-1, NF- $\kappa$ B, and MAPKs and induction of inflammatory cytokines in macrophages infected with live *M. smegmatis*.

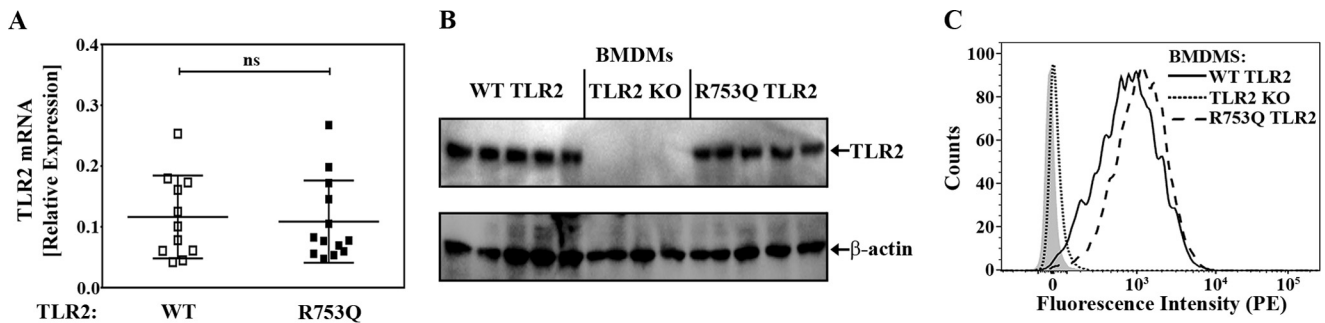
## Discussion

Genome-wide association studies have demonstrated the association of a microsatellite polymorphism in the intron of TLR2 and the R753Q TLR2 polymorphism with increased incidence of tuberculosis in several cohorts of patients (22–25, 41). Furthermore, the presence of TLR2 SNPs has been linked to infections with other mycobacteria, e.g. *M. bovis*, *M. avium*, and *M. abscessus* (26–29). Studies with PBMCs obtained from patients expressing the R753Q SNP showed attenuated NF- $\kappa$ B activation and cytokine release in response to stimulation with *Borrelia burgdorferi* lysates (42). We reported that the R753Q SNP alters the electrostatic potential of the TIR2 domain and impairs the ability of TLR2 to dimerize with TLR6 and recruit MyD88 in cells stimulated with TLR2 agonists or irradiated Mtb, leading to decreased cytokine expression (30). However, it has been unknown how the endogenous R753Q SNP affects TLR2 responses during infection of macrophages with live mycobacteria.

This paper demonstrates that exposure of HEK293 cells expressing transfected R753Q TLR2 to live *M. smegmatis* or *M. bovis* BCG resulted in deficient activation of p38 MAPK and NF- $\kappa$ B and impaired induction of IL-8. To confirm our results in macrophages expressing endogenous mutant TLR2, we engineered a new KI mouse strain harboring the mutant R753Q TLR2. Infection of BMDMs from R753Q TLR2 KI mice with *M. smegmatis* led to impaired activation of IRAK-1, MAPKs, and NF- $\kappa$ B and decreased induction of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 compared with the responses elicited by cells from WT mice, whereas TLR2<sup>-/-</sup> BMDMs exhibited the most pronounced deficiencies. Of note, we observed comparable levels of total and cell surface-associated WT and R753Q TLR2 proteins in macrophages, indicating that the effects of the R753Q SNP are not due to lower expression of the mutant TLR2. These results are consistent with conclusions obtained while studying the effect of the R753Q TLR2 SNP on manifestations of amyloidosis in patients with familial Mediterranean fever (43) and with our previous findings in HEK293 transfectants (30). However, there is a possibility that the R753Q SNP could affect dynamic trafficking of TLR2 between the plasma membrane,

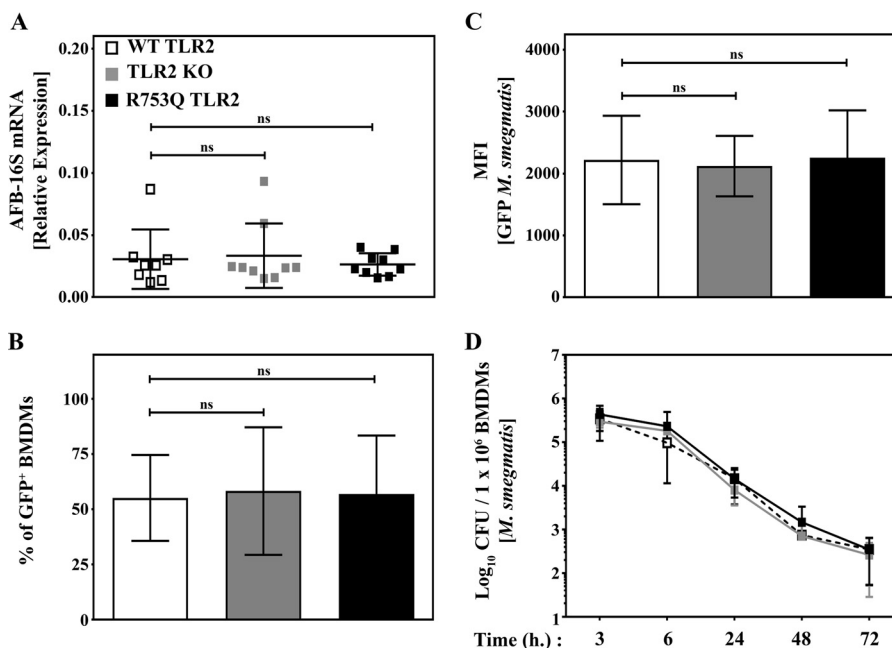


**Figure 4. Macrophages from R753Q TLR2 KI mice exhibit attenuated cytokine production during infection with *M. smegmatis*.** BMDMs from WT, TLR2 KO, or R753Q TLR2 KI mice were infected for 2 h with *M. smegmatis* (m.o.i. 10), and the cells were washed and incubated in gentamycin-containing medium. *A*, RNA was isolated, converted to cDNA, and subjected to real-time PCR analysis with the respective gene primers. The data depict TNF- $\alpha$  mRNA levels (2 h after infection), IL-6 and IL-1 $\beta$  mRNA (24 h after infection), and IL-10 mRNA (6 h after infection). *B*, following *M. smegmatis* infection, TNF- $\alpha$ , IL-6, IL-10 (24 h after infection), and IL-1 $\beta$  (48 h after infection) levels were analyzed in cell-free supernatants by ELISA. Summary data (mean  $\pm$  S.D.,  $n = 3$ ) are depicted. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

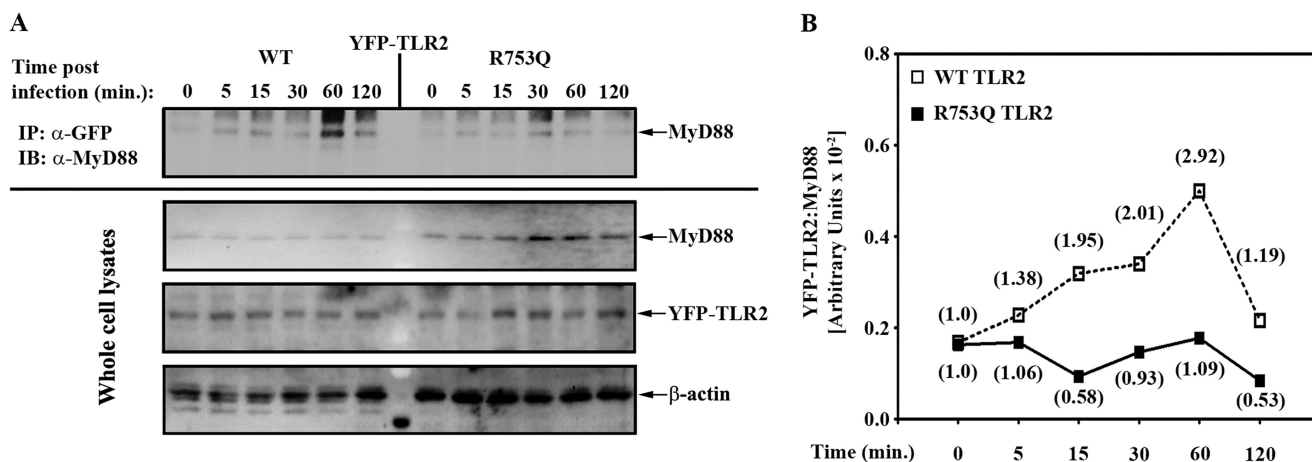


**Figure 5. The R753Q SNP does not affect TLR2 expression.** *A*, RNA was isolated from BMDMs obtained from WT or R753Q TLR2 KI mice, converted to cDNA, and analyzed by real-time PCR. Data (mean  $\pm$  S.D.) of the indicated number of mice from three experiments are shown. *ns*, not significant. *B*, whole-cell lysates were examined by Western blot analyses using Abs against total mouse TLR2 and  $\beta$ -tubulin. The results of five (WT and R753Q TLR2) and four (TLR2 KO) individual mice for each genotype are depicted. *C*, BMDMs from WT, KO, and R753Q TLR2 KI mice were stained with PE-conjugated anti-mouse CD282 (TLR2) Ab or isotype control IgG-PE, followed by FACS analyses of PE fluorescence. Isotype control staining profiles for WT BMDMs mice are shown on the left by a gray shadow (isotype control staining for the TLR2 KO and R753Q TLR2 BMDMs is not shown and exhibited a pattern identical to that of WT TLR2 BMDMs). The data of a representative experiment ( $n = 5$ ) are shown.

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**Figure 6. Macrophages harboring WT or R753Q TLR2 show similar bacterial burdens after infection with *M. smegmatis*.** BMDMs from WT, TLR2 KO, or R753Q TLR2 KI mice were infected for 2 h with *M. smegmatis* (m.o.i. 10). **A**, RNA was isolated, converted to cDNA, and examined by real-time PCR with the respective gene-specific primers to assess mycobacterium 16S rRNA expression. Data (mean ± S.D.) of the indicated numbers of individual mice from three experiments are depicted. *ns*, not significant. **B** and **C**, macrophages were incubated for 2 h with GFP-expressing *M. smegmatis* (m.o.i. 10), washed, and fixed, and bacterial uptake was assessed by FACS analyses judged by percentages of GFP-positive BMDMs (**B**) or by MFI values of GFP fluorescence (**C**). Summary results (mean ± S.D.) of three experiments are shown. **D**, cells were processed as in **B** and **C** and incubated for the indicated times in gentamycin-containing cDMEM. Cells were lysed, and serial dilutions of cell lysates were plated on bacterial agar dishes to determine cfu. The total number of cfu per  $1 \times 10^6$  cells was calculated, and the data (mean ± S.D.) of 8 mice/group are shown.



**Figure 7. The impact of the R753Q polymorphism on MyD88 recruitment to TLR2 in response to live *M. smegmatis*.** HEK293 cells stably expressing YFP-tagged human WT or R753Q TLR2 species were incubated for the indicated times with *M. smegmatis* (m.o.i. 50). **A**, YFP-TLR2 proteins were immunoprecipitated (IP) from whole-cell lysates with anti-GFP Ab, and MyD88 recruitment was analyzed by immunoblotting (IB) with anti-MyD88 Ab. Whole-cell lysates were examined by Western blot analyses using Abs against GFP, MyD88, and  $\beta$ -actin. The results of a representative experiment ( $n = 3$ ) are depicted. **B**, quantifications of the Western blot data shown in **A**. The data plotted are arbitrary values obtained by normalizing the band intensity for the YFP-TLR2-associated MyD88 to the band intensities of total YFP-TLR2 and then normalizing this to the band intensity of  $\beta$ -actin. The numbers in the brackets represent -fold induction values calculated by dividing arbitrary values for the intensities of bands in mycobacteria-infected cells by those measured in uninfected cells.

Golgi, and endosomes or its engagement of the co-receptors CD14, TLR1, TLR6, CD36, or integrins (9, 14, 44), requiring further studies.

Because TLR2 has been reported to regulate bacterial uptake and phagocytosis (34–39), we determined whether the R753Q SNP alters *M. smegmatis* uptake in the macrophage. Our results revealed a similar uptake of GFP-expressing *M. smegmatis* in BMDMs from WT, TLR2 KO, or R753Q TLR2 KI mice, as judged by comparable expression of 16S bacterial

RNA, numbers and percentages of GFP-positive macrophages, and levels of bacterial cfu. These data are consistent with previous reports showing comparable uptake of H37Rv *M. tuberculosis* and *M. bovis* BCG and similar percentages of bacilli in the cytoplasm of WT and TLR2 KO BMDMs (45). Thus, differences between WT versus R753Q TLR2 in eliciting signaling and cytokine expression during *M. smegmatis* infection are not due to altered levels of the mutant TLR2 or changes in bacterial uptake.

In response to stimulation with defined TLR2 agonists or during mycobacterial infection, TLR2 expression in macrophages is increased (46, 47). Interestingly, we demonstrate comparable increases in TLR2 levels in macrophages from WT or TLR2 R753Q KI mice infected with *M. smegmatis*. In addition to NF- $\kappa$ B, there are other transcription factors that bind to the promoter region of TLR2 and drive its expression, including Sp1, STAT5, and the Ets family members Sp1, Sp3, and, possibly, PU.1 (48–50). Thus, preserved induction of such transcription factors could account for the comparable up-regulation of TLR2 expression seen in WT versus R753Q KI macrophages. Furthermore, the TLR2 R753Q SNP is likely to represent not just a “loss-of-function” mutation but could uniquely affect different aspects of receptor signaling. Indeed, such a signaling mode is reported for another TLR2 SNP, R677W, whereby R677W TLR2-expressing monocytes respond to exposure to *Mycobacterium leprae* with impaired induction of TNF- $\alpha$  and IL-12 p40 but increased expression of IL-10 compared with cells expressing WT TLR2 (27). Notably, our preliminary results suggest that the R753Q TLR2 is likely to act by “reprogramming” and altering signaling, whereby the SNP inhibits phosphorylation of IRAK1, MAPK, and p65 NF- $\kappa$ B, without affecting PI3K-dependent phosphorylation of Akt (data not shown).

TLR2 engagement by pathogens involves its heterodimerization with TLR1 or TLR6 (51–53), leading to recruitment of the adapters Mal and MyD88 and myddosome assembly (20, 21). Efficient myddosome assembly is critical for optimal activation of IRAK-1, MAPKs, and NF- $\kappa$ B and induction of antibacterial immune responses. We reported that impaired responses of cells transfected with R753Q TLR2 to defined agonists or irradiated Mtb were due to the altered electrostatic potential of the mutant TLR2 domain, deficient TLR2-TLR6 assembly, and impaired recruitment of Mal and MyD88 (30). Using the HEK293 overexpression system, we demonstrate here that exposure of HEK293 cells expressing R753Q TLR2 to live *M. smegmatis* resulted in diminished ability of the mutant TLR2 to recruit MyD88 and activate IRAK-1. To the best of our knowledge, this is the first report uncovering impaired MyD88 recruitment to R753Q TLR2 and deficient IRAK-1 activation as the mechanistic basis of reduced cell activation in response to mycobacterial infection.

TLR2 undergoes tyrosine phosphorylation in its cytoplasmic domain (54), and two tyrosines, Tyr-616 and Tyr-761, play a critical role in TLR2-dependent NF- $\kappa$ B activation and cytokine expression (54, 55). It is currently unknown whether Tyr-616 or Tyr-761 are directly phosphorylated and which kinases could be involved in this process. It is noteworthy that the R753Q TLR2 SNP is in a close proximity to Tyr-761, suggesting that, during infection of macrophages with mycobacteria, this SNP may alter TLR2 tyrosine phosphorylation, which has been implicated in enabling efficient TLR2-TLR1 or TLR2-TLR6 signalosome assembly (30, 54). Supporting this notion, we previously reported deficient tyrosine phosphorylation of transfected R753Q human TLR2 in HEK293 cells stimulated with TLR2 agonists or irradiated Mtb, which correlated with impaired TLR2-TLR6 assembly (30). It is plausible that the R753Q SNP alters the TLR2 electrostatic potential and docking interface for protein tyrosine kinases and impairs TLR2 tyro-

sine phosphorylation. These processes could lead to deficient TLR2 assemblies with TLR6 or TLR1 upon infection of macrophages with live mycobacteria, translating into impaired recruitment of MyD88 and altered signaling. Experiments are ongoing to address this hypothesis. Our initial studies to address the functional significance of the TLR2 R753Q SNP *in vivo* revealed that mice harboring the TLR2 R753Q SNP *in vivo* infected with *M. smegmatis* exhibited increased bacterial burdens in the spleen and liver compared with WT animals (data not shown). These findings may suggest impaired killing of *M. smegmatis* *in vivo* in R753Q TLR2-expressing mice. Experiments are in progress to carry out comprehensive analyses of the impact of the R753Q TLR2 SNP on immune responses to mycobacterial species and infection morbidity and mortality *in vivo*.

Recently, Herrtwich *et al.* (56) have shown a novel role for TLR2 signaling in regulating the generation of polyploid macrophages, giant cells, and granuloma formation upon infection with *Mycobacterium* species. Chronic stimulation of TLR2 was found to promote macrophage polyploidy and suppress genomic instability by regulating Myc-induced DNA repair and activating unique gene expression signatures for metabolic and extracellular matrix-remodeling molecules (56). These events could promote a long-lived granuloma-resident macrophage differentiation program that regulates granulomatous tissue remodeling, which is critical in controlling Mtb infection (3, 4). It is an intriguing possibility that altered signaling by R753Q TLR2 could confer unique regulation of Myc-dependent DNA repair and expression profiles of genes involved in the regulation of metabolism and extracellular matrix remodeling, instructing the fate of granulomas in the lung. It is tempting to speculate that impaired TLR2-TLR6 heterodimerization leading to attenuated TLR2-mediated signaling in response to mycobacteria could be responsible for the increased incidence of TB in certain cohorts of patients carrying the TLR2 R753Q SNP. Interventions directed at corrections of impaired myddosome assembly exhibited by the mutant R753Q TLR2 species via application of agonistic peptides or small chemical agonists promoting TLR2-MyD88 complex formation could potentially provide new approaches for the treatment of patients that carry the R753Q SNP.

## Experimental procedures

### Abs, reagents, and cell lines

The following Abs were used in this study: anti-GFP (Thermo Fisher Scientific, Waltham, MA); anti-FLAG (Sigma-Aldrich, St. Louis, MO); anti-MyD88, anti-p65 NF- $\kappa$ B, anti-TLR2, and anti- $\beta$ -actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA); and anti-p-p38, anti-p-p65, anti-p-ERK1/2, anti-ERK1/2, anti-p38, anti-p-Akt, anti-Akt, and anti- $\beta$ -tubulin (Cell Signaling Technology, Inc., Danvers, MA.). S-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-Cys-Ser-Lys4-OH (Pam<sub>3</sub>CSK<sub>4</sub>) was obtained from InvivoGen (San Diego, CA). Zombie<sup>®</sup> Aqua cell viability dye, PE-labeled anti-mouse CD282 (TLR2) Ab, and PE-labeled rat IgG2a  $\kappa$  isotype control Ab were from BioLegend (San Diego, CA). HEK293T and HEK293 cells were obtained from the ATCC and maintained in complete

## R753Q TLR2 polymorphism and mycobacterial infection

DMEM supplemented with 10% FBS (HyClone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin (Thermo Fisher Scientific) (cDMEM).

### Generation of R753Q TLR2 KI mice

R753Q TLR2 KI mice were generated at Cyagen Biosciences (Santa Clara, CA) using C57BL/6J (B6) embryonic stem cells by standard gene-targeting techniques (Fig. 2A). In brief, to engineer the targeting vector, the 5' homology arm and 3' homology arm were amplified from bacterial artificial chromosome clone RP23-374N17 or RP23-158H14 from the C57BL/6J library as a template. The targeting vector was constructed by site-directed mutagenesis to replace Arg with Glu at the position of 753 (R753Q SNP) in exon 3 and was transfected into B6 embryonic stem cells. The targeting vector had a Neo selection cassette flanked by LoxP sites for antibiotic selection as well as diphtheria toxin A, which was used for negative selection to identify cells carrying the R753Q TLR2 SNP. After selection, the presence of the respective alleles was verified by Southern blot analysis and DNA sequencing of the PCR products generated using the primers 5'-TTTCTCTAGCCACCCATGTTCGCA-3' (forward) and 5'-TTCCGGGCAAATGGATCA-TTG-3' (reverse) (Fig. 2B and data not shown). Embryonic stem cells were injected into mouse blastocysts and transplanted into the uterus of Cre-expressing C57BL/6 mice to produce chimeric KI mice and to achieve removal of the Neo cassette. The chimeric heterozygous F1 mice were interbred to generate R753Q TLR2 homozygous mice. The mice were genotyped using DNA samples from tail snips and the primers 5'-AGTTCACACAAAATGCAGCCTGTG-3' (forward) and 5'-GGGTGGCTAGAGAAAGCATTGTGTA-3' (reverse), and PCR products were run on a gel to identify wild-type (191 bp) or R753Q TLR2 species (316 bp) (Fig. 2C).

### Mouse strains and macrophage isolation

WT B6 and TLR2 KO (B6.129-Tlr2<sup>tm1Kir/J</sup>) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in an environmentally controlled room in the UConn Health animal facility. Six-week-old female mice were used in the experiments. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee. Bone marrow cells were isolated from femora and tibiae of mice. To generate BMDMs, bone marrow cells were differentiated for 7 days in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FBS (HyClone), 2 mM L-glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 15% L929 cell-conditioned, M-CSF-containing supernatant, as reported previously (30).

### Bacterial cultures and infection

GFP-expressing *M. smegmatis* and *M. bovis* BCG were kindly provided by Drs. Volker Briken (University of Maryland College Park, College Park, MD) and Matyas Sandor (University of Wisconsin, Madison, WI), respectively. Bacteria were cultured in 7H9 broth containing 0.5% glycerol, 0.05% Tween 80, 10% oleic albumin dextrose complex enrichment, and 40  $\mu$ g/ml kanamycin. Bacteria were grown to semiconfluent cultures (optical density at 600 nm ranging from 0.4–0.8), washed in

PBS, allowed to settle for 10 min, and added to cells cultured in antibiotic-free DMEM at the respective m.o.i. for 2 h. Cells were washed in PBS and incubated in cDMEM supplemented with 100  $\mu$ g/ml gentamycin (Thermo Fisher Scientific) for the period of the assay.

### Plasmids and transfection

The pcDNA3-YFP-WT human (h) TLR2, pcDNA3-YFP-R753Q hTLR2, pELAM-luciferase (Luc), and p-thymidine kinase (TK)-*Renilla*-Luc plasmids were described previously (30). The R753Q SNP was introduced into the YFP-TLR2 expression vector by site-directed mutagenesis, and its presence was confirmed by sequencing, as published previously (30). For transient transfection, HEK293T cells were transfected with the respective plasmids, using the Lipofectamine 2000 transfection protocol as recommended by the manufacturer and as reported previously (30, 57). To generate stably transfected cell lines, HEK293 cells were transfected for 5 h with pcDNA3-YFP vectors encoding WT or R753Q TLR2s, recovered for 24 h, and selected for 2 weeks in cDMEM containing G418 (1 mg/ml). Cells were plated in 100-mm tissue culture dishes (for immunoprecipitation), 6-well plates (to generate cell extracts for immunoblotting), or 24-well plates (for gene expression, cytokine secretion, and reporter assays) as described previously (30, 57).

### Assessment of bacterial uptake by flow cytometry

To examine bacterial uptake, BMDMs ( $1 \times 10^6$  cells/well in 24-well plates) were incubated for 2 h with GFP-expressing *M. smegmatis* (m.o.i. 10) in antibiotic-free RPMI 1640 medium, washed with PBS, and incubated in cDMEM containing 100  $\mu$ g/ml gentamycin on ice or at 37 °C for the indicated times. Cells were detached by scraping, washed in ice-cold PBS containing 3% FBS, stained with Zombie<sup>®</sup> Aqua cell viability dye (BioLegend), fixed for 15 min in 3.7% formaldehyde, and analyzed by FACS on an LSRII flow cytometer (BD Biosciences) to measure GFP fluorescence. The data were analyzed using FlowJo software (Tree Star). BMDMs were gated using forward scatter *versus* side scatter, single cells were gated using forward scatter area *versus* forward scatter height, and then live cells were gated as Zombie<sup>®</sup> Aqua-negative events. Live BMDMs were then assessed for GFP-positive events as well as total GFP MFI.

### Assessment of cell surface TLR2 expression by flow cytometry

To examine surface TLR2 expression, BMDMs were stained for 30 min on ice with PE-labeled anti-mouse CD282 (TLR2) or PE-labeled rat IgG2a  $\kappa$  isotype control Abs. Cells were washed with PBS, stained with Zombie<sup>®</sup> Aqua cell viability dye, fixed for 15 min in 3.7% formaldehyde, and analyzed by FACS on a LSRII flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (Tree Star). Briefly, BMDMs were gated using forward scatter *versus* side scatter, then single cells were gated using forward scatter area *versus* forward scatter height, and then live cells were gated as Zombie<sup>®</sup> Aqua-negative events. Live BMDMs were then assessed for TLR2 expression by measuring PE expression as seen by MFI.



### Isolation of RNA and real-time PCR

Total RNA was prepared using the TRIzol protocol (Thermo Fisher Scientific), residual genomic DNA was digested with DNase, and RNA was repurified as recommended by the manufacturer. cDNA was prepared from 1  $\mu$ g of total RNA using the reverse transcription system (Promega) and examined by real-time PCR with the following primers: human IL-8, 5'-CACCGGAAGGAACCATCTC-ACT-3' (forward) and 5'-TGCACCTTCACACAGAGC-TGC-3' (reverse); human hypoxanthine-guanine phosphoribosyltransferase, 5'-ACCAGTCAACAGGGGACATAAAAAG-3' (forward) and 5'-GTCTGCATTGTTTTGCCAGTGTC-3' (reverse); mycobacterial acid-fast bacilli-16S, 5'-GCGTGCTT-AACACATGCAAGTC-3' (forward) and 5'-TCCTCCTGAT-ATCTGCGCATTTC-3' (reverse); mouse TLR2, 5'-CCGAAAC-CTCAGACAAAGCG-3' (forward) and 5'-TCACACACCCC-AGAAGC ATC-3' (reverse); mouse GAPDH, 5'-GCTGACC-TGCTGGATTACATT-3' (forward) and 5'-GTTGAGAGAT-CATCTCCACCA-3' (reverse); mouse TNF- $\alpha$ , 5'-CCCAGGC-AGTCAGATCATCTTC-3' (forward) and 5'-GCTTGAGGG-TTTGCTACAACATG-3' (reverse); mouse IL-1 $\beta$ , 5'-CTGG-TGTGTGACGTTCCCAT-3' (forward) and 5'-GATTCTTT-CCTTTGAGGCCCA-3' (reverse); mouse IL-6, 5'-TCAGGA-AATTTGCCTATTGAAAATTT-3' (forward) and 5'-GCTT-TGTCTTTCTTGTTAT CTTTTAAGTTGT-3' (reverse); and mouse IL-10, 5'-ATTTGAATTCCCTGGGTGAGAAG-3' (forward) and 5'-CACAGGGGAGAAATCG ATGACA-3' (reverse). Reaction mixtures were processed on a MyIQ real-time PCR machine (Bio-Rad). The data were analyzed by the  $2^{-\Delta\Delta CT}$  method (58) or expressed as a ratio of gene of interest normalized to the respective housekeeping gene (hypoxanthine-guanine phosphoribosyltransferase or GAPDH) as reported previously (57).

### Co-immunoprecipitation and Western blot analyses

Cell lysates were prepared as described previously (30, 57). In brief, cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5 mM NaF, 2 mM sodium orthovanadate, 1 mM PMSF, and complete protease inhibitors). Cell lysates were cleared by centrifugation at 13,000 rpm for 10 min, and insoluble debris was discarded. For co-immunoprecipitation, cell lysates were precleared for 4 h at 4 °C with protein G-agarose beads (25  $\mu$ l of 50% slurry per sample, Roche Applied Science) 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  $\beta$ -glycerophosphate, 50 mM NaF, 1 mM DTT, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, and protease inhibitor mixture (Roche Applied Science). Thereafter, protein G-agarose beads were added (45  $\mu$ l/sample), and incubation continued for 4 h. Beads were washed five times with lysis buffer and suspended in Laemmli sample buffer (50 mM Tris-Cl (pH 6.8), 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 5% 2-mercaptoethanol). Proteins were separated by SDS-PAGE on 4–20% mini gels (Thermo Fisher Scientific), transferred to polyvinylidene fluoride membranes (Bio-Rad), blocked, and probed with the respective Abs, as described pre-

viously (30). The protein bands were quantified using the National Institutes of Health ImageJ software package and expressed as values of phosphorylated proteins normalized to total species or to  $\beta$ -actin or tubulin.

### Assessment of intracellular bacterial cfu

BMDMs were plated in 24-well plates ( $1 \times 10^6$  cells/well), grown overnight, incubated for 2 h with *M. smegmatis* (m.o.i. 10) in antibiotic-free RPMI 1640 medium, washed with PBS, and incubated in cDMEM containing 100  $\mu$ g/ml gentamycin on ice or at 37 °C for the indicated times. Cells were lysed for bacterial counts in PBS containing 0.05% Tween 80 (59). The number of cfu per  $1 \times 10^6$  cells was determined by plating 10-fold serial dilutions of cell lysates on Middlebrook 7H10 plates complemented with 0.5% glycerol and 10% oleic albumin dextrose complex enrichment (59, 60). For selective medium, 40  $\mu$ g/ml kanamycin was added. Plates were incubated at 37 °C, and cfu were counted after 2 days.

### Analyses of cytokine secretion

Supernatants from cell stimulated with TLR agonists or infected with *M. smegmatis* were cleared by centrifugation, and cytokine levels were determined using ELISA kits (BioLegend) according to the recommendations of the manufacturer. The ELISA kits had the following limits of detection: IL-1 $\beta$ , 16 pg/ml; IL-6, 4 pg/ml; IL-10, 16 pg/ml; and TNF- $\alpha$ , 4 pg/ml.

### NF- $\kappa$ B reporter assays

NF- $\kappa$ B reporter assays were performed as described previously (30, 57). In brief, 293/TLR2 cells expressing WT or R753Q TLR2 species were grown in 24-well plates to reach 70% confluence and transfected with pELAM-Luc (300 ng/well) and pTK-RL (10 ng/well) plasmids (the total plasmid amount was adjusted to 400 ng/well with pcDNA3.1) using Lipofectamine 2000. After recovery for 48 h, cells were treated for 16 h as indicated in the figure legends and lysed in a passive lysis buffer (Promega). Firefly *versus* *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega) on an LB 9507 luminometer (Berthold Technologies).

### Statistical analysis

Experimental results were processed by the GraphPad Prism software package (GraphPad Software, San Diego, CA) using one-way analysis of variance with Tukey's post hoc test, and the data were expressed as mean  $\pm$  S.D.

*Author contributions*—G. P. conducted the experiments, analyzed the results, and wrote the manuscript. R. P. performed several of the NF- $\kappa$ B reporter experiments shown in Fig. 1. A. E. M. conceived the idea for the project, conceptualized the research, analyzed the data, and edited and finalized the manuscript written by G. P.

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