The Toll/Interleukin-1 receptor (TIR) domain of the Toll-like receptors (TLRs) plays an important role in innate host defense signaling. The TIR-TIR platform formed by the dimerization of two TLRs promotes homotypic protein-protein interactions with additional cytoplasmic adapter molecules to form an active signaling complex resulting in the expression of pro- and anti-inflammatory cytokine genes. In order to generate a better understanding of the functional domains of TLR2 we performed a random mutagenesis analysis of the human TLR2 TIR domain and screened for TLR2/1 signaling deficient mutants. Based upon the random mutagenesis results, we performed an alanine scanning mutagenesis of the TLR2 DD loop and part of the $\alpha$D region. This resulted in the identification of four residues crucial for TLR2/1 signaling: R748, F749, L752 and R753. Computer assisted energy minimization and docking studies indicated three regions of interaction in the TLR2/1 TIR docked heterodimer. In Region I, residues R748 and F749 in TLR2 DD loop were involved in close contacts with G676 in the TLR1 BB loop. Since this model suggested that steric hindrance would significantly alter the binding interactions between DD loop of TLR2 and BB loop of TLR1, G676 in TLR1 was rationally mutated to Ala and Leu. As expected, in vitro functional studies involving TLR1 G676A and TLR1 G676L resulted in reduced PAM3CSK4 mediated NF-$\kappa$B activation lending support to the computerized predictions. Additionally, mutation of an amino acid residue (TLR2 D730) in Region II also resulted in decreased activity in agreement with our model, providing new insights into the structure-function relationship of TLR2/1 TIR domains.

The first line of defense against any invading microbe is provided by the Innate Immune system. Cells of the innate immune system sense and respond to microbial products via the Toll-like receptor family. Toll-like receptors (TLR) are an evolutionarily conserved family of cell surface molecules that participate in innate immune recognition of pathogen-associated molecular patterns (PAMPs) (1). To date 11 mammalian homologues of these receptors have been found (TLR1-11) (2) and individual members of the TLR family recognize a diverse array of microbial components. For example, the first human toll like receptor to be identified, TLR4, senses lipopolysaccharide (LPS) while TLR2 on the other hand senses diacylated or triacylated lipopeptides after heterodimerizing with either TLR6 or TLR1, respectively. The primary function of the TLRs is to alert the immune system to the presence of pathogenic microorganisms. Upon recognition of specific microbial components these receptors turn on a complex series of signaling events leading to the production of numerous immunologically important cytokines, chemokines, and effector molecules. Additionally, microbial products also induce the production of pro-inflammatory cytokines, such as IL-1, TNF-$\alpha$, and IL-12 and the
expression of co-stimulatory molecules on professional antigen presenting cells that are necessary for the activation of T and B cells. Thus, in addition to directly controlling the microbial infection, the innate immune response is also instructive to the adaptive immune response (2).

The conserved cytoplasmic TIR (Toll/IL-1 receptor) domains of the IL-1 and Toll-like receptors are the critical focal point for the generation of ligand-induced cytoplasmic signaling cascades. It is generally believed that the TIR domains serve to promote homotypic protein-protein interactions between receptor chains and with additional cytoplasmic adapter molecules to form an active signaling complex. For signaling all the TLRs utilize one or more of the four known TIR-containing adaptor molecules: MyD88, TIRAP/MAL, TRIF, and TRAM (reviewed in (3;4)). Despite the critical role of the TIR domain in coordinating the initial cytoplasmic signaling events relatively little is known regarding how homotypic TIR-TIR interactions are formed. Several studies have pointed to the important role of a conserved proline within the conserved “BB loop” as being critical for the generation of downstream signals. Indeed, mutation of this proline to a histidine in TLR4 is responsible for the loss of LPS-responsiveness in the C3H/HeJ mouse (5) and acts as a dominant negative mutant. Mutation of the conserved residue within TLR2 (P681) was demonstrated to result in an inability to recruit MyD88 (6) but surprisingly has no effect on the ability of TLR4 to bind MyD88 (7). A peptidomimetic based on the BB loop of the MyD88 (8) clearly showed the role of BB loop in signaling of a TIR domain protein (IL-1) as the mimic could block downstream signaling. Recently, Loiarro et al (9) reported that an eta-peptide derived from the BB-loop region of the MyD88 resulted in the inhibition of homodimerization of MyD88 and thereby signaling.

Given the obvious importance of the TIR domain in the control and coordination of innate immune responses, it is surprising that, with the exception of the BB loop, there is a relative lack of information regarding functional domains within the cytoplasmic portion of Toll receptors that are essential for signaling activity. Potential clues as to the importance of several conserved amino acids within TIR domain were provided by Slack et al who performed alanine scanning mutagenesis of a number of residues within the three conserved TIR domain boxes in the type I IL-1 receptor (10). Of the twelve individual mutations made, four resulted in decreased cell surface expression and of those four; only two could be demonstrated to have decreased abilities to signal for NF-κB or SAPK activation. Ronni et al published a detailed alanine-scanning mutagenesis study of the TLR4 TIR domain which identified two structural surfaces that were required for TLR4-dependent signaling in macrophages (11). However, no information was provided regarding a molecular basis for the loss of function. Finally, Tao et al. have provided crystallographic data demonstrating in the crystal packing the TIR domains of C713S mutant of TLR2 formed asymmetric dimers and that the critical BB loop can adopt different conformations within the structure (PDB accession 1O77) (12). Interestingly, of the five-chains in the crystallographic asymmetric unit, two molecules showed interaction between the αD helix and DD loops of one with the αB helix and BB loop of another. Besides packing forces, the interacting surface was also held together by an inter-chain S-S linkage, but the authors argued for a minimal role of the latter. In all the crystal and homology modeled structures of the TIR domains, the DD loop is located on the opposite side of the BB regions (6) (12) (7). Since TLR2 is not functional as a homodimer and considering the conserved homology in the BB region amongst TLRs, this observation and supporting functional studies led them to suggest that TLR1 or TLR6 can form similar heterodimeric structures with TLR2. It is important to note here that Xu et al reported that gel-filtration and dynamic light-scattering experiments carried out to understand the oligomerization state of the isolated TIR domains in solution indicated a low affinity for self-association of the TIR domains (6). Additionally, based on molecular modeling studies, Dunne, et al., (7) predicted that the DD loops of adaptor molecules (Mal and MyD88) could be involved in molecular recognition and subsequent signaling. In summary, a clear understanding of the role of the DD loop of the TLR2 molecule in innate immunity is still elusive. In this study we report a
structural and functional analysis of the TLR2 TIR domain and based on computational and experimental methods propose a model for how the TLR2 DD loop may interact with the TLR1 BB loop.

Materials and Methods:

DNA, Plasmids and Reagents: pcDNA5 FRT/TO-TLR2 was generated by moving the TLR2 coding region as a HindIII and BamHI fragment from pcDNA3.1-TLR2 (13) to the same sites in pcDNA5FRT/TO. pDHA-TLR2 was generated by PCR amplifying the TLR2 gene from pcDNA3.1 TLR2 using primers with ApaI (5’-AAGGGCCCTCTCCAAGGAAGAATCTCCTC-3’) and PstI (5’-AACTGCAGCTAGACTTTATCGACGTTCT-3’) restriction enzyme sites at N and C terminus respectively and ligating to the same sites in pDisplay HA mouse TLR6 (14) (a gift from D. Underhill, Institute for Systems Biology, Seattle, WA). Wild type TLR1 plasmid (pFLAG-CMV-TLR1) was a gift from P. Tobias (The Scripps Research Institute, Dept of Immunology, La Jolla, CA). pFLAG-CMV2 human MyD88 was made by inserting the human MyD88 coding region into the HindIII and SmaI sites of pFLAG-CMV2. The human MyD88 coding region was PCR amplified using forward primer with HindIII (5’-TTATAGCTAGCTCTCCAGGAAGAATCCCTC-3’) and a blunt ended reverse primer (5’-AATTTCTAGATCAGGGCAGGGACAAGGCTGTC-3’). All the point mutations were made using Quikchange Site Directed Mutagenesis kit from Stratagene (La Jolla, CA). Plasmids were verified by restriction mapping and sequencing (University of Virginia Biomolecular Research Facility, Charlottesville). The MIP-3α luciferase reporter plasmid was a gift from A.C. Keates (Harvard Medical School, Boston, MA), IL-8 luciferase was a gift from N. Mukaida (Kanazawa University, Japan), NF-κB luciferase and pEGFPN1 were obtained from Clontech (Mountain view, CA). PAM3CSK4 was obtained from EMC Microcollections (Tübingen, Germany). All other reagents were obtained from Sigma (St. Louis, MO).

Random Mutagenesis: Random mutations were generated using GeneMorph PCR Mutagenesis kit (BD Biosciences). Using PCR primers Forward: (5’-TGATCTGTCTCAGGGGTC-3’) and Reverse: BamHI (5’-AAGGATCCCTAGGACTTTATCGACGCTCTCAG-3’) a 554 bp region of TLR2 containing the TIR domain was amplified under mutagenic conditions resulting in 1 to 3 random mutations per 500 bp. The resulting PCR product was digested with PpuMI and BamHI then cloned at the same sites in pcDNA5-TLR2. Individual clones were then screened for correct restriction pattern and tested for their ability to signal in response to PAM3CSK4 in HEK 293 transient transfection system. Clones resulting in reduced activity were sequenced using a TLR2 internal primer (5’-GCAATATACCTGTTGACTC-3’) to identify the mutations.

Quantitative reverse transcriptase (RT)-PCR: Total RNA was purified using the Trizol reagent (Invitrogen, Carlsbad, CA). Reverse Transcription (RT) of 0.5 µg total cellular RNA was performed in a final volume of 20 µl containing 1X final first-strand buffer, 1 mM each dNTPs, 20U placental RNase inhibitor, 5 µM random hexamers, and 9U Moloney murine leukemia virus RT (Invitrogen, Carlsbad, CA). After incubation at 37°C for 45 min, the samples were heated for 5 min at 92°C to end the reaction and stored at –20°C until PCR use. cDNA (2 µl) was subjected to real-time, quantitative PCR using the MJ Research Opticon system with SYBR Green I (Molecular Probes, Eugene, OR) as a fluorescent reporter. Duplicate PCR reactions were performed for each sample, and the average threshold cycle number was determined using the Opticon software. Levels of MIP-3α and IL-8 expression normalized to HGPRT levels were determined using the ΔΔCT method. Data are thus expressed as arbitrary units. Sequences of primers used: MIP-3α (F: 5’-CTGGCAATGAGGCTGTA-3’, R: 5’-ACCTCCAACCCGCAAGGT-3’), IL-8 (F: 5’-GGCAGGCTCTTATTGC-3’, R: 5’-GGGGTGAAAGGTGGTGGAAAGT-3’) and hypoxanthine guanine phosphoribosyl transferase (HGPRT) (F: 5’-TTGGAAAAGGGTGTTATTGCCCTCA-3’, R: 5’-TCCAGCAGGTACGAAAGA-3’).
Cells, Cell culture, Transfection and FACS: HEK 293 cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM (Mediatech, Herndon, VA) + 10% fetal bovine serum (FBS; Hyclone, Logan, UT). The HEK293/FpIn cell line which was engineered for use with the FpIn recombinase system was purchased from Invitrogen (Carlsbad, CA) and maintained in RPMI + 10% fetal bovine serum plus Zeocin (Invitrogen, Carlsbad, CA) as recommended. HEK 293 cell line was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer’s recommendations. Stable Cell Lines of pDHA-TLR2 clones were made by transfecting HEK 293 cell lines with respective DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and selecting with G418. For making stable cell lines of pcDNA5/FRT/TO-TLR2 clones, the HEK 293/FpIn cells were transfected in the same way as described above with the respective pcDNA5-TLR2 DNA along with recombinase expressing plasmid pOG44 (Invitrogen, Carlsbad, CA) in 1:9 ratio. Hygromycin-B (Invitrogen, Carlsbad, CA) resistant clones were picked and screened for surface expression with FACS.

To confirm the surface expression of WT or mutant TLR2 on stable cell lines, cells were washed with cold PBS, blocked with cold 0.1% BSA in PBS and stained with PE-conjugated monoclonal antibody against TLR2 (Y-11; Santa Cruz Biotecnology, Inc., Santa Cruz, CA) for 2 hrs followed by the addition of 20 µl ProteinA sepharose bead slurry (Pierce Biotechnology Inc., Rockford, IL). After washing in lysis buffer, the beads were suspended in 2X SDS gel loading dye, heated at 95°C for 10 mins followed by centrifugation at 12,000g for 15 min. Samples and dual color Precision plus Protein standards (BioRad, CA) were run on 10% PAGE and transferred to nitrocellulose membrane. HA-tagged TLR2 was detected using anti-HA (262K) mouse monoclonal antibody (Cell Signaling Technologies, Inc., Danvers, MA) and Flag-MyD88 with anti-FLAG M2 mouse monoclonal antibody (Sigma, St. Louis, MO). Secondary antibody was HRP conjugated anti mouse IgG (Cell Signaling Technologies, Inc., Danvers, MA). Blots were developed using ECL plus western blotting detection system, scanned and analyzed by Storm 840 and using software ImageQuant 5.2, all from Amersham Biosciences, (GE health care).

Computational Methodology

Energy Minimization: All the calculations were performed using the precompiled executables of Tinker molecular modeling suite of programs v. 4.0 for windows platform (15;16). The PDB coordinate files 1FYW and 1FYV were used as the starting structures for the TIR domain of the TLR2 and TLR1 molecules, respectively (6). Prior to energy minization of these structures, the covalent seleniums were replaced by sulphur atoms. The corrected structures were then minimized using NEWTON program and all-atom
AMBER 99 force-field to a root-mean square (RMS) gradient convergence of 0.05 kcal/mole/Å. The calculations were carried out in *implicit* dielectric conditions of 4.0 to mimic the receptor-like conditions where the role of water and salts can be presumed minimal (17;18). The resultant coordinates were confirmed to lack any racemisation of chiral centers or *cis-trans* isomerisation during optimization. A comparative plot of the backbone dihedrals confirmed no large alterations occurred in the structures during optimization for both the molecules. The minimized coordinates were then used for graphical analysis and docking studies.

**Protein-Protein Docking and Co-minimization:**

High resolution rigid body protein-protein docking between the TIR domains of TLR1 and TLR2 was done employing Global Range Molecular Matching (GRAMM) program v. 1.03 (19;20). Employing a grid-step of 1.7, repulsion factor of 30.0, atomic radius for potential range, gray mode projection and 10 degrees for angle of rotations, 500 lowest energy matches were written out. From this output, only those docked structures were sorted which satisfied two conditions: 1) the β-sheet backbone of TIR domains of TLR2 and TLR1 were in same plane and 2) the N-termini were in parallel orientation i.e. towards the membrane. The most stable low energy structure satisfying the imposed requirements (\(E - E_0 < 2\) kcal/mole) was considered for co-minimization experiments. For co-minimization, *implicit* water conditions were used by considering a dielectric value of 80. A cubic box size of one edge equal to 250 Å was used and the van der waals calculations were cut-off at 15 Å. Employing Newton program and all-atom AMBER99 force-field, the coordinates of the TLR2/1 complex were minimized to a RMS gradient convergence of 0.05 kcal/mole/Å. As before, the final coordinates were confirmed to lack any racemisation of chiral centers or *cis-trans* isomerisation during optimization and were used for analyzing close-contacts between the molecules.

**RESULTS and DISCUSSION:**

**Random Mutagenesis and Functional Screening of TLR2 TIR Domain:**

The only detailed structure/function analysis of any TLR TIR domain was done for TLR4 by Ronni et al (11) and very little is known about the amino acid residues and sub-domains of the human TLR2-TIR which are essential for function. Thus, we decided to undertake a detailed structure/function analysis of the TLR2 TIR domain. Unlike the alanine substitution approach used by Ronni et al (11) we decided to perform random mutagenesis followed by a functional screen to identify the crucial residues involved in signaling. Random mutagenesis of TIR-TLR2 (amino acids 607 to 784) was done using PCR based commercially available kit and the resulting product was cloned back into pcDNA5-TLR2. PCR conditions were optimized to result in 2 to 3 mutations per 500 bp (for details see materials and methods). The resulting random mutagenic (RM) clones were then screened for their abilities to activate an NF-κB Luc reporter construct in response to PAM3CSK4 (TLR2 agonist) in the HEK293 transient transfection assays. Clones which demonstrated reduced activity (compared to WT) were sequenced to identify the mutation(s). This screen resulted in the identification of numerous RM clones with reduced activity, 17 of which are shown in Table I. The inactive mutants can be basically grouped in three sets: First, clones that had mutations resulting in the stop codons within the coding region which include RM 27, 44, 51, 53, 54, 60 and 61. Signaling in these mutants is blocked implying the need of complete integrity of the TIR domain in TLR2 for the signaling to occur. The second set of clones had mostly single or multiple mutations in and around the well characterized BB loop. Under this category the mutants were RM5, RM18, RM37, RM38, RM57, RM62 and RM73. These mutants also show aberrant signaling supporting the previously pointed out crucial role of the integrity of BB loop for receptor functionality (6). The third group of clones RM6, RM30 and RM67, had mostly single or multiple mutations outside the BB loop. Of all these random mutant clones we chose RM18, RM 30 and RM67 for a more detailed analysis. These clones were stably transfected into HEK 293 Flp-In cells and checked for surface expression by FACS using the TL2.1 antibody. All the stable clones expressed approximately equivalent levels of TLR2 on the surface (Figure 1A). These clones were then examined by real-
time RT-PCR analysis for their abilities to induce IL-8 (Figure 1B) and MIP-3α (Figure 1C) mRNA in response to PAM3CSK4. These data indicated that the expression of both genes was almost completely inhibited in RM18 and RM30 and significantly repressed (~80%) in case of RM67. RM18 contains a single mutation in the BB loop (I685F) which, based upon previous reports of the critical role of the BB loop in mediating TLR2/MyD88 interactions, would be expected to be inactive (6). The reasons for the loss of activity observed for the other two clones are less clear. RM30 is a double mutant (F701V and K743E) and RM67 which has a single mutation L762Q which also decreases receptor activity significantly. Interestingly, according to the crystal structure 1FYW the amino acids in both these mutants i.e. K743 and L762 lie on the opposite face of the TIR domain from BB loop whereas F701V is buried deep inside the molecule, is not surface exposed, and is in fact a valine in TLR4. L762 is a conserved residue amongst various TLRs and is present at the base of the αD region whereas K743 is present in the DD loop of TLR2 (6). In the TLR2 molecule, the less conserved DD loop is also very mobile, as evident from the high R values for that region in the crystallographic data. Though Dunne et al have suggested that the adaptor molecules like Mal and MyD88 might bind/dock via their DD loop, no such argument has been reported for TLR2 molecule (7;21). As mentioned earlier, based on the contact surface amongst two chains in the C713S mutant TLR2 crystal, Tao et al implied that DD loop of TLR2 can interact with the BB loop of the another TLR to form TLR2:TLRx heterocomplex (12). However, no work identifying the key residues involved in the DD loop mediated TLR2 activity has been reported to date. Given the potential important role of the DD loop in mediating TLR2-dependent responses we decided to perform an alanine scanning mutagenesis of the TLR2 TIR domain in this region.

Alanine Scanning Mutagenesis of DD Loop of TLR2:

The DD loop is present as a flexible loop on connecting the fourth α-helix and fourth β-sheet of the TIR domain. Using a site directed mutagenesis approach, all the amino acids in the DD loop region (E738 to F749) were individually changed to alanine, except residue 744, that being alanine itself. When tested for ability to signal, two of the mutants (R748A and F749A) were found to have reduced ability to induce NF-κB Luc construct in response to PAM3CSK4 in the transient transfection assay. The activities of R748A and F749A were approximately 60% and 40% less, respectively, as compared to the wild type TLR2 (Figure 2A). Surprisingly, the K743A substitution had no significant negative effect on NF-κB activation in contrast to the effects observed above with the double F701V/K743E (RM30) mutant. The reason for this effect is unclear but may suggest that although not solvent accessible the F701 may play an essential role in stabilizing TIR domain secondary structure and/or the additional mutation of the surface K743 to E alters the DD loop region by reversing the electrostatic potential. Since F749 was at the end of the DD loop we generated mutations to screen five residues further downstream in αD region, i.e. C750 to K754. Notably, this series also included R753, whose naturally occurring mutation (polymorphism) to glutamine has been shown to result in decreased recognition of some bacterial peptides and also predispose to Staphylococcal and Mycobacterial infections (22). When screened for activity in response to PAM3CSK4, two of the mutants L752A and R753A were found to show reduced activity (Figure 2A). All these clones were checked for surface expression by FACS and found to be similarly expressed on the surface (Figure 2B), thereby ruling out this reason for their inability to signal. We further tested some of the mutants (R748A, F749A, L752A and R753A) for their abilities to induce MIP-3α and IL-8 promoter activities in transient transfection assays. Similar to the results of the screening with NF-κB Luc all mutants were found to have reduced ability to activate these promoters in response to PAM3CSK4 (Figure 2C). We also generated cell lines for these four mutants by stably transfecting them in HEK 293 cells. Individual clones were then screened for surface expression of mutant TLR2 protein using FACS and clones which gave surface expression equivalent to the WT-TLR2 were used for further study (Figure 3A). These mutant TLR2 cell lines were then stimulated with PAM3CSK4 for 8 hours and the levels of IL-8 and MIP-3α mRNA were not determined.
analyzed by RT-PCR (Figures 3B and 3C, respectively). MIP-3α and IL-8 expression for all the four mutants (R748A, F749A, L752A and R753A) were found to be reduced, which correlated with the transient transfection assays. As a result of our alanine scanning mutagenesis of the DD loop and downstream region we identified four crucial residues which appeared to be required for optimal signaling in response to PAM3CSK4. Our results for the TLR2 DD loop are in general agreement with those reported by Ronni, et al. in their alanine-scanning mutagenesis of the human TLR4 protein (11). Results from that study also indicate that the double mutants EK775-776AA and QQ781-782AA show reduced NF-κB Luc activity (<10% of WT); these residues correspond to DD loop residues in the TLR4 TIR domain. Although the DD loop is so mobile that one can not resolve it well in the crystallographic analysis, and its sequence is not well-conserved amongst the TLR family, it nevertheless appears to mediate the formation of an active signaling complex for at least two members of the family: TLR2 and TLR4. Herein, our work identified four residues in DD loop and αD helix of TLR2 which played critical roles in signaling via the TLR2/1 heterotypic complex.

Computer Assisted Docking of TIR domains of TLR2 and TLR1:

In order to gain structural insights into how the DD loop region may play a role in TLR2 signaling, we performed molecular modeling studies of the interaction of TLR2 with its heterologous partner TLR1. The crystal structures of both the TLR1 and TLR2 TIR domains have been previously resolved (6). The current understanding of the TLR2 signaling complex is that it forms a heterodimer with TLR1 to sense the synthetic lipopeptide PAM3CSK4, resulting in the recruitment of two adapter proteins MyD88 and MAL/TIRAP to the cytoplasmic TIR domain. Previously, MyD88 has been shown to interact in vitro with the TLR2 BB loop (6) and computerized docking studies suggest that both the monomers of MyD88 and MAL/TIRAP interact with the TLR2 BB loop (7). Furthermore, as stated earlier, crystallographic evidence from TLR2 homomultimers suggests that the DD loop and BB loop may form points of contact between two molecules (12). Based on these previous reports we hypothesized that the DD loop side of the TLR2 TIR domain might be interacting with TIR domain of TLR1 and that an inability to form such interaction might be the reason for the decreased activity observed with the TLR2 DD loop alanine mutants. To test our hypothesis we performed computer-assisted docking studies of the two TIR domain interactions. The previously resolved crystal structures of TLR2 and TLR1 PDB files 1FYW (TIR-TLR2) and 1FYV (TIR-TLR1) were used as the starting molecules. As described in the methods section, both molecules were individually energy minimized to a RMS gradient convergence of 0.05 kcal/mole/Å. This was followed by a high resolution rigid body protein-protein docking between the TIR domains of TLR1 and TLR2 using GRAMM program. Using a grid-step of 1.7, repulsion factor of 30.0, atomic radius for potential range and gray mode projection to calculate interacting surfaces provided good selection criteria. Using only 10 degrees for angle of rotations allowed us to map the surface in the lowest possible grid. Five-hundred lowest energy combinations were generated from the rigid body docking. Graphical analysis of docking output indicated a high (87 structures out of 100 lowest energy combinations) propensity for the TLR1 molecule to interact with the DD loop face of the TLR2 molecule. In order to sort out functionally meaningful orientations representing the complex between TIR domains of TLR2 and TLR1, we postulated that the N-terminal ends of both the components should be oriented towards the cell membrane. The 9th lowest energy docked complex was compatible with our assumption. Here, it is of importance to mention that identical results were obtained on docking minimized coordinates of TLR2 on the template of minimized coordinates of TLR1 and vice versa. This low energy complex was minimized again by employing a cubic BOX condition (a = 250 Å). During minimization, implicit dielectric conditions representing aqueous surroundings were employed. The final minimized heteromeric complex was used to analyze the interacting surface and is shown in Figure 4A.

Detailed analysis of this structure indicated three possible interacting faces between the two molecules. In Region I, the TLR2 DD loop
residues R748 and F749 were closely associated with G676 of TLR1 BB loop (Figure 4C). This contact region was in agreement with the possibility suggested by Tao et al (12). Analysis suggested that this region in the energy minimized complex was predominantly stabilized by three non-conventional C-H…O bonds, namely: two bifurcated TLR1 G676 Cα-Hs…O=C TLR2 R748 and a TLR1 G676 C=O…H-Cα TLR2 F749 with distances of 2.9 and 2.9, and 3.0 Å, respectively. Since in solution, these molecules will undergo substantial motion, we abstained from over-analyzing our static H-bonding features. Interestingly in our model of the heterotypic complex, the critical P675 in the BB loop of TLR1 was positioned close to the DD loop of TLR2. We speculate that as shown for TLR2, a single mutation of this Proline to Histidine will drastically reverse the electrostatic potential of the contact point and negatively effect the TLR2/1 mediated immune response. Another observation worth noting is the unique backbone conformational features across the G676 residue. In the minimized complex, φ and ψ torsion angles for this Gly were found to be 65° and -4°, respectively. All other natural L-α-amino acids will generally find this backbone geometry unfavored. Whether this is the very reason behind the evolutionary conservation of Gly at this position in the BB loops of TLRs and adapter proteins will remain an interesting query for structural biologists.

As discussed above, TLR2 amino acid residues R748 and F749 were independently demonstrated in the functional studies to be important for optimal TLR2-mediated responses. This functional correlate provides additional confidence in the validity of the computerized docking studies. Additional modeling experiments in which the R748 and F749 were singly substituted for alanines confirmed the disruption of this “native-like” interaction. Additionally we observed that L752 and R753 are intricate members of the αD helical region. Since Ala also has a high propensity for forming α-helix, as expected, we did not observe any loss in the helical architecture of the region in the mutants. While, the role of Leu side-chain could not be determined from the modeling data, the positively charged guanidine group of the Arg side-chain played a major role in stabilizing the Q747RF749 region of the TLR2 DD loop. Hence in the R753A mutant studied, the truncation of the charged group of Arg led to conformational changes across the QRF region which eventually led to an altered contact topology between the two proteins during co-minimization. Notably, polymorphism of residue R753 (R753Q) has been linked with the susceptibility to Staphylococcal, Borellia sp, and Mycobacterium sp. and has also been associated with acute rheumatoid fever in children (23-27). Thus our modeling studies also suggest a potential explanation for the observed decreased activity of the R753Q polymorphism.

Two other regions of interfacial contact between the two proteins were named as region II and III. In region II, most noticeably the TLR2 D730 comes in close proximity to H646 and N700 of TLR1 via a network of interwoven backbone and side-chain hydrogen bonds. In fact our modeling data showed a strong hydrogen bond (2.3 Å) between the side-chain OδN700 of TLR1 and backbone NH of H646 which appears to play a role in maintaining the tertiary structure of the TLR1 in the complex. The same Oδ of N700 was also involved in the intermolecular hydrogen bonding with the backbone NH of the Asp 730 in the TLR2 molecule (2.7 Å). The third component in this network was the strong intermolecular electrostatic interaction between the oppositely charged side-chains of His646 of TLR1 and Asp730 of TLR2 (2.3 Å). Close inspection also suggested that there was another region of possible contact between the two molecules. This region termed as region III mainly suggested interaction between the Tyr737 of TLR1 and the C-tail helix of the TLR2 molecule. Considering various possible intermolecular interactions through space which might result in stabilization of the complex and probable flexibility of the loop preceding the C-terminal helix in TLR2, we postulated that the
side-chain of Y737 might interact favorably with the backbone carbonyl of N777 in a C=O·π stacking fashion. Thus we identified three regions of close contact in our mathematically proposed model to be validated experimentally using site-directed mutagenesis and resultant downstream signaling.

**Experimental validation of Regions II and III using rational mutations in TLR2**

Individual mutations of R748A, F749A, L752A and R753A in TLR2, and previously published reports about R753Q and critical P681H mutation in TLR2 upheld the observed contact region I of our model of the complex. To critically evaluate the regions II and III from our modeling results, we generated additional mutations in TLR2, which we reasoned to be involved in TLR1/TLR2 heterodimer formation. Mutations made in TLR2 were: D730A (possible interacting residue of TLR2 in region II) and N777A (possible interacting residue of TLR2 in Region III). In addition several multiple mutants were generated in order to assess possible cooperative interactions between the three regions: double mutants D730A/F749A, F749A/N777A, and triple substitution D730A/F749A/N777A. All of these mutants were tested for their surface expression by FACS in transient transfection system in HEK 293 cells and were found to be approximately equivalently expressed on the cell surface (Fig-5 B). These mutants were then tested for their ability to induce NF-κB, MIP-3α and IL-8 luciferase promoter constructs in HEK 293 transient transfection system in response to PAM3CSK4. Results shown in Figure 5A indicated that substitution of alanine for D730 reduced activity of all the three reporters by about 50% to 60% indicating that D730 may in fact participate in TLR1/TLR2 heterodimer formation as suggested by the computerized docking studies. On the other hand, we could not confirm a role for N777 in stabilizing the heterodimer as the alanine substitution had no effect on reporter activities. Both the double mutants D730/F749A and F749A/N777A resulted in reduced activity. In the case of F749A/N777A activity was equivalent to that of single mutation, F749A (approximately 40% reduction). The double D730/F749A substitution was slightly more effective at inhibiting reporter gene responses than either of the single mutants alone, suggesting the potential for cooperativity between Regions I and II in forming an efficient signaling complex.

The earliest event in TLR signaling is the recruitment of MyD88 into the receptor complex. The decreased signaling activity observed with our mutant TLR2 molecules would suggest that we have compromised the formation of an appropriate heterodimeric signaling complex of TLR2 and TLR1. In the light of these observations we postulated that the TIR domain mutants might be inhibiting the recruitment of the MyD88 into the receptor complex. To test our hypothesis we performed co-immunoprecipitation experiments. HEK 293 cells were transiently co-transfected with either HA tagged WT-TLR2 or HA-TLR2 double mutant (D730A and F749A) along with FLAG tagged MyD88. Prior to immunoprecipitation of HA-Tagged TLR2, cells were stimulated with PAM3CSK4 for 15 minutes to induce the redistribution of MyD88 to the receptor complex. Results as shown in Fig-5 C indicate that MyD88 was recruited to WT-TLR2 but not to the D730A/F749A double mutant. Two control samples (vector and FLAG-MyD88 alone) show that the interaction was specific to WT-TLR2 and there was negligible binding of FLAG-MyD88 to the anti HA antibody and the beads. These results indicate that the TLR2 TIR domain mutants described here likely block signaling by preventing the formation of an appropriate platform for the efficient recruitment of adapter proteins into the complex.

**Evaluating the role of G676 in TLR1 in TLR2/1 mediated signaling**

As detailed above, in our minimized docking model, the two backbones CαHs G676 of TLR1 were involved in a bifurcated non-conventional hydrogen bonding with R748 of TLR2. At the same time, the carbonyl group of G676 of TLR1 was involved in a similar interaction with the CαH of F749 of TLR2. If this observation is true, then addition of an alkyl group on the Gly residue will result in decrement in the efficiency of native-like complexation and hence the mediated signaling. Thus two mutations G676A and G676L were generated to test this hypothesis. We expected the Leu mutant to exhibit higher degree of suppression in activity because of bulkier side-chain than the Ala one. TLR1-G676A and TLR1-G676L were
tested in transient transfection assays in HEK 293 cells for their ability to induce NF-κB luciferase reporter construct in response to PAM₃CSK₄. Since HEK293 cells express low levels of endogenous TLR1 we tested the activity of the TLR1 mutants to act as dominant negative mutants. The results of these experiments shown in Fig-6 indicated that both the mutants (G676A and G676L) negatively affected the ability of the cells to respond to PAM₃CSK₄ as both the mutants inhibited NF-κB activation by approximately 60%. These results though did not exhibit the increased suppression from Ala to Leu substitution, but they definitely strengthened our in silico data and support the hypothesis that the interaction between the TLR1 and TLR2 TIR domains is facilitated by these residues in the BB loops and the DD loops of the two molecules, respectively.

Importantly, the docking of the two TIR domains as described here leaves the BB loop of TLR2 available for binding to MyD88 and/or TIRAP/MAL as suggested in the modeling studies of Dunne et al. (7). The results of our studies also suggest, as was previously suggested by Dunne et al., that the role of the BB loop in TLR signaling may be different between the different receptors. Clearly the P714H mutation in the TLR4 BB loop abolishes activity however; the mechanism behind that effect remains elusive as this mutant is still capable of binding MyD88 and TIRAP/MAL (7). In contrast, the corresponding mutation in TLR2 (P681H) results in the inability of the molecule to bind MyD88 (6). Based upon the studies reported herein and our modeling data discussed earlier, it is likely that the same mutation in TLR1 (P675H) would disrupt the ability of TLR1 and TLR2 to efficiently heterodimerize. Previously, Sandor et al., demonstrated using chimeric TLR1 and TLR2 molecules that the TIR domains of the two molecules are non-redundant (28). In that study, NF-κB activation in response to araLAM or PAM₃CSK₄ was observed only when both the TLR1 and TLR2 TIR domains were present. A recent study by Brown et al (29) used yeast two hybrid experiments to demonstrate that MyD88 binds to the TIR domain of TLR2 and not TLR1. Their finding is consistent with our results indicating that the TLR1 BB loop is essential for heterodimerization with TLR2. In the same study, Brown et al., could not demonstrate a functional interaction between the TIR domains of TLR1 or TLR6 and TLR2 providing additional evidence that the affinities of the individual TLR TIR domains for each other are quite weak. Likewise, we have been unable to demonstrate a functional interaction between TLR2 and TLR1 TIR domains in a mammalian two hybrid system (data not shown). These results are consistent with studies from Meng et al, which demonstrated that mutant TLR2 molecules lacking the entire cytoplasmic domain could still be co-immunoprecipitated with WT TLR2 (30). Together these data support a model wherein the primary region for dimerization between TLRs is located in the transmembrane region.

Finally, Brown et al (29) demonstrated a unique interaction between TLR1-TIR and HSP60 and HSP75. It will be interesting to determine the region of TLR1 to which these proteins bind in light of our studies indicating that the TLR1 DD loops remains accessible in the heterodimer. Our results support the conclusion that the TLR1 and TLR2 TIR domains are unique in their structures and provide for the first time a structural basis for the functional interaction between these two TLRs.
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1 Abbreviations used are: araLAM, ara-lipoarabinomannan; BSA, bovine serum albumin; FBS, fetal bovine serum; LPS, lipopolysaccharide; PAM3CSK4, tripalmitoyl cysteiny1 lipopeptide; RM, random mutant; SAPK, stress associated protein kinase; TIR, toll/IL-1R; TLR, toll like receptor; WT, wild type
Figure Legends

Figure 1. Random mutagenesis of the TLR2 TIR domain
A, Surface expression TLR2 Random Mutant (RM) clones stably expressed in HEK 293/FPln cells as determined by FACS using FITC conjugated TLR2.1 monoclonal antibody. B, Quantitative RT-PCR analysis of IL-8 (B) and MIP-3α (C) mRNA in TLR2 wild type (WT) and RM mutant HEK 293 cells in response to 8 hour stimulation with 100ng/ml of PAM3CSK4.

Figure 2. Alanine scanning mutagenesis of TLR2 DD loop and αD region
A, NF-κB Luciferase activities in transiently transfected HEK293 cells in response to eight hour stimulation with100ng/ml of PAM3CSK4. Activities are plotted as percent of the wild type (WT) +/- SEM of at least 3 experiments with each mutant. B, Surface expression of all the TLR2 alanine scanning mutants HEK 293 cells co-transfected with pEGFPN1 and TLR2 plasmids were analyzed by FACS. Data is expressed as Mean Fluorescence Intensity (MFI) of the TLR2-PE channel after gating on GFP positive cells. WT-1 is cloned in pcDNA5 and WT-2 in pDisplay. C, MIP-3α and IL-8 Luciferase activity of the five TLR2 alanine mutants found to have reduced NF-κB activity. Activities are plotted as percent of WT-TLR2 in response to eight hour stimulation with100ng/ml of PAM3CysK4. Data are the average of two experiments.

Figure 3. Effects of DD loop mutants on endogenous TLR2-responsive genes
A, Surface expression of TLR2 DD loop alanine mutants in stably transfected HEK 293 cells. B, Quantitative RT-PCR analysis of IL-8 (B) and MIP-3α (C) mRNA in TLR2 wild type and the DD loop mutant HEK 293 cells in response to 8 hour stimulation with 100ng/ml of PAM3CSK4.

Figure 4. Computer predicted docking model of TLR1 and TLR2 TIR heterodimer
A. Ribbon diagram showing structural features including the proximity of TLR1 BB and TLR2 DD loop. B, Color coded surface topology visualization of the docked TLR1&TLR2 TIR heterodimer indicating G676 (TLR1 BB loop) (red) in close proximity with R748 and F749 (TLR2 DD loop) (magenta), molecules and amino acids are color coded for clarity. C, Reverse view of the structure in Fig.4B showing two other crucial residues: R753 (green) which, although on surface, is not involved in TLR1 interaction and L752 which is not surface accessible (see zoomed inset where TLR2 is set as transparent). D. Ribbon diagram showing three regions of interactions, Region I with TLR1 BB and TLR2 DD loops in close proximity, Region II contains D730 (TLR2), H646 and N700 (TLR1) and Region III (behind Region II in present view) contains N777 (TLR2) and Y737. See zoomed inlets for detailed interactions.

Figure 5. Effect of alanine substitutions in Regions II and III on TLR2 activity
A. Activities of single and multiple alanine mutants in TLR2. HEK293 cells were transiently co-transfected with the indicated promoter/luciferase reporter (NF-κB, MIP-3α, or IL-8) and the indicated TLR2 mutant. Activities of the various luciferase constructs are plotted as the percent activity versus wild type TLR2 in response to 8 hour stimulation with 100ng/ml of PAM3CSK4. Data are averages of 2 experiments with each combination of reporter and TLR2 mutant. B. Surface expression of all the TLR2 mutants described above by FACS using PE tagged TLR2.1 monoclonal antibody. HEK 293 cells were co-transfected with pEGFPN1 and the indicated TLR2 plasmids. Data is expressed as Mean Fluorescence Intensity (MFI) of the TLR2-PE channel after gating on GFP positive cells. C. TLR2 double mutant (D730A and F749A) does not recruit MyD88. HEK293T cells were transiently co-transfected with indicated plasmids. 48 hrs post transfection cells were stimulated with PAM3CysK4 for 15mins. Cell lysates were immunoprecipitated with a polyclonal antibody against HA epitope tag. Immunoprecipitation samples and whole cell lysates were separated on 10% SDS-PAGE and analyzed by western blotting. The membrane was cut into two pieces and the upper blot was probed with anti HA (for
HA-WT-TLR2 or the double mutant) and the lower with anti FLAG antibody (for FLAG-MyD88). The arrows indicate the TLR2 and MyD88 bands.

Figure 6. Inhibition of TLR2 responses by mutagenesis of TLR1
HEK293 cells were co-transfected with NF-κB luciferase reporter and the indicated TLR2 and/or TLR1 expression plasmids. Luciferase activities were determined following 8 hour stimulation with 100 ng/ml PAM3CSK4. Data are expressed as relative luciferase units +/- std. dev. of triplicate transfections and are representative of two independent experiments.
| Group | Name     | Residues Mutated in TLR2 | Corresponding Residue(s) in TLR4 | NF-κB Luc Activity (%WT) of RM Clones |
|-------|----------|--------------------------|----------------------------------|--------------------------------------|
| I (Truncated clones) | RM27     | Frame Shift at Q 747    | Q 781                           | 4.9                                  |
|       | RM44     | M 756-STOP              | L 789                           | 5.6                                  |
|       | RM51     | M 620-STOP              | L 660                           | 3.7                                  |
|       | RM53     | Frame Shift at I 685    | I 718                           | 7.8                                  |
|       | RM54     | W 616-STOP              | F 656                           | 5.3                                  |
|       | RM60     | M620-STOP               | L 660                           | 12.5                                 |
|       | RM61     | Q 747-STOP              | Q 781                           | 9.3                                  |
| II (Clones with one or more BB loop mutations) | RM5      | I 693 F, K 695 E        | F 727, K 729                     | 82.6                                 |
|       | RM18     | I 685 F                 | I 718                           | 5.7                                  |
|       | RM37     | I 680 F, S 696 I, E 711 D | I 713, S 730, R 745           | 4.6                                  |
|       | RM38     | H 675 Q, K 695 L, K 759 M | H 708, K 729, N 792        | 4.1                                  |
|       | RM57     | P 669 L                 | P 702                           | 63.6                                 |
|       | RM62     | I 693 F                 | F 727                           | 52.8                                 |
|       | RM73     | H 613 N, Y 617 H, M 620 L, K 683 M, S 710 T | Y 652, H 657, L 660, V 716, S 744 | 9.3                                  |
| III (Clones with mutations outside BB loop) | RM6      | F 644 L                 | F 677                           | 83.9                                 |
|       | RM30     | F 701 V, K 743 E        | V 735, T 777                    | 23.8                                 |
|       | RM67     | L 762 Q                 | L 795                           | 40.4                                 |

Table-1

Phenotypes of TLR2 Random mutant (RM) clones. Clones are grouped in three groups, shown on the rightmost column based on the location of mutation(s). The second column shows the clone numbers. The third column shows the details of mutation(s) as identified by DNA sequencing. The fourth column indicates the residue(s) in TLR4 which correspond to the respective residue(s) in TLR2 RM clones. Extreme right column shows the NF-κB activation levels of the RM clones depicted as the Percent (%) of the wild type (WT) TLR2 response to PAM3CSK4 in HEK 293 transient transfection system.
Fig. 2
Fig. 3
Fig-5
Fig. 6
Structural and functional evidence for the role of the TLR2 DD loop in TLR1/TLR2 heterodimerization and signaling
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