Summary of the research work

According to the current understanding of TLR mediated signaling, post activation/ligand binding TIR domain in TLRs and adaptors come together triggering downstream signaling cascade (Akira and Takeda 2004). Despite the attempts being made to decipher the details of various components involved in TLR mediated pathway, little success has been achieved in getting structural insights into the mode of interactions prevalent at the TIR-TIR junctions of various interacting partners. Only direct structural evidence for TIR-TIR complex is in the form of crystal structure of TIR domain of hTLR10, physiological relevance of which is questioned, since the gel filtration profile shows the protein to be a monomer in solution (Nyman, Stenmark *et al.* 2008). Studies following this however, have taken TLR10-TIR crystal structure as the basis for deriving dimer models for hTLR4-TIR (Núñez Miguel, Wong *et al.* 2007; Bovijn, Ulrichts *et al.* 2012). In the TLR10-TIR dimer, residues from BB-loop and αC-helix contribute to interaction interface. While involvement of BB-loop in TIR domain dimerization has been well established, a report from our group for hetero-dimerizing TLRs (TLR1/2 TIR hetero-dimer) showed the role of DD-loop in TIR-TIR interaction (Gautam, Ashish *et al.* 2006).

In light of the above facts we were interested in checking whether the TIR domains in TLRs follow a universal mode of interaction, or the specificity in TLR mediated signaling is also reflected in the way different TLR-TIR domains interact with each other. We applied an *in-silico* approach towards this problem which included molecular modeling and docking, followed by filtering of the docked complexes with the help of in house perl codes. Since association at the TIR domain occurs subsequent to the association of extracellular domain of TLRs, we hypothesized that low energy alone cannot be the sole criteria for filtering out the most probable solution. Therefore, frequency *i.e.* the most preferred dock pose was kept as an additional criteria for the selection of final dimer model.

Study of the different kind of dimers derived for the TIR domains of TLRs shows that homo-dimers follow symmetric mode of interactions *i.e.* BB-loop of the two interacting partners come together at the dimer interface. The only exception to this is the heterotypic dimer derived for TLR4-TIR which shows asymmetric interactions between the BB-loop of one sub-unit and the DD-loop of another, in addition to the usual symmetric one. Two different modes of interactions found for hTLR4-TIR correlates with recent report by

ıgloff wherein he has projected two different dimers formed by the extracellular domain TLR4 which can lead to different modes of dimerization at the TIR domain as well ıngloff 2012). Another key observation was the extensive interface, seen in the dimer dels for TLR9 subfamily and TLR1/TLR2, which possibly eliminates the need for dging adaptor (Mal) at the dimer interface. There are reports which suggest that members TLR9 sub-family and TLR2/TLR1 are capable of triggering downstream signaling ·ough MyD88 pathway, independent of Mal (Wagner 2004; Kenny, Talbot *et al.* 2009).

Further analyzing the dynamicity of the TIR domain before and after dimerization vealed that the normalized mean square displacement (R^2) of residues from BB-loop, αC-lix/CD-loop which are involved at the dimer interface goes down upon dimerization. On e other hand R^2 for residues from DD-loop (in case of TLR3, TLR4-heterotypic dimer and LR9-TIR dimers), EE-loop (in case of TLR4-homotypic dimer and rest of the homo-ımerizing TLRs) goes up or does not show decrement even after dimerization. Similarly in ıse of hetero-dimerization too, loss in entropy of the residues in and around the dimer ıterface is compensated by increase in entropy of residues elsewhere. Another noteworthy bservation was that the residues from β-sheet core showed values close to 0 for R^2 which ıostly remained unchanged even after dimerization. While the dynamicity in the β-sheet ore of the domain was restricted yet the loss in entropy at the interaction interface gets ıropagated to the faces lying opposite to those involved at the interaction interface. This ıbservation led to the initiation of second part of this study wherein we have shown the ıresence of a network kind of architecture within the TIR domain of hTLRs.

We did MD simulations of the TIR domain in hTLRs to probe the structural characteristics of TIR domains. An analysis of molecular dynamic trajectories of the ten hTLR-TIRs showed the presence of a network within the TIR domain of TLRs by the virtue of which N-terminal portion interacts with the flanking residues of the BB loop and the central β-sheets, simultaneously. At the same time, the residues of the central β-strands form favorable contacts with the DD loop and C-terminal, thus completing a two-way circuit between the N- and C-termini. Importantly, the "hubs" of this communication network were found to be conserved in all human TLRs, which were housed within the β-sheet core of the domain.

Validation of our *in-silico* analysis was done using mutagenesis experiments conducted by Ronni *et al.* where some of the mutations in the β-sheet of the TIR domain rendered them non-functional (Ronni, Agarwal *et al.* 2003). To this the authors, and those following this study, came up with an explanation that mutations in the β-sheet residues disrupt the core of the domain (Núñez Miguel, Wong *et al.* 2007). However, in our CD and SAXS data analysis of the recombinant hTLR-TIRs and its mutants, we did not observe significant alterations in the shape profiles of native and mutant proteins. Further analyzing the contact maps for the mutants of human TLR4 (which were earlier reported to be functionally compromised) we observed, that the communication network significantly deviates from the native form. Same was also found in the mutants of hTLR2 and hTLR1. Additionally, comparison of the network maps between monomeric and dimeric forms of hTLRs, using hTLR2/1 and hTLR10/10 as examples, revealed that dimerization also alters the profile of the interactions. Finally, multiple sequence comparison brought forth that the "hub" residues in the TIR domain have remained conserved in diverse species *viz.* human, mice, chicken, zebra fish, frog and drosophila, upholding their significance in innate immune signaling.

In conclusion, we would like to emphasize that while all the homo-dimerizing TLRs prefer symmetric interactions at the dimer interface there is diversity in the form of other secondary structural elements involved in dimerization. Hetero-dimerizing TLRs however exhibit both symmetric and asymmetric interactions at the dimer interface. Additionally we found that the DD-loop adds diversity to the dimer interface *i.e.* the TLRs which form more than one kind of dimer involve DD-loop at the dimer interface. We further rule out the presence of any universal mode of interaction interface prevalent in hTLRs' dimerization as has been proposed earlier (Valkov, Stamp *et al.* 2011). Residues from the β-sheets of the TIR-domain show little/no-involvement in TIR domains' dimerization on the contrary, they are involved in communicating the signal from one end of the domain to the other (Singh, Pandey *et al.* 2013).

General Discussion

Right from the discovery of Toll-like receptors and establishment of their role in innate immune response, a great deal of effort has gone in gaining structural insights into the

LRs and their mode of association post ligand binding/activation. Mutagenesis has been ıe major resort for proving the role of different TLRs or structural elements or residues in :sponse to different ligands. Release of cytokines in response to activation of TLRs has een the primary readout taken for positive response. The question which we have here is; ow accurate is it to use the release of cytokines as the read out since they come at last step)f TLR mediated immune response. Number of molecules associate and dissociate in this)rocess and multitude to reactions occurs before the release of cytokines. Therefore :onsidering only lack of formation of suitable binary and ternary complexes to be the root :ause for the inadequacy in the release of cytokines, in our opinion could be an over-assumption. We believe there should be direct read-outs for confirming the role of different TLRs, more so for the involvement of different secondary structural elements.

Although through our *in-silico* analysis we have added new perspective to TLR mediated signaling, with emphasis on the TIR domain. The results however need to be further validated experimentally. Great challenge would be to get stable complexes of TIR domains as they form weak dimers and are present as monomers in solution. Getting the correct biological representative assembly and proving it to be so would be another bottleneck in validation of our hypothesis. We are right now working on this aspect of developing a suitable system to check our *in-silico* results.

My long two-pointed ladder's sticking through a tree
Toward heaven still,
And there's a barrel that I didn't fill
Beside it, and there may be two or three
Apples I didn't pick upon some bough.
But I am done with apple-picking now…

(Excerpts from After Apple picking; Robert Frost)