Effects of Single Nucleotide Polymorphisms on Toll-like Receptor 3 Activity and Expression in Cultured Cells

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Key words: TLR3, single nucleotide polymorphisms, reporter assay, cellular localization, ectodomain, molecular modeling, dominant negative.

Recognition of double-stranded RNA by Toll-like Receptor 3 (TLR3) will increase the production of cytokines and chemokines through transcriptional activation by the NF-κB protein. Over 136 single nucleotide polymorphisms (SNPs) in TLR3 have been identified in the human population. Of these, four alter the sequence of the TLR3 protein. Molecular modeling suggests that two of the SNPs, N284I and L412F, could affect the packing of the leucine-rich repeating units in TLR3. Notably, L412F is reported to be present in 20% of the population and is higher in the asthmatic population. To examine whether the four SNPs affect TLR3 function, each were cloned and tested for their ability to activate the expression of TLR3-dependent reporter constructs. SNP N284I was nearly completely defective for activating reporter activity and L412F was reduced in activity. These two SNPs did not obviously affect the level of TLR3 expression or their intracellular location in vesicles. However, N284I and L412F were underrepresented on the cell surface, as determined by flow cytometry analysis and were not efficiently secreted into the culture medium when expressed as the soluble ectodomain. They were also reduced in their ability to act in a dominant negative fashion on the wild-type TLR3 allele. These observations suggest that the N284I and L412F affect the activities of TLR3 needed for proper signaling.

Innate immunity is responsible for the initial detection and response to pathogen infection. The process can be generally divided into several major steps, starting with the recognition of pathogen molecules by cellular receptors, the activation of signaling cascades, resulting in changes in gene expression, and production of effector and co-stimulatory molecules. The degree of activation of the innate immunity process can not only dictate the outcome of infection, but also the degree of T- and B-lymphocyte activation in the adaptive immunity pathways. Receptors that recognize the microbial components are localized in the plasma membrane, in intracellular vesicles, or in the cytoplasm and include the Toll-like receptors (TLRs). At least eleven TLRs have been identified in the mammalian genome.

TLR3 recognizes poly(I:C), a synthetic double-stranded (ds) RNA analog, as well as viral dsRNA, presumably formed during viral infection. TLR3 binding to cognate ligands modulates downstream cytokine and chemokine production through the activation of the transcription factor NF-κB, which translocates to the nucleus to modulate gene expression. A role for TLR3 in viral infection has been suggested based on the demonstration that TLR3 knockout mice were unable to mount a full response to cytomegalovirus infection, perhaps by contributing to cytopathic T cell response after the initial infection. TLR3 is localized primarily in intracellular vesicles, although some cell-surface expression is observed in human embryonic kidney cells.

The structures of the TLR3 ectodomain (ECD) produced in insect cells have been elucidated by X-ray crystallography by two groups and the
structure of the protein produced in human cells has been analyzed using electron microscopy in conjunction with 3D image reconstruction.\textsuperscript{12} The TLR3 ECD is shaped as a solenoid horseshoe, characteristic of proteins with multiple leucine-rich repeats (LRRs).\textsuperscript{10-13} Furthermore, at least two glycosylation sites have been shown to be important for TLR3 activity in transfected HEK 293T cells\textsuperscript{12, 14}.

Studies of bacterial infection of \textit{Drosophila} lines homozygous for a wild chromosome 2 documented that SNPs in a number of molecules associated with microbial recognition and response are linked with the susceptibility to bacterial infection.\textsuperscript{15} These studies demonstrate that genetic makeup of individuals will affect the ability to survive infections. Specifically, genetic variations in the TLRs have been correlated with disease susceptibility, including sepsis, immunodeficiencies, atherosclerosis and asthma.\textsuperscript{1-3}

For TLR3, polymorphisms at nt 2593C/T, 2642C/A, 2690 A/G have been reported to be linked to Type 1 diabetes in South African populations.\textsuperscript{16} These studies are primarily correlative. In this work, we examined the distribution of SNPs in the human population and determined whether four known human SNPs that we predicted to cause changes in the TLR3 protein molecule will affect TLR3 structure and function in cell-based assays.

Experimental Procedures

\textit{Analysis of the evolutionary conservation of the SNPs} - Fourteen sequence homologs to human TLR3 were identified using the PipeAlign package\textsuperscript{17}, which identifies a cluster of homologs ranging from fish to mammals. The sequences were subsequently aligned using M-Coffee.\textsuperscript{18} The resulting alignment was manually refined and used to direct the alignment of the corresponding coding sequences using PAL2NAL.\textsuperscript{19} A maximum likelihood tree for the amino acid data set was determined using the PHYML program.\textsuperscript{20} Site-specific variation rates of the TLR3 amino acid sequences were calculated using the Bayesian method as implemented in Rate4site.\textsuperscript{21} Evolutionary conservation scores and functional/structural importance of each residue were assessed using Conseq.\textsuperscript{22}

Site-specific rate variations at the nucleotide level were calculated using maximum likelihood as implemented in the codeml program of the PAML package,\textsuperscript{23} which estimates synonymous versus non-synonymous substitution rates (Ka/Ks, or ω) of the aligned nucleotide data set. Bayesian posterior probabilities of each codon being positively selected were assessed using either a simple or a complex model: M03, single ω ratio modeled with three categories of sites, or M08, single ratio with ten categories of sites with a discrete β distribution.\textsuperscript{24}

\textit{Molecular modeling} - The model of the TLR3 ectodomain was based on the crystal structure determined by Bell et al.\textsuperscript{11} \textit{(PDB code 2A0Z)}. The transmembrane helix was predicted using Phobius (http://phobius.cgb.ki.se/). The cytosolic portion was modeled based on sequence homology with a similar domain from human TLR1 whose crystal structure was determined (PDB code 1FYV, alignment not shown). The structural fragments were subsequently assembled to form a single model with the Quanta molecular modeling package (version 2000, Accelrys).

\textit{Plasmid construction} - The wild-type (WT) TLR3 plasmid was the one previously described in Sun et al.\textsuperscript{12} Plasmids containing SNPs were made by site-directed mutagenesis using oligonucleotides annealed to the target sequence and the QuikChange kit (Stratagene Inc., San Diego CA). Sequences of the oligonucleotides will be made available upon request. All of the constructs containing SNPs were sequenced in their entirety to confirm that no unintended mutations were made.

The sequence of the cyan fluorescent protein and yellow fluorescent protein were fused to the last coding codon of the WT TLR3 to generate plasmids pTLR3-CFP and pTLR3-YFP constructs, respectively. Briefly, the CFP and YFP DNA sequence was amplified via PCR from the plasmids PAmCyan1-C1 and pZsYellow1-C1 (Clontech Inc., Mountain View, CA) using primers to introduce an \textit{EcoR}I site on the 5’ end and a Xhol site on the 3’ end of each CFP and YFP. The primer sequences will be made available upon request. The resulting fragments were cloned in frame at the C-terminal end of TLR3 in pcDNA using existing restriction sites. The TLR3-CFP and TLR3-YFP plasmids

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were then used as a template for site directed mutagenesis to create the N284I-CFP, L412F-CFP, N284I-YFP and L412F-YFP SNP constructs. Alternately N284I constructs were created using the internal restriction sites NheI and AflII to swap the WT section of the TLR3 sequence with the same region of untagged N284I-TLR3.

TLR3 ΔTIR mutant was generated by amplifying TLR3 DNA sequence corresponding to 756 aa from the N-terminus and cloned using Nhe I and XhoI restriction sites. The resultant clone was used as template for generating N284I ΔTIR and L412F ΔTIR.

Cell-based assays - HEK 293T cells were harvested from an actively growing culture and plated in Costar White 96-well plates at 4.4 x10^4/ml for transfection. When the cells were ~60 to 80% confluent, they were transfected with a mixture of the Lipofectamine 2000 reagent (Invitrogen Inc., San Diego, CA) and plasmids pNF-kB-Luc (30 ng, Stratagene Inc., La Jolla, CA), pFL-TLR3 (15 ng), and phRL-TK (5 ng, Promega Corp., Madison, WI) that, respectively, code for the firefly luciferase reporter, full-length WT and/or TLR3s with mutations in the ectodomain, and the Renilla luciferase transfection control. The cells were incubated for 24 h to allow expression from the plasmids. Poly(I:C) (2.5 µg/mL, unless stated otherwise) was then added to appropriate sets of transfected cells to induce TLR3-dependent NF-κB activity. After another incubation for 24 h, the cells were harvested using the Dual Glo Luciferase Assay System reagents (Promega). Luminescence was quantified using the FLUOstar OPTIMA Plate Reader (BMG Labtech, Inc).

Western blots - 293T cells were transiently transfected with WT, mutant TLR3 or with control pcDNA as described above. Unless stated otherwise, the cells were lysed 36 h after transfection using passive lysis buffer (Promega Inc.) and sonicated to degrade chromosomal DNA. Equal amounts of proteins from each sample, as determined by staining with Coomassie Blue, were separated on NuPAGE 4-12% bis-tris gel (Invitrogen) and blotted onto PVDF membrane. The anti-TLR3 antibody IMG315A (Imgenex Inc.) was used in western blots since the mAb used in the FACS analysis was unable to detect denatured TLR3. The blots were developed with peroxidase conjugated secondary antibodies and ECL-plus western blotting detection system (Amersham Biosciences).

FACS analysis - FACS analyses were performed with 293T cells grown in 6-well collagen-coated plates (BD Biosciences) at a concentration of 2x10^6 cells/well. The cells were transfected with 1 µg of the appropriate plasmids using Lipofectamine 2000 (Invitrogen Inc.). For staining of cell surface-associated TLR3, the cells were harvested 24 h after transfection and washed twice with ice-cold FACS buffer (1X PBS containing 10 mM Phosphate, 150 mM NaCl, pH 7.4; + 3% fetal bovine serum + 0.04% sodium azide) before suspension at ~2x10^7 cells/mL in FACS buffer. The cells were stained for 30 minutes at 4°C with 1 µg PE-labeled anti-human TLR3 mAb (clone TLR3.7, purchased from eBioscience, San Diego, CA) or a negative control mouse IgG1 control antibody. The antibodies were added to aliquots of the cells in 96 well plates and incubated for 30 min on ice in the dark. The cells were washed twice with FACS buffer to remove unbound antibody, then resuspended in FACS buffer. Viaprobe (BD Biosciences) was added to the cultures to exclude dead cells. The cells were transferred to the appropriate tubes and analyzed using a FACS Calibur machine (BD Biosciences). For intracellular staining, cells transfected for 24 h were fixed and permeabilized by incubation in Cytofix/Cytoperm™ buffer (BD Biosciences) for 40 minutes at 4°C. The cells were then washed and stained in Perm/Wash™ buffer (BD Biosciences) as above. After staining, the cells were washed twice in Perm/Wash buffer then resuspended in FACS buffer for acquisition. Data analysis was performed using FCS Express (De Novo Software, Thornhill, Ontario, Canada).

Microscopy – Cells were plated on LabTek II CC2 treated chamber slides (Nunc Intl., Naperville, IL) and transfected with plasmids in Lipofectamine2000 (Invitrogen, Carlsbad, CA). CFP, YFP, TLR3-CFP/YFP, N284I-CFP/YFP, and L412F-CFP/YFP were all visualized 24 hours post transfection with a Zeiss Axioplan fluorescent microscope. TLR3, N284I and L412F constructs not tagged with a fluorescent protein were also visualized via immunofluorescence. Briefly, cells transfected for 24 h were removed from the incubator and rinsed with PBS before being fixed...
with 4% formaldehyde in PBS and permeabilized with 0.1% Triton X-100. After this treatment cells were incubated at room temperature, protected from light, for at least 1 h in anti-TLR3 FITC conjugated monoclonal antibody (Imgenex, San Diego, CA). Cells were washed and counterstained with Hoechst 33342 dye (Molecular Probes, Eugene, OR) before mounting in a buffered glycerol aqueous mounting medium. Cells were then imaged with a Zeiss Axioplan fluorescent microscope.

RESULTS AND DISCUSSION

SNPs of TLR3 - Sequencing of genomic DNA from individuals of different ethnic groups has provided insight into the relationship between gene polymorphisms and disease. The recent elucidation of the X-ray structure of the human TLR3 ectodomain allowed the ability to model whether or not mutations in TLR3 may cause structural changes. At least 136 single nucleotide polymorphisms (SNPs) exist in the TLR3 gene.\(^{25}\) Seven SNPs are within protein-coding sequences, but only four (N284I, Y307D, L412F, S737T) resulted in amino acid substitutions (Fig. 1A). The four mutations are found in independent genetic backgrounds and are not linked. Furthermore, L412F is a prevalent allele, represented in ~20% of the population sampled (Fig. 1A).\(^{25}\) Also, N284I and L412F affect residues that are conserved in the TLR3 orthologs from human to fish whereas the remaining Y307D and S737T affect residues conserved only in mammals (Fig. 1B).

Analysis of the functional significance of the SNPs - Relative functional significance of amino acid residues can often be determined by combining the information for sequence conservation and protein structures in a method called evolutionary tracing.\(^ {26}\) We performed such analysis using the seven SNPs that are located in the TLR3 protein coding sequence. In the TLR3 amino acid data set, evolutionary tracing using Conseq identified N284, L412 and A888 as the most conserved residues (Fig. 1A).

Using the Bayesian method, we calculated the \(\omega\) values of the seven SNP sites under two rate variation models. The result was obtained using the single ratio model plus three categories of sites to model the \(\omega\) ratios among site (M03, Fig 1A) or single ratio plus ten categories of sites with a discrete \(\beta\) distribution (M08, data not shown). The M03 model (\(\text{lnL}=-25,334\)) appears to fit the data set better than the M08 model (\(\text{lnL}=-25,339\)). A \(\chi^2\) test indicates a significant difference between the two models with \(p < 0.001\). However, both models indicate that the relative substitution rates of L204, N284, L412 and A888 were the lowest and approached zero. Y307, F459 and S737 were less conserved and had higher substitution rates. However, none of the rates were >1, suggesting that the residues were all neutrally selected and not under the influence of positive selection.

Since the four non-synonymous SNP residues were not selected as the sites for functional innovation during evolution, mutations at these sites in the human population tend to be either deleterious or neutral depending on the extent of sequence conservation. N284 and L412 have the highest purifying selection pressure, and, therefore, mutations at these sites should be disruptive on the structure and function of the protein.

Frequencies of the SNPs in human populations – Genotype information to determine the distribution of the four SNP alleles in human ancestry was extracted from the InnatelImmunity (IIPGA) and Hapmap websites\(^ {27,28}\) and formatted for Convert\(^ {29}\) (Fig. 1C).

The demographics for the sampling were as follows: The European and African American data from the HapMap project was taken from families, meaning parent:child sampling groups. Therefore, the effective population size of the sample is smaller and may bias the allele frequencies in these populations. The African group is composed of 90 individuals from Sub-Saharan Africa from the HapMap data and 24 African American individuals from the IIPGA data. The data for the Asian population came from random unrelated individuals, half Chinese (from Beijing area) and half Japanese (from Tokyo) and are from the Hapman site. The European population consists of European Americans (90 from Hapmap and 23 from IIPGA). Some gaps in the data such as Y307D in the Asian population were assumed to be missing data rather than assumed to be WT.

One potentially important feature revealed by these studies is that the L412F allele frequency is 41% in the asthmatic population tested\(^ {28}\) (Fig. 1C).
This is significantly higher than the allele frequency of European American (26.97%) and the Asian (30.1%) populations and the overall population (20%). While this result suggests a possible link to asthma, we note that the number of asthmatics analyzed is relatively low (n =18) (Fig. 1C).

Molecular modeling of the SNPs – The interesting distributions of the TLR3 SNPs, especially the L412F SNP led us to ask whether the resultant changes in the TLR3 protein sequence would affect the function of TLR3. We first modeled the four SNPs for possible effects on TLR3 structure. Three SNPs, N284I, Y307F and L412F, exist within the TLR3 ectodomain, whose structure is known, while S737T lies outside of the ectodomain, whose structure is unknown. Therefore, we modeled the entire structure of TLR3 in order to examine the possible effects of all four SNPs. A helical conformation was assigned to the transmembrane domain and the cytosolic domain model was based on sequence homology with the closely related cytosolic domain of the TLR1 molecule (Fig. 2A).

N284 is at the end of the type VI β-turn regions (consensus motif position N10) of LRR 10, on the concave surface of the TLR3 ECD, facing the inner portion of the solenoid. The side chain of this asparagine forms five hydrogen bonds to amide and carbonyl groups of nearby residues from the same and neighboring LRR motif.10,11 We predicted that a change to an isoleucine at this position could partially destabilize the solenoid structure (Fig. 2B).

Y307 is an exposed residue located at the concave surface of the ectodomain stacked with Y283 in the exposed inner surface of the solenoid. A change to an aspartate should alter the positions of the side chain of R331, although R331, would remain as surface-exposed residues (Fig. 2B).

L412 is near the concave surface of the TLR3 ectodomain, but it is buried within the solenoid. Mutant L412F, like N284I, could alter the hydrophobic interaction environment by the more bulky substitution. We predict that this substitution would destabilize the solenoid structure and may also affect potential glycosylation of neighboring residue N413, which was observed to have N-acetylglucosamines attached in the crystal structure of TLR3 ectodomain.10,11

Lastly, S737 lies within an exposed portion of the model for the TIR domain and is not predicted to interact with other residues of the TIR domain. These modeling results, along with the observations that N284 and L412 are invariant in all species (Fig. 1B), suggest that these two SNPs could affect TLR3 activity.

Effects of SNPs on TLR3 activity - To determine whether four SNPs that alter TLR3 amino acids affect function, we used a cell-based assay to assess TLR3-dependent activation of a firefly luciferase reporter driven by an NF-κB-dependent promoter.12 The HEK 293T cells do not have detectable endogenous levels of TLR39,12, therefore the activity is dependent on the transfected TLR3. Furthermore, the addition of poly(I:C) is required to activate firefly luciferase activity, demonstrating that the assay responds to exogenous ligand (Fig. 3A). All results are measured as a ratio of the firefly luciferase to a transfection control, the Renilla luciferase expressed from the herpesvirus thymidine kinase promoter and plotted as fold induction in the presence of poly(I:C) over untreated control. For clarity, the results will be stated as “reporter activity”. We routinely obtained between four to ten-fold reporter activity with WT TLR3 when the cells were induced with poly(I:C). While there is some variation that could be due to the cell growth, the signal above background is easily discernable in all experiments.

Y307D and S737T had the same level of activity as the WT (Fig. 3A), L412F had reduced reporter activity by approximately 30% and N284I reduced activity to background (Fig. 3A). To determine whether the reduced activity observed were statistically significant, P values were determined for the difference in activity of SNP mutants in comparison to WT TLR3. P values less than 0.05 are considered statistically significant. The P value of the activities of N284I and L412F relative to WT were both less than 0.001. Y307D and S737T had P values of 0.5 and 0.2 respectively (Fig. 3A). Similar results are reproducible in over a dozen independent experiments.

To confirm the activity of the TLR3 containing the SNPs, a reporter construct driven by the IFN-stimulated response element (ISRE) was additionally
tested (Fig. 3B). In comparison to the WT, N284I (P value 0.01) was almost comparable to the background level and L412F (P value 0.03) had reduced activity. Y307D (P value 0.3) and S737T (P value 0.2) had activity not significantly different from WT. Hence, the relative activities of the four SNPs to each other and to the WT control are consistent. This result was reproducible in three independent experiments. Thus, the cell-based assay results confirmed the modeling prediction that perturbations to residues buried within the TLR3 solenoid imposed by L412F and N284I had the most detrimental effects on TLR3 activity.

**TLR3 expression** - The SNP mutations could affect the expression/stability of the TLRs or have a more specific effect. To examine whether the SNPs affected protein expression, a western blot of transfected HEK cells was performed (Fig. 3C). A band (denoted by an asterisk) that is nonspecifically recognized by the mAb used serves as an internal loading control. The levels and migration of the four SNP mutant proteins were comparable to WT (Fig. 3C). Since the migration of TLR3 is affected by the state of glycosylation, this result suggests that the SNPs do not grossly alter the levels or modification states of TLR3.

We examined whether the amount of plasmid expressing N284I and L412F would affect activity when compared with WT TLR3. Plasmid amounts from 1 to 50 ng increased the activity of WT TLR3 (Fig. 4A). However, with all plasmid concentrations tested, N284I and L412F were lower in activity in comparison to the comparable amount of WT TLR3. Similarly, increasing poly(I:C) concentration did not restore the activity of L412F or N284I relative to WT (Fig. 4B). Even with 50 μg/ml polyIC, L412F was still defective compared to WT (data not shown). These results demonstrate that while N284I is almost completely inactive L412F could have reduced affinity to the ligand. Furthermore, the ligand-binding surface of TLR3 was recently mapped to a charged surface of TLR3 to LRR 17 to 20, which is C-terminal to L412F.

**Effects of SNPs on TLR3 localization** – In 293T cells, TLR3 is primarily localized in intracellular vesicles, with some expression on the cell surface. To determine whether N284I or L412F are grossly affected in their cellular localization in comparison to WT, we performed in situ localization studies with permeabilized cells using a fluorescently-labeled mAb IMG-325C (Fig. 5). Two types of staining were observed, a hazy fluorescence around the periphery of the cell and cytoplasmic punctate forms. Some hazy fluorescence was also observed with 293T cells transfected with pcDNA, suggesting that at least some of the fluorescence is due to nonspecific binding of the mAb (Fig. 5). The cell surface fluorescence from TLR3 is thus difficult to quantify by microscopic analysis and will be examined in more detail later. As for the intracellular staining, the punctate forms are likely acidic vesicles, as their staining partially overlap with the staining pattern of acidic membranes stained by Lysotracker (Supplemental Fig. 1). Similar punctate forms were observed with N284I and L412F (Fig. 5), suggesting that the gross intracellular locations of the two SNP version of TLR3 are not changed. However, we cannot rule out a more subtle change in the intracellular localization of the SNP mutants. It is also possible that transfections could have differentially affected the localization of endocytic vesicles as well.

To confirm the location of the WT, N284I, and L412F by a method that does not require the fixation of the cells, we fused the yellow fluorescent protein (YFP) or the cyan fluorescent protein (CFP) to the C-terminal end of WT TLR3, N284I, and L412F. The fusion proteins were expressed, in transfected cells, as detected by Western blots (data not shown). However, to our disappointment, the fusion of WT TLR3 to either fluorescent protein resulted in poor activation of NF-κB expression after poly(I:C) (Supplemental Fig. 2A), suggesting that the fusion proteins altered the ability to respond properly to poly(I:C). To ensure that the fusion proteins do retain some activities of TLR3, we tested them for the ability to suppress the activation of NF-κB expression by the WT TLR3 in an assessment for dominant negative effect. When co-transfected at a six molar excess of the WT TLR3, both the YFP and the CFP fusions could interfere with WT TLR3 activity (Supplemental Fig. 2B). We thus proceeded to compare the intracellular localization of the fusions to TLR3 and to the two SNP mutants. Again, punctate spots were observed in all three samples, confirming that the SNP mutations N284I and
L412F are not grossly altering the intracellular distribution of TLR3 (Supplemental Fig. 2C).

**Functional analysis of SNP ECDs** – To further elucidate how N284I and L412F affected function, we attempted to express them as a soluble ectodomain in HEK293-T cells. The plan was to secrete the ECDs into the medium for purification and subsequent biochemical characterizations. As controls, the WT ECD and the Y307D ECD were expressed and purified. However, ECDs from N284I and L412F were not found to secrete to the same degree as WT (Fig. 6A). This was a surprising result given that the full-length version of these mutants expressed to similar levels as WT TLR3 (Fig. 3C). The mutations did not render the ECDs unstable, as the intracellular levels of the ECDs from WT, Y307D, N284I and L412F were comparable at different time points (Fig. 6A). These results were reproducible in two independent experiments. This indicates that the mutations are not detectably affecting the translation and/or stability of the proteins, but that SNPs N284I and L412F affect the normal processing/secretion of TLR3.

**Cell surface expression of the SNPs** - Given that the signals for proteins expressed on the cell surface are usually diffuse in the in situ staining experiments and difficult to visualize, we performed fluorescence-activated cell sorting (FACS) to quantitatively monitor full-length TLR3 and the four SNP alleles in intact cells (Fig. 6B). In all, more than 30,000 cells were analyzed in each measurement and the results were performed four independent times and were consistent in all experiments. The cell surface expression of the mutants Y307D and S737T were similar to that of WT and significantly above that of cells transfected with N284I and L412F which had lower surface expression (Fig. 6B top). To ensure that this lack of surface localization is not due to lack of expression of protein itself, we did intracellular FACS analysis and observed that all the SNPs had similar levels of expression as the WT (Fig. 6B bottom). Western analysis of total cell extract shown in Fig 3C, also confirmed that the protein expression of all the SNPs were similar to WT.

While it is not clear how the cell surface expression of TLR3 could influence TLR3 activity, the observation that the two SNPs are localized differently in the FACS analysis and are affected in their ability to secrete suggests that the cell surface expression of N284I and L412F may be related to signaling events downstream. One caveat of these results is that biochemical analysis of the N284I and L412F ECDs will be difficult, given that they cannot be produced in a form convenient for purification.

**Dominant negative effects of SNPs** – To further explore how N284I and L412F can affect TLR3 activity, we tested them for their ability to act as a dominant negative to WT TLR3 (Fig. 7A). Ranjith-Kumar et al.\textsuperscript{31} has documented that the dominant negative activity is useful to genetically analyze TLR3-TLR3 interactions. We used a TLR3ΔTIR (missing residues 756 to 904 of the TIR domain) as a control because it was previously shown to be a dominant negative of WT TLR3\textsuperscript{33}. As expected, ΔTIR reduced the activity of the WT TLR3 to 20% when transfected at two molar excess of WT TLR3. A six molar excess of TLR3ΔTIR reduced the activity of WT to 10%.

Since L412F retained ~70% of the ability of WT to induce NF-κB luciferase, it would be difficult to discern a potential dominant negative effect. This problem does not apply to N284I and we tested its presence at 2, 4, or 6 molar excess to WT TLR3 (Fig. 7A). In these experiments, all cells were transfected with the same amount of DNA, using the empty vector, pcDNA to adjust the total DNA concentration. At a two or six-fold molar excess of N284I to WT TLR3, robust WT TLR3 activity was observed, indicating that N284I cannot act as a dominant negative suppressor of TLR3 activity (Fig. 7A).

To confirm that N284I had lost the ability to interact with other copies of TLR3, we constructed a ΔTIR version of N284I (Fig. 7B). L412F was also tested in this context since the ΔTIR versions is incapable of activating NF-κB reporter activity. N284IΔTIR was expressed at levels comparable to ΔTIR-WT, as determined by western blots (Fig. 7B, inset). However, unlike ΔTIR-WT, N284IΔTIR lost the ability to act in a dominant negative manner (Fig. 7B), corroborating the results in Fig. 7A. L412FΔTIR was also reduced in its ability to act as a dominant negative, although a small reduction of
WT TLR3 activity was observed at six molar excess of L412F TIR. TLR3 and other TLRs likely act as dimers that undergo a conformational change(s) to form a signaling competent complex. The ligand-binding surface of TLR3 was recently mapped to an asparagines-rich surface of TLR3 that spans residues N515 to N541. Therefore, we do not expect that N284 and L412 will directly impact ligand binding. Instead, likely mechanism for the dominant negativity is due to the formation of inactive dimers by WT and a mutant TLR3. Since N284I and L412F are affected in their ability to act as dominant negatives, these SNP mutations may, directly or indirectly, affect the ability to form dimers with WT TLR3.

_N284I and L412F ECDs can modulate TLR3 activity_ - Since the N284I and L412F ECDs are not efficiently secreted into the medium, we examined whether they affect WT TLR3 activity in the NF-κB reporter assay. As expected, none of the ECDs were able to activate reporter gene expression since these constructs lack the transmembrane and the intracellular domain (ICD) (Fig. 8A). When co-expressed in HEK293T cells along with WT TLR3, we observed that the ECDs of WT, N284I, and L412F increased TLR3 activity in a concentration-dependent manner (Fig. 8B). P values were calculated for the increase in activity and were less than 0.05 for WT and SNP ECDs when present at 6X amounts. This activity of the ECDs is in strong contrast to the full-length N284I and L412F, which are reduced for activity and did not stimulate the activity of a second copy of WT TLR3. Furthermore, since the WT, N284I and L412F ECDs all had similar stimulatory effects on the co-transfected full-length TLR3, the stimulatory effect is not caused by the SNP mutations.

Given that the WT ECD was secreted better than the N284I and L412F ECDs, we examined the induction of TLR3 activity as a function of the culture medium (Fig. 8C). A stimulatory effect was observed whether or not the medium into which ECDs were secreted was removed prior to induction by poly(I:C). P values were less than 0.05 in all cases except for WT ECD in fresh medium, which was 0.053. We note that the increase in activity was found only upon expression of ectodomain of TLR3 lacking the membrane anchor region. One possible explanation for these results is that the ectodomain may interact with a cellular factor that can modulate TLR3 activity.

_Final Comments_. We have characterized the properties of four SNPs that affect the amino acid sequence of TLR3. SNPs Y307D and S737T are localized in two relatively less conserved residues and did not affect TLR3 activity. In contrast, N284 and L412 are residues that are absolutely conserved in all species from mammals to fish and molecular modeling suggests that N284I and L412F should affect the packing of the solenoid-shaped ECD of TLR3. In terms of function, N284I is nearly completely defective for TLR3 activity while L412F is only partially defective.

Based on the inability to normally secrete the TLR3 ECDs with the N284I and L412F mutations, and the decreased cell-surface abundance of these TLR3 proteins by FACS analysis, we speculate that cell surface expression of TLR3 may be required for activation of downstream signaling. Funami et al. and Nishiya et al. recently demonstrated that the intracellular localization of TLR3 in bone marrow-derived macrophages requires a 23 amino acid sequence (E727 to D749) in the linker region between the transmembrane domain and intracellular TIR domain. Our results extend the requirements for cell surface expression of TLR3 to include residues in the central portion of the TLR3 ECD. It is currently unknown whether TLR3 gains access to pathogen ligands. Our observation that there is a correlation between cell surface expression and the activation of downstream gene expression could have direct implications for the mechanism of ligand recognition.

At this point, the biochemical assays for TLR3 are not sufficiently advanced to determine whether N284I or L412F are affecting ligand binding, adaptor protein binding and/or potential dimerization of TLR3 molecules. However, we do have genetic results from the cell-based assays to speculate on possible effects of the SNPs on the mechanism of action. Based on the dominant negative analysis, we speculate that N284I and L412F affect the normal dimerization of TLR3, perhaps as a consequence of the improper targeting of the N284I and L412F proteins. In addition, the TLR3 ECDs can stimulate WT TLR3 activity, perhaps by titrating out a
negative factor. This activity is not affected by the SNPs.

If these results for N284I and L412F activity in cell-based assays could be extrapolated to a person, these two SNPs would likely not affect the normal function of the WT copy of TLR3 in a person heterozygous for the alleles. In this vein, the L412F allele is particularly interesting since it is over-represented in individuals with asthma (Fig. 1C). This correlation remains to be examined further and may indicate a linkage between TLR3 and human disease. It is possible, however, an alteration in the activity of one molecule could lead to an advantage in other circumstances, as seen with mutations in the HIV co-receptor, CCR5.

ACKNOWLEDGEMENTS

We thank members of the Kao and Mbow labs especially Jarrat Jordan for constructive comments and suggestions throughout this work. JX thanks the Welch Foundation for financial support.

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FIGURE LEGENDS

Fig. 1. Analyses of single nucleotide polymorphisms that affect the human TLR3 polypeptide-coding sequence. A) Properties of the SNPs within the coding regions of TLR3. The arrow denotes a change in the WT amino acid residue. This information is compiled from the data from Lazarus et al.25 from the website: http://snpper_chip.org/bio/show-gene/TLR3. Site specific rate variations of the TLR3 amino acid sequences were calculated using the Bayesian method as implemented in Rate4site.21 Evolutionary conservation scores and functional/structural importance of each residue were assessed using Conseq.22 B) Alignments of the sequences encompassing the four SNPs that altered TLR3 amino acid sequence. The numbers in parentheses denote the position of the first residue in the sequence shown. The four SNP residues that are the subjects of this study are in bold letters. C) The distribution of the four SNP alleles that change the TLR3 polypeptide sequence and their frequency in human populations of different ancestry and the database of asthmatic individuals. SNP informations were retrieved from the Innate Immunity PGA, NHLBI Program in Genomic Applications. URL: http://innateimmunity.net/PGAs/InnateImmunity/TLR3/, [12/2006].

Fig. 2. A molecular model of TLR3 and the local environment of the four SNP residues. A) A structure model of the full-length human TLR3 protein based on the structure of Bell et al.31, with the positions of the four SNP mutations highlighted. B) Local structures of the four SNP residues (in magenta) in the WT (left) and mutated forms (right). The yellow dashed lines in N284 represent hydrogen bonds and the dashed line in Y307 represents an aromatic ring stacking interaction between Y283 and Y307.

Fig. 3. Activities and expression of the WT and SNP alleles of TLR3. Results of TLR3-dependent reporter expression from NF-kB (A) or ISRE promoters (B) in transfected HEK 293T cells. Each bar represents the average and standard error from an assay with six replicates. The same trends are observed in four independent assays, each with six replicates. The identities of the TLR3 alleles transfected into cells in the presence of 2.5 µg/ml poly(I:C) (PIC) are shown below the horizontal axis. Statistical significance for the difference in activity was analyzed by determining the P value and is given above each bar of the SNP mutants. C) Western blot analysis of the expression of WT TLR3 and the four SNPs. Cell lysates were harvested 24 h after transfection of HEK 293T cells. The western blot was probed with monoclonal antibody IMG-315A and detected using the ECL reagent. A strongly reacting band unrelated to TLR3 (denoted with an asterisk) is recognized by this antibody in our blots and serves as an internal control for protein loading.

Fig. 4. Additional characterizations of the effects of SNPs on TLR3 activity. A) Effects of different plasmid concentrations on the TLR3 activation of luciferase activity in the HEK 293T cells. B) Effects of increasing poly(I:C) (PIC) concentration on the ability of the two SNP mutants to activate luciferase reporter expression. For both panels, the error bars represent one standard error in the mean of the measurements.

Fig. 5. In situ localization of TLR3. Typical images of the localizations of WT TLR3 and SNP mutants N284I and L412F. HEK 293T cells transfected with the appropriate plasmid were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with FITC-labeled monoclonal antibody IMG-315A and Hoechst 33342 to visualize the nuclei. The DNA used to transfect the cells are shown to the left of the cell images.

Fig. 6. SNPs N284I and L412F affect the secretion of the TLR3 ECD and cell surface expression of full-length TLR3s. A) Western blot analysis of the secreted ECDs and total cell lysates from select SNPs and WT TLR3 at different time points. B) Analysis of TLR3 and the SNP mutants in transfected 293T cells in permeabilized and non-permeabilized cells. The upper panel shows histogram overlays of permeabilized cells stained with PE-labeled isotype control antibody (filled in grey) or anti-TLR3 antibody (open black). The lower panel shows staining of non-permeabilized cells. The percent positive is shown in the upper right of each panel. The percent positive was calculated by subtracting the isotype control from the
sample on a channel by channel basis. The monoclonal antibody recognizing TLR3, TLR3.7 is from eBioSciences Inc. (San Diego, CA). The signals from a nonspecific, but matched, immunoglobulin isotype of TLR3.7 serves as a background control.

**Fig. 7.** Ability of N284I and L412F to act as dominant negatives. A) Analysis of the full-length N284I to inhibit the activity of WT TLR3. The WT TLR3 plasmid was present at 15 ng per transfection. The molar excess of the plasmid expressing N284I or the known dominant negative, TLR3ΔTIR relative to WT TLRs are noted below the bars. B) The ability of truncated versions of the SNP alleles, N284I-ΔTIR and L412F-ΔTIR, to act as dominant negatives. The WT TLR3 plasmid was present at 15 ng per transfection and the molar ratios of the competitors are shown as “Fold Comp”. Inset: Western blot probed with monoclonal antibody IMG-315A and detected using the ECL reagent demonstrating the expression of the ΔTIR versions of the WT TLR3 and the two SNP alleles. For both panels, the error bars represent one standard error in the mean of the measurements.

**Fig. 8.** ECDs from WT TLR3 and the SNPs can stimulate TLR3 activity. A) A control experiment demonstrating that the ECDs from WT TLR3 and the four SNPs are unable to activate NF-κB reporter gene expression. B) Co-expression of the ECDs from WT and SNP mutants can increase the activity of the full-length TLR3. ECD is present at twofold or sixfold the molar ratio of the full-length TLR3 as indicated. C) Effects of the ECDs on TLR3 activity in either fresh DMEM medium or the same medium that contains excreted ECDs. For both panels, the error bars represent one standard error in the mean of the measurements. The significance of increase in activity is analyzed by calculating the P value and is given on top of each bar.

**Supplemental Fig. 1.** Localization of GFP fusions of TLR3, N284I and L412F along with control GFP. HEK293T cells were transfected with fusions constructs indicated on the top of the images. The images of the live cells were visualized with filters detecting TLR3 fused to GFP or the lysotracker dye that stains acidic organelles. WT TLR3 and those with SNP mutations are localized to intracellular compartments stained with Lysotracker, suggest that these proteins are localized on the acidic compartments. Unfused GFP control that should be present throughout the cytoplasm was visualized as a control for the specific localization of TLR3-GFP fusion.

**Supplemental Fig. 2.** A) Effects of cyan fluorescent protein and yellow fluorescent protein fusions at the C-terminus of TLR3 on the ability to activate reporter expression in transfected 293T cells. B) Effects of the fusion proteins to dominantly negative suppress wt TLR3 activity. The competitor plasmid is present at a six-fold molar excess of the wt TLR3 plasmid. C) Localization of YFP or CFP fusions of TLR3, N284I and L412F. HEK293T cells were transfected with fusions constructs indicated at the upper right corner of the images. Fluorescence in the live cells was detected using the FITC filter (left column) and without a filter (brightfield, right column). TLR3-YFP and TLR3-CFP show distinct localization to an intracellular compartment. The SNP fusions show a similar localization pattern.
Fig. 1.

### Table A

| Nuc. change (codon position) | Amino acid change | Minor allele frequency | Rate variant score | Conserv. score |
|-----------------------------|-------------------|------------------------|-------------------|---------------|
| G612A (3rd)                 | L204              | 0.5                    | 0.136             | 4             |
| A851T (2nd)                 | M284 → I         | 0.42                   | -1.191            | 9             |
| T9190 (1st)                 | Y207 → D         | 0.056                  | 0.610             | 3             |
| C1224T (1st)                | L412 → F         | 0.056                  | -0.956            | 9             |
| T1277C (3rd)                | F459              | N.D.                   | 2.246             | 1             |
| T2208A (1st)                | T737 → T         | 0.70                   | 0.433             | 3             |
| C2664T (3rd)                | A888              | 0.6                    | -1.094            | 9             |

### Table B

| Species | (202) SYEKLH (305) LIDHNK (410) KHEHL (723) NHVNH |
|---------|--------------------------------------------------|
| Homo    | SYEKLH | LIDHNK | KHEHL | NHVNH |
| Pan     | SYEKLH | LIDHEK | KHEHL | NHVNH |
| Joes    | SYEKLH | LIDHNK | KHEHL | NHVNH |
| Kattus  | SYEKLH | LIDHNK | LHEHL | NHVNH |
| Mas     | SYEKLH | LIDHNK | LHEHL | NHVNH |
| Thasigu | SYEKLH | LIDHNK | KHEHL | NHVNH |
| Tettrodon | SYEKLH | LIDHNK | KHEHL | NHVNH |
| Parichn | SYEKLH | LIDHNK | KHEHL | NHVNH |
| Carcassius | SYEKLH | LIDHNK | KHEHL | NHVNH |
| Branchios | SYEKLH | LIDHNK | KHEHL | NHVNH |
| Oncorhynchus | SYEKLH | LIDHNK | KHEHL | NHVNH |
| Mascheni | SYEKLH | LIDHNK | KHEHL | NHVNH |
| Icthusus | SYEKLH | LIDHNK | KHEHL | NHVNH |

### Table C

| Ancestry | (N) | M284 | T9207 | L412 | T737 |
|----------|-----|------|-------|------|------|
| Asian    | 89  | 0.50 |       | 26.97| 0    |
| European | 113 | 0    | 30.1  | 0    |
| African  | 114 | 0.45 | 0.88  | 2.19 |
| Overall  | 363 | 0.43 | 0.56  | 19.97|
| Asthmatic| 36  | 0    | 41.67 | 0    |

| Ancestry | (N) | M284 | T9207 | L412 | T737 |
|----------|-----|------|-------|------|------|
| Asian    | 89  | 0.50 |       | 26.97| 0    |
| European | 113 | 0    | 30.1  | 0    |
| African  | 114 | 0.45 | 0.88  | 2.19 |
| Overall  | 363 | 0.43 | 0.56  | 19.97|
| Asthmatic| 36  | 0    | 41.67 | 0    |
Fig. 2
Fig. 3

A. Reporter: NF-κB luciferase

B. Reporter: ISRE luciferase

C. TLR3 -
Fig. 4

A

B
| IMG-325C | Brightfield | FITC | DAPI | Merge |
|----------|-------------|------|------|-------|
| pcDNA    | ![Brightfield](image1) | ![FITC](image2) | ![DAPI](image3) | ![Merge](image4) |
| TLR3     | ![Brightfield](image5) | ![FITC](image6) | ![DAPI](image7) | ![Merge](image8) |
| N284I    | ![Brightfield](image9) | ![FITC](image10) | ![DAPI](image11) | ![Merge](image12) |
| L412F    | ![Brightfield](image13) | ![FITC](image14) | ![DAPI](image15) | ![Merge](image16) |
Fig. 6
Fig. 8

A

B

C

P values:

Field induction

Field induction

Field induction

TLR3 (induced in fresh DMEM)

TLR3 (induced in same medium)
Effects of single nucleotide polymorphisms on toll-like receptor 3 activity and expression in cultured cells

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J. Biol. Chem. published online April 13, 2007

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