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Cell Surface Trafficking of TLR1 Is Differentially Regulated by the Chaperones PRAT4A and PRAT4B*[S]

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Background: TLR trafficking must be regulated to control ligand accessibility for effective pathogen recognition. The TLR1 I602S polymorphism is deficient in surface trafficking.
Results: A TLR1 trafficking motif is interrupted by the 602S polymorphism. PRAT4A and PRAT4B differentially regulate TLR trafficking.
Conclusion: A mechanistic basis for deficient TLR1 602S surface trafficking is provided.
Significance: Differential receptor trafficking is a potential mechanism for controlling inflammation, based on TLR availability.

The subcellular localization of Toll-like receptors (TLRs) is critical to their ability to function as innate immune sensors of microbial infection. We previously reported that an I602S polymorphism of human TLR1 is associated with aberrant trafficking of the receptor to the cell surface, loss of responses to TLR1 agonists, and differential susceptibility to diseases caused by pathogenic mycobacteria. Through an extensive analysis of receptor deletion and point mutants we have discovered that position 602 resides within a short 6 amino acid cytoplasmic region that is required for TLR1 surface expression. This short trafficking motif, in conjunction with the adjacent transmembrane domain, is sufficient to direct TLR1 to the cell surface. A serine at position 602 interrupts this trafficking motif and prevents cell surface expression of TLR1. Additionally, we have found that ER-resident TLR chaperones, PRAT4A and PRAT4B, act as positive and negative regulators of TLR1 surface trafficking, respectively. Importantly, either over-expression of PRAT4A or knock-down of PRAT4B rescues cell surface expression of the TLR1 602S variant. We also report that IFN-γ treatment of primary human monocytes derived from homozygous 602S individuals rescues TLR1 cell surface trafficking and cellular responses to soluble agonists. This event appears to be mediated by PRAT4A whose expression is strongly induced in human monocytes by IFN-γ. Collectively, these results provide a mechanism for the differential trafficking of TLR1 I602S variants, and highlight the distinct roles for PRAT4A and PRAT4B in the regulation of TLR1 surface expression.

Toll-like receptors (TLRs)[2] are central elements of the innate immune system that provide a first line of immune defense against infectious agents. The direct recognition of bacterial, fungal, or viral components induces TLR activation and results in the cellular expression and release of immune mediators (1). TLRs play indispensable roles in bridging the innate and adaptive immune systems by inducing the expression of genes encoding cell adhesion molecules, proinflammatory cytokines and chemokines, as well as the co-stimulatory molecules and antigen presentation machinery required for T cell activation (2).

Humans possess 10 TLR family members, numbered 1 through 10, subsets of which are expressed in leukocytes and a wide variety of tissue types. TLRs can be broken down into two main groups based upon their function and phylogenetic relationship. TLRs 3, 7, 8, and 9 are grouped according to their ability to recognize various bacterial and viral nucleic acids. The remaining TLRs predominantly sense bacterial and fungal cell surface components. For example, TLR4 and TLR5 are sensors of bacterial LPS and flagellin, respectively. The TLR2 subfamily, comprised of TLRs 1, 2, 6, and 10, mediates immune responses to a variety of microbial cell wall components including lipoproteins and glycolipids. Cell signaling by all TLRs is initiated by the coordinate binding of ligand, which induces receptor dimer formation. While most TLRs signal as homodimers, TLR2 signals by forming heterodimers with either TLR1, 6, or 10 in an agonist-dependent fashion. For example, cell activation by triacylated bacterial lipoproteins occurs through coordinate binding by TLR1 and TLR2.

The subcellular localization of each TLR is vital for the appropriate recognition of microbial components leading to cell activation (3, 4). TLR3, 7, 8, and 9 are endosomally localized, providing proximity to bacterial and viral nucleic acids generated during viral replication or released following microbial degradation. Conversely, TLR1, 2, 6, and 10 are displayed on the plasma membrane where they are best available to contact bacterial and fungal cell wall constituents (5). TLR localization is dynamic and surface TLRs traffic to phagosomes during uptake of particulate microbes (6).

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[2] The abbreviations used are: TLR, Toll-like receptor; TIR, Toll/interleukin-1 receptor domain; ER, endoplasmic reticulum; PRAT4, protein associated with TLR4; LPS, lipopolysaccharide; RFP, red fluorescent protein; Mtb, *M. tuberculosis*; IFNγ, interferon-γ; shRNA, short-hairpin RNA; TNFα, tumor necrosis factor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

A variety of receptor regions have been identified as trafficking signals which direct TLR transport from the endoplasmic reticulum to either the cell surface or endosomal compartments. These trafficking motifs include the transmembrane domains of TLR4, 7, and 9, and the cytoplasmic linker region of TLR3 (7–9). Interruption of these sequences and the associated trafficking mechanisms prevents proper localization of TLRs, thereby disrupting accessibility to ligands and blocking recognition and responses to invading pathogens. In addition, several ER-resident chaperones have been shown to control subcellular receptor distribution (10). Gp96, PRAT4A, PRAT4B, and UNC93B1 are examples of such chaperones and their disruption can ablate proper localization of specific TLRs and induce hyporesponsiveness to their respective agonists (11–16).

We have recently identified a frequent single nucleotide polymorphism in TLR1 (I602S), which greatly inhibits trafficking of the receptor to the cell surface (17). Aberrant surface display of the I602S variant is associated with a marked inability of cells to respond to soluble TLR1 agonists (17, 18). Interestingly, disease association studies have identified TLR1 602S as a key protective allele against both tuberculosis and leprosy (17, 19–24).

In this report, the trafficking deficiency of TLR1 602S is used as a model to identify the regulatory elements and chaperones required for TLR1 trafficking. We have found that the transmembrane domain and a short intracellular region interrupted by the 602S polymorphism is necessary for plasma membrane localization of TLR1, and is sufficient to drive surface display of TLR9. Additionally, we have found that the ER chaperones PRAT4A and PRAT4B differentially regulate trafficking of all surface-displayed TLRs, and that the physical interaction between TLR1 602S and PRAT4B may be responsible for the aberrant localization of this common TLR1 variant.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK 293T and COS7 cells were grown in RPMI 1640 media (Cellgro) supplemented with 10% fetal bovine serum (Thermo Scientific), penicillin/streptomycin (Cellgro), and 20 mM L-glutamine (Cellgro). Primary human peripheral blood mononuclear cells were isolated by ficoll-paque (GE Healthcare) gradient centrifugation. Following thirty minutes of monocyte attachment to tissue culture plates in RPMI 1640 media, extraneous leukocytes were washed and discarded. Primary human monocyte-derived macrophages were generated by stimulating monocytes with 50 ng/ml M-CSF (R&D Systems) for 7 days. During cytokine induction studies, monocytes were stimulated with 20 ng/ml IFNγ (Miltenyi Biotec, Thermo Scientific) for 24 h.

DNA Constructs and PCR Mutagenesis—TLR constructs were cloned into pFLAG-CMV-1 (Sigma Aldrich) or HA-epitope pDisplay (Invitrogen) expression plasmids. Receptor point mutants and truncation mutants were generated by site-directed mutagenesis and PCR amplification. Chimeric receptors were created using PCR SOEing, described previously (25, 26). Truncation mutants are named according to the missing portion of the receptor (*i.e.* TLR1 Δ603-C is a receptor in which the region comprising amino acids 603 to the end of the C terminus are deleted). Chimeric receptors are named according to the portion of TLR9, which is replaced with TLR1 sequence. For example, TLR9/1 TM is a TLR9 mutant in which the transmembrane domain of TLR9 has been replaced with that of TLR1. TLR9/1 TM-Cyto replaces TLR9 residues 810–1032 with that of the transmembrane domain and cytoplasmic domains of TLR1 (amino acids 571–786). TLR9/1 TF replaces amino acids 844–849 of TLR9 with that of the trafficking motif of TLR1 (amino acids 602–607). TLR9/1 TM replaces amino acids 823–843 of TLR9 with that of TLR1 (amino acids 581–601). Finally, TLR9/1 TM-TF replaces aa823–849 of TLR9 with the transmembrane and trafficking motif of TLR1 (amino acids 581–607).

PRAT4A and PRAT4B-FLAG cDNA clones were expressed from within the pCMV6-XL5 vector (OriGene). Two anti-PRAT4B shRNAs were synthesized (Integrated DNA Technologies) and cloned into pSilencer 3.1-H1 Neo vectors (Ambion) targeting the following: Sequence 1, AAGAGGAAGAGACACGTGCCT; Sequence 2, AAACTTGGACTGGAAAGGAGA. Respective scrambled control sequences were: Scramble 1, GCGGGGAAACGGCGATAATACA; Scramble 2, GAGAGGGAACAACGGTATAAT. Cells were transfected using Fugene 6 (Roche) or TransIT 2020 (Mirius) reagents overnight, followed by replacement with fresh media for 24 h before harvesting cells for flow cytometry or immunoblotting. Batch-derived, stable cell lines were generated by transfecting HEK 293 cells with pSilencer vectors encoding neomycin resistance, followed by selection with 500 μg/ml G418 for 4 weeks.

Flow Cytometric Analysis—Surface expression of TLRs was measured using a triple-step staining procedure. Cells were blocked with flow buffer (10% rabbit serum and 0.3% NaN_3 in PBS), then stained using a primary mouse anti-FLAG M2 antibody (Sigma Aldrich) or mouse anti-HA.11 (Covance), followed by biotinylated anti-mouse Fab fragment (Jackson ImmunoResearch), and finally, streptavidin-conjugated Alexa Fluor-647 or Alexa Fluor-488 (Invitrogen). Staining steps were performed for 30 min on ice, followed by a wash with flow buffer. Intracellular expression was assessed by fixing cells in 4% paraformaldehyde, permeabilizing in 0.25% Triton X, and using the above staining protocol. Stained cells were analyzed using a BD FACS Canto flow cytometer. using FCS Express V3 (De Novo Software).

Microscopy—COS7 fibroblasts were seeded onto chambered slides (Lab-Tek), transfected, and stained using the above method. The endoplasmic reticulum was visualized using pDsRed2-ER (Invitrogen). Images were acquired using a Carl Zeiss LSM510 laser-scanning confocal microscope using appropriate filter sets.

ELISA—Enzyme-linked immunosorbent assays of IL-6 from macrophage supernatants were performed using an IL-6 Cyto-Set kit (Invitrogen) according to the manufacturer's protocol. Cells were stimulated with TLR agonists for 24 h. PAM_3CSK_4 (50 ng/ml), *Mycobacterium tuberculosis* membrane fraction (500 ng/ml), and zymosan (1×10^7 particles/ml).

Immunoblotting and Co-immunoprecipitation—Lysates were prepared by detaching HEK 293T cells using ice-cold 10 mM EDTA, followed by lysis in RIPA buffer. Protein samples were

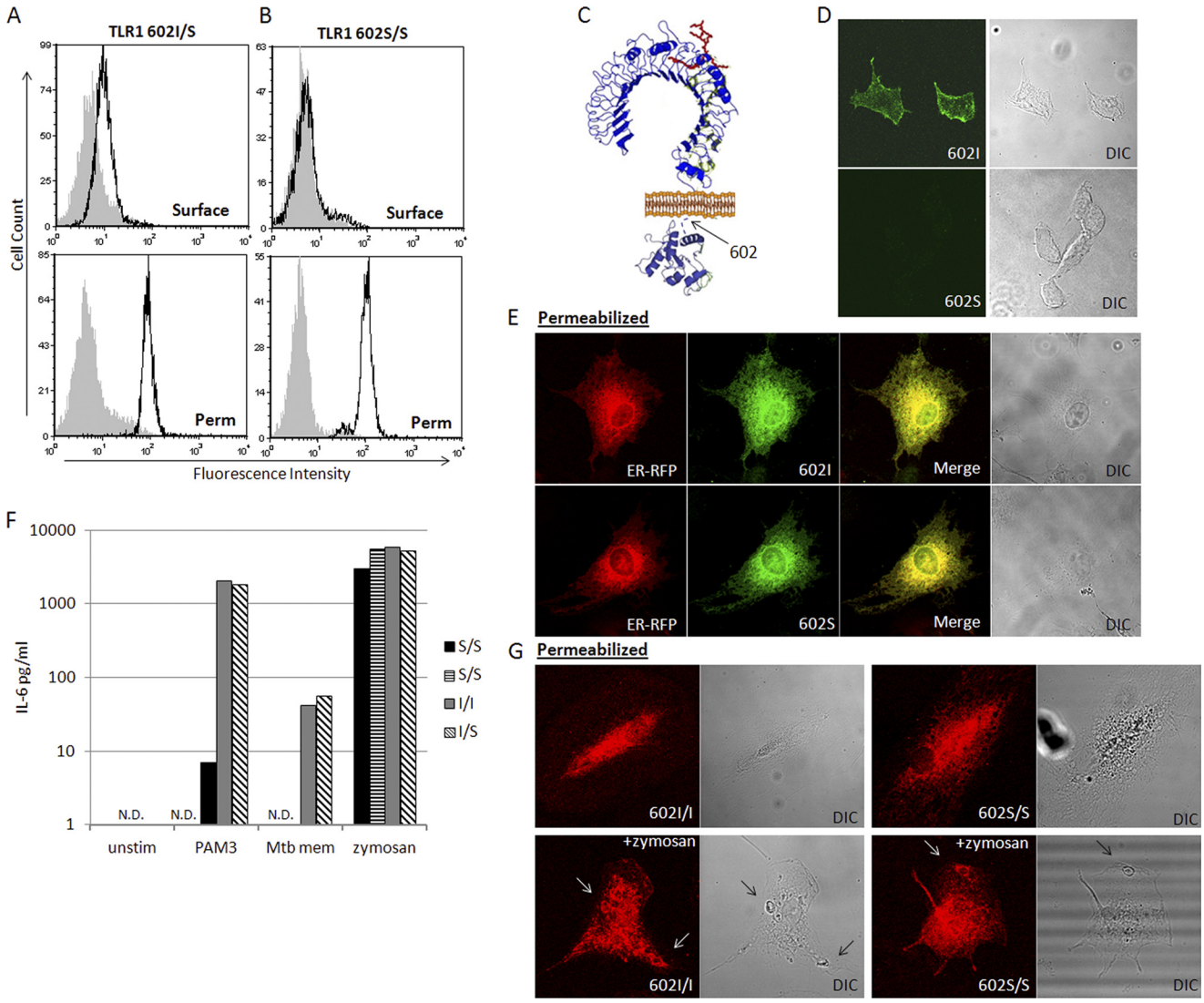

FIGURE 1. **TLR1 I602S exhibits impaired TLR1 surface trafficking.** TLR1 staining of intact (surface) and permeabilized cells (perm) was assessed by flow cytometric analysis using primary human monocytes from individuals of two TLR1 genotypes: 602 I/S (*A*) and 602 S/S (*B*). *Filled histograms* represent isotype controls. *C*, position of the 602 polymorphism is marked on the crystal structure of TLR1 (adapted from Ref. 41). *D*, COS7 cells were transiently transfected with FLAG-tagged TLR1 602I or TLR1 602S and stained to detect FLAG. Surface TLR1 (*green*) was visualized by scanning laser confocal microscopy. *E*, COS7 cells were co-transfected with the indicated FLAG-tagged TLR1 variants and an ER-localized, red fluorescent protein (ER-RFP, *red*). Cells were permeabilized and stained for intracellular TLR1 as indicated (*green*). Co-localization of ER and TLRs is denoted by *yellow signal* in the merged image. *F*, primary human macrophages from blood donors of the three different TLR1 602 genotypes were stimulated with various TLR1 agonists as indicated (*N.D.*; not detectable, *unstim*; unstimulated, *PAM3*; Pam_3CSK_4, *Mtb* mem; *M. tuberculosis* membrane fraction). IL-6 secretion was measured from culture supernatants by ELISA. *G*, intracellular TLR1 (*red*) was visualized in resting primary human macrophages from donors of different TLR1 602 genotypes as indicated (*top row*). Human macrophages were also stimulated with zymosan particles (shown by *arrows*) to detect recruitment of TLR1 to phagosomal compartments (*bottom row*).

run in 10% SDS-PAGE gels. For Western blotting, primary mouse anti-FLAG (Sigma), mouse anti-HA, rabbit anti-human PRAT4A (Imgenex), rabbit anti-human PRAT4B (Santa Cruz Biotechnologies), or rabbit anti-human actin (Thermo Scientific) antibodies were incubated 1:1000 in 5% milk-TBST for 1 h. Secondary HRP-conjugated anti-isotype antibodies were incubated 1:10,000 for 1 h. Proteins were detected by chemiluminescence (Pierce). Anti-FLAG pull-downs were conducted using M2 affinity gel (Sigma).

RESULTS

TLR1 I602S Exhibits Impaired TLR1 Surface Trafficking—We have previously demonstrated that TLR1 602S exhibits hyporesponsive function compared with the TLR1 602I variant (17). This study revealed that this phenotype resulted from impaired surface trafficking and was not due to a reduction in total cellular TLR1 602S protein expression (Fig. 1, *A*, *B*, *D*). The deficiency in surface trafficking is not restricted to the monocyte cell type, as staining of whole blood leukocytes shows an identical phenotype for TLR1 602S in lymphocytes and granulocytes (supplemental Fig. S1).

To define the subcellular localization of both TLR1 variants, COS7 cells were transfected with vectors encoding N-terminal FLAG-tagged versions of either TLR1 602I or TLR1 602S, along with red fluorescent protein fused to an endoplasmic reticulum retention motif (ER-RFP). Transfected cells were subsequently permeabilized, stained for TLR1, and visualized by confocal microscopy. Both receptor variants exhibit high levels of intra-

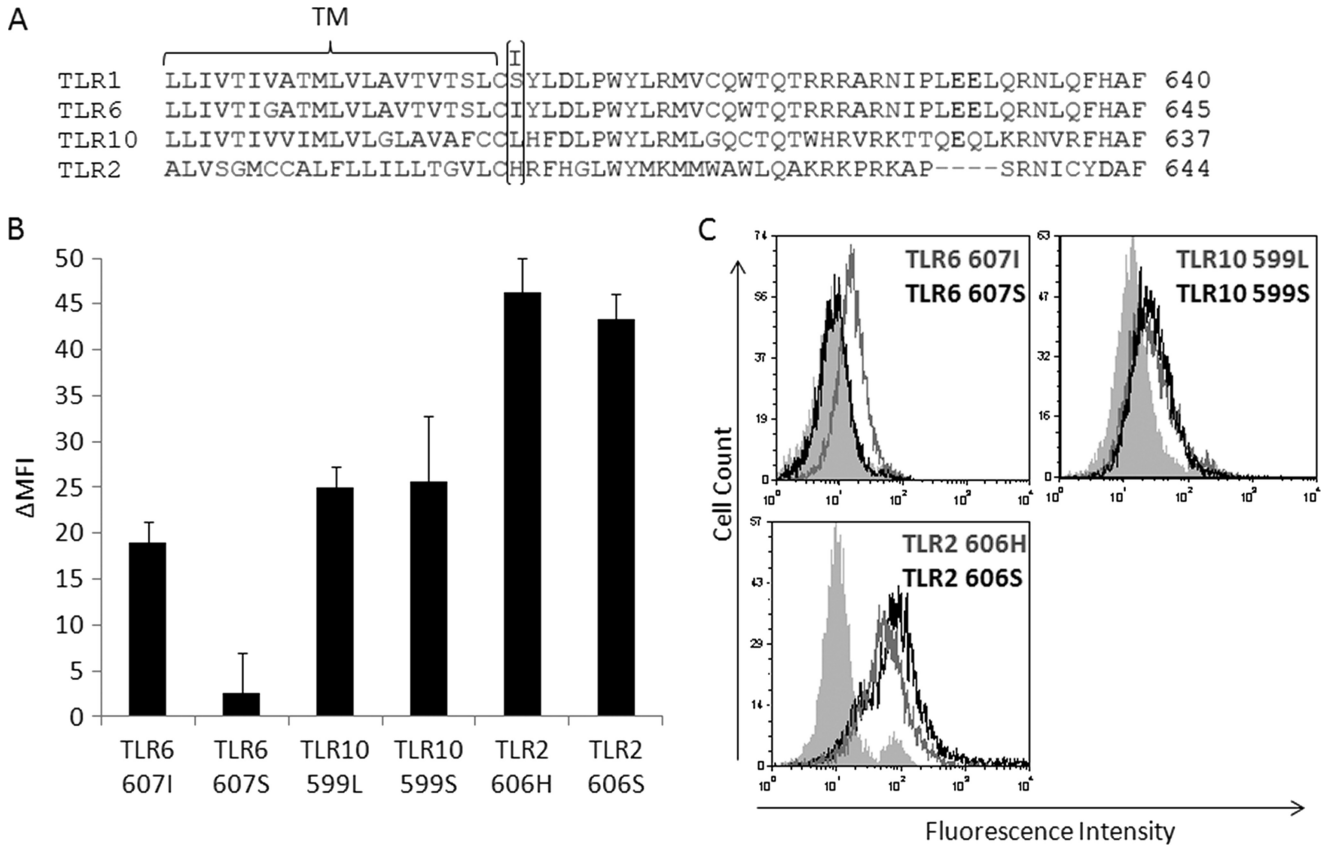

FIGURE 2. **An equivalent serine substitution of TLR6 mimics the trafficking deficiency of TLR1 602S.** *A*, ClustalW alignment of amino acids forming the transmembrane domain (*TM*) and a portion of cytoplasmic domain from members of the TLR2 subfamily. Position 602 is bracketed. *B*, HEK 293T cells were transfected with wild type receptors or serine point mutants of TLR6, TLR10, and TLR2. The change in mean fluorescence intensity (ΔMFI) of transfected cells was measured by flow cytometry. Error bars represent the standard deviation of triplicate transfections. *C*, representative histograms show surface TLR expression (*filled curve*; empty vector, *gray line*; wild type, *black line*; serine mutant).

cellular expression in a perinuclear compartment and both variants co-localize with the endoplasmic reticulum (Fig. 1*E*). This result further suggests that the lack of surface expression of TLR1 602S results from improper anterograde transport from the ER to the plasma membrane.

We have previously shown that primary human monocytes homozygous for the TLR1 602S allele are deficient in mediating responses to soluble TLR1 agonists PAM_3CSK_4 and *Mycobacterium tuberculosis* membrane fraction compared with monocytes that possess at least one TLR1 602I allele (17). This differential response between TLR1 variants is also observed in primary human monocyte-derived macrophages (Fig. 1*F*). However, a particulate TLR1 agonist, zymosan, induces equivalent secretion of IL-6 from macrophages of all TLR1 genotypes (Fig. 1*F*). Underhill *et al.* previously demonstrated that TLR2, the heterodimeric partner for TLR1, is rapidly recruited to the zymosan phagosome in murine macrophages (6). To determine if stimulation of TLR1 602S is mediated by similar phagosomal recruitment, primary human macrophages were stimulated with zymosan particles and intracellular TLR1 was visualized by confocal microscopy. As shown in Fig. 1*G*, both TLR1 602I and TLR1 602S localize to the zymosan phagosome. Overall, these results suggest that phagosomal recruitment enables the TLR1 602S variant to mediate responses to particulate agonists.

An Equivalent Serine Substitution of TLR6 Mimics the Trafficking Deficiency of TLR1 602S—Among human TLRs, TLR1 and TLR6 are most homologous, followed by TLR10. These three TLRs are tandemly arranged on chromosome 4 suggesting that they arose from gene duplication events. Similar to TLR1, TLR6 and TLR10 heterodimerize with TLR2 in response to microbial ligands (27, 28). Sequence alignment reveals that a conserved hydrophobic amino acid (TLR6 607I and TLR10 599L) resides at a homologous position to that of TLR1 602I (Fig. 2*A*). To determine if an equivalent serine substitution has a similar phenotype to TLR1 602S, surface expression of a TLR6 607S mutant and a TLR10 599S mutant was measured by flow cytometry. A similar point mutant was also generated in TLR2 (H606S). As shown in Fig. 2, *B* and *C*, TLR10 L599S and TLR2 H606S have unchanged surface expression, while an I to S mutation of amino acid position 606 in TLR6 abrogates plasma membrane receptor localization. Total expression of each mutant was verified by flow cytometry (supplemental Fig. S2). Taken together, these results show that the serine substitution of the TLR1 602S variant generates the same trafficking deficiency in TLR6 but not in more distantly related TLRs.

Trafficking of TLR1 Is Sensitive to Nonconserved Substitutions at Position 602—To characterize the amino acid side chains of residue 602 that are required for proper surface trafficking, additional point mutants of TLR1 were created by site-directed mutagenesis and examined for cell surface expression. Substi-

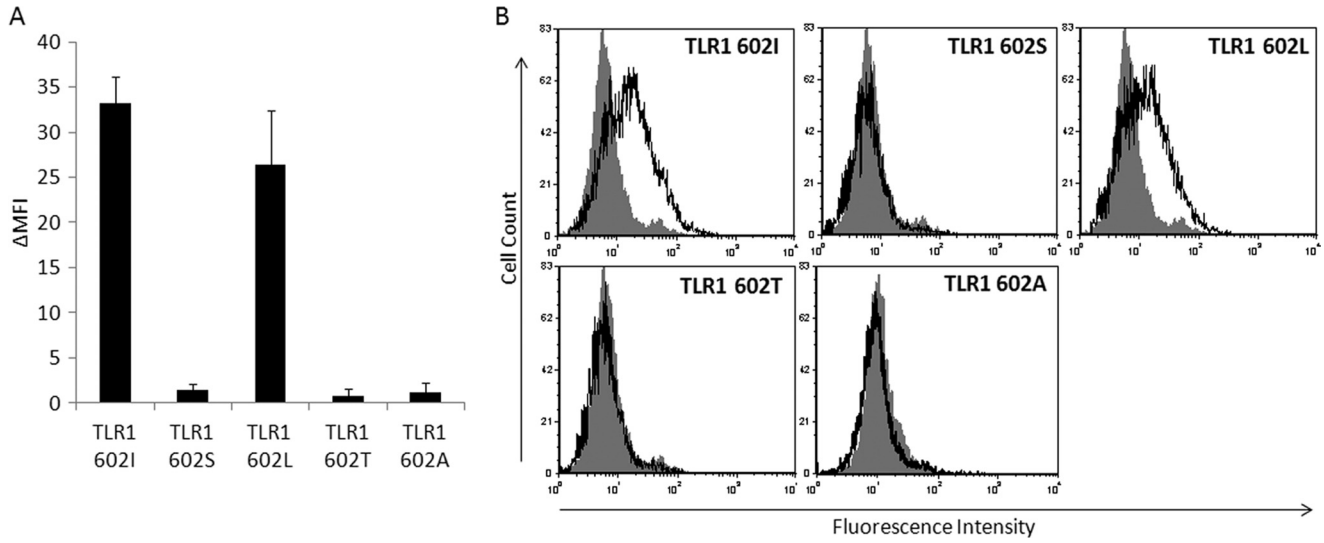

FIGURE 3. **Trafficking of TLR1 is sensitive to nonconserved substitutions at position 602.** *A*, HEK 293T cells were transfected with the indicated TLR1 point mutants and surface expression was assessed by flow cytometry. Error bars represent the standard deviation of triplicate transfections. *B*, representative histograms show surface TLR expression (*filled curve*; empty vector, *black line*; TLR).

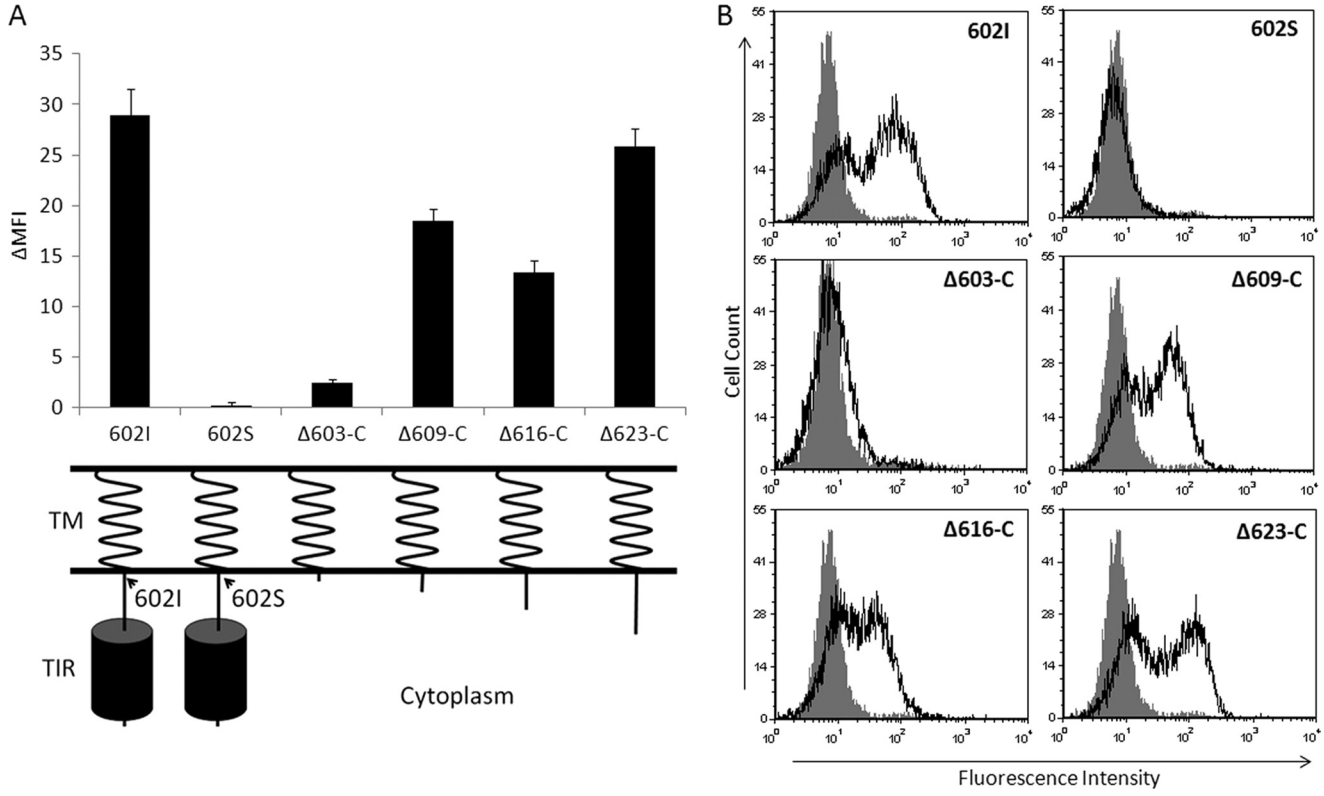

FIGURE 4. **A short cytoplasmic domain extending from position 602 is required for surface trafficking of TLR1.** *A*, HEK 293T cells were transfected in triplicate with the indicated TLR1 602I truncation mutants (shown schematically). Surface expression was assessed by flow cytometry. Bars represent the mean and standard deviation of triplicate transfections. *B*, representative histograms show surface TLR expression (*filled curve*; empty vector, *black line*; TLR).

tuting a conserved hydrophobic leucine residue at position 602 had little effect on TLR1 surface expression (Fig. 3, *A* and *B*). To determine whether the trafficking defect is a result of phosphorylation at 602S, we substituted a threonine residue at this position. TLR1 602T was found to be absent on the plasma membrane. Introduction of a neutral alanine residue at position 602 also resulted in a deficiency in surface trafficking, suggesting that proper TLR1 localization is generally sensitive to nonconserved mutations at this site (Fig. 3, *A* and *B*). All of the mutants exhibited equivalent levels of intracellular expression (supplemental Fig. S3).

A Short Cytoplasmic Domain Extending from Position 602 Is Required for Surface Trafficking of TLR1—To determine the minimum sequence required for TLR1 surface trafficking, truncation mutants of the receptor's cytoplasmic domain were generated (Fig. 4*A*). These mutants were expressed in HEK 293T cells and their surface trafficking was examined by flow cytometry. To be certain that loss of surface trafficking of the

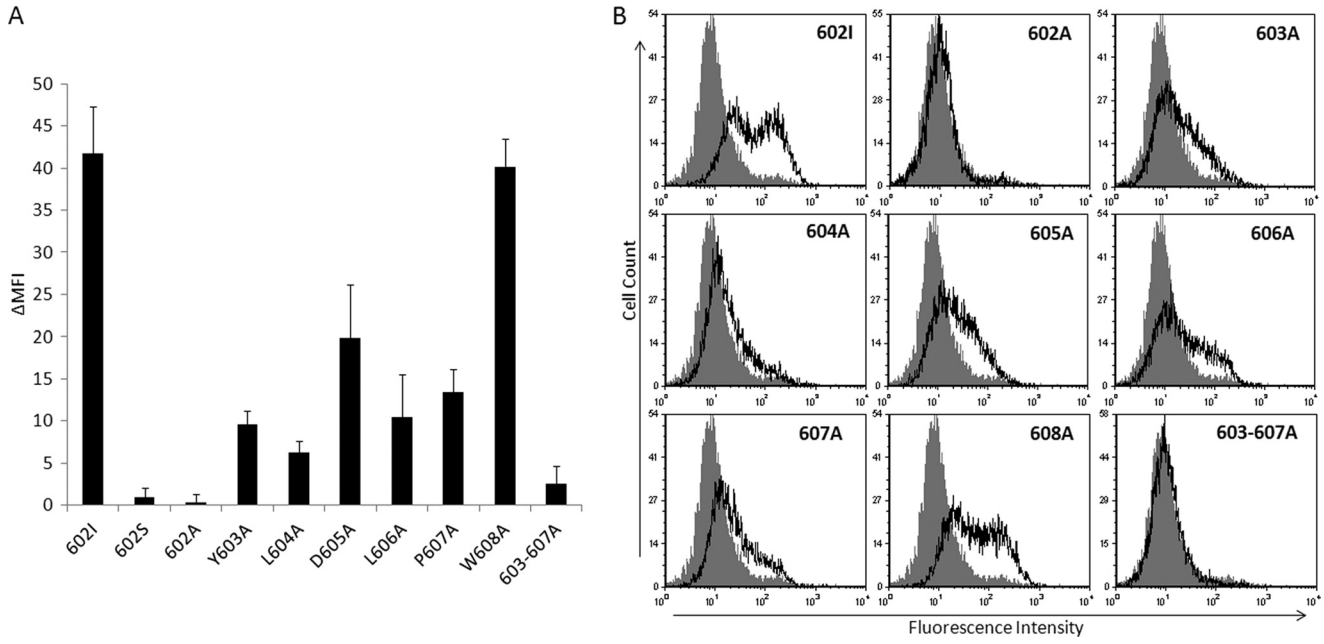

FIGURE 5. **Cytoplasmic residues 602–607 contribute to surface expression of TLR1.** *A*, FLAG-tagged alanine scan mutants of TLR1 were transfected into HEK 293T cells as indicated and surface expression was measured by flow cytometry. The average change in mean fluorescence intensity (ΔMFI) of triplicate transfections with standard deviations is shown. *B*, representative histograms show surface TLR expression (*filled curve*; empty vector, *black line*; TLR).

mutants was not the result of abrogated protein expression, intracellular receptor levels were verified for each mutant (supplemental Fig. S4). Truncation of the entire cytoplasmic domain of TLR1, beginning at position 603Y (TLR1 Δ603-C), resulted in the loss of cell surface trafficking. However, deletion of amino acids 609Y to the end of the C terminus (TLR1 Δ609-C) has little effect on plasma membrane localization compared with the full-length TLR1 602I variant. As expected, truncations which preserve larger portions of the cytoplasmic domain (TLR1 Δ616-C and TLR1 Δ623-C) also have little effect on trafficking. These data suggest that a short cytoplasmic region proximal to the transmembrane domain and extending from position 602–608 is required for surface trafficking of TLR1.

Cytoplasmic Residues 602–607 Contribute to Surface Expression of TLR1—To determine the importance of individual amino acids in this short cytoplasmic region, an alanine scan was performed across residues 602–608, and the ability of the resulting point mutants to traffic to the cell surface was assessed by flow cytometry. TLR1 602A exhibited an identical trafficking defect to that of TLR1 602S, while mutation of individual residues 603–607 to alanine resulted in significantly reduced surface expression (Fig. 5, *A* and *B*). Cell surface expression of TLR1 W608A was similar to that of TLR1 602I, suggesting that this residue is not necessary for efficient surface display. Replacement of all the residues from 603–607 with alanine (TLR1 603–607A) resulted in a fully deficient trafficking phenotype. Intracellular expression of all of the mutants was verified (supplemental Fig. S5). These results, in combination with the truncation mutants, highlight an essential, internal trafficking motif for localization of TLR1 to the plasma membrane.

The Transmembrane Domain and Adjacent Residues 602–607 of TLR1 Are Sufficient to Drive Surface Expression of TLR9—To define the minimal TLR1 trafficking motif sufficient for driving surface expression, we generated chimeras between TLR9, an endosomally localized receptor, and TLR1 (TLR9/1). Surface expression of these mutants in HEK 293T cells was assessed by flow cytometry. Intracellular expression of all the chimeric receptors was verified (supplemental Fig. S6). As expected, wildtype TLR9 is not expressed on the cell surface (Fig. 6, *A* and *B*). However, TLR9 can traffic to the cell surface when the transmembrane domain and entire intracellular region is replaced with that of TLR1 (TLR9/1 TM-Cyto). Neither the trafficking domain alone (TLR9/1 TF) nor the transmembrane domain alone (TLR9/1 TM) of TLR1 is sufficient to enable TLR9 to traffic to the cell surface. However, both domains of TLR1 together in the context of TLR9 (TLR9/1 TM-TF) enables surface expression. Importantly, surface expression of both TLR9/1 TM-Cyto and TLR9/1 TM-TF is lost upon introduction of the 602S mutation (Fig. 6). Together these results show that the transmembrane domain and trafficking motif of TLR1 is sufficient to drive surface expression of TLR9. Additionally, a chimeric receptor containing this minimally sufficient TLR1 trafficking domain retains the differential trafficking of the I602S polymorphism.

The ER Chaperone, PRAT4A, Positively Regulates TLR Surface Trafficking—Protein-associated-with-TLR4 (PRAT4A) was originally identified as a positive regulator of TLR4 cell surface expression and was later shown to play a similar role in TLR1 trafficking (12, 13). Given this, we hypothesized that the deficient trafficking phenotype of TLR1 602S may result from disrupted guidance from this chaperone. To examine this hypothesis, we measured the effects of PRAT4A expression on

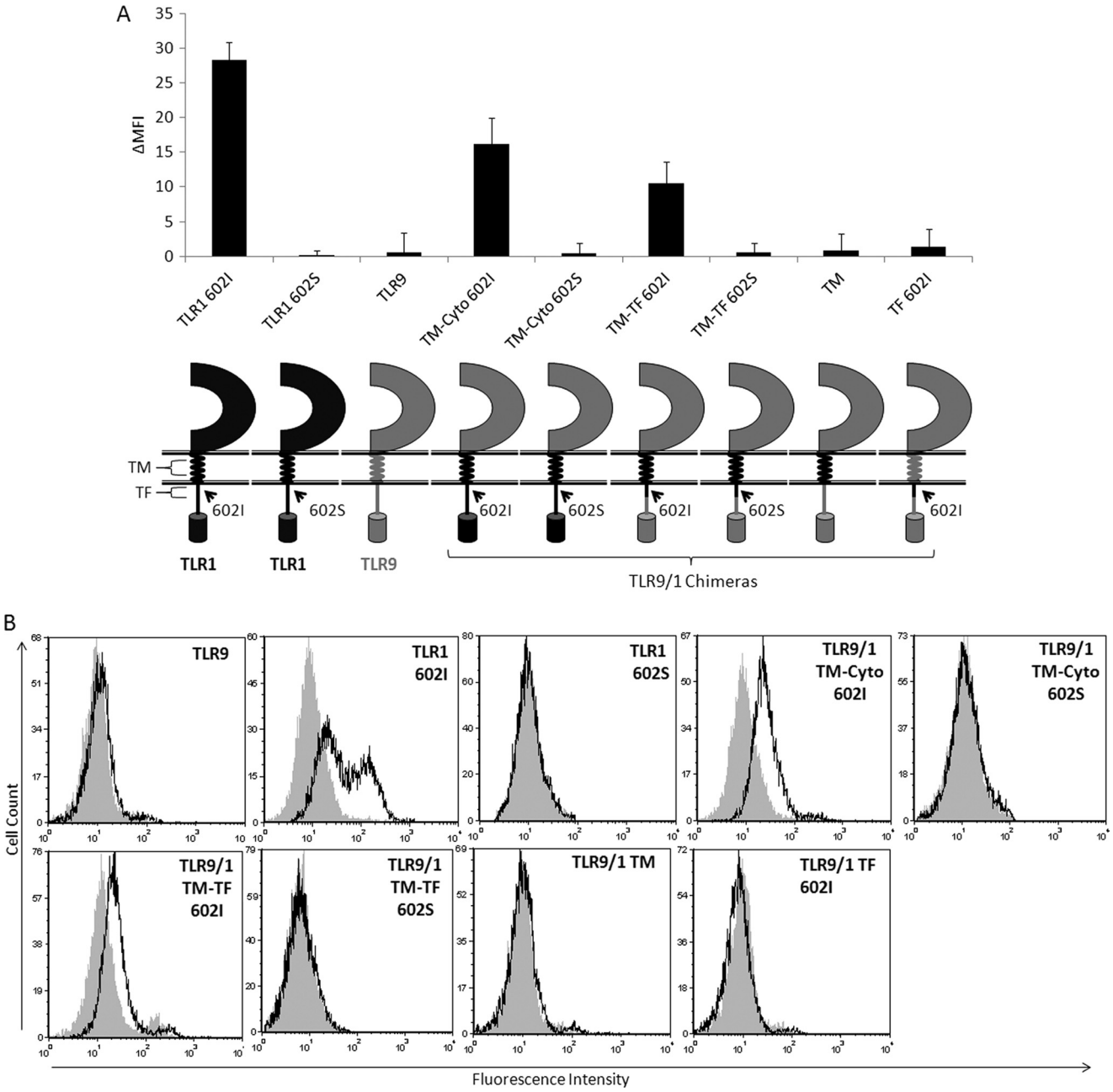

FIGURE 6. **The transmembrane domain and adjacent residues 602–607 of TLR1 are sufficient to drive surface expression of TLR9.** *A*, HEK 293T cells were transiently transfected with FLAG-tagged TLR9/1 chimeric receptors as indicated and surface expression was measured by flow cytometry. Schematic diagram of TLR9/1 chimeras in which regions of TLR9 (*gray*) are replaced by specific domains of TLR1 (*black*) are shown. Chimeras contain either the 602I or 602S polymorphism, as indicated (*TM*; transmembrane domain, *TF*; trafficking domain, *cyto*; cytoplasmic domain). Bars represent the mean and standard deviation of triplicate transfections. *B*, representative histograms show surface TLR expression (*filled curve*; empty vector, *black line*; TLR).

TLR1 surface trafficking. Gp96, another ER-resident TLR chaperone shown to cooperate with PRAT4A, was also examined (29). Overexpression of gp96 in HEK 293T cells did not affect surface trafficking of either TLR1 variant (Fig. 7*A*). However, increased levels of PRAT4A boosted plasma membrane expression of TLR1 602I and enabled recruitment of some TLR1 602S to the cell surface (Fig. 7, *A* and *B*).

We subsequently investigated the effects of PRAT4A and gp96 over-expression on other TLRs. Surface expression of both TLR2 and TLR4 were potently up-regulated by PRAT4A (Fig. 7, *A* and *B*). Interestingly, gp96 over-expression reduced TLR2 surface levels and slightly down-regulated TLR4 surface expression, but did not affect the other surface TLRs (data not shown). PRAT4A could not induce surface trafficking of TLRs known to localize intracellularly, as no significant increase in surface TLR3, TLR7, TLR8, or TLR9 was detected. Surface TLR3 appeared to be slightly up-regulated by gp96 overexpression (data not shown).

Because increased availability of PRAT4A overcomes the TLR1 602S trafficking deficiency, we hypothesized that the trafficking phenotype was linked to interruption of PRAT4A-TLR1 602S interaction. To assess this, both variants were transfected

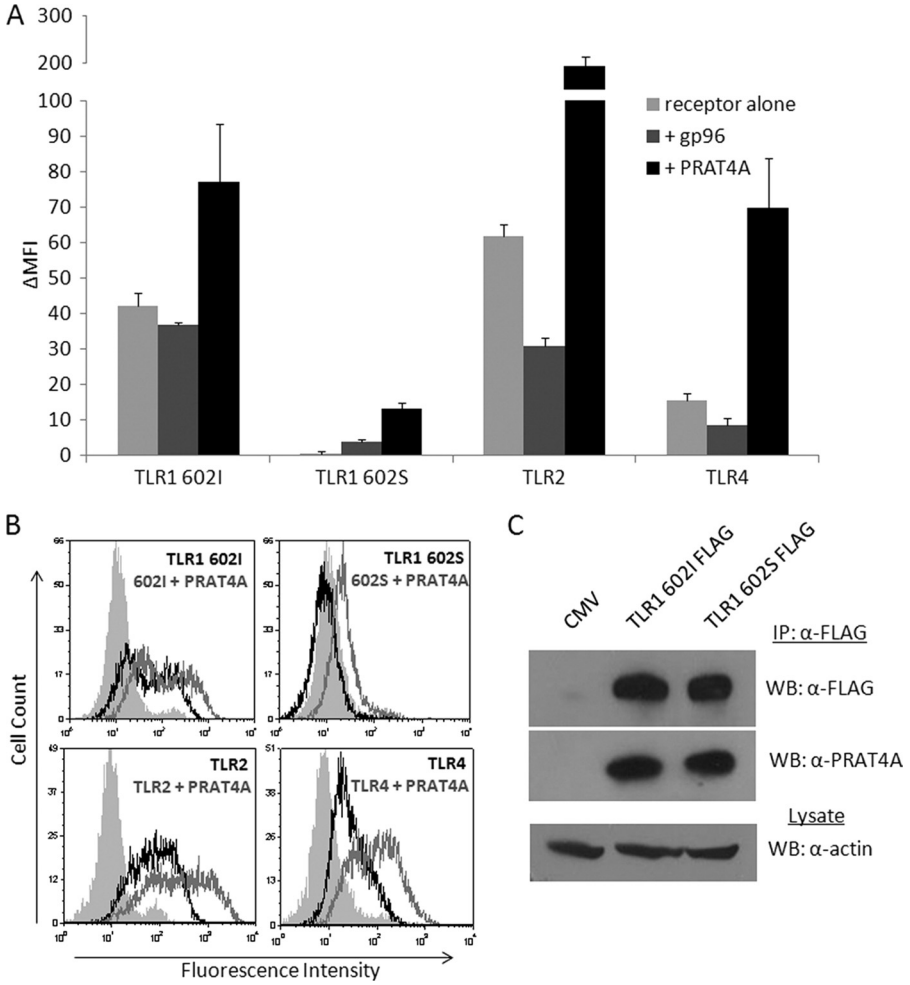

FIGURE 7. **The ER chaperone, PRAT4A, positively regulates TLR surface trafficking.** *A*, HEK 293T cells were co-transfected with TLR1 602I, TLR1 602S, TLR2, or TLR4 and the ER chaperones, gp96 or PRAT4A. Surface levels of TLRs were assessed by flow cytometry. The average change in mean fluorescence intensity (ΔMFI) and standard deviation of triplicate transfections are shown. *B*, representative histograms show surface TLR expression (*filled curve*; empty vector, *black line*; TLR alone, *gray line*; TLR + chaperone). *C*, HEK 293T cells were transfected with the indicated FLAG-tagged TLR1 variant and cell lysates were prepared. Anti-FLAG beads were used to pull down TLR1 and Western blots were probed for levels of immunoprecipitated TLR1 as well as co-immunoprecipitated PRAT4A. Actin levels in cell lysates support equivalent protein loading.

into HEK 293T cells and co-immunoprecipitations were performed to assess efficiency of protein-protein interaction. Pull-down of FLAG-tagged TLR1 revealed, however, that both 602I and 602S receptor variants interact equally with PRAT4A (Fig. 7*C*).

PRAT4B Negatively Regulates TLR1 Surface Expression—PRAT4B, a second ER chaperone which shares 44% identity with PRAT4A, has also been identified as a regulator of TLR4 surface expression (12). To assess the role of PRAT4B in trafficking of the TLR1 variants as well as other TLRs, we performed similar experiments to those using PRAT4A. Fig. 8*A* and *B* shows flow cytometric analyses of TLR surface expression where receptors have been co-transfected with a PRAT4B vector. Overexpression of PRAT4B potently reduced trafficking of TLR1 602I, as well as TLR2, TLR4, TLR6, and TLR10 (data not shown). PRAT4B may be regulating TLR surface trafficking by affecting intracellular protein expression, as over-expression of the chaperone greatly reduced intracellular levels of all ten TLRs (supplemental Fig. S7). Because of the negative regulatory role PRAT4B appears to play in TLR1 surface trafficking, we hypothesized that the deficiency in TLR1 602S localization was due to an enhanced interaction with this chaperone. To test this, we performed co-immunoprecipitations examining the level of protein-protein interaction between PRAT4B and the TLR1 variants. HEK 293T cells were transfected with PRAT4B-FLAG and either HA-tagged TLR1 602I, TLR1 602S-HA, or TLR4-HA. TLR4 was readily detected by anti-HA immunoblotting of lysates immunoprecipitated with anti-FLAG beads, confirming the previously described interaction between PRAT4B and TLR4 (Fig. 8*C*). Similarly, TLR1 602S also co-immunoprecipitated with PRATB, while TLR1 602I was almost undetectable on the anti-HA immunoblot. These results suggest that TLR1 602S exhibits a stronger interaction with PRAT4B than does TLR1 602I (Fig. 8*C*).

Knock-down of PRAT4B Rescues the TLR1 602S Phenotype—To verify the role of PRAT4B in the regulation of TLR1 trafficking, we used shRNA vectors to knock-down PRAT4B and assessed surface localization of both TLR1 variants. Immunoblotting experiments confirmed the efficacy of two anti-PRAT4B shRNAs to knock-down PRAT4B following transient transfection (Fig. 9*C*) and in stable cell lines (Fig. 9*D*). As previously

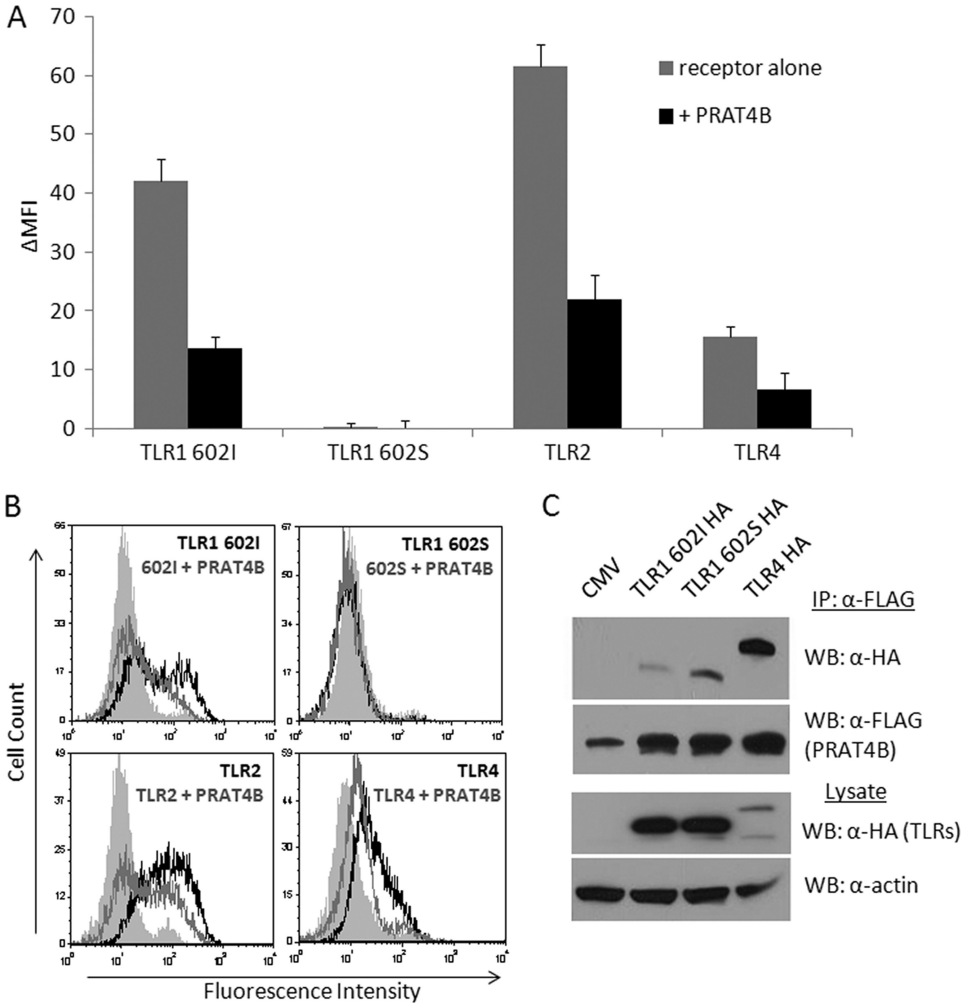

FIGURE 8. **PRAT4B negatively regulates TLR surface expression.** *A*, HEK 293T cells were co-transfected with TLR1 602I, TLR1 602S, TLR2, or TLR4 and the ER chaperone, PRAT4B, as indicated. Surface levels of TLRs were assessed by flow cytometry. The average change in mean fluorescence intensity (ΔMFI) and standard deviation of triplicate transfections are shown. *B*, representative histograms show surface TLR expression (*filled curve*; empty vector, *black line*; TLR alone, *gray line*; TLR + PRAT4B). *C*, HEK 293T cells were co-transfected with either TLR1 602I-HA, TLR1 602S-HA, or TLR4-HA, and PRAT4B-FLAG. Cell lysates were incubated with anti-FLAG beads to pull down PRAT4B and probed for levels of co-immunoprecipitated TLRs. Lysates confirm equivalent protein loading (actin) and equivalent production of both TLR1 variants.

shown by Konno *et al.*, knock-down of PRAT4B reduced surface expression of TLR4 (supplemental Fig. S8) (12). Conversely, surface levels of TLR1 602S were induced upon co-transfection with the anti-PRAT4B shRNAs, while TLR1 602I trafficking remained unaffected (Fig. 9, *A* and *B* and supplemental Fig. S9). Scrambled control shRNAs failed to affect trafficking of either TLR1 variant or TLR4. Taken together, these results confirm that PRAT4B positively regulates TLR4 surface expression but plays a negatively regulatory role in the surface expression of TLR1. Additionally, PRAT4B has a greater negative effect on TLR1 602S compared with TLR1 602I perhaps as a result of a stronger physical interaction with the former variant.

Interferon-γ Up-regulates TLR1 Surface Trafficking and PRAT4A Expression in Primary Human Monocytes—It is well established that IFNγ primes macrophages for TLR-mediated responses and many recent studies have linked this phenotype to integration of the IFNγ and TLR signaling pathways (30). In addition, IFNγ stimulates surface expression of TLR1, TLR2, and TLR4 in human peripheral blood cells (31–33). We assessed the effect of IFNγ on surface expression of different TLR1 602 variants using primary human peripheral blood monocytes either lacking or possessing a TLR1 602I allele. As seen in Fig. 10, *A* and *B*, surface trafficking of TLR1 is induced in individuals regardless of the TLR1 602 genotype. Interestingly, surface TLR1 from 602S/S homozygotes reached levels equivalent to that of resting TLR1 602I monocytes after 36 h of IFNγ stimulation. In addition to induction of surface TLR1 expression, flow cytometric analysis of permeabilized cells revealed an increase in intracellular TLR1, and qRTPCR analysis showed an early induction of TLR1 mRNA (Fig. 10*C*). To determine if surface-expressed TLR1 602S is capable of signaling, primary human monocytes were stimulated with PAM_3CSK_4 in the presence or absence of IFNγ for 24 h, followed by measurement of TNFα release (Fig. 10*D*). As expected, PAM_3CSK_4 was unable to stimulate TNFα secretion from 602S homozygotes, but efficiently activated TLR1 602I monocytes. Co-treatment with IFNγ strongly induced TNFα secretion from all monocytes, with levels of TNFα production from TLR1 602S homozygotes nearing that of TLR1 602I cells. These data show that IFNγ rescues cell surface trafficking of TLR1 602S and

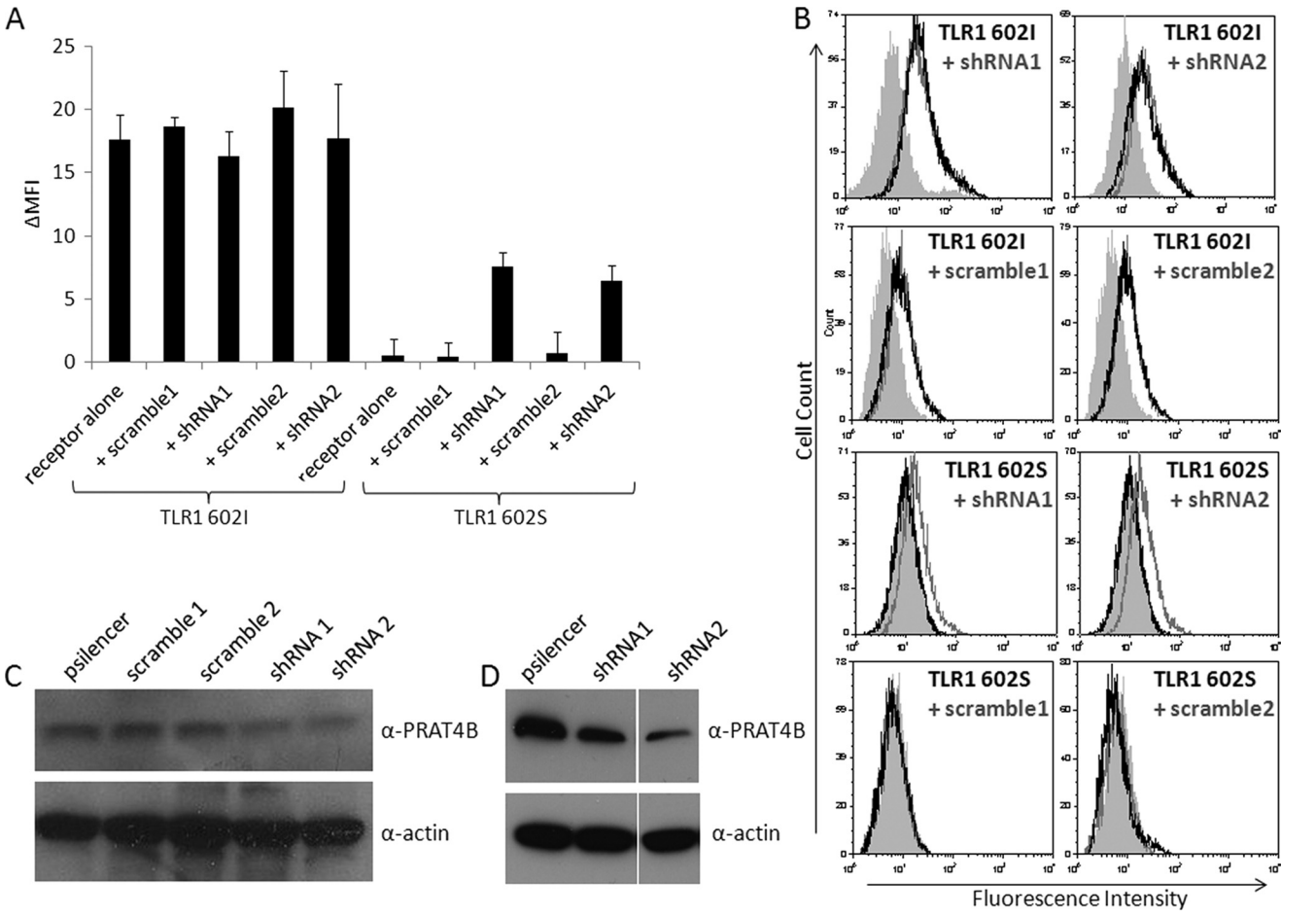

FIGURE 9. **Knockdown of PRAT4B rescues the TLR1 602S phenotype.** *A*, TLR1 602I or TLR1 602S was transiently transfected into HEK 293T along with shRNA expression vectors targeting PRAT4B (shRNA1, shRNA2) or scrambled shRNA expression vectors (scramble1, scramble2) as indicated. TLR1 surface expression was measured by flow cytometry. Bars represent the average change in mean fluorescence intensity (ΔMFI) and standard deviation of triplicate transfections. *B*, representative histograms show surface TLR expression (*filled curve*; empty vector, *black line*; TLR alone, *gray line*; TLR + shRNA). *C*, cell lysates from similar transient transfections were collected and probed by immunoblot for PRAT4B to assess shRNA-mediated knockdown of the chaperone. Actin levels support equivalent protein loading. *D*, HEK 293 cells were transfected with empty vector (psilencer) or anti-PRAT4B shRNAs and stable batch-derived lines were selected. PRAT4B levels from cell lysates were assessed by immunoblot.

enables the receptor to transduce inflammatory signals to soluble agonists.

Given that both PRAT4A over-expression and IFNγ stimulation are able to rescue the TLR1 602S phenotype, we examined the effects of IFNγ on PRAT4A protein expression. Primary human monocytes were stimulated with increasing concentrations of IFNγ for 24 h, followed by a Western blot of PRAT4A in cell lysates. IFNγ stimulation greatly increased PRAT4A protein levels in a dose dependent manner (Fig. 10*E*). A similar analysis revealed no effect on expression of PRAT4B by IFNγ (Fig. 10*F*). Together, these results suggest that IFNγ rescues TLR1 602S surface trafficking through induction of PRAT4A expression.

DISCUSSION

The subcellular distribution of each TLR is orchestrated in a way to ensure that ligand accessibility leads to cell activation. Trafficking of TLRs is dynamic, reflecting the fact that microbial agonists are diverse in terms of their accessibility and physical nature. Soluble microbial agonists in the extracellular milieu activate immune cells through cell surface receptor signaling, while particulate agonists are internalized within endosomal or lysosomal compartments where intracellular TLRs are poised to sense released nucleic acids. Importantly, the subcellular localization of TLRs also directs specific innate immune signaling pathways and responses. For example, upon LPS stimulation at the cell surface, TLR4 is recruited to lipid rafts where signaling pathways that drive classic proinflammatory responses are engaged. However, upon endocytosis, TLR4 recruits a different set of adaptors and signaling pathways that mediate production of type 1 interferons (34, 35). Similar to TLR4, TLR2 is recruited to cholesterol-rich lipid rafts along with TLR1 or TLR6 and associated co-receptors, in response to soluble agonists (36). These TLRs also co-localize in phagosomes where they are thought to sample phagosomal cargo (6, 37, 38). These membrane segregation events enable efficient and coordinated signaling responses by concentrating agonists, receptors, and even signaling adaptors to defined membrane compartments (reviewed in Refs. 4, 5).

We previously reported that a common polymorphism in TLR1, I602S, prevents trafficking of TLR1 to the cell surface

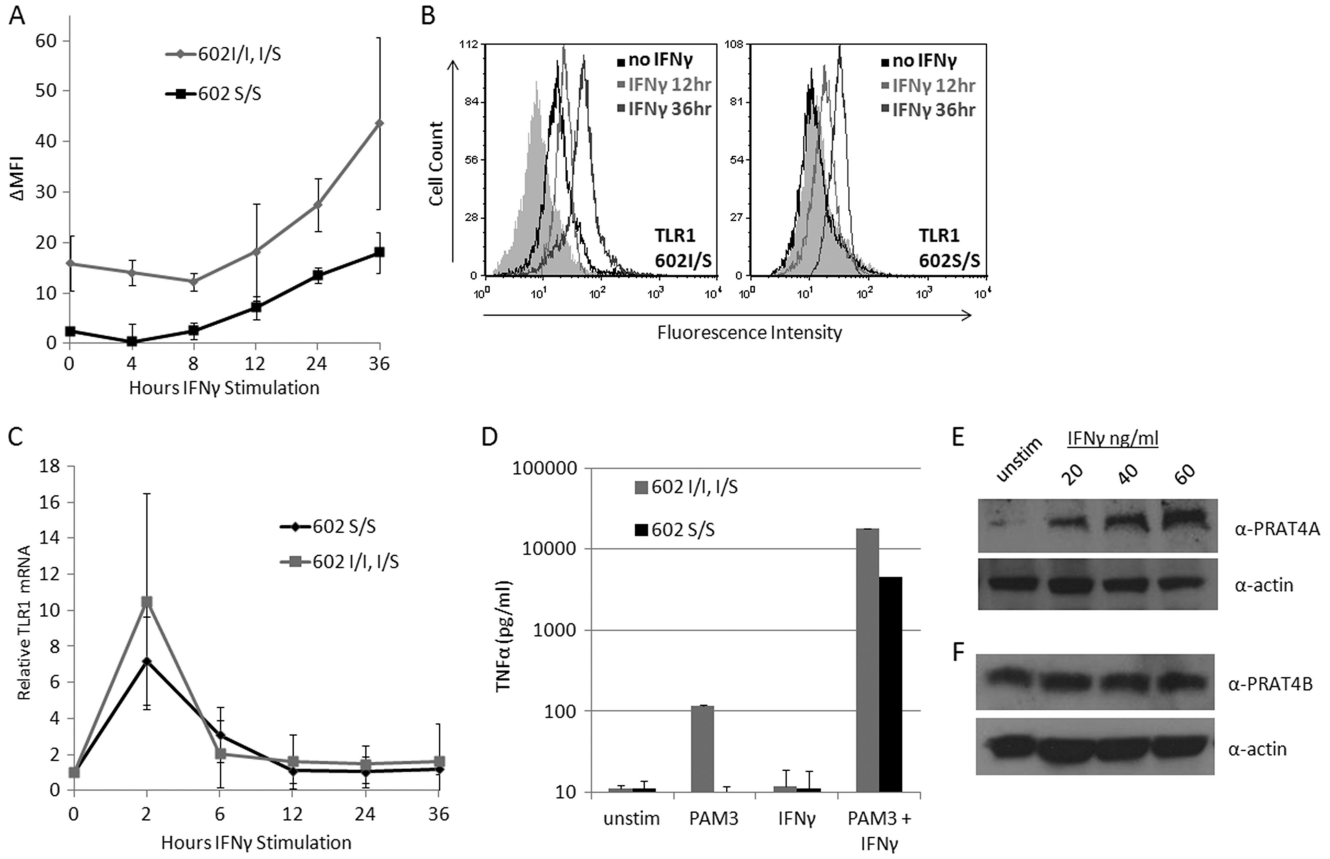

FIGURE 10. **Interferon-γ up-regulates TLR1 surface trafficking and PRAT4A expression in primary human monocytes.** *A*, human monocytes from either TLR1 602S homozygotes or TLR1 602I individuals were stimulated with IFNγ (20 ng/ml) over a 36-hour time-course. Surface expression of TLR1 was measured by flow cytometry. *Error bars* represent the standard deviation from three different donors. *B*, representative histograms show monocyte surface TLR expression (*filled curve*; isotype control, *black line*; no IFNγ, *silver line*; 12 hr IFNγ, *gray line*; 36 hr IFNγ). *C*, primary human monocyte mRNA was collected over the same time-course, and relative levels of TLR1 transcripts were measured by qRTPCR (compared to GAPDH mRNA). Values were normalized to that of unstimulated monocytes. *Error bars* represent the standard deviation from three different donors. *D*, to assess the signaling competency of IFNγ-rescued TLR1 602S, monocytes were simultaneously co-incubated with the TLR1/2 agonist, PAM_3CSK_4 (50 ng/ml), in the presence or absence of IFNγ (20 ng/ml) for 24 hours. TNFα secretion was measured by ELISA. *Error bars* represent the standard deviation from three different donors. Human monocytes were isolated from blood donors and stimulated with increasing concentrations of interferon-γ as indicated. Cell lysates were probed for PRAT4A (*E*) or PRAT4B (*F*).

and is associated with greatly diminished responses to soluble TLR1 agonists (17). Here, we show that the cell surface trafficking defect persists despite high TLR1 602S expression in the ER. Surprisingly, we have found that the TLR1 602S variant is able to traffic to zymosan containing phagosomes. Accordingly, macrophages homozygous for TLR1 602S, which are unable to respond to soluble TLR1 agonists, retain the ability to respond to particulate zymosan. The mechanism which underlies TLR1 602S trafficking to phagosomes remains unknown but could involve direct trafficking from the ER thus bypassing the need for surface expression.

We have found that amino acid position 602 is part of a short, cytoplasmic region of TLR1, which is located proximal to the transmembrane domain, and is required but not sufficient for surface localization. Mutagenesis of any amino acid in this cytoplasmic trafficking motif reduces surface trafficking, but none are as deleterious as I602S. Similar to TLR1, TLR3 contains a sequence within the region between the transmembrane and TIR domains that directs proper localization (7). However, at 23 amino acids in length, the trafficking region of TLR3 is much larger and, unlike TLR1, is sufficient on its own to direct TLR3 to endosomal compartments. In contrast to TLR3, the transmembrane domain alone is sufficient to target TLR4, TLR7, or TLR9 to their distinct subcellular locations (7–9). Using chimeric receptors, we have found that both the short 6 amino acid cytoplasmic trafficking domain and the transmembrane domain of TLR1 are required and sufficient to drive surface receptor expression. Importantly, the I602S mutation ablates surface trafficking even in the context of a chimeric TLR. Taken together, TLR1 is unique in its requirement for both cytoplasmic and transmembrane trafficking domains for proper localization.

PRAT4A is an ER-resident chaperone that is required for surface trafficking of TLR4/MD2 and cellular responses to LPS (12). In PRAT4A-deficient mice, cell surface expression of TLR1, TLR2, and TLR4, and endosomal localization of TLR9 are completely disrupted (13). As expected, macrophages from these mice exhibit abrogated cytokine responses to respective agonists of these TLRs, as well as ligands for TLR6 and TLR7, but not TLR3. We have observed that overexpression of PRAT4A enhances surface expression of several TLRs including TLR1 602I, TLR2, and TLR4. Surprisingly, we found that overexpression of PRAT4A rescues surface trafficking of TLR1 602S, suggesting that PRAT4A is a limiting component in the trafficking of this receptor variant to the plasma membrane. The fact that TLR1 602S retains the ability to localize to

endosomal compartments suggests that the involvement of PRAT4A in this trafficking event is distinct from that of surface trafficking. In support of this idea, it was reported that proper endosomal localization of TLR9 requires an interaction with a gp96-PRAT4A complex (29) and it has been suggested that the way in which endosomal TLR9 utilizes PRAT4A may be different from that of cell surface TLRs (10).

PRAT4B, a homolog of PRAT4A, has also been reported to promote TLR4 surface expression (12). Unexpectedly, both PRAT4B overexpression and knock-down studies show that this chaperone acts to inhibit TLR1 surface expression. PRAT4B interacts more strongly with TLR1 602S than with TLR1 602I, suggesting that this chaperone could be responsible for the differential surface trafficking of these two receptor variants. How the serine polymorphism promotes a preferential interaction between PRAT4B and TLR1 602S is unclear but could involve conformational changes and/or additional, as yet unidentified, proteins in the immunoprecipitated receptor complexes. Interestingly, PRAT4B expression has been associated with increased susceptibility to sepsis, Kawasaki disease, and general infection (39, 40). Thus, the fact that overexpression of PRAT4B inhibits TLR trafficking may have *in vivo* relevance for a number of inflammatory diseases.

We and others (17, 19–24) have shown that the TLR1 602S allele is protective against mycobacterial infection, including leprosy, leprosy reversal reaction, and tuberculosis. Most strikingly, an unbiased genome-wide array of 1500 individuals identified TLR1 602S ($OR = 0.31$, $p < 0.001$) as one of two alleles that conferred the greatest protection against leprosy, the other being an allele of MHCII (19). IFNγ, a key component of immunity to mycobacteria and other intracellular pathogens, is released by helper T cells and potently increases microbicidal functions of macrophages, including phagosome maturation and oxidative burst. We have found that surface expression of both TLR1 602I and 602S is enhanced in primary human monocytes stimulated with IFNγ. Importantly, this induction of surface TLR1 enables monocytes from TLR1 602S homozygotes to mediate responses to soluble TLR1 agonists. This enhancement is associated with an increase in mRNA and intracellular TLR1 protein. In addition, the up-regulation of TLR1 by IFNγ correlates with a large increase in intracellular PRAT4A protein expression, suggesting that induction of this positively regulating TLR chaperone may play an important role in monocyte/macrophage priming by IFNγ. The basis for the protective role of TLR1 602S in the context of mycobacterial disease is the subject of ongoing studies in our laboratory.

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