

**UNDERSTANDING THE RELATION OF CANCER AND
INFLAMMATION BY CONSTRUCTING STRUCTURAL
TOLL-LIKE RECEPTOR PATHWAY**

by

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ABSTRACT

Toll-like receptor (TLR) pathway is one of the major pathways that give rise to inflammation, which is the first line of defense against pathogens. Although it is essential for host defense, inflammation also contributes to almost all phases of tumor development. TLR pathway plays a key role in inflammation-cancer crosstalk, but the exact mechanisms how they contribute to this remain elusive. Construction of structural TLR pathway provides insights into its roles in this crosstalk. The classical node-and-edge representation of pathways provides the big picture in a simple way, but it is incomplete. Structural pathways can help complete missing parts of such diagrams: they can help in understanding how an upstream signal is transduced to downstream; how higher-order oligomerization modes of proteins can influence their function; how mutations, inhibitors or antagonists affect the signaling and change cellular outcome; and which alternative parallel pathways can be activated simultaneously. Constructing structural pathways is a challenging task. In this dissertation, I constructed structural network of TLR pathway by employing the powerful PRISM (PRotein Interactions by Structural Matching) algorithm and available mutational/biochemical data. I built the structural network of TLR pathway; its regulatory pathways; and also other pathways that have important roles in inflammation-cancer relation, such as different cytokine pathways, and CASP8, and TRAF3 centered pathways. The architectures that I obtained provide the structural basis for TLR clustering upon stimulation and assembly of key signaling complexes, such as “TIR domain signalosome”. They also demonstrate that almost all downstream parallel pathways of TLRs are competitive and clarify decisions at pathway branching points. I showed that several negative regulators restrict TLR signaling by interfering with the formation of the key interactions and signalosomes. I also performed *in silico* mutagenesis analysis to characterize the effects of oncogenic mutations and found that some mutations that fall on the interfaces disrupt the interactions with their targets and enable constitutive activation of NF- κ B, which might lead to chronic inflammation, which promotes oncogenesis. Our results help to understand the crosstalk between cancer and inflammation from a structural perspective.

ÖZET

Toll-like reseptör (TLR) yolağı, patojenlere karşı ilk savunma mekanizması olan iltihaba yol açan başlıca yolaklardan biridir. İltihap savunma mekanizması için gerekli olmasına rağmen, kanser gelişiminin bütün evrelerine katkıda bulunur. TLR yolağı, iltihap-kanser ilişkisinde çok önemli rol oynar ama buna nasıl katkı sağladığına dair kesin mekanizmalar bilinmemektedir. TLR yolağının yapısal networklerinin inşaaası, TLR'nin bu ilişkideki rolünü anlamamıza yardımcı olur. Yolakların klasik düğüm-ve-kenar şeklindeki simgelenmelerini, büyük resmi basit bir şekilde sunar ama bu simgeler eksiktir. Yapısal networkler, bu simgelerin eksiklerini tamamlar: yukarıdan gelen bir sinyalin aşağıya nasıl iletildiğini; yüksek oligomerizasyon modlarının proteinin fonksiyonunu nasıl etkilediğini; mutasyonların, inhibitörlerin ve antagonistlerin sinyalizasyonu ve hücredeki sonucu nasıl değiştirdiğini; ve hangi paralel yolakların eş zamanlı aktive olabileceklerini anlamamıza yardımcı olur. Yapısal yolakların inşaaası zor bir iştir. Biz bu tezde, PRISM (Protein Interactions by Structural Matching) algoritmasını ve mevcut mutasyonel/biyokimyasal verileri kullanarak, yapısal TLR yolağını inşaa ettik. TLR yolağının yapısal networkünü; onun regülasyonunu sağlayan yolakları; ve sitokin, CASP8 ve TRAF3 gibi iltihap-kanser ilişkisinde önemli olan diğer yolakların yapısal networklerini inşaa ettik. Burada elde ettiğimiz yapılar, TLR'lerin uyarıldıkları zaman kümeleşmelerine ve "TIR domain signalosome" gibi önemli çok-proteinli komplekslerin kurulumuna yapısal dayanak sağlar. Ayrıca, nerdeyse bütün paralel TLR yolaklarının birbirleri ile rekabet ettiklerini gösterir, ve yol ayrımlarındaki kararları açıklar. Birçok negatif TLR regülatörlerinin, önemli protein etkileşimlerini ve çoklu-protein komplekslerinin oluşumunu engellediğini gösterdik. Ayrıca, *sanal ortamda* mutasyon analizi ile onkojenik mutasyonların etkisine baktık ve bağlanma ara yüzeylerinde bulunan bazı mutasyonların protein etkileşimlerini bozduğunu, NF- κ B'nin konstitütif aktivasyonunu sağladığını ve bu sayede onkogenezi destekleyen kronik iltihaba yol açtığını bulduk. Sonuçlarımız iltihap-kanser arasındaki ilişkiyi yapısal perspektiften olarak anlamamızı sağlar.

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NOMENCLATURE

<i>PDB</i>	Protein Data Bank
<i>PPI</i>	Protein-Protein Interaction
<i>PRISM</i>	PRotein Interactions by Structural Matching
<i>PRR</i>	Pattern recognition receptors
<i>PAMPs</i>	Pathogen-associated molecular patterns
<i>DAMPs</i>	Damage-associated molecular patterns
<i>LRR</i>	Leucine rich repeats
<i>LPS</i>	lipopolysaccharide
<i>TIR domain</i>	Toll/IL-1R domain
<i>MyD88</i>	Myeloid differentiation factor 88
<i>Mal</i>	MyD88 adaptor-like (also known as TIRAP)
<i>TRIF</i>	TIR domain containing adaptor inducing interferon- β (TICAM1)
<i>TRAM</i>	TRIF-related adaptor molecule (also known as TICAM2)
<i>BCAP</i>	B-cell adaptor for PI3K
<i>SARM</i>	sterile α and heat-armadillo motifs
<i>TNFR</i>	Tumor necrosis factor receptor
<i>TRAF</i>	TNFR-associated factor
<i>DD</i>	Death domain
<i>DED</i>	Death effector domain
<i>CASP8</i>	Caspase 8
<i>cIAP</i>	cellular inhibitors of apoptosis
<i>DISC</i>	Death inducing signaling complex

Chapter 1

INTRODUCTION

Inflammation, the first line of defense against pathogens can contribute to all phases of tumorigenesis, including tumor initiation, promotion and metastasis. Within this framework, the Toll-like receptor (TLR) pathway plays a central role in inflammation and cancer crosstalk. Although extremely useful, the classical representation of this, and other pathways in the cellular network in terms of nodes (proteins) and edges (interactions) is incomplete. Structural pathways can help complete missing parts of such diagrams: they demonstrate in detail how signals coming from different upstream pathways merge and propagate downstream, how parallel pathways compensate each other in drug resistant mutants, how multi-subunit signaling complexes form and in particular why they are needed and how they work, how allosteric events can control these proteins and their pathways, and intricate details of feedback loops and how kick in. They can also explain the mechanisms of some oncogenic SNP mutations. Constructing structural pathways is a challenging task. This dissertation mainly focuses on constructing structural TLR pathway to shed light on their mechanism of action in tumor microenvironment. Presented results may help to understand the crosstalk between cancer and inflammation better.

The outline of this dissertation is as follows:

In the second chapter, I provide a comprehensive review on cancer and inflammation crosstalk, TLR signaling and its regulation, cytokines and their roles on cancer, and lastly constructing structural networks.

The third chapter presents the structural TLR pathway that I built. Here, I present a model for the large TIR-domain signalosome assembly, which demonstrates how downstream oligomerization triggers TLR clustering upon stimulation. Atomic details of the interactions revealed that parallel downstream pathways with opposing consequences are competitive at almost each branching point of the TLR pathway, such as Mal and TRAM recruitment, or TRAF6, TRAF3, and FADD, resulting in either pro-inflammatory, anti-inflammatory, or death pathways. The structural details allow us to observe the effects of mutations on the interactions.

The fourth chapter focuses on negative regulation of TLRs from a structural standpoint. Even though TLR pathway is integral to inflammatory defense mechanisms, its excessive signaling may be devastating. Cells have acquired a cascade of strategies to regulate TLR signaling by targeting protein-protein interactions (PPIs), or ubiquitin chains, but the details of the inhibition mechanisms are still unclear. Here, I provide the structural basis for the regulation of TLR signaling. Structural data suggest that (i) TIR domain-containing regulators interfere with TIR domain signalosome formation; (ii) major deubiquitinases prevent association of TRAF6 and TRAF3 with their partners, in addition to removing K63-linked Ub chains that serve as docking platform for downstream effectors; (iii) alternative downstream pathways of TLRs also restrict signaling by competing to bind common partners through shared binding sites. Missense mutations that fall on interfaces of negative regulators disrupt the interactions with the targets, thereby enabling constitutive activation of NF- κ B, and contributing to chronic inflammation, autoimmune diseases and oncogenesis.

In Chapter 5, I concentrate on some key orchestrators of TLR pathway, such as CASP8, TRAF3, and RIPK1 and model their pairwise interactions. In the binary interactions, I include all partners of these proteins, regardless of whether those are involved in TLR pathway or not. This way, I was able to elucidate the crosstalk of TLR pathway with other cellular signaling pathways and predict the possible ultimate cellular outcome.

In Chapter 6, I focused on different cytokine pathways from a structural perspective. I provide examples for pro-inflammatory and anti-inflammatory cytokines and their roles in tumor microenvironment. I map cancer-related structural pathways of cytokines, and focus on examples illustrating the usefulness of structural data in understanding cytokine function; the role of aberrant cytokine expression in pathogenesis of autoimmune diseases and cancer; the importance of oligomerization modes of cytokines and their receptors; and the benefit of viral cytokine antagonists in designing peptide inhibitors of cytokines.

Chapter 7 explains the methods I employed in this study. I describe how I construct the structures of the PPI complexes, how I obtained the models of monomeric proteins whose

structures haven't resolved yet; and how I evaluated the effects of mutations on interactions.

Finally, Chapter 8 presents the conclusion. I conclude this thesis by emphasizing that structures of PPIs are crucial for having a comprehensive dynamic picture of the cell; a better understanding of the pathogenesis mechanisms of diseases; and development of better drugs.

Chapter 2

LITERATURE REVIEW

In this chapter, a comprehensive review of the studies related to structural PPI networks and their applications are presented. First, PPIs and structural PPI networks and how to build them are described. Then, the crosstalk between cancer and inflammation, TLR pathway and cytokine pathways are reviewed. Finally, the main contributions of this thesis are described.

2.1. Constructing Structural Networks: Merits and Challenges

Proteins may form homo-oligomers, hetero-oligomers, transient or permanent complexes to perform their functions. Better understanding of molecular basis of the protein functions necessitates characterization of the PPIs at the atomic level. PPIs can be determined by high-throughput experiments, such as yeast-two-hybrid, protein arrays, pull-down experiments, and mass spectrometry. However, these techniques do not provide how proteins interact [1]. Construction of the whole structural interactome is one of the major objectives of structural biology [2-4]. Thus, there is no wonder that a major effort by the scientific community has targeted the modeling of PPIs. The availability of structural data relating to the mode of protein interactions is crucial to the understanding of how function is executed, and its control. When tiled together in the framework of the cell, it helps obtain the information flow from the extracellular space, through the membrane, the cytoplasm, and into the nucleus, to turn genes on and off. Signaling is not linear: pathways merge and diverge, signals integrate, and the proteins, which are the nerve centers of these pathway crossings, are frequently involved in cancer. Mutations deregulating these proteins are often responsible for turning on, and keeping on, entire pathways. They can also deregulate proteins that act as repressors of over-expression and activity.

The community has invested much effort in constructing networks and in devising simple and clear ways of presenting them. The most common among these is the so-called node-and-edge representation, where nodes are the proteins and edges their

interactions. This representation has been extremely useful since it essentially provides an overview of which proteins interact, in much the same way as a reference guide. At the same time, such network diagrams also suffer from limitations [3], since the information that they provide is incomplete, and does not allow in-depth understanding of the processes and their regulation. In contrast, structural networks which piece together the structures of individual proteins are much more powerful [5]. Structural networks allow understanding of how pathways cross through key hub proteins; which domains are involved in the interactions and which residues. They provide insight into the question of whether the pathways are controlled allosterically; how constitutive gain- and loss-of-function mutations can turn the protein on or off; and how parallel pathways can take over during drug resistance. Structural pathways are also very useful in drug discovery, because they can help forecast the global effects on the cell [6-9]; and they can help in selecting drug targets which are not the direct 'ailing' dysfunctional protein [10, 11]. Such drugs exploit conformational ensembles [12, 13]; they may also make use of network dynamics [14].

Constructing the structural network of major pathways in the cell is challenging: it entails putting together available structures (determined by crystallography, NMR, and high quality models) when data about their interactions exist in the literature, and when these data are missing, predicting them. It necessitates modeling the linear pathways as well as pathway integration and branching; it also necessitates predicting if the protein can act as a dimer – homo or hetero, and possible oligomerization modes. To grasp the enormity of the challenge, recall that hub proteins, such as p53, can interact with tens or hundreds of partners; Raf can work as a monomer and as a dimer; and EGFR has two dimeric states, symmetric (inactive) and asymmetric (active). However, the interaction surfaces are often unknown [3, 15-17].

So why are structural pathways important? Structural pathways predict new interactions, not observed in 'classical' pathway maps; in particular those involving scaffolding proteins; they allow identification of parallel pathways in drug resistant mutants; they may discover positive feedback loops, altering core processes, as in the case of inhibitors of apoptosis (IAP) [18]. They also provide interaction details, which

allow identification of oncogenic mutations; help drug discovery; and allow assembling multi-subunit signaling complexes, such as the key complex MyD88.

2.1.1. An Overview of the Rationale and Methods for Constructing Structural Pathways

Constructing pathways [3, 5, 19, 20], in particular structural pathways, is challenging. First, experimental structural data are limited, which necessitates exploiting homology models. Homology models lead to increased noise in the predictions [21]. Second, the accuracy of high-throughput data relating to interactions is unclear. Third, ideally, conformational changes in the protein that take place following allosteric events should be taken into account. For example, binding of ligands to the leucine rich repeats (LRR) facing the endosomal lumen of TLR9 dimer induce allosteric changes in the cytoplasmic TIR domains of the dimer [22]. These conformational changes are responsible for activating TLR9 signaling rather than receptor dimerization [22]. The presence of post-translational modifications (PTMs) such as phosphorylation, methylation, and acetylation may also lead to conformational changes. For instance, IRAK1 goes through a conformational change following phosphorylation by IRAK4 [23]. In the unphosphorylated state, IRAK1 is in a closed-inactive state and cannot associate with MyD88 through its death domain and TRAF6 through its C-terminal domain. When it is phosphorylated by IRAK4, it undergoes a conformational transition to an active open form. In the active state, IRAK1 can associate with both MyD88 and TRAF6 [23]. In another example, C/EBP protein, which plays an important role in inflammatory cytokines transcription [24], undergoes a conformational change upon phosphorylation and gets activated [25]. Conformational changes, regardless of their origin – allosteric binding or PTMs – may alter the interactions with partner proteins. Fourth, a structural pathway does not provide the protein availability at any given time; for example, some proteins are expressed only during certain phases of the cell cycle. Similarly, protein localization can also come into play. If a protein is restricted to the nucleus, it cannot interact with another protein present in the mitochondria.

Structures of proteins can be obtained by experimental techniques, such as X-ray crystallography, NMR, and electron microscopy (EM). X-ray and NMR provide high-resolution structures of macromolecules, whereas EM offers low-resolution of larger macromolecular assemblies. Although the number of resolved protein structures increases rapidly with the advances in the experimental techniques, structural space of the whole proteome is still far from complete [26]. The number of experimentally identified interactions is much greater than the number of resolved PPI structures [2]. Therefore, computational approaches can fill in the gaps that experiments cannot resolve [5, 27-29]. Computational modelling of residue-level details of the PPIs is a promising way to elucidate the comprehensive interactome and overall dynamic picture of the cells [3-5, 26, 27, 30].

There are various computational approaches to forecast the structures of PPIs, through co-evolution, co-expression, sequence similarity, and structural similarity [2, 29, 31, 32]. These methods can be grouped into two major classes: ab initio docking and template-based docking [33-35]. Ab initio docking, which treats proteins as rigid bodies in their native form, does not require any prior knowledge and depends on shape and electrochemical complementarity [1]. However, template-based docking (TBD) employs the experimentally determined structures (templates) to model the structures of query proteins (targets) [27, 36, 37]. Detailed assessment studies demonstrated that TBD strategies generate more reliable results [27, 35] and they are more successful at predicting structures of PPIs that undergo conformational changes upon binding if both bound and unbound forms of the template structures are provided [34, 38, 39]. In addition, it is more convenient to use TBD in large-scale proteome-wide applications, since they lower the computational cost and have less false-positive rates [39].

There are different evaluation criteria relating targets to templates in TBD: overall sequence similarity, sequence similarity of the predicted interface, the presence of sequence and structural motifs derived from known interactions and the presence of co-evolving residues in the interface, global structural similarity, local structural similarity, and combination of both sequence and structural similarities [27, 28, 34, 37,

[40](#)]. Methods based on global similarity have been successfully used [\[40\]](#) in cases where more than 30% similarity exist [\[41\]](#). Many of the proteins, however, do not have sufficient sequence identity, thus, such methods are not applicable at large scale. Using previous CAPRI targets, Rodrigues *et al*, however, showed that poor models built by using templates as low as 20% sequence identity, can still yield acceptable predictions (within 3 Å iRMSD), provided that the interface information is reliable [\[42\]](#). Local similarity based methods (interface-based methods), on the other hand, can be applied to cases of low sequence identity to obtain high-quality predictions. These methods have become more popular for large-scale predictions particularly after the studies reporting that protein interfaces are sufficient to cover most PPIs [\[43\]](#).

2.1.2. Predicting and modeling PPIs using template interfaces

The binding sites of proteins are conserved in protein structure space [\[44, 45\]](#). That is, different proteins use the similar interfaces to interact with their partners (as displayed in **Figure 2.1**); thus, previously identified interfaces from structures of protein complexes can be used to predict novel interactions. If the surfaces of two proteins of interest structurally match with the template interface, theoretically these two proteins can interact. Therefore, instead of aligning the entire structures, partial alignment can be performed to relate targets to templates: template interface structures are aligned to global structures of the targets. After superimposition of the monomers to the templates, the constructed models are evaluated by scoring functions. The success of TBD methods depends on whether the interface is present in the template set.

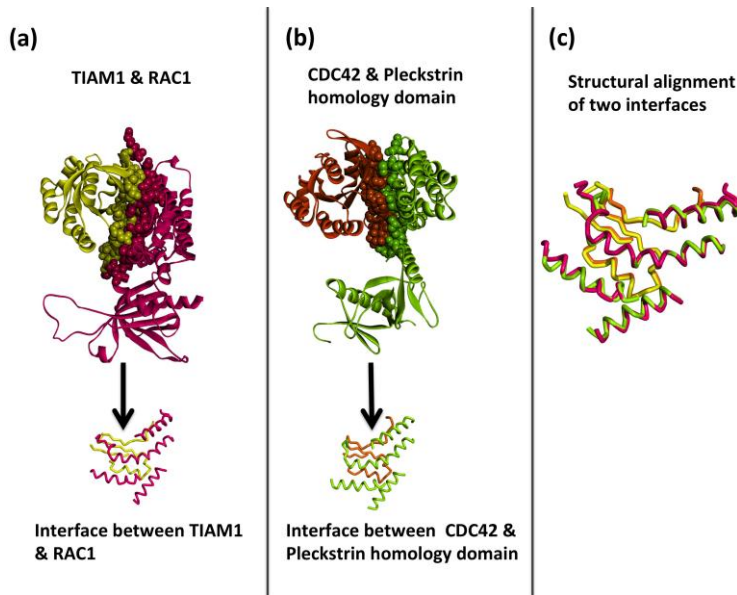


Figure 2.1: Structurally similar interfaces. (a) Interaction between TIAM1 and RAC1 (PDB_ID: 1foe, chains A and B). (b) Interaction between CDC42 and Pleckstrin homology domain (1ki1, chains C and D). The interface between TIAM1 and RAC1 is structurally very similar to the interface between CDC42 and Pleckstrin homology domain. Although the global structures of the proteins differ, they use similar binding surfaces to interact with their partners. (c) Structural alignment of these two interfaces by MultiProt [46]. The two interfaces align almost perfectly (with 1.02 Å RMSD), thus, these interfaces are alike structurally, even though their sequences vary [47].

To address the modeling of the inflammation and cancer network, I use PRISM (PRotein Interactions by Structural Matching) [36, 48, 49]. PRISM is the first algorithm to predict the structures of PPI complexes by employing only interface structure similarity (not necessarily fold similarity), without sequence or global structural similarity [31], later extended with flexible refinement [36]. The template library comprises 22,604 non-redundant template interface structures constructed from the cocrystallized complex structures deposited to PDB [50]. The strength of the interaction is estimated with the number of the contacts that interacting proteins make and their electrochemical complementarity. Its success is assessed in [39] using Docking Benchmark [51] and in [35] with other approaches. Overall, it finds acceptable models for most cases with low conformational changes upon binding. In

certain cases, PDB possesses structures for multiple conformations of the same protein. Additionally, some proteins undergo large conformational changes upon binding and computational prediction/modelling tools perform very poorly in such cases. Availability of different conformations, such as bound, unbound, and post-translationally modified states increases the success of TBD methods. Kuzu et al. [38] showed that even the difficult cases with large conformation changes in the docking benchmark could be accurately predicted if structures of multiple conformations are utilized.

The difficulty in the prediction is compounded by the fact that a particular protein can interact with other proteins via the same or different interfaces (**Figure 2.2**). If a protein interacts with its two partners through the same interface, these two interactions are mutually exclusive. Likewise, if the interfaces overlap, those interactions are also mutually exclusive. Conversely, if two partners bind to a particular protein through different interfaces, with no overlap, these interactions can co-exist [52-54].

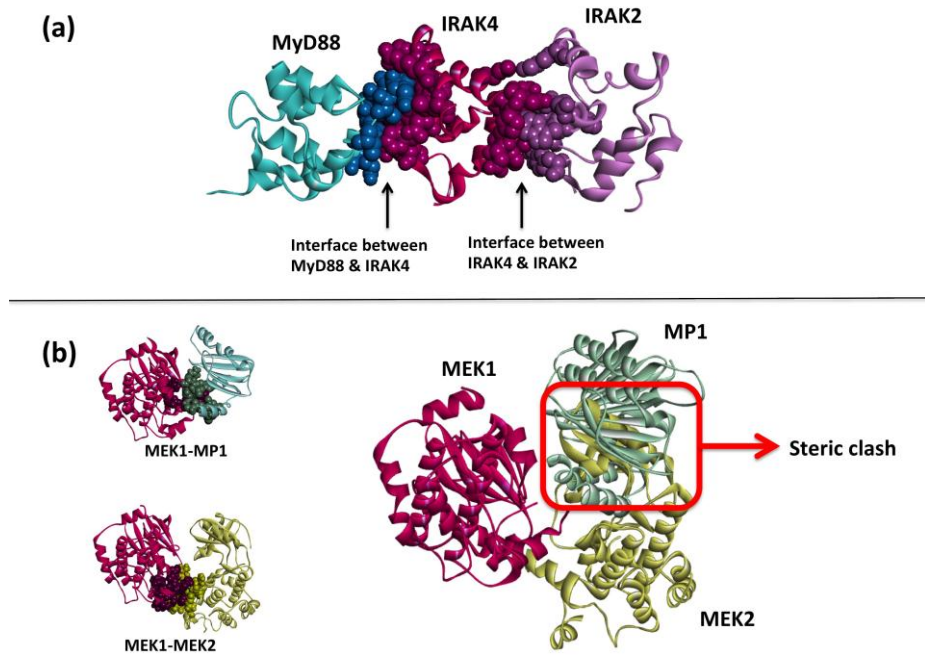


Figure 2.2: Shared and different interfaces. (a) IRAK4 interacts with MyD88 and IRAK2 through different interfaces (PDB_ID: 3mop: F, J, N chains). Thus, these two interactions can co-exist. (b) Modeled interactions of MEK1 protein from in MAPK pathway, with MP1 and MEK2 [4]. MP1 and MEK2 interact with MEK1 through shared interfaces. Therefore, these two interactions cannot take place simultaneously because of the steric clash. (PDB_IDs: 1s9i, 3e8n, 1sko, and 3dv3). [47].

As I noted above, hub proteins interact with tens or hundreds of partners. Due to the limited surface area of a protein, a single protein cannot interact with all of its partners simultaneously. Structural pathways provide the knowledge of which interactions can take place simultaneously and thus the respective pathways co-activated/inhibited, and which are mutually exclusive [52]. Even though the structures of many of these proteins are available in the PDB, and of some in multiple forms (e.g. IRF3 and MAPK), their interactions provide the cellular network, which ultimately highlights how the cell is regulated. **Figure 2.2** depicts some of these key proteins and their interactions.

2.2. Cancer and Inflammation

Inflammation by innate immunity, which is required to fight microbial infections, heal wounds, and maintain tissue homeostasis, can lead to the hallmarks of cancer [55-57]. Several recent studies suggested that inflammation has an important role in all phases of tumor development, including tumor initiation, tumor promotion, invasion, metastatic dissemination, and evading the immune system [55, 56, 58]. Inflammation causes cellular stress and may trigger DNA damage or genetic instability [58], and chronic inflammation can contribute to primary genetic mutations and epigenetic mechanisms that initiate malignant cell transformation [57-59]. Tumor-promoting effects of inflammation alter tissue homeostasis, predisposing individuals to cancer [58, 60, 61]. The connection between inflammation and cancer has been established by Virchow already in 1863, who noticed that cancers originate at sites of chronic inflammation and tumors have “lymphoreticular infiltrate” [57, 58, 62, 63]. Inflammation establishes a tissue microenvironment, which tolerates tumor growth and metastasis by setting immunosuppressive mechanisms [58]. Therefore, inflammation not only induces carcinogenesis but also makes immune cells incapable of destroying tumor cells. A key initiator of a major pathway leading to inflammation is the TLR pathway (displayed in **Figure 2.3**).

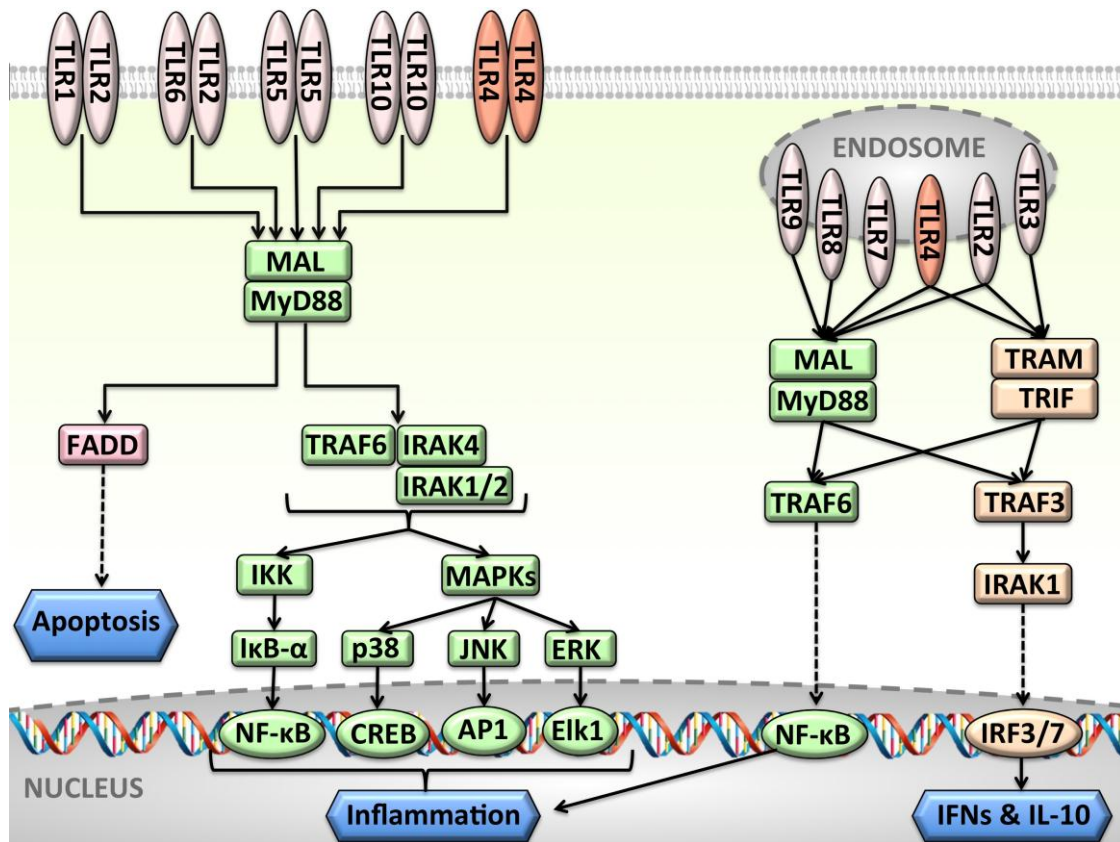


Figure 2.3: Toll-like receptor pathway (adapted from literature [64-66]), in traditional node-and-edge representation: nodes represent proteins and edges represent interactions between proteins. TLR pathway is complicated and has many branches. Stimulation of TLRs propagate the signal through two parallel paths: MyD88-dependent path (green), which leads to production of pro-inflammatory cytokines, and TRIF-dependent path (orange), which gives rise to transcription of antiviral proteins – interferons – and anti-inflammatory cytokine IL-10. MyD88-mediated pathway also has three branches, namely TRAF6- (green), TRAF3- (orange) (downstream of endosomal TLRs), and FADD-dependent (pink) downstream pathways. For space limitation, I showed TLR on endosomal membrane as monomers, but they also dimerize upon stimulation [67].

Inflammatory cells supply growth factors to maintain proliferation, survival factors to escape from apoptosis, pro-angiogenic factors and extracellular matrix (ECM) modifying enzymes that enable angiogenesis, invasion and metastasis [55].

Inflammatory cells can also secrete reactive oxygen species (ROS) that induce mutations, lead to failure of DNA repair, activation of oncogenes, and ultimately cancer [55, 57, 58]. ROS further activates inflammatory genes and takes part in tumorigenesis which is regulated by c-MYC, K-Ras, and Wnt signaling pathways [55].

The first evidence that connected inflammation with cancer was that inflammatory diseases such as inflammatory bowel disease (IBD) increase cancer susceptibility [68]. Additional evidence comes from the tumor microenvironment, which has inflammatory cells, cytokines and chemokines. In addition, long term administration of non-steroidal anti-inflammatory drugs (NSAIDs) leads to a decrease in the number of relapses and newly acquired tumors [55]. Moreover, pathways of inflammation function downstream of oncogenic mutations (MYC, RAS, BRAF, and RET), suggesting that these oncogenic mutations lead to activation of an inflammatory pathway [69]. Last but not least, inhibiting inflammatory cytokines and chemokines such as TNF- α , IL-1 β , or essential transcription factors, such as NF- κ B and STAT3, decrease the appearance of cancer [61]. Most of these proteins that connect inflammation and cancer are also members of TLR-pathway.

The TLR pathway in **Figure 2.3** is presented in the classical node-and-edge form. As I noted above, such pathway diagrams are informative and have been enormously useful in compiling and laying out the interaction maps. As can be seen in **Figure 2.3**, they provide the interconnectivity between the proteins and outline the major signaling flow. Nonetheless, the information that they are able to provide is incomplete. Structural pathways can fill in gaps; add missing proteins and interactions, and provide the interaction details, which can help in figuring out parallel pathways, and the conformational mechanisms that control them. These are important for understanding function under physiological conditions and oncogenic mutations and drug resistance in disease.

The link between inflammation and cancer appears to stem from two pathways [70], the intrinsic and extrinsic inflammation pathways, as shown in **Figure 2.4**. Intrinsic inflammation is initiated by mutations that lead to activation of oncogenes and

inactivation of tumor suppressors (tumor-promoter role) [61]. On the other hand, in the extrinsic pathway, infection or inflammation precedes cancer and increases the risk of cancer (tumor-initiator role) [61]. The similarity between cancer tissue and inflamed tissue involves angiogenesis and tissue infiltrating leukocytes, such as lymphocytes, macrophages and mast cells [55, 56].

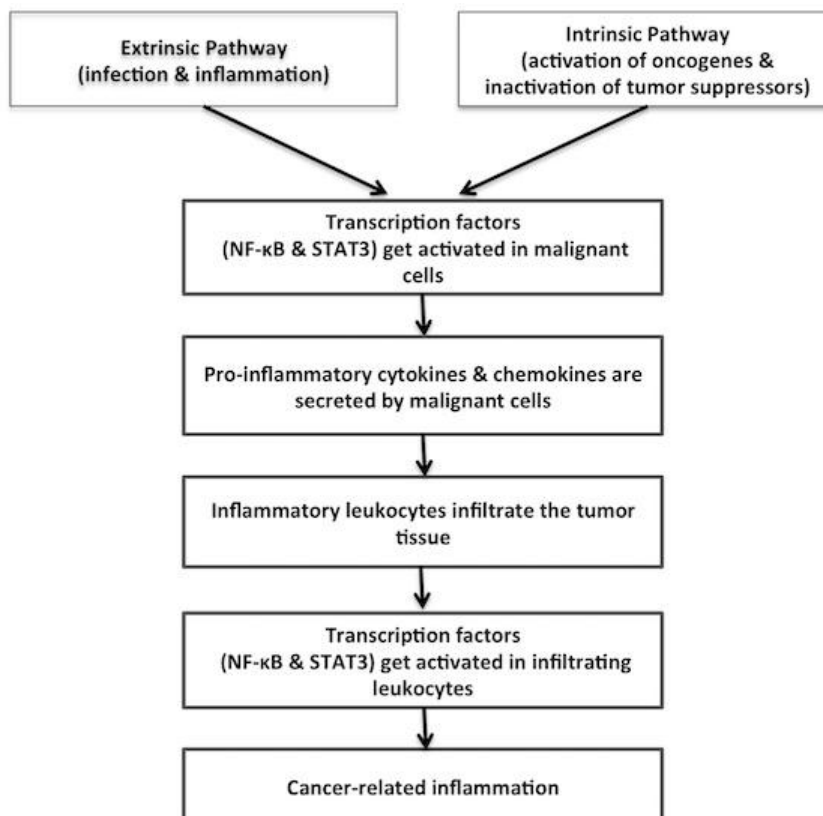


Figure 2.4: The extrinsic and intrinsic pathways of inflammation [47].

RAS, MYC, RET, and BRAF are among the oncogenes that stimulate inflammation [58]. Besides their roles in tumor initiation and promotion, oncogenes impact the relationship between the tumor and its microenvironment. The RAS-RAF signaling pathway is involved in most cancer types, and the activated proteins along the pathway induce the secretion of pro-inflammatory cytokines and chemokines [71]. Another oncogene, MYC, has a critical role in tissue remodeling of ECM [61, 72]. RET, a tyrosine kinase, can be constitutively activated by mutations even in the absence of its ligands.

As oncogenes, tumor suppressor proteins also modulate the production of pro-inflammatory elements. Transforming growth factor-1 beta (TGF- β), a tumor suppressor protein, accelerates tumorigenesis by increasing tumor-promoting inflammation. Other examples of such tumor suppressors are phosphatase and tensin homologue (PTEN) and von Hippel Lindau (VHL) proteins [61, 69, 73].

Tumors are not composed of solely homogenous cancer cells, but are rather heavily infiltrated by cells, both the innate and adaptive immune cells, like macrophages, and mast cells [56, 60]. In addition to immune cells, epithelial cells, endothelial cells, fibroblasts and stromal cells, infiltrate tumor tissue [55]. Mast cells produce compounds that preserve chronic inflammation and support tumor development [74]. Some of the inflammatory cells, namely, regulatory T cells (T_{reg}s), myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) have immune suppressive roles [56, 61]. MDSCs and TGF- β released by cancer cells may inhibit cytotoxic T cells and natural killer cells [56]. Inflammatory cells also secrete epidermal growth factor, EGF (tumor growth factor), vascular endothelial growth factor, VEGF (pro-angiogenic factor), and matrix degrading enzymes, which help tissue-remodeling, angiogenesis and metastasis [56].

2.3. Overview of TLR Signaling

TLRs orchestrate the innate and adaptive immune systems and have an essential role in inflammation [47]. The TLR pathway plays critical roles in almost every phase of tumor development [58]. Two opposing roles are attributed to TLRs: anti-tumor and pro-tumor actions [75]. TLR-induced inflammation promotes cancer via proliferative and anti-apoptotic factors [76]. TLRs are highly expressed in tumor cells and they confer chemoresistance, escape from immunity, and promote tumor growth [77, 78]. For instance, MyD88 and NF- κ B signaling through TLR4 on ovarian cancer cells enable these cells to escape from immunity and contribute to cancer progression [57, 79]. On the other hand, there is also evidence to the contrary: TLR activation of cancer cells helps to inhibit the proliferation of cancer cells [57]. These conflicting results demonstrate the complexity of TLR action on cancer cells. A complete structural TLR

pathway can help to forecast what will be the outcome: inhibition or promotion of tumor.

TLRs detect the presence of pathogens and initiate inflammation. TLRs belong to pattern recognition receptors (PRRs) and they recognize highly conserved motifs, pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) of gram-negative bacteria and damage-associated molecular patterns (DAMPs), such as high mobility group box-1 (HMGB1) protein or heat shock proteins [57, 59, 80, 81]. Hence, pathogen-derived compounds and endogenous molecules of damaged tissue serve as ligands for TLRs. TLRs are single-pass trans-membrane receptors with extracellular LRR and cytoplasmic Toll/IL-1R (TIR) domain [57]. TLRs are expressed in a variety of cell types, including innate immune cells, T and B lymphocytes (adaptive immune cells), endothelial cells, epithelial cells, fibroblasts and cancer cells [57]. Up to now, 11 mammalian and 13 murine TLRs have been identified [57]. Some of the TLRs are found on plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10), whereas others are located on endosomal membranes (TLR2, TLR3, TLR4, TLR7, TLR8, TLR9) [57, 82]. Upon binding with their ligands on extracellular LRR, TLRs form homo- or hetero-dimers, recruit some TIR domain-containing adaptor proteins to their cytoplasmic TIR domains and activate downstream signaling pathways (see **Figure 2.3**) [57, 81]. They have six adaptor proteins, Myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (Mal, also known as TIRAP) [83], TIR domain containing adaptor inducing interferon- β (TRIF, also known as TICAM-1) [84], TRIF-related adaptor molecule (TRAM, also known as TICAM-2) [85], sterile α and heat-armadillo motifs (SARM) [86], and B-cell adaptor for PI3K (BCAP) [87]. MyD88 is the common adaptor for almost all TLRs, except TLR3 and also IL-1R family members [84]. Mal serves as a bridging adaptor between MyD88 and TLRs. Binding surfaces of both MyD88 and TLRs are electropositive, thus they repel each other. However, Mal, with an electronegative surface, brings MyD88 and TLRs together [83, 84, 88]. Likewise, TRAM acts as a bridge between TLR3 and TRIF.

TLR signaling induces expression of pro-inflammatory cytokines, interferons (IFNs) and interleukin-10 (IL-10, an anti-inflammatory cytokine). While IFN production suppresses cancer, pro-inflammatory cytokines promote it [89]. Upon stimulation, TLRs cluster in lipid rafts and follow one of two pathways, as displayed in **Figure 2.3** [64, 90]. In their MyD88-dependent pathway, the TLR TIR domains associate with TIR domains of MyD88 and Mal proteins. MyD88 TIR domain is connected through a long linker to its death domain (DD). Through its DD, MyD88 can initiate three downstream pathways (**Figure 2.3**). In the first, pro-survival inflammatory pathway, it recruits serine/threonine kinases IRAKs (Interleukin-1 receptor-associated kinases) to stimulate the TNF receptor-associated factor 6 (TRAF6), IKK complex and MAPKs, (e.g. ERK, JNK, and p38 [91]) and transcription factors NF- κ B, AP-1, and CREB [65, 92], which ultimately result in transcription of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α), and IL-1 β [61]. In the second path, MyD88 DD binds to TRAF3 instead of TRAF6 (only TLRs on endosomal membranes recruit TRAF3[93])[66]. TRAF3 is a negative regulator of TLR- and TNFR-mediated MAPK activation and has to be degraded for MAPK stimulation [66]. Instead of activating NF- κ B, it activates interferon regulatory factors (IRFs) [94]. In the third death path, MyD88 DD associates with FADD (Fas-associated death domain) protein, which leads to apoptosis. In the TRIF-dependent pathway, IRFs dimerize and get activated, producing IFNs. Whether Mal and TRAM bind to TLR4 competitively using the same interaction surface has been unknown [95], but several studies pointed out that they do [93, 95, 96]. This is important since it could explain the outcome of inflammation/cancer-related aberrations or mutations on the Mal and TRAM binding surfaces, or overexpression of either of these. It was suggested that upon engagement of TLR4 with its cognate ligand LPS, these two pathways are activated sequentially: first the MyD88-dependent and then the TRIF-dependent [64, 84, 93]. They proposed a mechanism in which TLR4 on the plasma membrane first associates with Mal and MyD88, leading to NF- κ B activation. Then, TLR4 is endocytosed and gets associated with TRAM and TRIF, leading to transcription of IFN- α and IFN- β .

2.4. Negative Regulation of TLR Signaling

Defects in TLR signaling predispose individuals to infections [97]. Over-activation can result in chronic inflammatory and autoimmune diseases and contribute to oncogenesis [57, 87, 97, 98]. To retain a delicate balance between activation and inhibition, and avoid detrimental effects of excessive inflammation, TLR signaling is strictly regulated [99, 100]. I aim to better understand the mechanisms of negative regulation of the TLR pathway to obtain insights into how the balance is established and the immunological homeostasis is achieved.

Negative regulation of TLR signaling takes place at multiple steps, ranging from extracellular soluble decoy TLRs, to transmembrane and intracellular inhibitors such as those that contain TIR-domain [87, 99]. At almost each step in the TLR signaling cascade, protein interactions or ubiquitin (Ub) chains are targeted by one or more inhibitors (**Figure 2.5**). The presence of several checkpoints and the redundancy of negative regulators suggest that regulation can be achieved by a cascade of regulators, indicating that a particular inhibitor may be essential but not enough to fine-tune signaling [99], or that different regulators may take action in different tissue types or at different times. While some inhibitors are constitutively expressed, others are upregulated upon TLR stimulation. TLR-induced apoptosis comes into play when negative regulators fail and the cell becomes overactive [99]. Alternative downstream paths of TLR signaling are competitive [67], limiting signaling through the temporally outcompeted pathways. I focus on cytoplasmic and transmembrane regulators to elucidate the mechanisms through which they attenuate TLR signaling and the resulting inflammation.

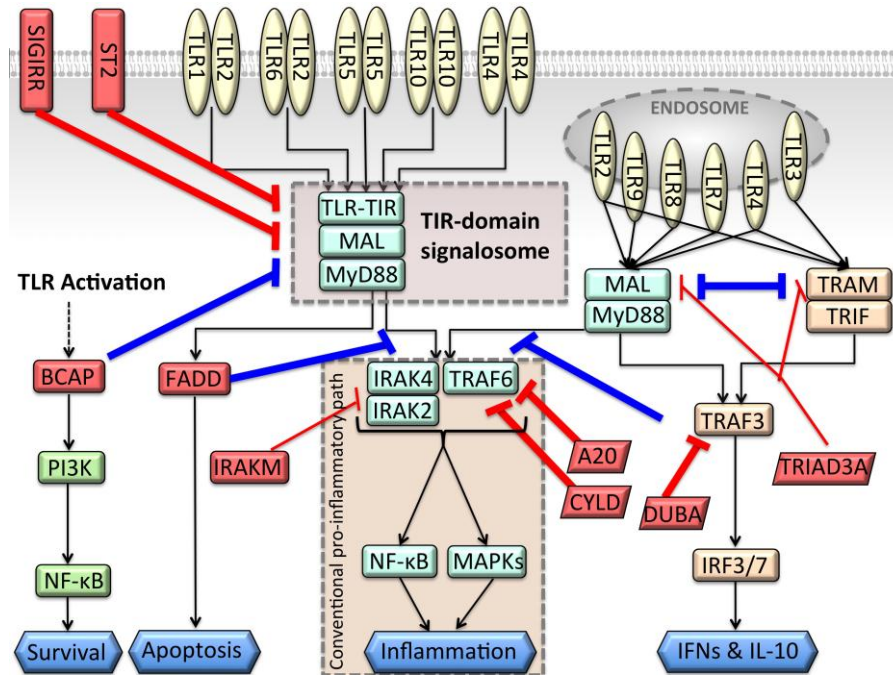


Figure 2.5: Negative regulators take action at almost all steps of TLR signaling. Red-labeled edges show the negative regulators that turn-off the TLR signaling. The bolded red edges are the interactions that are modeled here. Blue-labeled edges are negative regulations from the parallel downstream paths to the conventional pro-inflammatory path. They switch the signaling path, not terminate the signal [101].

2.5. Structural Pathways of Cytokines may Illuminate Their Roles in Regulation of Cancer Development and Immunotherapy

Cytokines are complex messengers that enable crosstalk between tissues and the immune system. They also underlie events in tumor initiation and progression [102]. Cytokines, their receptors, downstream consequences, as well as their upstream pathways that promote cytokine production are important in malignant transformation. **Figure 2.6** displays node and edge networks of some major cytokines. Structural details of these pathways can provide novel insights to the mechanisms of cytokine-induced oncogenesis.

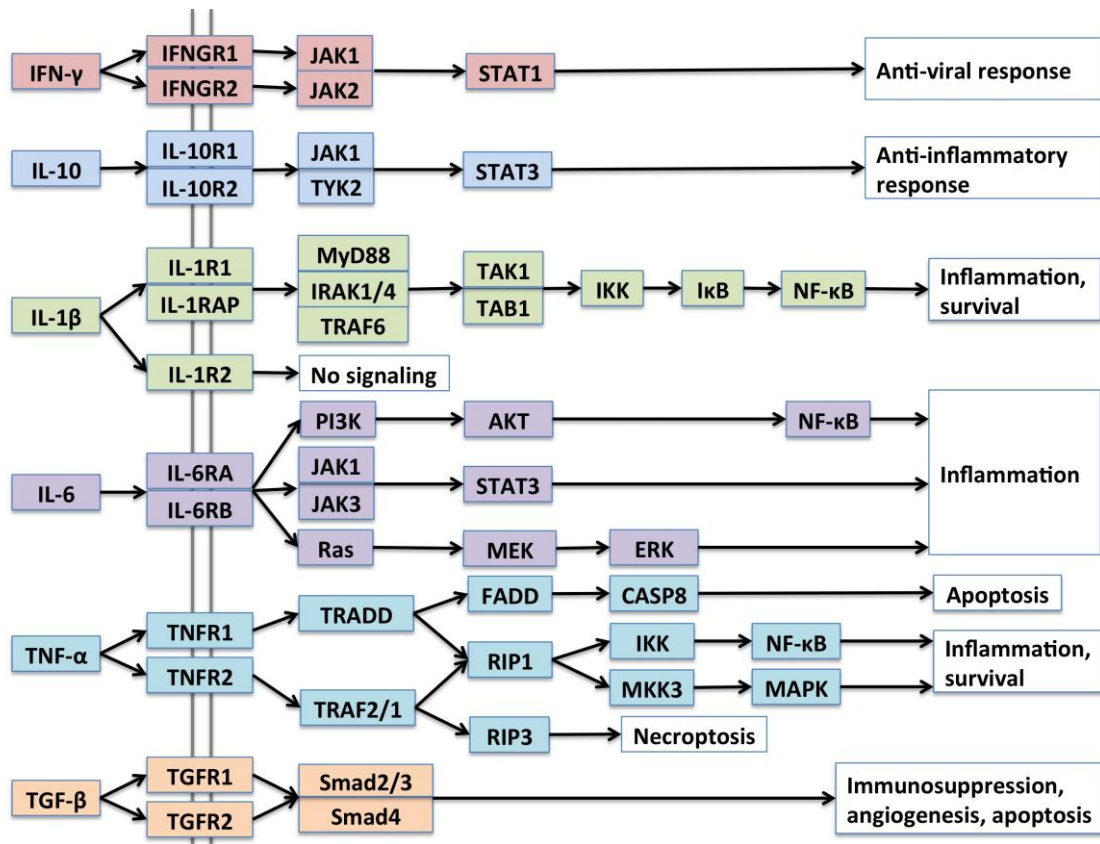


Figure 2.6: Pathways of major cytokines, in node-and-edge representation [103].

Cytokines are grouped into nine categories according to Kyoto Encyclopedia of Genes and Genomes (KEGG): chemokines, interferons (IFNs), tumor necrosis factors (TNFs), transforming growth factor- β (TGF- β) family members, interleukin-1 (IL-1) family members (such as IL-1 α , IL-1 β , and IL-18), IL-10 family members, IL-17 family members, hematopoietic growth factors (such as IL-1, G-CSF, GM-CSF), and platelet derived growth factors (PDGFs) [104]. Cytokine receptors are classified into seven subclasses: Type-I cytokine receptors (IFN- α R, IFN- β R, G-CSFR, GM-CSFR, etc.), Type-II cytokine receptors (IFN-GR, IL-10R), TGF- β receptors, TNF receptors, immunoglobulin superfamily receptors (IL-1R), G-protein coupled receptors, and IL-17 receptors [105].

Cytokines are a diverse group of signaling molecules that are produced as a result of infection, inflammation, injury, and cellular stress [106]. They activate immune cells and regulate their development, differentiation, and migration; they stimulate host

immune responses to control stress and minimize cellular damage, and thus restore homeostasis [104, 106, 107]. These messenger proteins are either secreted or are membrane-bound, allowing communication of immune cells with each other in a paracrine, autocrine or endocrine fashion [104, 105]. Membrane-bound forms of cytokines allow communication through direct cell-cell contact, but secreted cytokines permit rapid spread of the signal to other tissues [105]. They act locally or systemically to coordinate acute or chronic inflammatory responses.

Cytokines have complex and even opposing roles in the immune system [105]. They are either pro- or anti-inflammatory. Pro-inflammatory cytokines, such as IL-1 β and TNF- α , induce inflammation as a result of infection or injury; anti-inflammatory cytokines, like IL-10 and TGF- β , suppress the activity and production of pro-inflammatory signals [108], limiting inflammation and host damage. While not the individual cytokines, their overall balance determines cell fate: stimulate inflammatory response or suppress it [108]. Different cytokine combinations give rise to distinct consequences, such as inflammation, proliferation, and angiogenesis [104]. Imbalances in cytokine expression or signaling contribute to malignant transformation [106].

Cytokines stimulate host immune responses not only against pathogens, but against tumors [105]. Host-derived cytokines can inhibit tumor progression as well as promote proliferation, decrease apoptosis, and foster invasion and metastasis [106]. They are important orchestrators of cancer-related inflammation [102, 109]. Tumor cells perturb tissue cytokine expression and these alterations in cytokine levels recruit infiltrating leukocytes to the tumor microenvironment, which in turn contribute to extra cytokine production [58, 106, 110, 111]. Cytokines are pleiotropic, allowing a particular cytokine to act on various cell types; they may also have opposite effects on distinct target cells [104, 106, 108], depending on other cytokines present in the local environment. Cytokines are redundant, with multiple cytokines having similar activities [104, 106, 108, 112], allowing them to compensate for each other in deficiency or drug resistance.

The immune system has dual roles in cancer: it eradicates cancer cells when it recognizes tumor antigens and provokes anti-tumor immune response. On the other hand, the immune system selects for resistant cancer cells best fit to survive in immunocompetent hosts and promote its growth, which is also called “cancer immunoediting” [113]. During primary tumor development, immune surveillance eliminates tumors, but established cancers provoke immune tolerance [114, 115]. Tumors in immunodeficient host are “unedited” and more immunogenic; when transplanted to another host with intact immune system, they alert anti-tumor immunity. However, they are “edited” in immunocompetent host, allowing them to escape the immune system [113-115].

Cytokines play critical roles in the pathogenesis of autoimmune and auto-inflammatory diseases and cancer. Nevertheless, they are also used as therapeutic agents in such diseases. Much effort has been invested in cancer immunotherapy, exploiting cytokines in cancer treatments to activate immune responses against cancer [105]. Manipulation of cytokine levels can be used to stimulate immune responses [106]. Several cytokines have been used in clinical trials, and Type-I IFNs were proposed to be beneficial for treatment of cancer [105]; however, cytokine redundancy hinders such efforts [105].

In order to investigate the role of cytokines in carcinogenesis, cytokine-deficient mice were used in several studies [106, 115-120]. For instance, lack of IL-1 decreases tumor invasiveness and angiogenesis [105] and IL-1R antagonists suppress tumor growth [120]. TNF- α -deficient mice are resistant to skin tumor [118]. Thus, pro-inflammatory cytokines IL-1 and TNF- α promote tumorigenesis. In contrast, IFN- γ contributes to prevention of carcinogen-induced sarcomas [115] and its loss enhances tumor formation [106]. IL-10 deficiency increases IL-1 levels, promoting cancer in the absence of IL-10 [121]. Thus, anti-inflammatory cytokines IFN- γ and IL-10 protect the host against cancer. However, the inter-relationship between pro- and anti-inflammatory cytokines and oncogenesis is still not entirely clear. TGF- β acts to inhibit cancer in normal and pre-cancerous cells, although cancer cells lose tumor-inhibitory effects of TGF- β , invade and metastasize to other tissues [122].

2.6. Main contributions

Structures of PPIs depict how signal is relayed to downstream effectors, how regulation is achieved, and whether parallel pathways can be activated simultaneously. They also help in figuring out the mutation mechanisms and in drug discovery. Despite the recent advances in experimental procedures to determine the structures of proteins, there are still a large percentage of proteins, particularly PPIs, whose structures yet to be determined. Computational tools help address this problem: enrich the available structural data for PPIs, and analyze the effects of mutations on the interactions and pathways. PRISM is a powerful tool to model structures of PPIs. In this study, I utilized PRISM algorithm, available experimental mutagenesis/biochemical data, and crystal structures to construct the structural network of TLR and other cancer-related pathways.

I provided structural models for the key multi-protein complexes, such as “TIR-domain signalosome” and death inducing signaling complex (DISC). Although there are some models available for the individual pairwise interactions in TIR domain signalosome, structure for the entire complex is not present. Together with the myddosome crystal structure, our TIR domain signalosome model, and downstream interaction models allowed us to explain how TLRs cluster at lipid rafts upon activation, whether parallel downstream paths are mutually exclusive, and how oncogenic mutations facilitate oncogenesis through constitutive activation of NF- κ B. I also showed that most of the negative regulators of TLR pathway interfere with the formation of key signalosomes and interactions. Through our models, I provided the structural basis of the crosstalk between TLRs with other pathways, such as Fas and TNFRs.

Besides TLR pathway, I also modeled other cancer-related pathways, namely CASP8, TRAF3 and cytokine pathways. Through our models I could explain how CASP8 gets activated when it is recruited to the DISC complex and how its mutations resists cell death in tumor tissues. I also structurally clarify how/why stimulated and unstimulated cells leads to distinct outcomes.

The architectures introduced in this dissertation may assist structure-based drug design studies for chronic inflammatory and autoimmune diseases and also cancer. Formation or inhibition of some interactions contributes to pathogenesis of certain diseases. Therefore, PPI architectures I present may guide scientists where to target the inhibitors on target protein.

Chapter 3

STRUCTURAL TLR PATHWAY

This chapter presents the structural TLR pathway that I constructed by employing PRISM. I included not only the binary interactions of proteins, but also the multimeric signaling complexes to reveal a comprehensive picture of the TLR signaling. At almost all levels of TLR signaling, proteins oligomerize to form large multimolecular assemblies. Upon activation, TLRs form clusters at lipid rafts [123, 124]; TIR-domain containing adaptors form the TIR-domain signalosome; DD-containing proteins form the myddosome [90]; and TRAF6 forms an infinite network by trimerization of their TRAF-C domains and dimerization of their RING-domains [125]. Oligomerization of proteins facilitates execution of cellular functions by amplifying signals and allowing more efficient enzymatic reactions [126]. In support of this, it has been demonstrated that induced TLR4 clustering activates TLR4 even in the absence of its ligand LPS [124].

TLR1, TLR2, TLR10, Mal, MyD88, TRAM, TRIF, and IL-1RAPL have resolved structures of their TIR-domains. Among these, TLR10 (PDB_ID: 2j67), C713S mutant TLR2 (PDB_ID: 1o77), and IL-1RAPL (PDB_ID: 1t3g) are in homodimer form. Each TIR domain is composed of five central β -strands (A-through-E) and six surrounding α -helices (A-through-E) [127]. The loops that connect helices and strands are named by the elements that they link. Although the overall structure is similar across TIR domains, their loops vary [128]. TIR-domain containing proteins associate through TIR-TIR interactions and form multimeric signalosomes. Several models were proposed and almost all point to the importance of the BB-loop [83, 85, 95, 127, 129-131]. These studies identify interface residues by mutagenesis. However, there are some contradictions among the studies relating to interface residues that are involved in TIR-TIR interactions. For instance, the C747S mutation is said to inhibit TLR4 homodimerization [95], whereas other studies suggested that blockage of C747 by a small molecule TAK-242 (resatorvid) inhibits TLR4 signaling not because it interferes with TLR4 dimerization, but because it abolishes TLR4-Mal and TLR4-TRAM interactions [129]. I draw two conclusions from these findings: some mutations may

be allosteric and are not necessarily on the interface, or there is more than one binding mode.

Mutagenesis studies identified interface residues and led to structural models of some of the binary interactions of TIR-domain signalosome [83, 85, 95, 127, 129-131] but not of the entire complex. Here, I model the MyD88- and TRIF-dependent signalosomes by exploiting the powerful PRISM algorithm [36, 49]. The architecture that I obtain provides the structural basis for TLR clustering through formation of a TIR-domain signalosome with 8 MyD88 molecules and a helical myddosome crystal structure with 6 MyD88 (**Figure 3.1**). Our binary interaction models are compatible with available experimental data. Significantly, our results reveal how regulation at key anti- and pro-inflammatory signaling checkpoints takes place, providing insight into TLR and MyD88 signaling decisions.

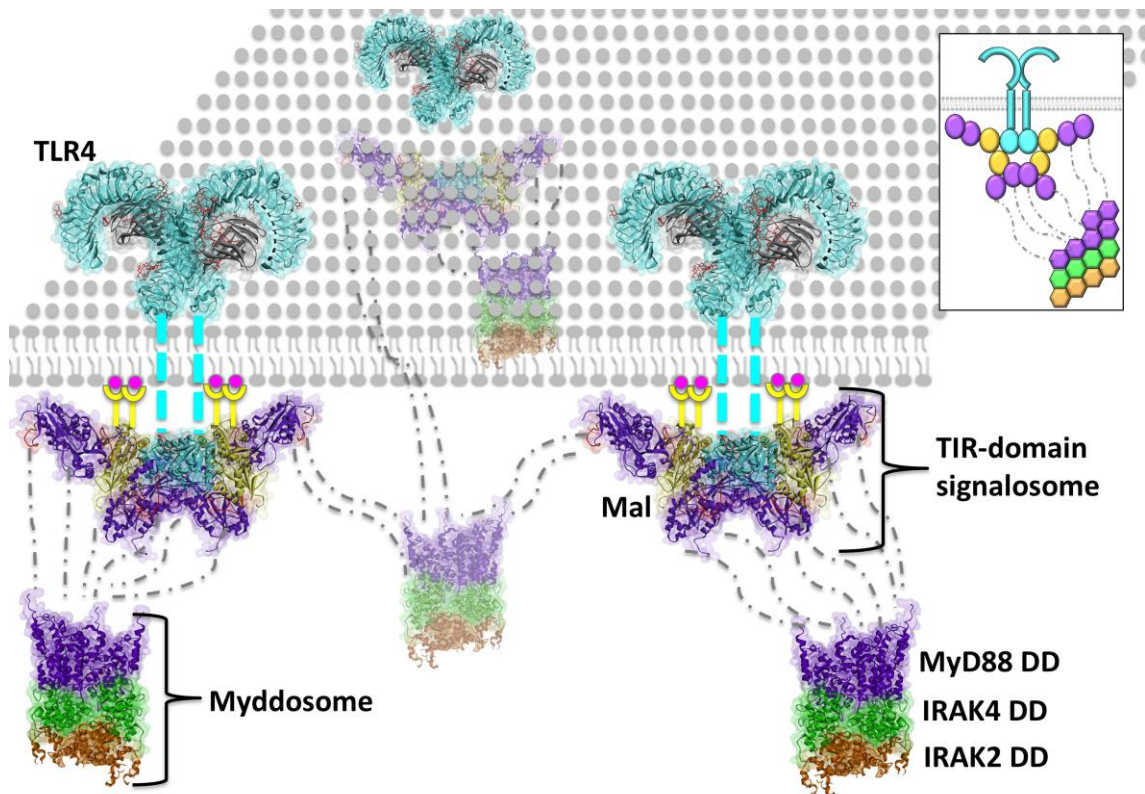


Figure 3.1: 3D schematic view of TIR-domain signalosome, myddosome and TLR clustering. It is known that TLRs cluster on lipid rafts, but they cannot tetramerize due to the steric hindrance of their ectodomains [126, 132]. Oligomerization of the downstream proteins may hold TLRs together. Here, all TIR domains are in dimer form, TLR4, Mal, and MyD88. A TLR4-dimer recruits two Mal-dimers, which in turn recruit four MyD88-dimers. In the myddosome complexes, there are six MyD88 molecules, four IRAK4 and four IRAK2. The box at the upper right corner shows the cartoon version of the model (circles correspond to TIR domains and hexagons correspond to DDs). The PDB_ID of the myddosome complex is 3mop: MyD88 death domains 3mopBCDE, IRAK4 death domains 3mopGHIJ, IRAK2 death domains 3mopKLMN. Pink circles are PI(4,5)P₂, which are enriched in lipid rafts and N-terminal region of Mal associates with it [67]. Cyan represents TLR4, yellow represents Mal, purple represents MyD88, green represents IRAK4, and orange represents IRAK2.

3.1. Multimeric TIR Domain Signalosome Assembly and TLR Clustering

TLRs' clustering is crucial for efficient signaling, but they cannot form clusters through tetramerization or higher order oligomerization due to steric hindrance of their ectodomains [126, 132]. Instead, oligomerization of downstream proteins may hold TLRs in close proximity via a linked network mesh. I built MyD88- and TRIF-dependent TIR domain signalosomes, comprising TLR4/Mal/MyD88 or TLR4/TRAM/TRIF. The signalosome models are meshed through the myddosomes whose crystal structure is available (**Figure 3.1**). The linker region (45 residue-long) of MyD88 between its TIR and DD is essential for the TLR clustering. A splice variant of MyD88, so called MyD88s (short MyD88), lacking the interdomain linker region has been shown to inhibit NF- κ B activation [133] and this outcome is attributed to its inability to recruit IRAK4, which is necessary for nucleation of the myddosome assembly. In support of this, our model suggests that without myddosome formation, TLRs cannot cluster and no signal is relayed to downstream effectors.

In our MyD88-dependent signalosome, all TIR domains are in dimer form: a TLR4-dimer recruits two Mal-dimers, which in turn recruit four MyD88-dimers. Different signalosome schemes, with varying stoichiometries of Mal and MyD88 were proposed before: some of them show 2 Mal and 2 MyD88 molecules per TLR4 dimer in the signalosome [134, 135], whereas others include 2 Mal and 4 MyD88 (2 dimers) [136]. However, studies clearly revealed that both Mal [127] and MyD88 [137, 138] should be in dimeric form to assist the signaling by serving as a binding platform. Different signalosome schemes, with varying stoichiometries of Mal and MyD88 will give rise to different mesh-like structure scenarios. Below I outline the step-by-step construction of TIR domain signalosomes.

3.1.1. TLR4 Dimerization

Upon stimulation, TLRs form homo- or hetero-dimers with their Leucine Rich Repeats (LRRs) and TIR domains [96]. The structure of the TLR4 TIR-domain has not yet been resolved. I built its model by the I-TASSER server (residues 672-818) [139]. The crystal structure of the TLR1 TIR domain (PDB_ID: 1fyv) was used as the template.

The model has 1.29Å RMSD with TIR domains of other TLRs over 111 residues and other TLRs have 1.22Å RMSD over 112 residues with each other. Several models have been proposed for the TLR4-TLR4 interaction based on mutagenesis [95, 96, 129] with disagreements among these with respect to interface residues [95, 129]. Such diverse findings for the interface region may suggest different binding modes for TLR4 dimerization. Also, the presence of other partners, like Mal, may change the TLR4 binding mode preference. In line with this idea, I found three different TLR4-homodimer organizations (**Figure 3.2**). Details of the interactions are in **Table A.1**.

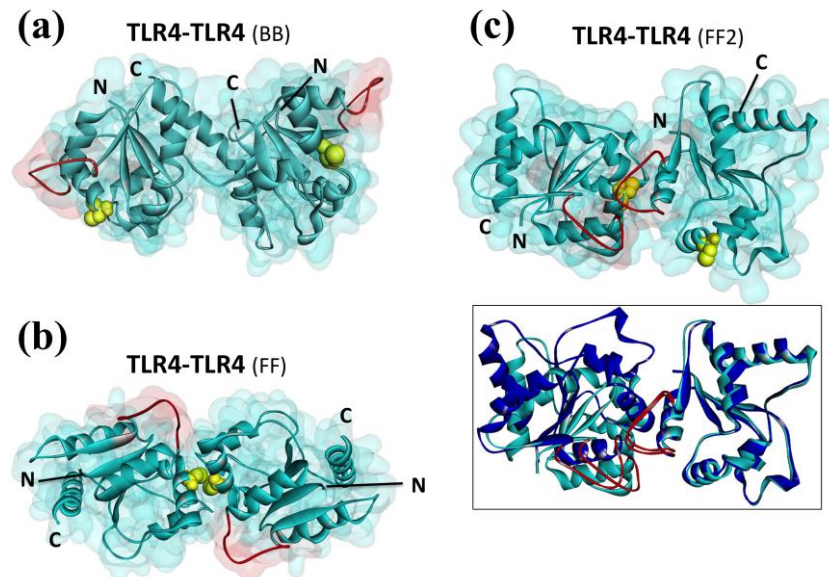


Figure 3.2: TLR4 homodimer models. (a) Back-to-back TLR4 dimer (BB). (b) Face-to-face TLR4 dimer (FF), which is very similar a previously suggested TLR4-dimer model [95] and crystal structure of the dimeric TIR domain of IL-1RAPL (1t3g.pdb) [140]. (c) Another face-to-face dimer (FF2) in which BB-loops are in very close proximity. The box in the lower left corner shows the structural alignment of this TLR4-homodimer model with the one that is obtained by superimposition of TLR4 with TLR2 homodimer crystal structure (1o77_CD), (146 of 276 residues with 0.73 RMSD by multiport). Cyan color is TLR4 TIR domain, red-labeled regions are BB-loops, and yellow spheres are C747 residues on each TLR4 TIR domain, which are suggested to be involved in the interface. The dark blue dimer in the box is TLR4-dimer, which is obtained by superimposition with TLR2 dimer (1o77_CD) [67].

In the first potential TLR4-homodimer model (**Figure 3.2a**), BB-loops face opposite directions (back-to-back dimer, BB), contrary to what has been suggested before [95]. The second with BB-loops facing each other (face-to-face, FF) (**Figure 3.2b**) is very similar to a previously proposed TLR4-homodimer [95] and a crystal structure of the dimeric TIR domain of IL-1RAPL (1t3g.pdb) [140]. In addition, the C747 residues that have been suggested to be at the interface [95] are very close to each other. FF interface is the major interface for downstream signaling. The third model shown in **Figure 3.2c** is very similar to TLR2-homodimer crystal structure (1o77_CD), in which the BB-loops are in close proximity (face-to-face-2, FF2). Although this structure is very similar to a crystal packing TLR2-dimer it is not plausible with the downstream TIR-domain interactions, which have the mutation-indicated interface residues at the correct sites: meaning that this interface has steric clashes with downstream interactions. The homodimer captured in the crystal may not be the physiological conformation. I thus built the TIR-domain signalosome complexes for the BB and FF models.

3.1.2. Mal Dimerization

Mal has a TIR-domain and a small amino terminal localization domain, through which it can interact with phospholipids, particularly phosphatidylinositol-4,5 bisphosphate (PI(4,5)P2) that is enriched in lipid rafts [135]. Mal is a homodimer *in vivo* [83]. Recently, it was suggested that Mal dimerization facilitates its interactions with MyD88 and TLR4 by forming a binding platform [127]. Unlike other TIR-domains, Mal TIR-domain lacks a BB-loop, but has an extraordinarily extended AB-loop. BB-loops of other TIR domains correspond to a part of Mal's AB-loop [83, 127]. In Mal crystal structures (4fz5, 2y92, 3ub2, 3ub3, 3ub4, 4lqd), most of Mal's AB-loop residues (21 residues) are missing. The asymmetric unit of the crystal (3ub2.pdb) displays a symmetrical back-to-back Mal-dimer with the AB-loops facing the opposite direction [129], which has been suggested to be the physiological state [83]. I obtained a Mal-homodimer organization, which is very similar to the unit cell Mal-dimer (**Figure 3.3a**). Residues P155, W156, K158 and E190 that mutagenesis suggested to be involved in the interface of Mal-homodimer [83, 127] are in the interface of our

Mal-homodimer. In addition, the N-termini of the both monomers face the same direction, such that both could attach to the PI(4,5)P₂ in the membrane.

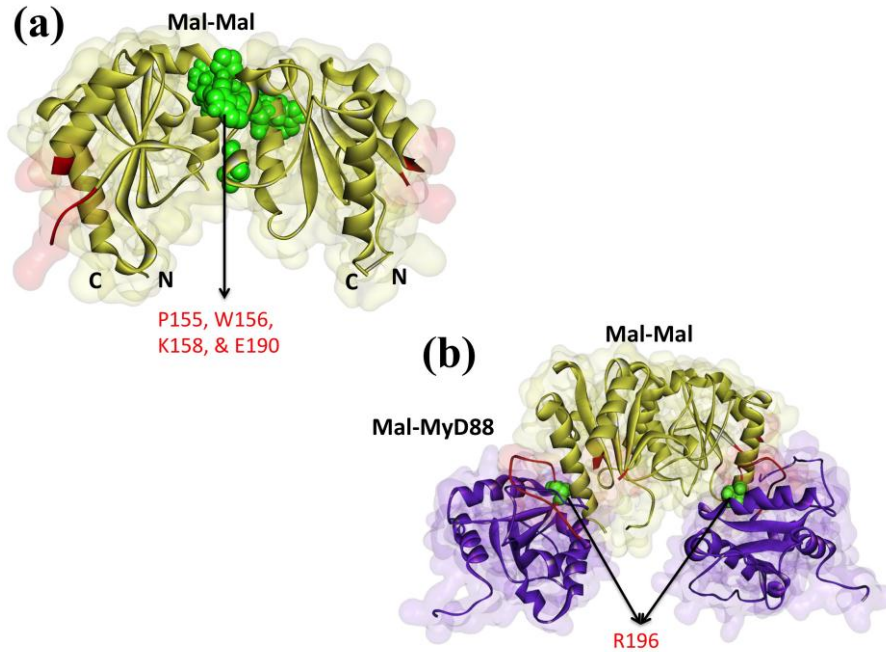


Figure 3.3: Interaction models of Mal-Mal, and Mal-MyD88. (a) Mal-homodimer model has the proposed interface residues (P155, W156, K158, and E190) (green spheres) [83, 127] at the correct site. Red-labeled regions are the residues of the AB-loop, the rest of the AB-loop residues (21 residues) are missing in the crystal structure. (b) Interaction of two MyD88 molecules with a Mal-homodimer. R196 residue of MyD88 was previously suggested to be at the interface [83, 130] and is represented as green spheres in the figure. I obtained this complex by superimposing binary interaction models of Mal-Mal and Mal-MyD88 [67].

3.1.3. TLR4-Mal Interaction

Like the TLR4-homodimer, I predicted several architectures for the TLR4-Mal interaction. The two TLR4-dimers (BB and FF) use different interfaces to interact with Mal TIR-domain, suggesting that distinct TLR4-Mal architectures are possible (Figure 3.4a & c). However, the previously suggested interface residues of Mal (R184, A185, & Y187) [127] are not at the correct site. When I superimpose the Mal-

homodimer on the TLR4-Mal complexes, I observed that both Mal monomers are in contact with TLR4 and one of the Mal monomers has the proposed interface residues at the correct site (**Figure 3.4b & d**). Had I not taken the Mal-homodimers into account, I would have concluded that none of the TLR4-Mal interaction models have the interface residues at the correct site. This underscores the importance of higher order oligomerization modes while deciphering signaling pathways.

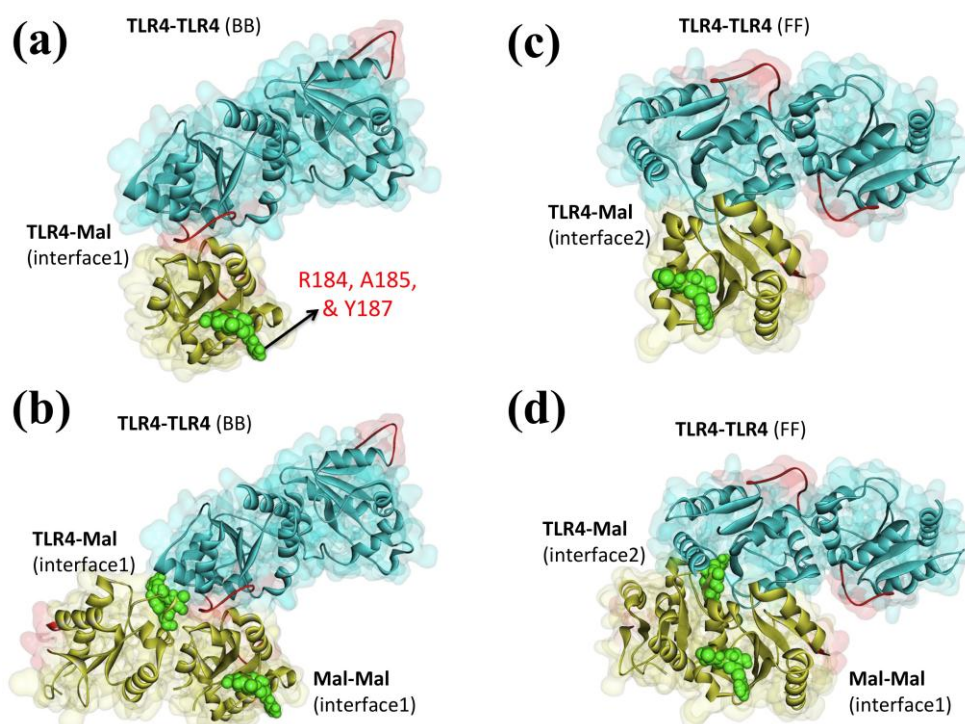


Figure 3.4: Interaction models of Mal-monomer (a & c) and Mal-dimer (b & d) with BB and FF TLR4-homodimer models. Yellow protein is Mal and green spheres show the proposed interface residues of Mal (R184, A185, Y187) [127], none of which are at the correct site in the monomeric-Mal-TLR interaction model. However, if dimerization of Mal is also taken into account, it is seen that both monomers are in contact with TLR4, one of which has the interface residue at the correct site [67].

3.1.4. Mal-MyD88 Interaction

Mal serves as a bridge between TLR4 and MyD88. Mutational analysis indicated that MyD88 R196 and R288 are at the Mal-MyD88 interface [83, 130]. However, these

two residues fall on opposite sides of MyD88, indicating that there is more than one bound conformation for the Mal-MyD88. Among several Mal-MyD88 architectures, only one features TLR4- and Mal-homodimers with R196 at the interface (**Figure 3.3**), when superimposed with TLR4-Mal interactions. **Figure 3.5** and **Figure 3.6** show the signalosomes of TLR4-Mal-MyD88 for the two possible TLR4-homodimers, FF and BB, respectively. As in TLR4-Mal interaction where the suggested interface residues are at the correct site only when Mal is in dimer form, R288 of MyD88 is in contact with TLR4 only if MyD88 dimerizes (**Figure 3.5b** and **Figure 3.7**).

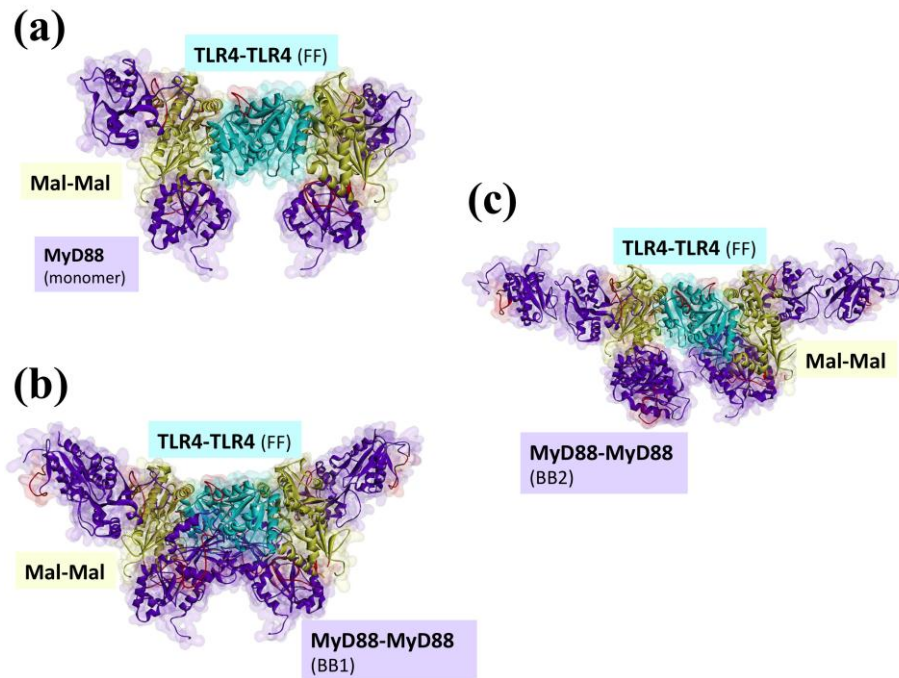


Figure 3.5: Possible TIR domain signalosome models for FF TLR4-dimer. (a) Interaction model of monomeric-MyD88 with TLR4 and Mal dimers. (b & c) MyD88-dependent TIR-domain signalosome models for FF TLR4-dimer. All proteins are in dimer form, including TLR4, Mal, and MyD88. It is known that dimeric MyD88 has higher affinity to stimulated TLRs due to their extended interfaces. In line with this, models (b & c) show that the second MyD88 of the MyD88-dimer is very close to TLR4. Especially in part-c, one of the MyD88 molecules in the dimer is bound to Mal, and the other is bound to TLR4. I obtained these complexes by superimposition of the binary interaction models of TLR4-TLR4, TLR4-Mal, Mal-Mal, Mal-MyD88 and MyD88-MyD88 [67].

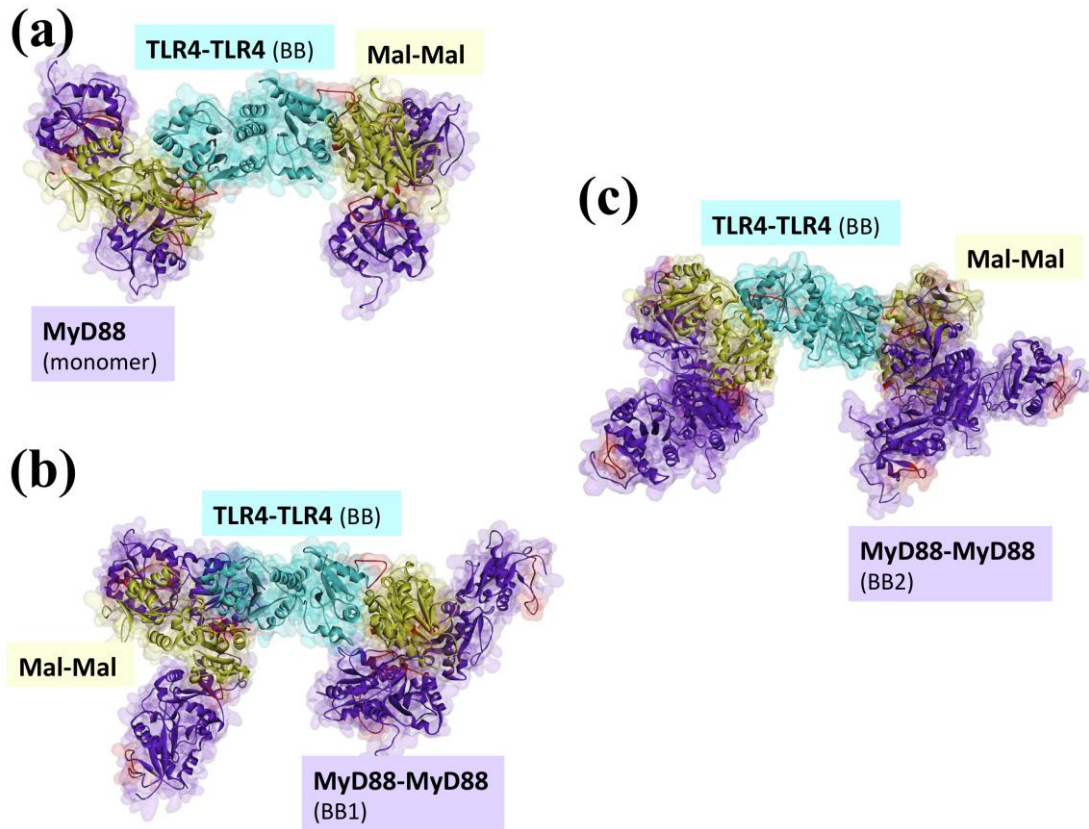


Figure 3.6: TIR-domain signalosomes with monomeric (a) and dimeric MyD88 molecules (b & c) for the BB TLR4 dimer. Oligomerization models of the large signalosome complex with two different back-to-back MyD88 dimers (BB1 and BB2). The TIR-domain signalosomes comprise a dimer of TLR4 interacting with two Mal-dimers, and four MyD88 dimers. I obtained this figure by superimposition of the binary interactions of TLR4-TLR4, TLR4-Mal, Mal-Mal, Mal-MyD88, and MyD88-MyD88 [67].

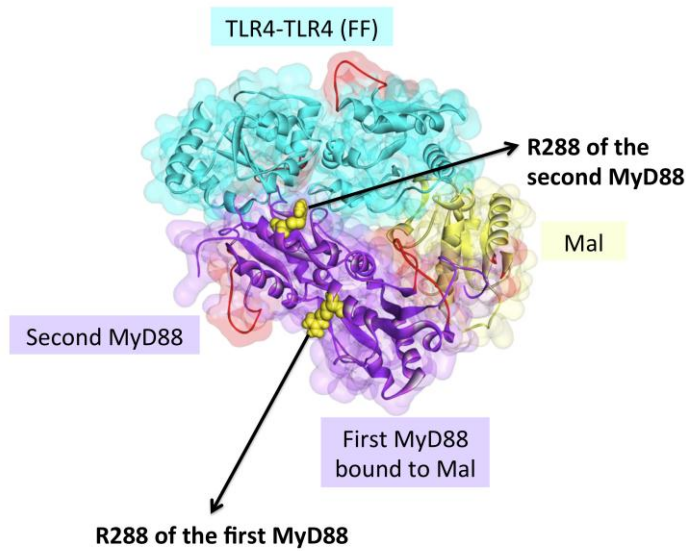


Figure 3.7: TLR4 dimer recruits Mal dimer, which in turn recruits MyD88-dimer (For simplicity only one Mal monomer is shown here). It has been suggested that MyD88-dimers have higher affinity to TLR4. The architecture I obtained supports this: when MyD88 dimerizes, one of the MyD88 monomers interacts with Mal, and the other is in contact with TLR4 itself. R196 and R288 of MyD88 have been shown to be at the Mal-MyD88 interface. Although R196 was at the Mal-MyD88 interface (**Figure 3.3b**), R288 is found at the TLR4-MyD88 interface [67].

3.1.5. MyD88 Dimerization

In order to form the myddosome, comprising MyD88, IRAK4 and IRAK2/IRAK1, MyD88 molecules should dimerize and oligomerize. Although variable stoichiometries have been observed (8:4:4, 7:4:4, 6:4:4) [90, 124] a more favored myddosome organization should have six MyD88 molecules [90]. The crystal structure of the helical myddosome involves six MyD88 DDs, four IRAK2 and four IRAK4 DDs [90]. The myddosome complex has four layers: layer-1 has four IRAK2 DDs, layer-2 has four IRAK4 DDs, layer-3 has four MyD88 DDs, and layer-4 has the next two MyD88 DDs. MyD88 dimerization through both its TIR and DD is necessary for assembly into myddosome [137]. Inhibition of the dimerization of its TIR domain by peptidomimetic compounds blocks the assembly of the myddosome [138].

Therefore, I include MyD88-dimer models in the TIR-domain signalosome (**Figure 3.5b**, **Figure 3.5c**, and **Figure 3.6**).

An earlier study suggested more than one binding mode for TIR domain MyD88-dimerization and formation of multivalent aggregates [132]. In line with this, I found four MyD88-dimer organizations (**Figure 3.8**), two FF and two BB. However, only BB dimers (**Figure 3.8a & b**) are possible for the TIR domain signalosome TLR4-Mal-MyD88 interactions. Some viruses employ TIR domain containing proteins (Tcp) to suppress TLR-mediated host immune response [132]. The crystal structure of a TIR domain dimer of TcpB of *Brucella* (4lqc.pdb) is also BB [141]. This may support the BB MyD88-dimer architectures as being feasible with the whole TIR-domain signalosome.

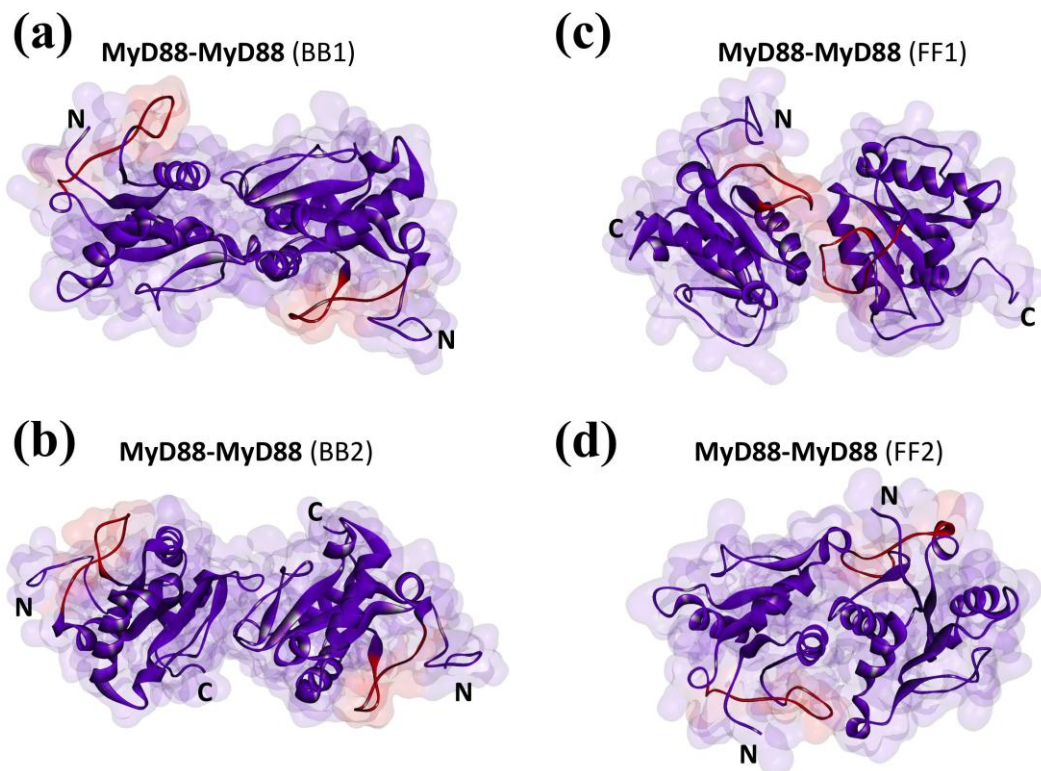


Figure 3.8: Four MyD88- dimer models: (a & b) back-to-back dimer models (BB1 and B2) and (c & d) face-to-face dimer models (FF1 and FF2). Only BB1 and BB2 are feasible with our TIR-domain signalosome models [67].

Figure 3.5b-c and **Figure 3.6b-c** display the superimposition of the BB MyD88-dimers with two possible TLR4-Mal-MyD88 interaction modes. MyD88-dimers have higher affinity for stimulated TLRs than monomeric MyD88 because of the extended interfaces of dimeric MyD88 TIR domains [132]. Remarkably, with our preferred (FF) TLR4-dimer model, if monomeric MyD88 is recruited to the TIR-domain signalosome, this MyD88 is away from TLR4 (**Figure 3.5a**), but if MyD88-dimers bind to the TIR-domain signalosome, two of the MyD88 molecules get very close to the TLR4 dimers (**Figure 3.5b & c**). In particular, in the assembly shown in **Figure 3.5b**, one MyD88 molecule of the MyD88-dimer is bound to Mal and the other to TLR4. **Figure 3.7** provides the details. MyD88-dimer may indeed have higher affinity for activated TLR4, because it is in contact with both Mal and TLR4 itself. Although the first MyD88 in **Figure 3.7** does not have any contacting residues with TLR4, the second MyD88 has 21 interacting residues according to the HotPoint server [142]. Consequently, a TLR4-dimer recruits two Mal-dimers and four MyD88-dimers. Although the stoichiometry of MyD88 in the myddosome complex was determined, there are no such data for the signalosome. Nevertheless, it was suggested that as long as MyD88 TIR domains are in a dimer form, it is not that critical how many MyD88 molecules are in the signalosome [132]. **Figure 3.1** provides an overview of the TLR/Mal/MyD88 signalosome, myddosome and TLR clustering.

3.1.6. TRAM Dimerization

Similar to Mal, which is a binding adaptor and associates with phospholipids in the membrane, TRAM is also a bridging adaptor and is attached to the membrane via its myristoyl group [64]. TRAM homodimerization is crucial for recruitment of TRIF [85]. In our TRAM-dimer architectures, residue H117 is at the interface, as suggested earlier [85] (**Figure 3.9**). This dimer is similar to TLR10 homodimer in the crystal structure (2j67_AB) as shown in the box in **Figure 3.9**.

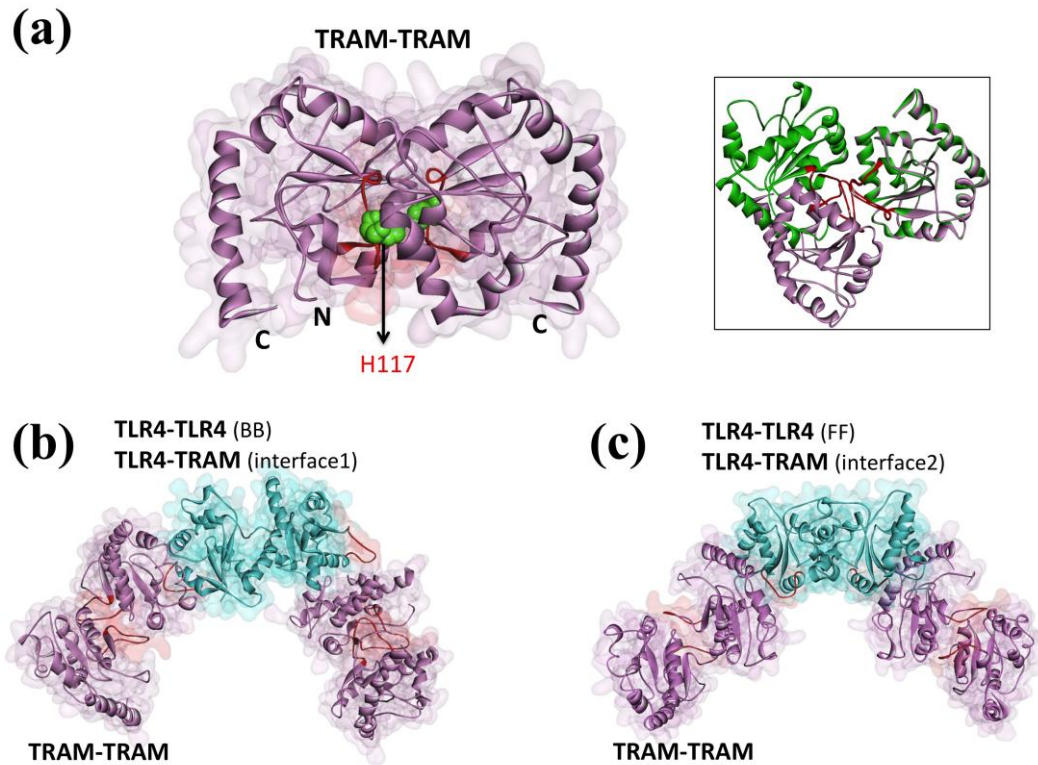


Figure 3.9: A detailed view of TRAM-homodimer model and its interaction with the two opposite TLR4 dimer models. (a) TRAM homodimer model, with BB-loops facing each other. Small box at the top right corner shows the superimposition of TRAM-homodimer model obtained by PRISM with TRAM-homodimer obtained by superimposition with TLR10 homodimer in the crystal structure (2j67_AB). Pink monomers are TRAM TIR domains; red-labeled regions are BB-loops of TRAMs; green spheres show the H117 residue of TRAM, which is suggested to be at the interface [85]. **(b & c)** TLR4-TRAM interaction model with BB and FF TLR4-homodimer models, respectively [67].

3.1.7. TLR4-TRAM Interaction

TRAM links TLRs to TRIF, just like Mal connecting TLRs to MyD88. The C747 residue of TLR4 was shown to be involved in TLR4-TRAM interface [83, 129]. However, this residue was also shown to be at the TLR4-homodimerization interface [95], suggesting different binding conformations. Supporting this assumption, I found different architectures for the TLR-TRAM interaction. For each TLR4-homodimer (FF

and BB), only one TLR4-TRAM which does not interfere with the TLR4-homodimer is possible (**Figure 3.9b & c**). None of them have the C747 residue of TLR4 at the TLR4-TRAM interface.

TRAM homodimer formation is required for TRIF recruitment. **Figure 3.9** displays the TLR4-homodimers that are bound to TRAM-homodimers. **Figure 3.10** shows TLR4-TRAM-TRIF interactions with no TLR4-homodimers. When I superimpose the Mal-homodimers onto these TLR4-TRAM, I observe that TRAM and Mal interactions are mutually exclusive in the BB TLR4-homodimer since they have overlapping binding sites on TLR4 (**Figure 3.11a**), but not with the FF TLR4 dimer (**Figure 3.11b**). Proteins that bind to the identical or overlapping interfaces on a protein will have a steric clash and thus cannot bind simultaneously [[143](#)].

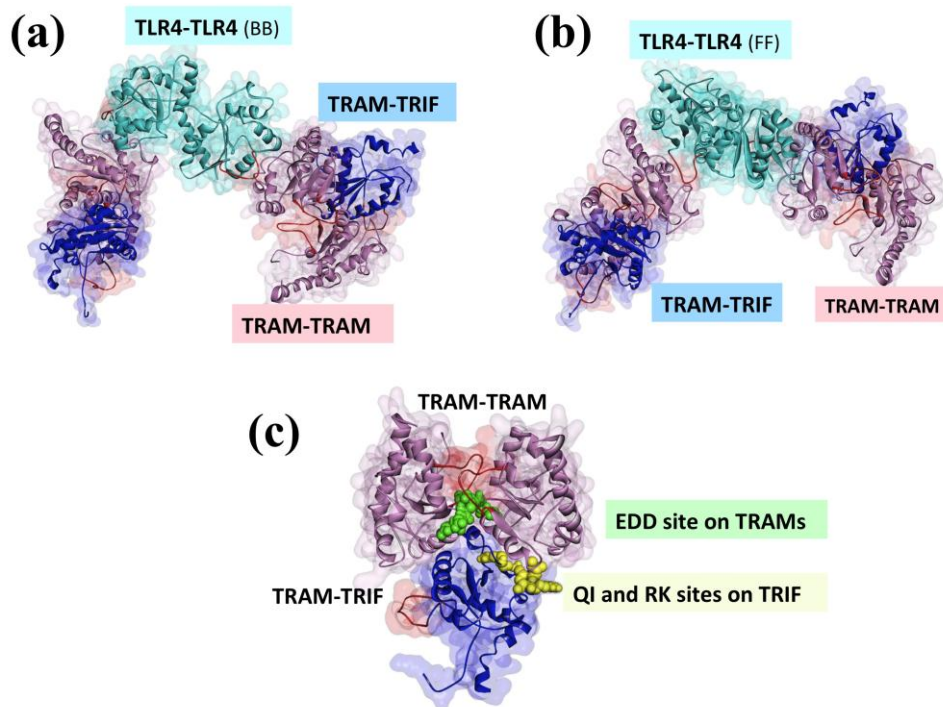


Figure 3.10: TRAM-TRIF interaction and TRIF-dependent TIR-domain signalosome for BB and FF TLR4 dimer. (a) TRIF-dependent TIR-domain signalosome model of BB TLR4-dimer, which involves TRAM-dimer and TRIF-monomer. (b) TRIF-dependent TIR-domain signalosome model for FF TLR4-dimer. (c) TRAM-TRIF interaction model. The residues that are proposed to be at the TRAM-TRIF interface include Q512, I519 (QI-site), R522, K523 (RK-site) of TRIF

and T155, S156 (TS-site), E87, D88, D89 (EDD-site) of TRAM [85]. The TRAM-TRIF interaction model has QI-, RK- and EDD-sites at the interface, but not the TS-site. Yellow spheres are residues Q512, I519 (QI site), and R522, K523 (RK-site) of TRIF; green spheres are residues E87, D88, D89 (EDD-site) of TRAM proteins in the homodimer. These residues are suggested to be at the interface by a recent study [85], which is in accordance with our model. This TRAM-TRAM-TRIF interaction model is possible with both FF and BB TLR4 homodimer models. Cyan protein is TLR4; pink is TRAM; blue is TRIF; and red-labeled regions are BB-loops [67].

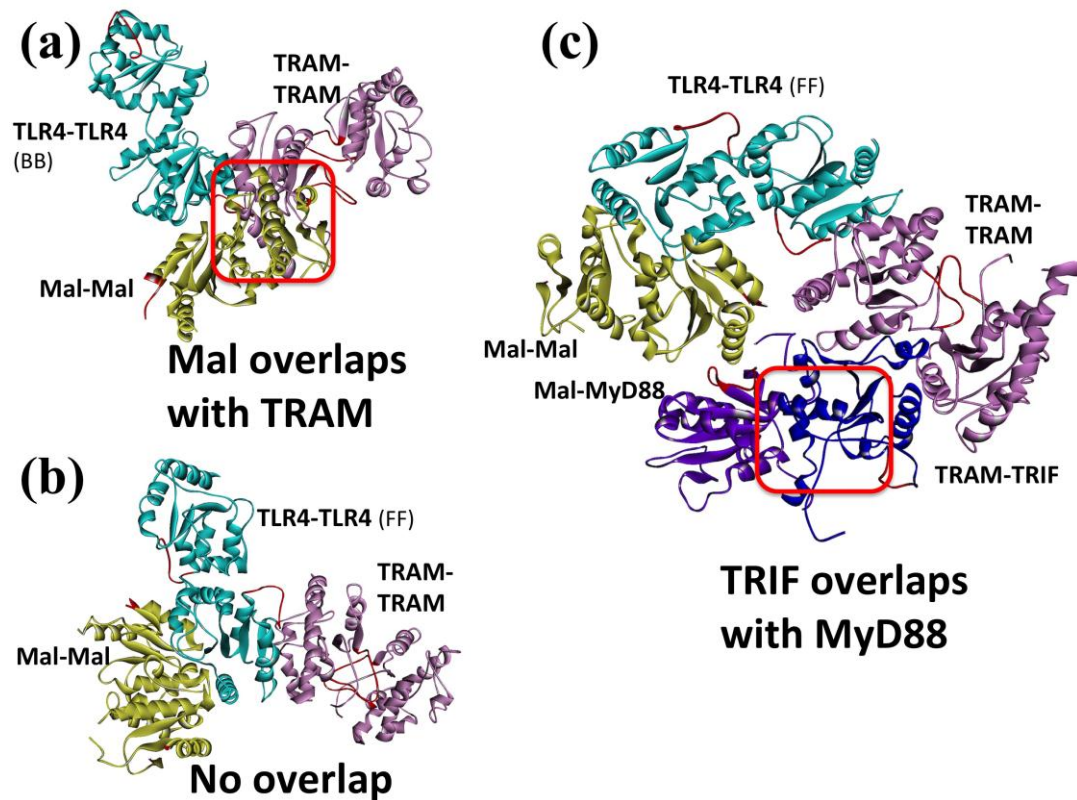


Figure 3.11: MyD88- and TRIF-dependent downstream TLR pathways are mutually exclusive. (a) TRAM-homodimer has a steric clash with Mal-homodimer when superimposed to BB TLR4-homodimer model, and thus they are mutually exclusive: either Mal or TRAM homodimers can bind to TLR4 at any time. TRAM and Mal interactions are mutually exclusive in BB TLR4-homodimers and this is in line with the findings of several studies [93, 95, 96]. This indicates that MyD88-dependent pro-inflammatory and TRIF-dependent anti-inflammatory pathways are competitive. (b) TRAM-homodimer does not overlap with Mal-homodimer when

superimposed to FF TLR4-homodimer model. **(c)** MyD88 overlaps with TRIF on TLR4: the FF TLR4-homodimer model has steric clashes of MyD88 and TRIF when superimposed Mal-MyD88 and TRAM-TRIF [67].

3.1.8. TRAM-TRIF Interaction

TRAM acts as a scaffold bringing TLRs and TRIF together. This assembly is a key upstream branching step in the interferon and anti-inflammatory pathway (**Figure 2.3**). Monomeric TRIF is able to bind to TRAM homodimer [85], suggesting that there is no need for TRIF dimerization. The residues that are proposed to be at the TRAM-TRIF interface include Q512, I519 (QI-site), R522, K523 (RK-site) of TRIF and T155, S156 (TS-site), E87, D88, D89 (EDD-site) of TRAM [85]. The TRAM-TRIF interaction model is shown in **Figure 3.10** and it has QI, RK, and EDD-sites at the interface, but not the TS-site. When the MyD88-mediated signalosome is superimposed on the TRIF-mediated signalosome based on the FF TLR4 dimer (the major TLR4-dimer for signaling), MyD88 and TRIF present a steric clash (**Figure 3.11c**). Both BB and FF TLR4 dimers have steric hindrance when MyD88- and TRIF-dependent signalosomes are superimposed. This is in line with the findings of several studies [93, 95, 96]. Importantly, this indicates that MyD88-dependent pro-inflammatory and TRIF-dependent anti-inflammatory pathways are competitive and thus restrict the activation of one another. This may offer a means of regulation to the TLR signaling. However, it is important to note that these parallel paths only switch the function: while negatively regulating one path, they positively regulate others downstream of TLRs.

3.2. Interactions of Downstream Players with DD of MyD88

Clustering of MyD88 DDs initiates the oligomerization of the myddosome complex. Besides IRAK4 and IRAK2, MyD88 DDs also associate with TRAF6 [144], TRAF3 [89], and FADD [145, 146]. Schematic representations suggested that downstream proteins, like TRAF6, interact with IRAK1/2, but not with MyD88 [65, 126]. However, a recent study revealed that there is also a direct interaction between MyD88 and TRAF6 and abrogation of this interaction inhibits NF- κ B activation [144]. In line with

this, I observed that TRAF6 prefers to bind to MyD88 when the whole myddosome is given as a target instead of monomeric MyD88 or IRAK2. That is, although there are favorable interactions of TRAF6 with both monomeric MyD88 and monomeric IRAK2 (**Figure 3.12**), when the whole myddosome is taken into consideration, TRAF6 selects MyD88. As I stated before, higher order oligomerization is important for function and should be considered in modeling. I select the myddosome-TRAF6 interaction, with TRAF6 bound to MyD88, instead of the monomeric IRAK2-TRAF6. **Figure 3.13** illustrates the interaction of TRAF6 with MyD88; the detailed **Figure 3.14** shows that the myddosome-TRAF6 organization in which the TRAF-C domain of TRAF6 is in contact with DDs of two MyD88 molecules (two layers of MyD88), one interaction is major, with hotspots and the other further stabilizing the complex. The interface is similar to the concave TRAF binding site with peptides as observed in TRAF6-CD40 (1lb6.pdb) [147], TRAF2-TRADD (1f3v.pdb) [148], and TRAF2-OX40 (1d0a.pdb) [149]. The TRAF-C region of TRAF6 needs to trimerize to function [125] and TRAF6 trimerization is possible with this myddosome-TRAF6 architecture (**Figure 3.14**). Previous studies showed that TRAF3 also associates with MyD88-IRAK4-IRAK1 complex [150]. Similar to TRAF6, TRAF3 also associates with two MyD88 proteins (two layers of MyD88) in the myddosome (**Figure 3.14**). MyD88 binds to the concave site on the TRAF-C region of TRAF3, as previously observed in other interactions such as TRAF3-CD40 (1fll.pdb) [151], TRAF3-BAFFR (2gkw.pdb) [152], TRAF3-LMP1 (1zms.pdb) [153], and TRAF3-Cardif (4ghu.pdb) [154].

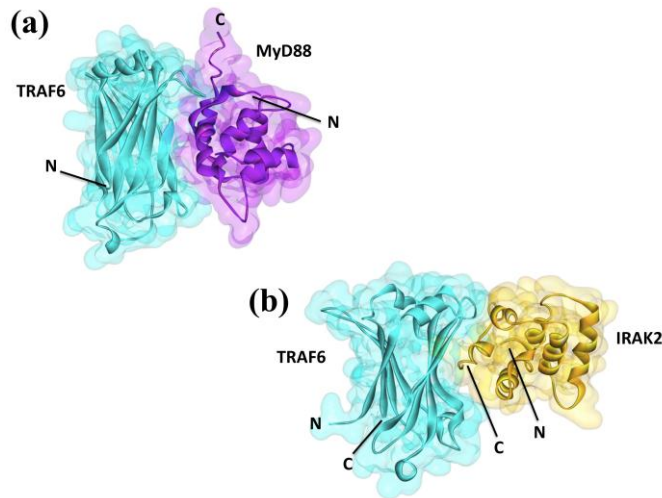


Figure 3.12: Interaction models of TRAF6 with monomeric MyD88 and IRAK2 [67].

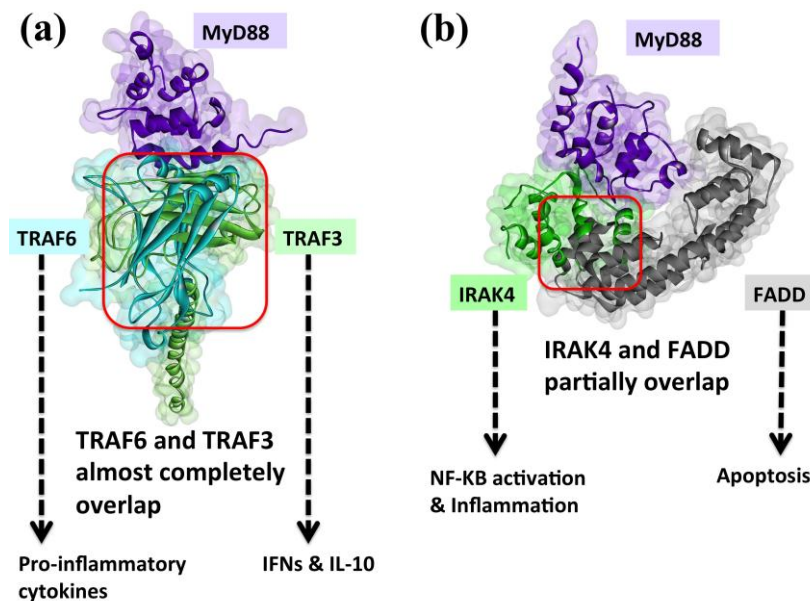


Figure 3.13: MyD88 interaction models with the downstream orchestrators reveal that the three parallel downstream paths are competitive. (a) TRAF6 (1lb5:A) and TRAF3 (1fil:A) binds to almost completely overlapping interfaces on MyD88 DD (3mop:F), thus they are mutually exclusive. **(b)** IRAK4 (3mop:J) and FADD (2gf5:A) bind to overlapping interfaces on MyD88 DD (3mop:F), thus they compete to bind to MyD88. MyD88-IRAK4 interaction is not PRISM prediction, where the crystal structure of the complex is available (3mop:FJ) [67].

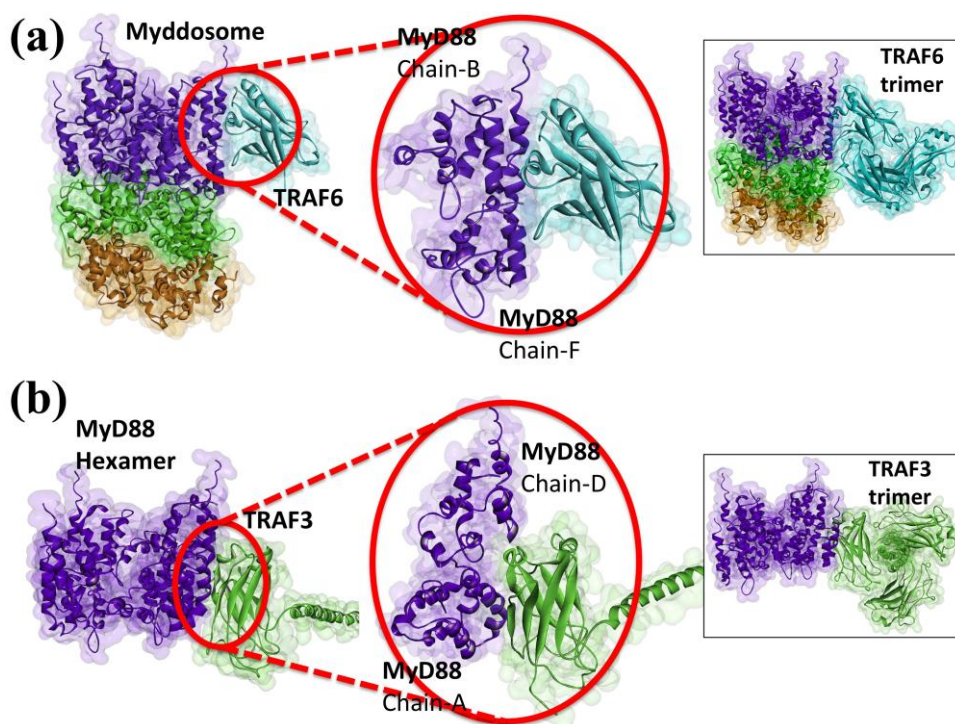


Figure 3.14: Interaction models of MyD88 with TRAF6 and TRAF3. (a) Myddosome-TRAF6 interaction model. TRAF-C domain of TRAF6 prefers to bind to MyD88 when the whole myddosome structure is given as a target. The interface is very similar to the concave TRAF binding site with peptides as observed in TRAF6-CD40 (1lb6.pdb) [147], TRAF2-TRADD (1f3v.pdb) [148], and TRAF2-OX40 (1d0a.pdb) [149]. TRAF6 is in contact with two monomers of MyD88 (two layers of MyD88) in the myddosome assembly. In order to execute its function, TRAF6 needs to trimerize and TRAF6 trimerization is also possible with our model. (b) Interaction model of TRAF3 with hexameric MyD88 in the myddosome. Like TRAF6, TRAF-C region of TRAF3 is in contact with two MyD88 monomers (two layers of MyD88) [67]. The interface is very similar to the concave TRAF binding site observed in other interactions, such as TRAF3-CD40 (1fll.pdb) [151], TRAF3-BAFFR (2gkw.pdb) [152], TRAF3-LMP1 (1zms.pdb) [153], and TRAF3-Cardif (4ghu.pdb) [154].

Endosomal TLRs can signal through both TRAF3 and TRAF6. TRAF6 signaling activates the classical NF- κ B pathway, leading to expression of pro-inflammatory cytokines [150]. On the other hand, TRAF3, a negative regulator of MAPKs and the alternative NF- κ B pathway [66] induces production of anti-inflammatory cytokine IL-

10 [94, 150] (**Figure 2.3**). I observed that TRAF6 and TRAF3 bind to almost completely overlapping interfaces on MyD88 (**Figure 3.13a**). This may assign a new regulatory role for TRAF3 in TLR signaling: the presence of TRAF3 restricts the activation of NF- κ B and give rise to production of IFNs and IL-10.

FADD is another protein interacting with MyD88 DD [144]. It is a negative regulator of TLR signaling by suppressing LPS-induced NF- κ B activation through possible competition with IRAK4 for binding to MyD88 [145, 146]. Considering the MyD88-FADD organization and the MyD88-IRAK4 crystal structure, MyD88 exploits (partially) overlapping surfaces to interact with IRAK4 and FADD (**Figure 3.13b**). This organization explains why FADD hinders IRAK4 binding to MyD88 and thus myddosome assembly. In addition, Fas activation promotes TLR signaling and chronic inflammation [145]. If Fas and TLRs are activated simultaneously, activated Fas sequesters FADD and liberates MyD88, allowing constitutive inflammation as shown in **Figure 3.15** [146].

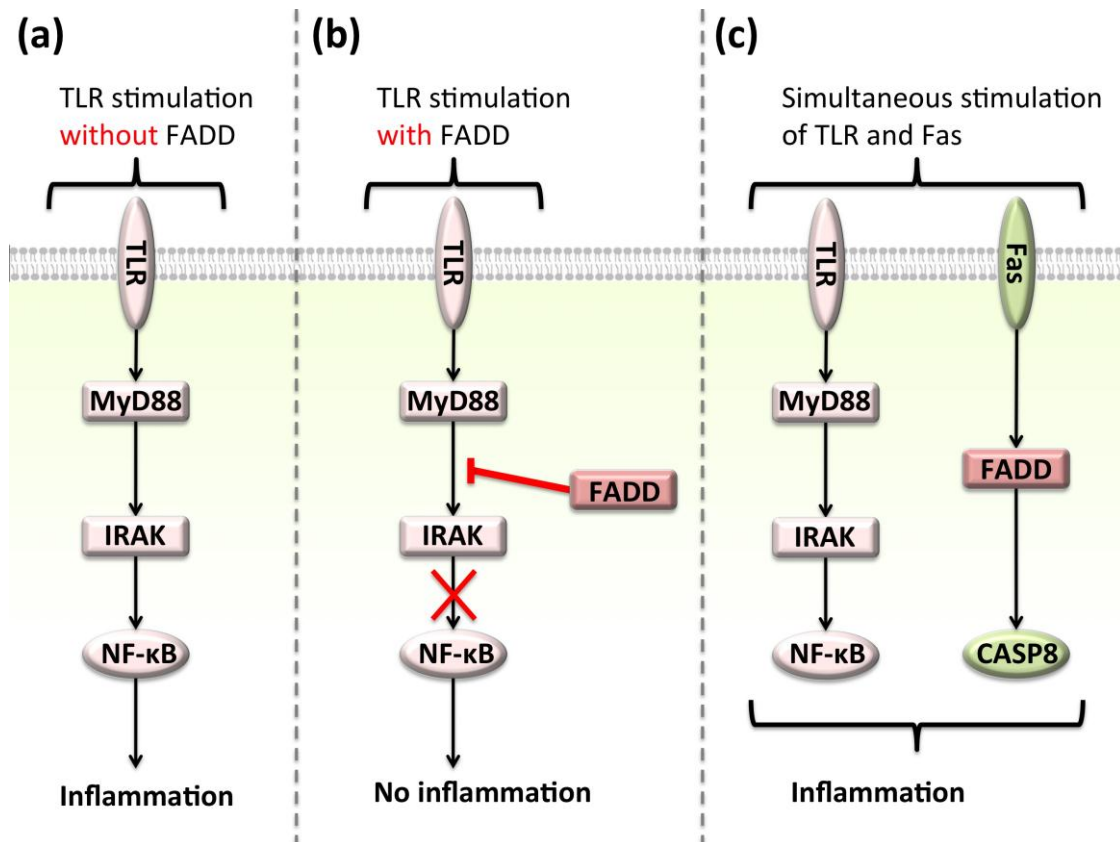


Figure 3.15: Constitutive inflammation takes place when TLRs and Fas are activated simultaneously. Adapted from [146] **(a)** When there is no FADD available, conventional TLR signaling propagates and leads to inflammation. **(b)** In the presence of FADD, conventional downstream path is inhibited since MyD88-FADD interaction hinders MyD88-IRAK4 interaction. **(c)** When Fas receptor is activated, it recruits FADD and prevents MyD88-FADD interaction, thereby allowing constitutive inflammation.

Remarkably, the C27* nonsense mutation on FADD protein, which is clinically observed in lung squamous carcinoma with 0.21 frequency (according to the TCGA data) [155], abolishes the MyD88-FADD interaction (**Figure 3.16**) can be explained by this architecture. Since FADD can no longer occupy the MyD88 binding site, TRAF6 and IRAK4 are able to bind. This activates MAPKs, which induce production of pro-inflammatory cytokines, and prevents induction of apoptosis. This may clarify how the C27* mutation on FADD contributes to initiation or progression of tumor. Another mutation, the R34H missense mutation on FADD, observed in stomach

adenocarcinoma, falls just next to the interface region (**Figure 3.16**). Our model suggests that this mutation decreases the affinity of FADD to MyD88 and may block TLR-mediated apoptosis.

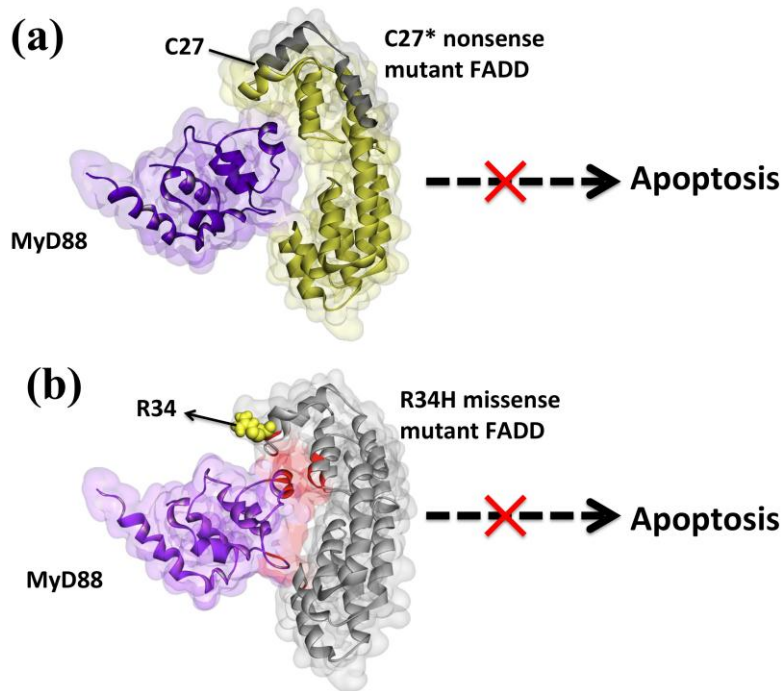


Figure 3.16: A nonsense and a missense mutation on FADD prevents FADD-MyD88 interaction. (a) C27* nonsense mutation on FADD abolishes its interaction with MyD88, since large portion of the interface would be missing in the mutant FADD (yellow-labeled part on FADD). (b) R34H missense mutation on FADD falls just next to interface region and decreases the affinity of FADD to MyD88 and may block apoptosis. Red-labeled residues are the interface residues and Arg-34 residue is labeled with yellow [67].

Taken together, the TLR4 architectures indicate that all TLR's parallel downstream pathways are competitive (**Figure 3.17**). MyD88- and TRIF-dependent pathways downstream of a single TLR4-dimer cannot be activated simultaneously due to shared binding site. If MyD88 is recruited to the activated TLRs, it uses its TIR domain to interact with the bridging adaptor protein Mal/TIRAP and its DD to interact with the other downstream partners, IRAKs, TRAF6, TRAF3, and FADD. These trigger three alternative parallel pathways and lead to distinct/opposing outcomes.

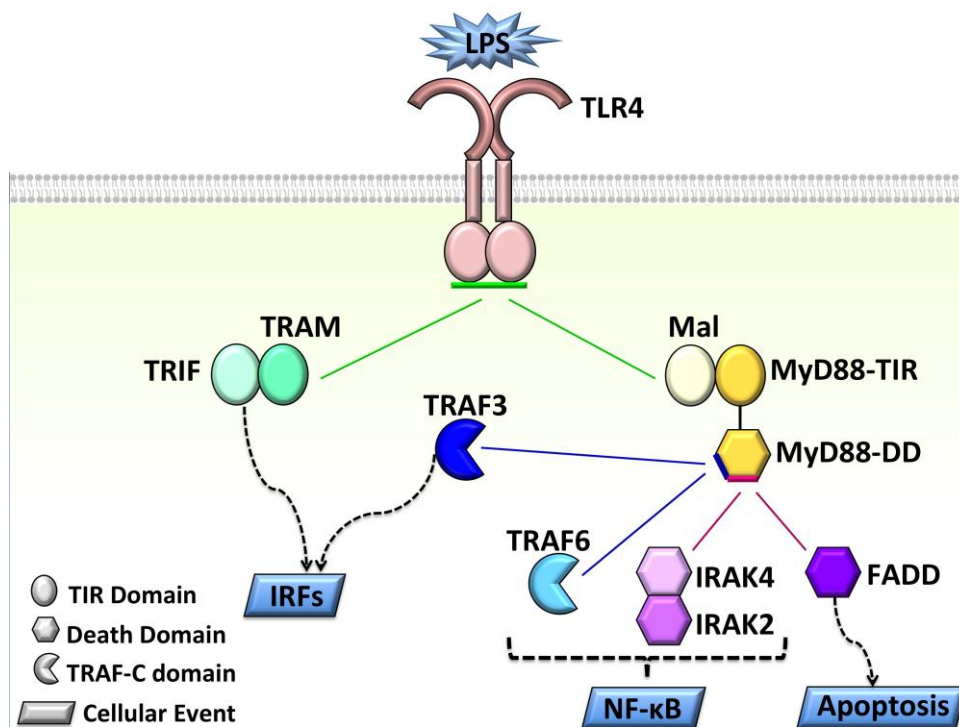


Figure 3.17: Parallel downstream pathways of TLRs, which lead to distinct outcomes, are mutually exclusive. Green arrows shows that TRIF- and MyD88-dependent paths cannot be activated simultaneously due to shared binding sites on TLR4-dimer or steric hindrance. Blue arrows demonstrate that TRAF6 and TRAF3 bind to overlapping interfaces on MyD88 DD (downstream of endosomal TLRs). Pink arrows show that IRAK4 and FADD will have steric clash when they bind to MyD88 at the same time. The three branches of TLR pathway, namely pro-inflammatory, interferon and anti-inflammatory, and apoptotic paths are mutually exclusive [67].

To conclude, considerable effort has been invested in the quest for the entire TIR signalosome assembly, including its clustered architectures. This problem is significant since TLR activation involves clustering and signalosome formation. As I show here, the architectures may clarify TLR4 physiological signaling control and how it can go wrong in disease. I present the two, MyD88-dependent and TRIF-dependent TIR-domain signalosome assemblies. I exploit experimental mutational data in every step in the construction. TLR activation through its ectodomain dimerization can elicit the proinflammatory, anti-viral and anti-inflammatory, and

apoptosis pathways. Binding to Mal/MyD88 or TRAM/TRIF is the step making the first cellular decision. Our results suggest that steric hindrance of Mal and TRAM in a TLR/TRAM/TRIF assembly and Mal/MyD88 and TRIF leads to competitive binding to TLR's TIR domain. Our results further reveal that parallel downstream pathways with opposing consequences are competitive at almost each branching point of the TLR pathway, beyond Mal and TRAM recruitment. TRAF6, TRAF3, and FADD, whose recruitment results in pro-inflammatory, anti-inflammatory, and death pathways respectively, present similar scenarios. Our signalosome architectures with 8 MyD88 molecules are important, since they provide the basis for obtaining an insight into how TLR4 clusters. The 8 MyD88 molecules connect into multiple 6 MyD88 molecules in myddosome helical signaling units, and unveil downstream oligomerization clusters formed by stimulated TLRs. We speculate that the long MyD88 linker is critical for TLR clustering, and could be its *raison d'être*. The clusters, and their multivalent network, particularly through TRAFs [[126](#)], allow efficient signaling, even with reduced TLR concentration since it enables signal amplification. Finally, our models can help explain the mode of action of relevant human mutations [[156](#), [157](#)].

Chapter 4

NEGATIVE REGULATION OF TLR PATHWAY

This chapter presents the models that I propose to understand the regulatory mechanisms in TLR pathway. There are several negative regulators in TLR pathway and I found most of them interfere with the binary interactions or large multi-molecular complexes to restrict TLR signaling.

Through cooperative cellular network linkages, multivalent oligomeric proteins display efficient enzymatic reactions and amplify signal [126, 158]. These large protein assemblies are formed either by direct PPIs or by Ub chains attached to proteins that serve as a dynamic, allosteric scaffold. These scaffolds not only bring proteins together; the shifting landscape that they orchestrate preorganizes the ensembles for subsequent binding and catalytic events [159]. The signal that these elicit propagates across the network [160]. Cells acquired strategies to block cellular functions such as inflammation by targeting proteins, PPIs and Ub chains. At almost each step of the TLR signaling cascade, one or more regulators take action to constrain NF- κ B activation. In addition, parallel downstream paths also restrict inflammation since they compete with the conventional MyD88-dependent pro-inflammatory pathway [67] (**Figure 4.1**). Below, I describe the negative regulators focusing on the structural basis of how they forestall signaling.

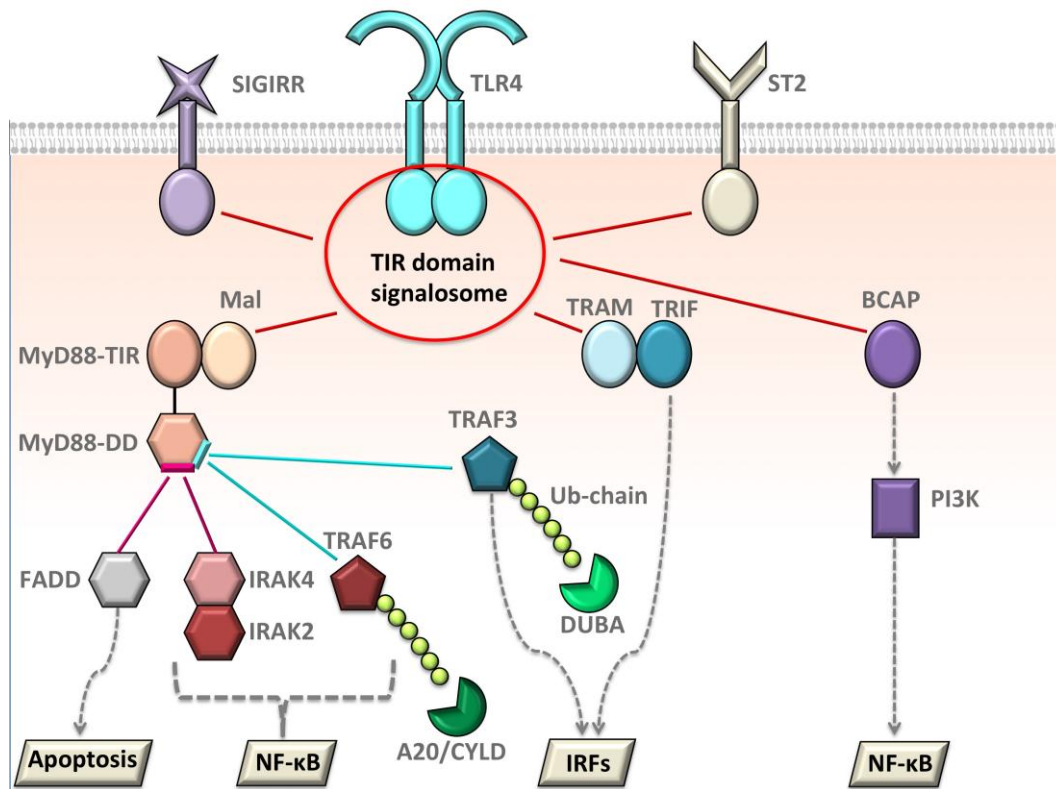


Figure 4.1: The competition in the TLR pathway due to overlapping binding sites. Most of the negative regulators and the proteins that lead to distinct parallel paths of TLRs compete with one another to bind to a common partner. The TIR domain-containing proteins all compete to bind to each other; IRAK4 and FADD compete to bind to overlapping interfaces on DD of MyD88; TRAF6 and TRAF3 have shared interfaces on MyD88-DD. Deubiquitinases, such as A20, CYLD, and DUBA removes Ub chains from TRAF6 and TRAF3 [101].

4.1. TIR Domain Containing Negative Regulators

Upon stimulation, TLRs dimerize and recruit TIR domain-containing adaptors to form either MyD88- or TRIF-dependent TIR domain signalosomes. In our recent study [67], I have provided structural models for these two signalosomes. I showed that they are competitive and cannot form simultaneously on the same receptor dimer. Apart from Mal, MyD88, TRAM, and TRIF, there are also other TIR domain-containing proteins, which act as negative regulators of TLR signaling. These include BCAP [98], SIGIRR

[161], and ST2 [162], which are suggested to interfere with the assembly of TIR domain signalosome. Below, I explain the regulation mechanism of these three TIR domain containing negative regulators structurally and provide models for how they impede TLR signaling.

4.1.1. BCAP (B-cell adaptor for PI3K)

Besides MyD88- and TRIF-dependent downstream paths, the PI3K pathway can also be activated upon TLR stimulation and serve as another parallel pathway of TLRs [98, 163]. Although the mechanism of TLR-mediated pro-inflammatory NF- κ B and MAPK activation has been investigated extensively, the stimulation mechanism of PI3K α , a lipid kinase, is still unknown [87]. BCAP is a TIR domain-containing adaptor protein that links TLRs to PI3K/Akt pathway [87]. BCAP needs to be tyrosine-phosphorylated at its YXXM motif in order to interact with the SH2 domain of the regulatory subunit (p85) of PI3K α [97, 98]. TLR activation increases the phosphorylated BCAP in the membrane which helps recruit activated PI3K to the membrane where its lipid substrate, phosphoinositide-4,5-bisphosphate (PIP2), is found [98]. If TLR is stimulated and the BCAP level is low, elevated expression of pro-inflammatory cytokines, such as IL-12 and IL-6, ensues [87, 98, 99]. Although full-length BCAP (BCAP-L) can bind to both Mal and MyD88 and suppress NF- κ B activity, truncated BCAP (BCAP-S) that lacks TIR domain is unable to do so [87, 97], suggesting that its TIR domain is critical for TLR regulation. BCAP recruitment and the resulting PI3K activation serve as a checkpoint to limit TLR-induced inflammation [98]. BCAP represses MyD88-dependent NF- κ B and MAPK activation and there might be two different scenarios for this: in the first, BCAP interferes with the TIR domain signalosome [87, 164]. In the second, BCAP may allow signalosome formation and function downstream of MyD88 but still interfere with conventional downstream NF- κ B pathway; since Akt is not activated in the absence of MyD88 in macrophages [87] and MyD88 is vital for the recruitment of PI3K p85 regulatory subunit [163].

Our results show that the TIR domain of BCAP can form potential complexes with all TIR-domain containing proteins in TLR signaling, including TLR4, Mal, MyD88,

TRAM, and TRIF (**Figure 4.2**). The details of each of the interaction architectures are given in **Table A.2** and **Table A.3**. Some of these interactions interfere with TIR domain signalosome formation, supporting the first scenario. For instance, if BCAP binds to TLR4, it abolishes TLR4-TRAM interaction due to steric clash of BCAP and TRAM, preventing TRIF-dependent signal relay (**Figure 4.2b**). Moreover, if it binds to Mal, due to overlapping binding sites the Mal-BCAP interaction prevents Mal homodimerization, which is vital for MyD88-dependent signalosome formation [127] (**Figure 4.2c**). Similar to MyD88, TRIF can also mediate PI3K activation [165], but the IL-10 level does not change in the absence of BCAP [98]. BCAP-TRAM interaction abrogates TRAM homo-dimerization (**Figure 4.2e**), which facilitates TRIF recruitment by serving as a binding platform [85] and the BCAP-TRIF association disrupts TRAM-TRIF interaction (**Figure 4.2f**).

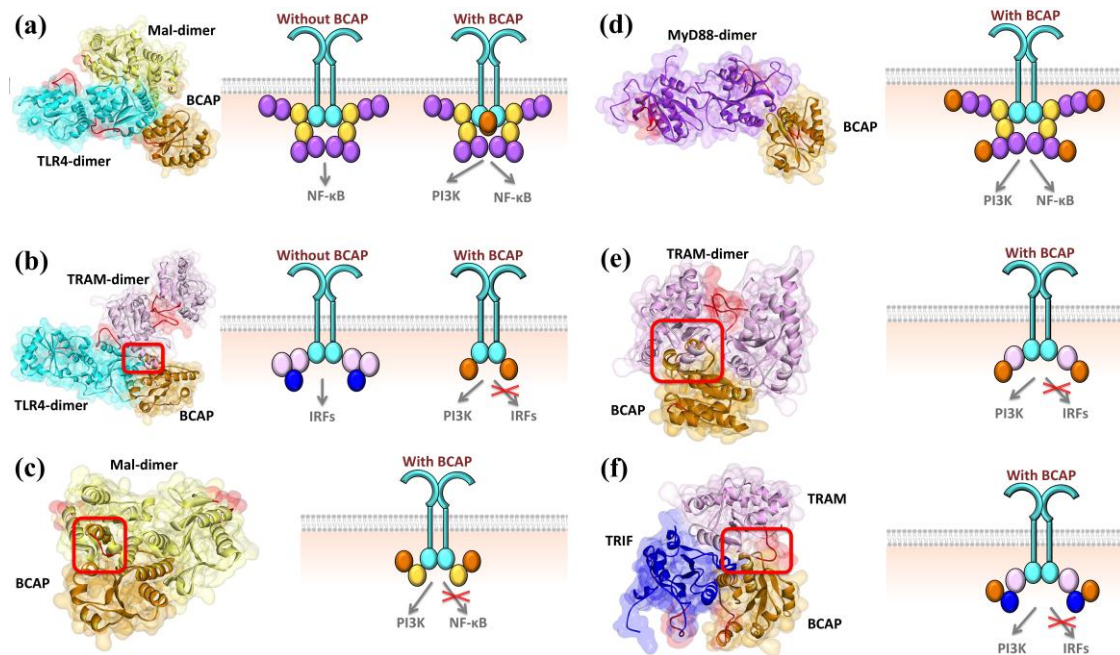


Figure 4.2: BCAP interactions with all TIR domain-containing proteins in TLR signaling. (a) BCAP interaction with TLR4 allows TLR4 dimerization, TLR4-Mal interaction, Mal-dimerization and the entire MyD88-dependent signalosome formation. (b) BCAP interaction with TLR4 has steric clash with TLR4-TRAM binding therefore these two interactions are mutually exclusive: if BCAP binds to TLR4, TRAM cannot. (c) BCAP interaction with Mal hinders its dimerization and the assembly of MyD88-dependent signalosome. (d) BCAP-MyD88 interaction enables

the signalosome formation. **(e)** BCAP-TRAM interaction interferes with TRAM-dimerization and prevents TRIF-dependent signalosome assembly. **(f)** BCAP-TRIF interaction blocks TRIF-TRAM interaction due to overlapping binding sites. Red boxes indicate the location of clash and therefore those complexes are not possible. The red-labeled regions are the BB-loops of the proteins. In all parts, the left-hand side figure shows the structure of PPI complexes obtained by PRISM, and the right-hand side schematic figure shows the TIR domain signalosome and how the negative regulators affect the assembly of signalosome. Circles in the schematics represent the TIR domains: Cyan represents TLR, yellow represents Mal, purple represents MyD88, light pink represents TRAM, blue represents TRIF and orange represents BCAP [101].

In addition to interfering with the formation of TIR-domain signalosome, our results indicate that the second scenario is also plausible. For instance, BCAP-TLR4 enables MyD88-dependent signalosome formation, as it does not abolish TLR4-dimerization or TLR4-Mal interaction (**Figure 4.2a**). Moreover, the interaction of BCAP with MyD88 does not intrude into MyD88-dimerization interface and allow signalosome formation (**Figure 4.2d**). So, if BCAP does not disrupt signalosome formation and functions downstream of MyD88, how does it inhibit NF- κ B? Since BCAP is a large protein (811 residues), it was suggested that it may interfere with the formation of the myddosome structure, which is composed of death domains (DDs) of MyD88, IRAK4 and IRAK2/1 [90]. Our results indicate that when MyD88 binds to BCAP through its TIR domain, it may not be able to oligomerize with other MyD88 and IRAK molecules through its DD due to steric clash that BCAP may cause. Assembly into large complexes and receptor clustering has been shown to activate signaling cascades even in the absence of essential ligands [124], probably via allosteric activation.

Structural details of the interactions allow us to characterize the mechanisms of oncogenic mutations. I mapped the clinically observed cancer mutations onto interfaces of the PPI architectures that I generated and performed *in silico* mutagenesis. There are 12 oncogenic mutations on BCAP that fall onto the interface residues (**Table A.4**). Our *in silico* mutagenesis analysis revealed that 7 of these mutations abolish BCAP interactions with either one of 5 TIR domain-containing

proteins (TLR4, Mal, MyD88, TRAM, and TRIF). These mutations are observed in melanoma, breast, lung, head and neck, colorectal and stomach cancers. They do not cluster at a specific location on the BCAP structure; rather they disperse almost over the entire BCAP structure (**Figure 4.3**). A recent study showed that disease-causing mutations often change the number of Hydrogen-bonds and salt bridges in comparison to non-disease-causing counterparts [166]. In line with this, I found that the E50K mutation on BCAP decreased the number of intermolecular H-bonds by 1 and salt-bridges by 2 in the BCAP-TLR4 interaction. Moreover, K139fs frameshift mutation reduced the number of salt bridges from 7 to 4 in the BCAP-TRAM interaction. These decreases in intermolecular bonds may also contribute to loss of the interaction with the mutant BCAP. Additionally, there is also a nonsense mutation (G9*) on BCAP seen in stomach cancer (**Table A.5**) that causes the loss of BCAP and its interactions. Attenuation of the interactions liberates the conventional TLR downstream pathway that leads to NF- κ B and MAPK activation and pro-inflammatory cytokine production. Without a checkpoint to restrict TLR signaling, constitutive production of inflammatory cytokines leads to chronic inflammation, which promotes tumorigenesis.

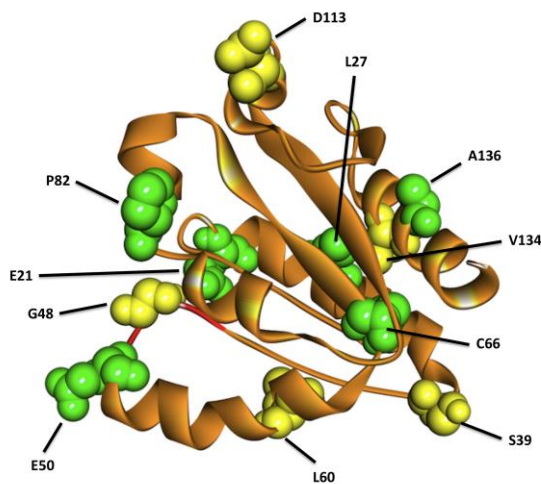


Figure 4.3: Clinically observed BCAP mutations that fall onto interfaces of BCAP interactions with other TIR domain-containing proteins. The mutations do not cluster at a particular location on 3D structure. The green-labeled residues abolish the interactions when they get mutated, but the yellow-labeled ones do not [101].

4.1.2. SIGIRR (Single Immunoglobulin IL-1R-related Molecule)

An orphan receptor SIGIRR (also known as TIR8) has a single extracellular Ig domain, a transmembrane domain, a cytoplasmic TIR domain, and an unusually long tail (95 residues), which is missing in other TIR domain containing receptors [99, 167, 168]. SIGIRR inhibits NF- κ B activation induced by only TIR domain containing receptors, like TLR and IL-1R1, but not the others such as TNFR that do not possess a TIR domain [99]. Increased production of inflammatory cytokines and chemokines has been reported in SIGIRR-deficient mice after IL-1 and LPS administration [161]. Mice lacking SIGIRR have been shown to be more prone to develop intestinal tumors [168]. The mechanism of inhibition of TLRs by SIGIRR remains unclear [169]. While its extracellular Ig and intracellular TIR domains restrain IL1 signaling by blocking the interaction between IL-1R1 and IL-1RAP, only its TIR domain inhibits TLR signaling [170]. In particular, the BB-loop of its TIR domain appears important in association with TLRs, since deletion of the BB-loop abolished the inhibitory role of SIGIRR [168, 171]. The BB-loop is conserved among TIR domain-containing proteins [85].

SIGIRR could either interfere with the organization of TIR domain signalosome [168-170] or block the translocation of this complex from the receptor [161]. It can form heterodimers with TLR4 [169] and decrease the recruitment of MyD88 and IRAK4 to activated TLR4 [170]. I found that SIGIRR can interact with all TIR domain-containing proteins in the TLR pathway (**Figure 4.4**). SIGIRR's BB-loop is not involved in its interactions with Mal and TRAM, but close to the interface region in the MyD88, TLR4, and TRIF interactions. Similar to BCAP, SIGIRR interactions with Mal, TRAM, and TRIF interfere with Mal-homodimerization (**Figure 4.4c**), TRAM-homodimerization (**Figure 4.4e**), and TRIF-TRAM (**Figure 4.4f**) interactions, respectively; hence, they prevent the signalosome assembly. In addition to the MyD88-dependent pathway, SIGIRR also modulates the TRIF-dependent pathway, since it also inhibits TLR3 signaling [172, 173], which signals only through TRIF, but not MyD88. The architectures suggest that the mechanism of this inhibition is by SIGIRR blocking TRAM-homodimerization (**Figure4.4e**), TLR4-TRAM (**Figure4.4b**), and TRIF-TRAM (**Figure4.4f**) interactions.

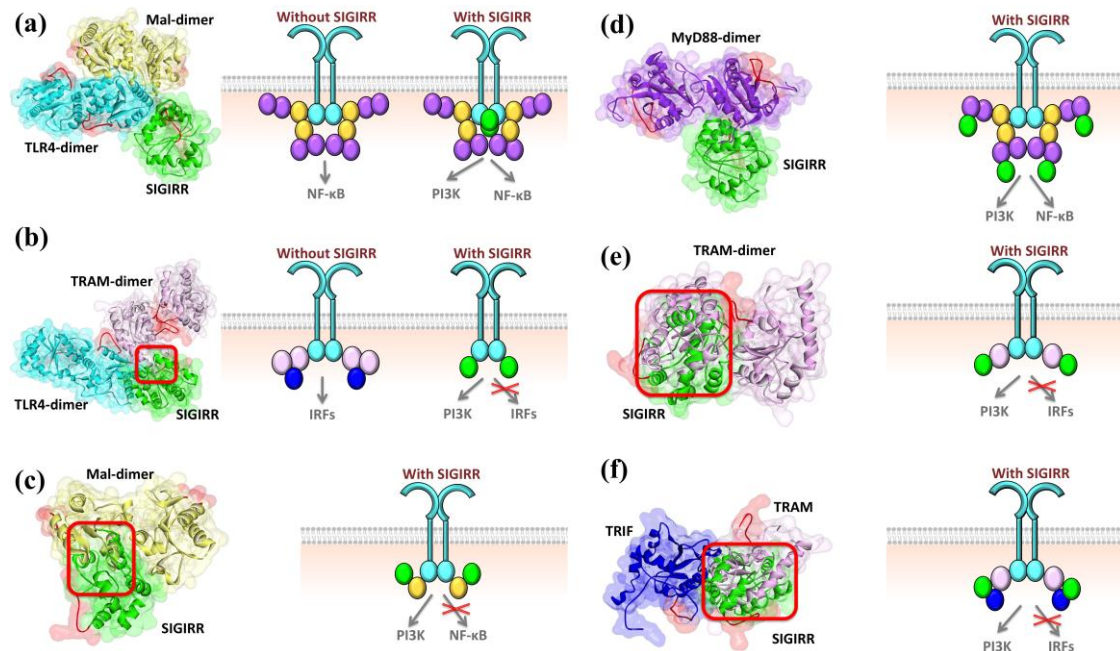


Figure 4.4: SIGIRR interactions with all TIR domain-containing proteins in TLR signaling. (a) SIGIRR interaction with TLR4 allows TLR4 dimerization, TLR4-Mal interaction, Mal-dimerization and the entire MyD88-dependent signalosome formation. (b) SIGIRR-TLR4 interaction has steric clash with TLR4-TRAM binding therefore these two interactions are mutually exclusive: if SIGIRR binds to TLR4, TRAM cannot. (c) SIGIRR interaction with Mal hinders its dimerization and the assembly of Myd88-dependent signalosome. (d) SIGIRR-MyD88 interaction enables the signalosome formation. (e) SIGIRR-TRAM interaction interferes with TRAM-dimerization and prevents TRIF-dependent signalosome assembly. (f) SIGIRR-TRIF interaction blocks TRIF-TRAM interaction due to overlapping binding sites. Red boxes indicate the location of clash. The red-labeled regions are the BB-loops of the proteins. In all parts, the left-hand side figure shows the structure of PPI complexes obtained by PRISM, and the right-hand side schematic figure shows the TIR domain signalosome and how the negative regulators affect the assembly of signalosome. Circles in the schematics represent the TIR domains: Cyan represents TLR, yellow represents Mal, purple represents MyD88, light pink represents TRAM, blue represents TRIF and green represents SIGIRR [101].

TLR-SIGIRR (**Figure 4.4a**) and MyD88-SIGIRR (**Figure 4.4d**) architectures are feasible with the MyD88-dependent signalosome formation. Although it has been proposed to hinder MyD88 homodimerization [168, 169], which is vital for myddosome formation [132, 137], our model suggests that it abolishes neither MyD88-dimerization nor signalosome organization. So, if TLR4 or MyD88 are the proteins that SIGIRR interact with and they do not interfere with the signalosome, how does it hinder the transmission of the signal from TLR4 to NF- κ B? The answer could lie in the inhibition of the translocation of this complex from the receptor [161]. This strategy is also utilized by other negative regulators of TLRs, such as IRAK-M, which prevents the dissociation of the myddosome structure from the receptor complex [100, 174].

SIGIRR has only 2 clinically observed oncogenic mutations in the interface (**Table A.4**). Only one of the mutations (L282M) falls on the SIGIRR-Mal and SIGIRR-TRIF interfaces and it abrogated the SIGIRR-TRIF interaction. It also has a nonsense mutation (Q111*) and a frameshift mutation (P2fs) (**Table A.5**) that produce truncated SIGIRR lacking a TIR domain and abolish its interactions presented here.

4.1.3. ST2 (ST2L)

ST2 (IL1RL1) is also an orphan receptor with cytoplasmic TIR domain [99]. It inhibits NF- κ B activation in response to IL-1R1 and TLR stimulation [162]. Overexpression of ST2 prevents activation by TLR4- but not TLR3-induced NF- κ B (TLR3 utilizes only TRIF-dependent downstream path). In addition, ST2 co-immunoprecipitates with Mal and MyD88, but not with TRIF [162]. This suggests that ST2 has inhibitory roles only in the MyD88-dependent pathway of TLRs.

As with other TIR domain-containing regulators, I obtained possible interaction models of ST2 with all TIR domain-containing proteins in TLR signaling (**Figure 4.5**). Although its interactions with TLR4 (**Figure 4.5a**), MyD88 (**Figure 4.5d**), and TRAM (**Figure 4.5e**) allow homodimerization of these proteins, its interactions with Mal and TRIF abolish Mal-homodimerization (**Figure 4.5c**) and TRAM-TRIF interaction (**Figure 4.5f**). Although it may not interact with TRAM or TRIF under physiological

conditions (another protein may prevent the association or the proteins may not be co-expressed), PRISM still finds these interactions.

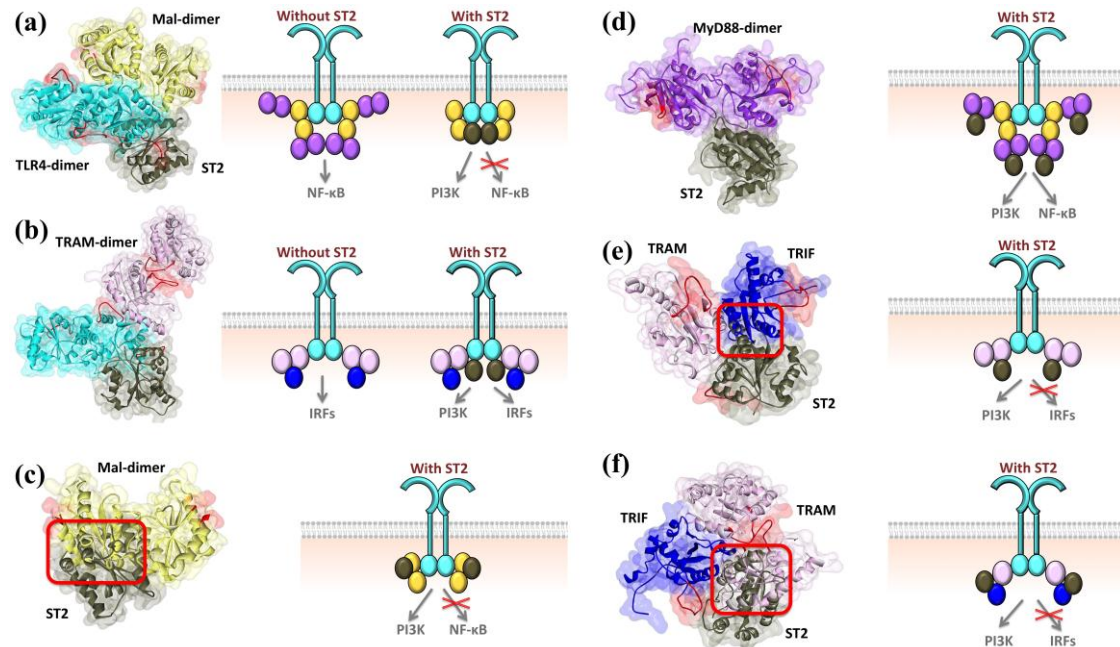


Figure 4.5: ST2 interactions with all TIR domain-containing proteins in TLR signaling. (a & b) ST2 interaction with TLR4 allows TLR4 dimerization, Mal-dimerization, and TRAM dimerization and the formation of both MyD88- and TRIF-dependent signalosomes. (c) ST2-Mal interaction hinders its dimerization and the assembly of Myd88-dependent signalosome. (d) ST2-MyD88 interaction enables the signalosome formation. (e) ST2-TRAM interaction interferes with TRAM-TRIF. (f) ST2-TRIF interaction blocks TRIF-TRAM interaction due to overlapping binding sites. Red boxes indicate the location of clash. The red-labeled regions are the BB-loops of the proteins. In all parts, the left-hand side figure shows the structure of PPI complexes obtained by PRISM, and the right-hand side schematic figure shows the TIR domain signalosome and how the negative regulators affect the assembly of signalosome. Circles in the schematics represent the TIR domains: Cyan represents TLR, yellow represents Mal, purple represents MyD88, light pink represents TRAM, blue represents TRIF and dark grey represents ST2 [101].

In mice, ST2 loses its inhibitory action in a proline-to-histidine mutation at position 431 of its TIR domain [99, 162]. This residue corresponds to P426 in the human homolog. P426 is found at the BB-loop of ST2, which is estimated by alignment of the ST2 structure with other TIR domains. This residue is on the TLR4-ST2 and TRIF-ST2 interfaces (**Figure 4.6**) and the P426H mutation abolishes TLR4-ST2 interaction. In addition to this mutation, there are 6 oncogenic mutations on the interfaces of ST2 (**Table A.4**) and 2 of them impede the ST2-TLR4 interaction. There are also 8 nonsense and frameshift mutations on ST2 (**Table A.5**), leading to loss of the TIR domain and all interactions with the TIR-containing proteins.

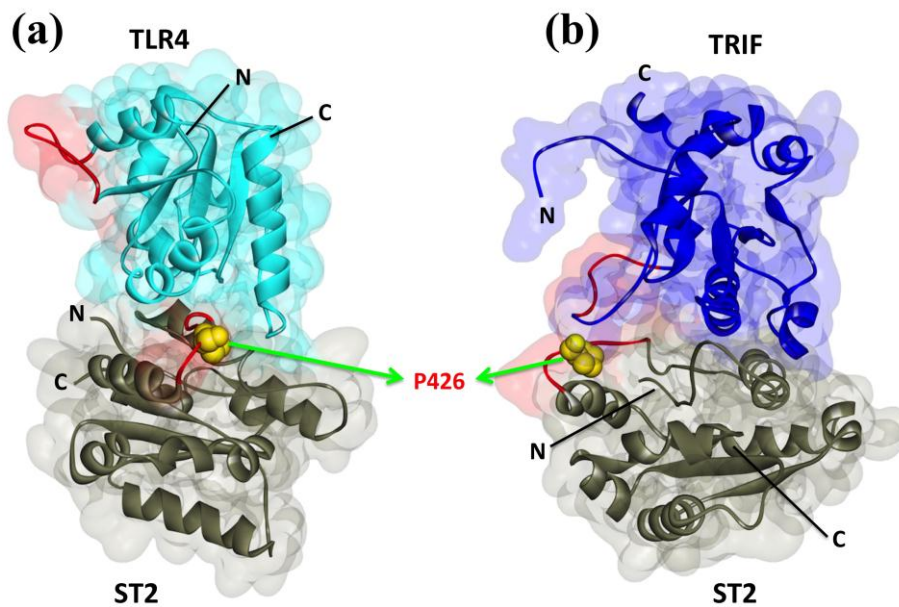


Figure 4.6: P426 residue of ST2 is at the interface of its interactions with TLR4 and TRIF. P426H mutation abolishes TLR4-ST2 interaction. The red-labeled regions are the BB-loops of the proteins [101].

4.2. Ubiquitinases and Deubiquitinases as Regulators of TLR Signaling

Ubiquitination is a reversible post-translational modification (PTM) and it is one of the most prevalent in TLR signaling and NF- κ B activation [175], like phosphorylation [176, 177]. Different ubiquitination modes may result in opposing outcomes, like

degradation or signaling of TRAF3 [66]. Ub chains serve as an anchor, assisting in the assembly of large protein complexes. For instance, the K63-linked Ub chain attached to TRAF6 recruits TAB2/3 and NEMO (IKK- γ); however, these proteins do not associate directly with TRAF6 itself. Since NF- κ B activation depends on ubiquitination, evolution developed Ub-dependent regulation to abate inflammation [178]. Some of the negative modulators of the TLR pathway are ubiquitinases that add K48-linked Ub chains to essential orchestrators. TRIAD3A catalyzes the K48-linked ubiquitination and leads to proteosomal degradation of Mal and TRIF [175]; and Nrdp1 adds K48-Ub to MyD88, inhibits the MyD88-dependent TLR pathway and preferentially enhances TRIF-dependent interferon (IFN) expression [179]. Some others are DUBs, such as A20 (also known as TNFAIP3), CYLD (Cylindromatosis D), and DUBA (Deubiquitinating enzyme A), that negatively modulate TLR signaling by removing the K63-linked Ub chains from proteins, such as TRAF6 and TRAF3 [175]. Below I demonstrate that these key DUBs turn-off TLR signaling because they prevent the binding of TRAF6 and TRAF3 to their partners in addition to removing their Ub-chains.

4.2.1. A20

A20 is a pleiotropic Ub editing enzyme: it has an N-terminal ovarian tumor (OTU) domain with DUB activity, and seven C-terminal zinc finger domains (ZF) – the fourth (ZF4) with E3 ligase activity [180, 181]. Although it is a DUB, it also catalyzes the addition of K48-linked Ub chains [182, 183]. It antagonizes TLR- and TNFR-mediated NF- κ B activation [183]. TRAF6 is a substrate for A20 in the TLR pathway [175]. A20 was also proposed to inhibit the association of TRAF6 (E3) with Ubc13 (E2) and UbcH5c (E2) [182]. A20 forms homodimers through the ZF4 domains and its higher-order oligomerization facilitates its deubiquitinase, E3 ligase and Ub-binding functions [180]. The A20 homodimer structures in the asymmetric unit of two pdb entries (3dkb_CF and 2vfj_BC) were suggested to be biological since mutation of the interface residues (M15A, R16E, and H351A) suppressed A20-dimerization [180]. I obtained similar homodimer with PRISM (**Figure 4.7a**).

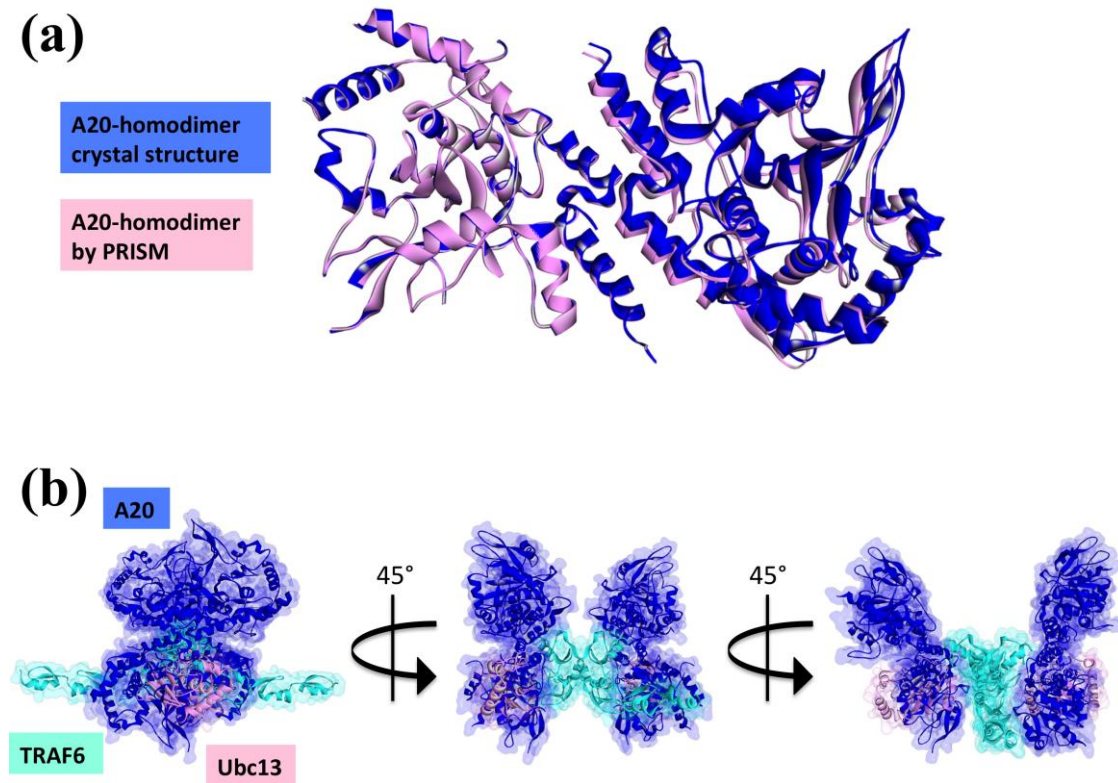


Figure 4.7: A20 needs to dimerize in order to execute its function. (a) I obtained A20-dimer structure that is very similar to the ones that are observed in asymmetric units of two pdb entries (3dkb_CF and 2vfj_BC), which were suggested to be biological. **(b)** Structure of TRAF6 interaction complex with A20-homodimer. This interaction interferes with TRAF6-Ubc13 interaction [101].

I found interactions between the RING domain of TRAF6 and the OTU domain of A20 (**Figure 4.8**) and this architecture also allows A20 homodimerization (**Figure 4.7b**). A20-TRAF6 interaction abolishes TRAF6-Ubc13 as proposed earlier [182, 183], since they have overlapping binding sites on TRAF6. C103, H256 and T97 residues on A20 were determined to be the catalytic residues [183]. The C103A mutation inhibits A20 catalytic function (C103A mutant of A20 was unable to de-ubiquitinate TRAF6) [183] and TRAF2-Ubc13 interaction [184]. However, this residue is not on the interface of TRAF6-A20 that I obtained. This result is expected since C103A mutant of A20 was shown to still bind to TRAF6 [185]. The reason for the blockage of TRAF2-Ubc13 interaction may be that the C103 residue is involved in the interface of

TRAF2-A20 complex; alternatively, mutation of this residue disrupts the stability of A20 and allosterically modifies its interaction sites. In addition to inhibition of E2-E3 interactions, A20 has also roles in degradation of E2 proteins (e.g. Ubc13) by attaching K48-linked Ub chains to E2, thus affecting E2-dependent E3 function [186]. I could not find clinically observed oncogenic mutations on A20 that fall onto A20-TRAF6 interface residues or non-sense/frameshift mutations that lead to truncated A20 without OTU domain the TCGA database.

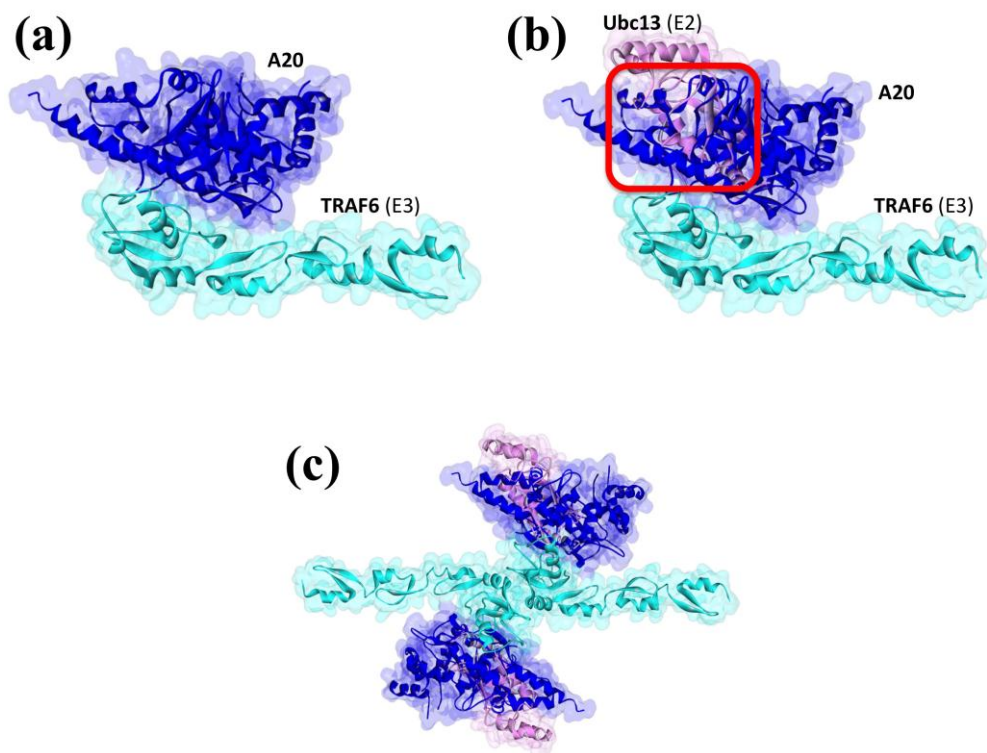


Figure 4.8: A20 interaction with TRAF6. (a) OTU domain of A20 interacts with RING domain of TRAF6. (b) Ubc13 and A20 have almost completely overlapping interfaces on TRAF6 and hence their interaction with TRAF6 are mutually exclusive. Red square indicates the location of clash. TRAF6-Ubc13 interaction is obtained from crystal structure (3hcu_AB). (c) TRAF6-dimer through its RING domain and its interactions with A20 and Ubc13 [101].

4.2.2. CYLD

CYLD is a Ub-specific protease (USP) with a tumor suppressive role. It removes K63-linked Ub chains from TRAF6 [175] and negatively regulates NF- κ B and expression of pro-inflammatory cytokines [187, 188]. To date, its NF- κ B inhibition mechanism has been unclear. CYLD interacts with TRAF6 [188], but it was also suggested to require some bridging adaptor proteins, like p62 (also known as sequestosome1) to interact with its substrates since its activity is reduced in the absence of p62 [187, 189, 190].

I found TRAF6 interactions with CYLD either via direct binding or via p62 (**Figure 9a & b**). CYLD has 3 CAP-Gly (Cytoskeleton-associated protein-glycine conserved) domains with similar global folds. The third is suggested to interact with TRAF2 [191, 192]. As to direct interaction, I found that the first CAP-Gly domain of CYLD binds to the RING domain of TRAF6. However, in the p62 adaptor case, the second CAP-Gly domain of CYLD interacts with p62, which interacts with TRAF6. Residues 225-255 of p62 were already identified as an interface that binds to TRAF6 [193] and this is in agreement with our TRAF6-p62 complex architecture. Both the direct and indirect (through p62) associations sterically hinder the TRAF6-Ubc13 interaction (**Figure 9c & d**). If p62-CYLD or CYLD-itself binds this interface on TRAF6, Ubc13 cannot approach that site and TRAF6 is nonfunctional.

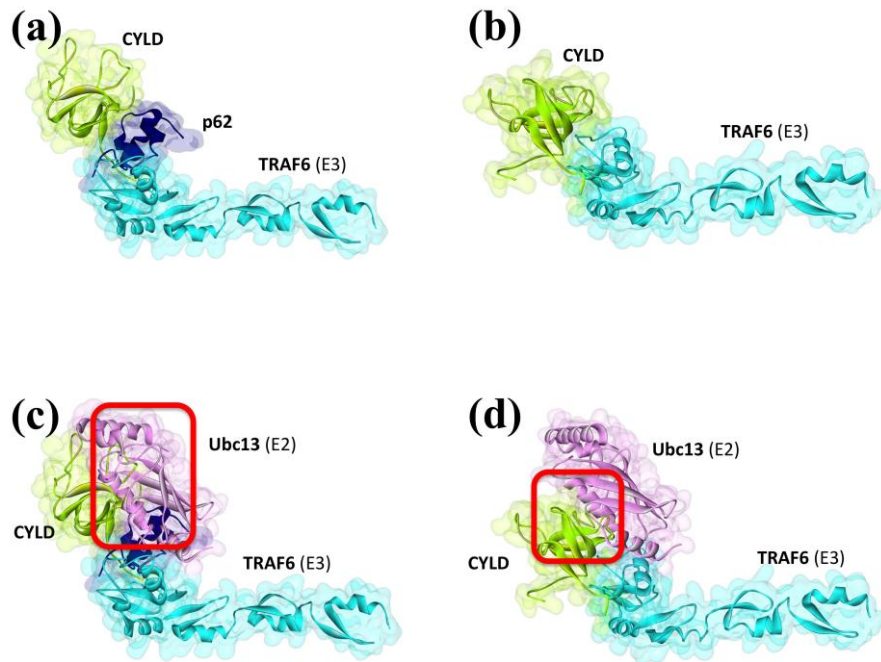


Figure 4.9: Architectures of direct and indirect interactions of TRAF6 with CYLD. (a) Indirect association of TRAF6 with CYLD through p62 bridging adaptor. (b) Direct interaction of TRAF6 with CYLD. (c) In the p62 case, both p62 and CYLD has steric clashes with Ubc13. (d) In the direct binding, CYLD has steric clash with Ubc13. Red squares indicate the location of clash. TRAF6-Ubc13 interaction is obtained from crystal structure (3hcu_AB) [101].

Why does TLR negative regulation have redundant players with the same function and the same substrate (TRAF6 with K63-Ub chain), such as A20 and CYLD? The timing of their action may address this question: CYLD is constitutively expressed, but A20 is not [187]. Alternatively, they may act in distinct cell types, or subcellular locations [194]. They may also cooperate to remove the Ub chains from TRAF6.

Seven missense mutations on CYLD correspond to the interface residues, but only one of them causes the loss of its interactions (p62-CYLD) according to our results (Table A.4). Additionally there are also 6 nonsense or frameshift mutations that lead to truncated protein without CAP-Gly or DUB domains (Table A.5). Since these mutations cause loss of the domains that are required for CYLD-TRAF6 or CYLD-p62 interactions, the truncated CYLD can no longer associate with these proteins and deubiquitinate TRAF6. Nonsense and frameshift mutations predominantly constitute

the mutations of CYLD in tumors [[185](#), [195](#)].

4.2.3. DUBA (OTUD5)

Similar to A20, DUBA (also known as OTUD5) is also an OTU domain family member cysteine protease. It suppresses IFN-I production in response to TLR activation by removing K63-Ub chains from TRAF3 and preventing recruitment of TBK1 and other downstream proteins, which are necessary for NF- κ B and IRF activation [[66](#), [175](#), [196](#)]. Interaction of endogenous TRAF3 and DUBA takes place upon TLR3 stimulation [[196](#)]. The D221, C224 and H334 are the catalytic residues of DUBA and C224S mutation on DUBA compromised its function (deubiquitinating TRAF3) [[196](#)]. There is less Ub alteration in TRAF3 lacking a RING domain [[196](#)], indicating that the RING domain of TRAF3 is probably the interaction region. However, I could not find interaction between RING domain of TRAF3 and catalytic OTU domain of DUBA. Instead, I found interaction with concave site on TRAF-C region of TRAF3 (**Figure 4.10**). This concave region is where TRAF3 interacts with CD40 (1fll.pdb) [[151](#)], BAFFR (2gkw.pdb) [[152](#)], LMP1 (1zms.pdb) [[153](#)], Cardif (4ghu.pdb) [[154](#)], and also MyD88 [[67](#)]. If DUBA also binds to TRAF-C region in addition to the RING domain of TRAF3, it attenuates TLR-induced IRF activation not only through its catalytic (DUB) function, but also through interfering with the TRAF3 interactions with other signaling proteins. There are no clinically observed oncogenic mutations on DUBA that fall onto DUBA-TRAF3 interface residues or nonsense/frameshift mutations.

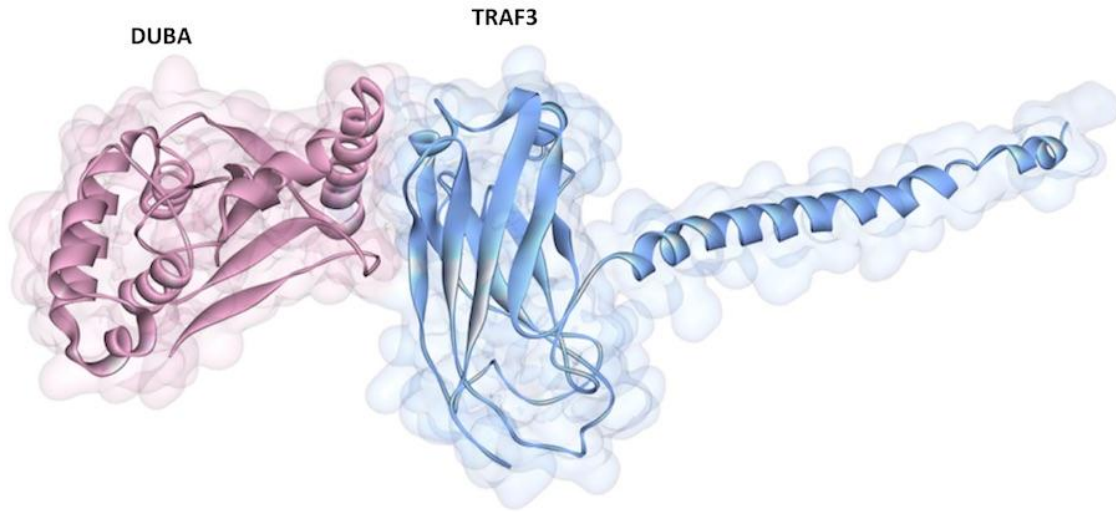


Figure 4.10: The architecture of the interaction between the OTU domain of DUBA and TRAF-C domain of TRAF3 [101].

4.3. MyD88s

Assembly of proteins into multimolecular complexes takes place at almost all levels of TLR signaling and is key for efficient regulated signaling across the cell [126]. Examples of such oligomeric structures include clustering of TLRs upon recognition of their ligands [123, 124]; TIR-domain signalosome; the myddosome complex formed by the DDs of MyD88 and IRAKs [90]; the infinite network of TRAF6 formed by its trimerization through TRAF-C domain and dimerization through RING domain [125]; and complexes formed with the help of poly-Ub chain scaffolds [182]. Induced clustering of TLRs activates the signaling even in the absence of their cognate ligands [124]. Therefore, formation of clusters and oligomerization is the main outcome that ligand binding triggers.

In Chapter 3, I showed that TLRs cluster via the formation of TIR-domain signalosome with 8 MyD88 molecules and helical myddosome structures with 6 MyD88. I proposed that the linker region (45 residue-long) of MyD88 between its TIR and DD plays critical roles in TLR clustering. MyD88s (short MyD88), a splice variant of MyD88, missing the interdomain linker region has been shown to inhibit

NF- κ B and MAPK activation [133]. Our model provides the structural basis for inhibition of TLR signaling by MyD88s.

4.4. Parallel Downstream Paths of TLRs

The TLR pathway activates different parallel downstream paths. The conventional downstream path of TLRs is the MyD88-dependent path, which involves TRAF6 and IRAKs to activate NF- κ B and MAPK and to produce pro-inflammatory cytokines. There are also TRIF-dependent, TRAF3-dependent, FADD-dependent, and BCAP-dependent paths, which lead to production of IFNs apoptosis, or activation of the PI3K pathway, respectively. Our recent study [67] suggest that almost all parallel pathways of TLRs compete with each other and thus restrict the activation of one another due to overlapping binding sites on a common binding partner (**Figure 4.1**). These parallel paths only switch the function: while negatively regulating one path, they positively regulate others. The negative regulators covered in this study, except BCAP, cease TLR signaling. Network flow algorithms might be helpful to understand the interplay between parallel paths in TLR pathway [197].

In conclusion, TLR pathway resembles a double-edged sword: On the one hand, it is vital for triggering proper host immune response against pathogens and its malfunction makes individuals more susceptible to infections. On the other, its overactivation results in excess inflammation, which is detrimental to the host. The cell has evolved several mechanisms to strike a balance between activation and inhibition of TLR signaling at almost each step of the pathway. Although several negative regulators have been identified, their inhibition mechanisms remain elusive. I provide the structural basis for the regulation of TLR signaling. Overall, I observed that negative regulators tend to interfere with the assembly of key signaling complexes, such as TIR-domain signalosome, compete with proteins to bind to their partners, and modify ubiquitination states and target them for proteosomal degradation. Similarly, parallel downstream paths compete with each other and restrain their activation. Several oncogenic mutations on negative modulators tend to disrupt their interactions with the key players in TLR pathway, liberating the conventional downstream paths and

constitutive activation of NF- κ B. Better understanding of negative regulation of TLR signaling holds promise for novel treatment strategies for autoimmune diseases and cancer.

Chapter 5

STRUCTURAL MODELING OF SOME OTHER KEY PATHWAYS OF CANCER

There are many pathways that take part in oncogenesis and inflammation-cancer crosstalk, such as Caspase 8 (CASP8), TRAF3 and RIPK1 centered pathways. These proteins have important roles in apoptosis, inflammation and necroptosis. Throughout my Ph.D. studies I also dealt with the structural modeling of PPIs in these pathways. One of the hallmarks of cancer is “resisting cell death” [56]. In this section, I begin with how structural details of CASP8 interactions illuminate its activation mechanism to trigger apoptosis and how its mutations contribute to “resisting cell death” in cancer.

Then, I continue with the pairwise interaction models for TRAF3 and RIPK1 in order to understand the molecular mechanisms of the communication of TLR pathway with other pathways. In a cell, more than one signaling cascade is active at a time and the communication and crosstalk of these pathways determines the ultimate cellular outcome: i.e. whether to survive, die, or produce inflammatory responses, etc. For instance, **Figure 3.15** demonstrates an example for the communication of TLR and Fas pathways. In the binary interactions of TRAF3 and RIPK1, I include all their partners, regardless of whether those are involved in TLR pathway or not. This allowed us to elucidate the crosstalk of TLR pathway with other signaling pathways such as TNFRs and predict the possible cellular outcome.

5.1. Architectures of CASP8 interactions with its partners

Apoptosis is a cellular suicide mechanism whose loss is one of the hallmarks of cancer [56]. There are two different types of apoptosis: intrinsic apoptosis, induced by intracellular damage or stress, and extrinsic apoptosis, stimulated by ligation of “death receptors” with their extracellular ligands [198]. Apoptosis is triggered by consecutive actions of a cascade of initiator and executioner caspases. Caspases are cysteine

proteases. They cleave their substrates after Asp residues. They are expressed as catalytically inactive zymogens or proenzymes [199]. They need to undergo cleavage events in order to get fully active [200, 201]. Initiator caspases dimerize, cleave, and autoactivate themselves and subsequently the executioner caspases, which in turn cleave several downstream substrates.

CASP8 is an initiator caspase, with a prodomain, consisting of two death effector domains (DEDs) and a catalytic C14 peptidase domain, which comprises a large subunit and a small subunit (p18 and p10, respectively) (**Figure 5.1**) [201]. It remains monomeric until it is recruited to large signaling complexes, such as the death inducing signaling complex (DISC) formed at the membrane, involving a death receptor, FADD and RIPK1 [201, 202]. In addition to triggering apoptosis, CASP8 also has inhibitory impact on inflammation [201].

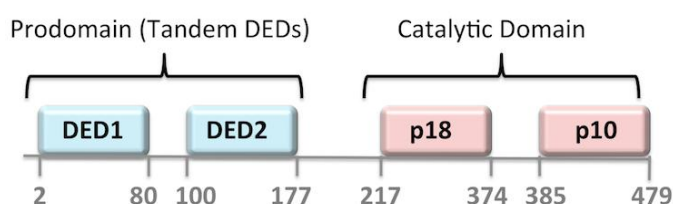


Figure 5.1: Domain structure of CASP8.

Apoptosis is tightly regulated by caspase inhibitors, such as cellular inhibitors of apoptosis (cIAPs). cIAPs are E3 ubiquitin ligases. They catalyze ubiquitination of CASP8 and RIPK1 and target them for degradation.

I aim to construct structures of CASP8 interactions with its partners to reveal how it gets activated when recruited to DISC, how it is regulated by cIAPs, and how its mutations affect the interactions and the cellular outcome.

It is known that CASP8 interacts with several partners, but not how. Structures of only CASP8-cFLIP (3h11AB) and CASP8-p35 (1i4eAB) interaction complexes are available in the PDB. The architectures of the other complexes are obscure. As can be seen in **Figure 5.2**, I provide structural insights to CASP8 binary interactions with its binding partners and to the effects of its mutations on these interactions.

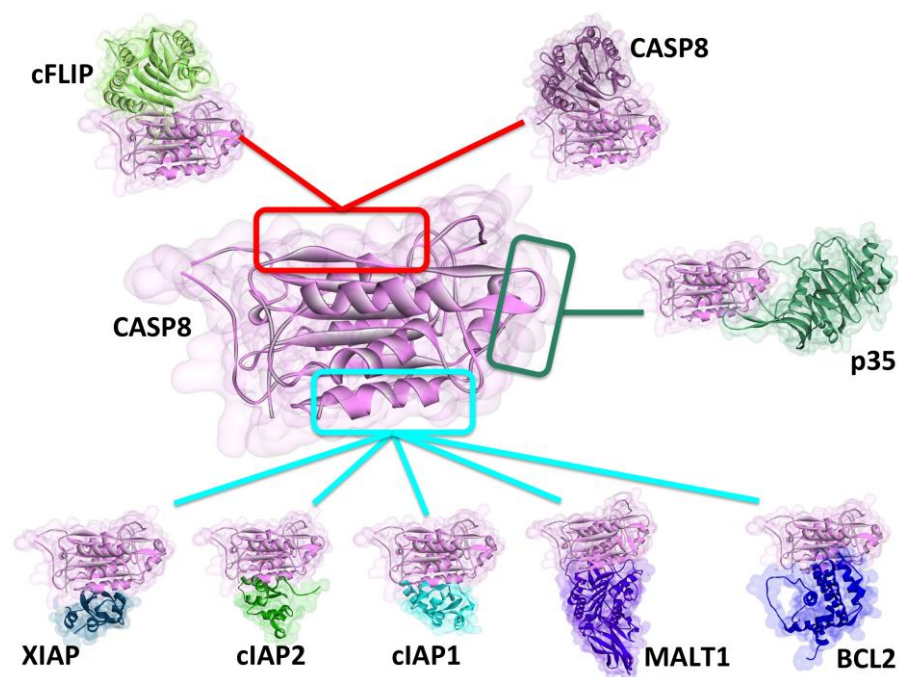


Figure 5.2: The structures of CASP8 and its interactions. The solid rectangles represent the interfaces. If the lines converge on the same rectangle, this means that the proteins bind to same interface on CASP8, hence they are mutually exclusive. All the interactions presented here are PRISM predictions, except CASP8-p35 and CASP8-cFLIP.

5.1.1. CASP8-homodimer

Caspases need to undergo homodimerization and a subsequent cleavage event in order to get activated and trigger apoptosis [201]. CASP8 also forms heterodimers with cFLIP (FLICE-like inhibitory protein), which is a catalytically inactive homolog of CASP8 that prevents induction of apoptosis by interfering with CASP8 dimerization [201]. I built the CASP8 homodimer structure (**Figure 5.3**). It is very similar to the CASP8-cFLIP complex, which already has a resolved 3D structure. The CASP8 homodimer interface forms a β -sheet extension. The binding energy score of this complex is very low (-242), compared to CASP8-cFLIP interaction (-85) (**Table A.6**). Since they share the same interface, if the cellular cFLIP concentration is high, CASP8-cFLIP heterodimers can form [201] rather than the CASP8 homodimers.

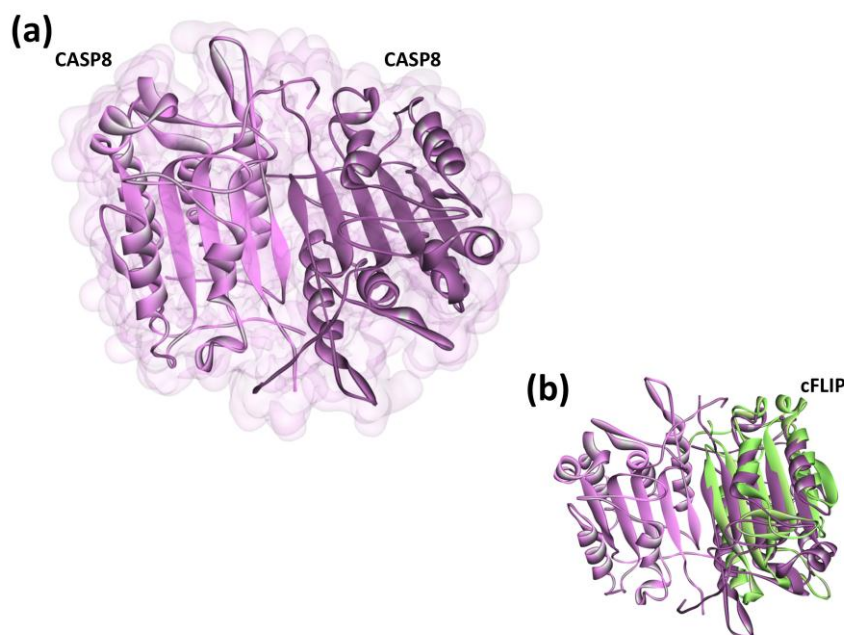


Figure 5.3: CASP8 homodimer and heterodimer. (a) CASP8-homodimer structure induces a β -sheet extension. (b) This homodimer structure is very similar to CASP8-cFLIP heterodimer (3h11_AB.pdb), as shown in the superimposition of CASP8-dimer and CASP8-cFLIP complex.

5.1.2. CASP8-FADD

When Fas death receptor recognizes Fas ligand, it recruits FADD and they form a helical DISC oligomer through their DDs, with the stoichiometry of 5:5, 6:5, and 7:5 (5:5 is the predominant one) [202]. Formation of higher order oligomers by proteins facilitates execution of the cellular functions since it amplifies the signal and allows more efficient enzymatic reactions [126]. Multi-protein oligomers may constitute a scaffold, bringing the components of cellular machinery into close proximity and enhancing the formation of the active conformer of the enzymes [203, 204]. Recruitment of CASP8 zymogen to DISC organization initiates proteolytic activation of CASP8, but it is not known how.

Part of the DISC complex has a resolved crystal structure (3oq9.pdb), consisting of 5 Fas and 5 FADD. CASP8 is also recruited to this structure and form the ternary DISC [205]. Death receptors have a DD, FADD has both DD and DED, and CASP8 has two

DEDs. Therefore, FADD serves as a bridge between the receptors and CASP8, by interacting through its DD and DED, respectively [206].

CASP8 has two consecutive DEDs (tandem DEDs), each consisting of 6 helices. However, their structure has not yet been solved. There is only one tandem DED-containing protein, viral MC159, whose structure is resolved (PDB_IDs: 2bbr, 2bbz, and 2f1s) [205, 207]. I modeled the tandem DEDs of CASP8 together by I-TASSER server [139] and found a possible interaction complex with the DED of FADD (**Figure 5.4a**). In this model, both DED1 and DED2 of CASP8 interact with FADD-DED. Previously, only DED2 of CASP8 (helices 2 & 5) was suggested to be in contact with FADD-DED (helices 1 & 4) [205, 208]. However, in our model they are not at the interface (**Figure 5.4b**). There is no experimental evidence regarding whether DED1 of CASP8 associates with FADD. It has also been demonstrated that F25 and L26 residues of FADD that reside in helix2, are involved in the interface of CASP8 interaction [205] and these residues are at the interface of our FADD-CASP8 model, where FADD contacts with DED1 of CASP8 (**Figure 5.4b**).

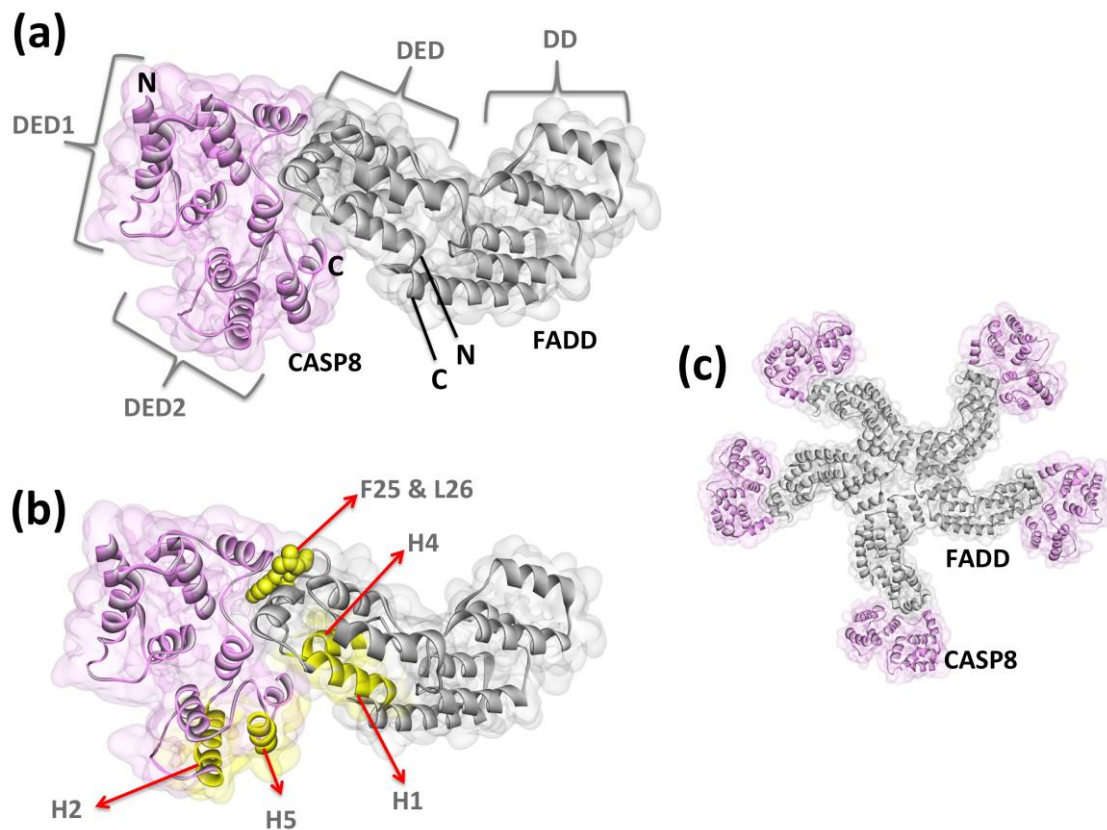


Figure 5.4: Architecture of CASP8-FADD interaction complex obtained by PRISM. (a) Both DEDs of CASP8 associate with DED of FADD. (b) Helices 2 & 5 of CASP8-DED2 and helices 1 & 4 of FADD-DED (yellow-highlighted helices) were suggested to be at the interface of CASP8-FADD interaction [205, 208]. However, they are not at the interface of our model. F25 and L26 residues of FADD (yellow spheres) were shown to be at the CASP8-FADD interface [205] and they are at the interface of the organization that I obtained by PRISM. (c) CASP8-FADD interaction structure by PRISM does not violate DISC assembly.

Our FADD-CASP8 interaction model does not violate the formation of the DISC complex. The superimposed structure of our FADD-CASP8 model with DISC can be seen in **Figure 5.4c**. This architecture has one CASP8 per FADD molecule. If this is the case in the cell, how does recruitment to DISC activate the CASP8 zymogen, and how does it facilitate the formation of active catalytic dimers of CASP8? DEDs of CASP8 are far apart in the DISC organization (**Figure 5.4c**). Since the entire CASP8 structure is not available, we don't know the orientations of the domains of CASP8

with respect to each other. We also don't know whether C14 peptidase domains of CASP8 molecules in DISC are close enough to form catalytic dimers. As suggested before, 1:1 stoichiometry of FADD and CASP8 does not explain the dimerization and activation of CASP8 [198]. A recent study illustrated that each FADD in the DISC associates with more than one CASP8 [198, 209], which may suggest that DEDs of pro-CASP8 also form dimers or higher order oligomers, in addition to its C14 domains. There is clear experimental evidence that isolated DEDs and DED-only isoforms of CASP8, such as isoform-6, can undergo homotypic DED-DED interactions and form "death effector filaments", which is sufficient to induce apoptosis in the presence of full-length CASP8 [210]. Possibly DISC assembly enables DED-DED interactions of CASP8, which in turn facilitates C14 dimerization. I also checked whether tandem DEDs of CASP8 can form dimers and found a possible dimer structure with PRISM (**Figure 5.5a**), in which DED2 of each CASP8 monomer is in contact (**Figure 5.5b**). However, this dimer structure of tandem DEDs is mutually exclusive with CASP8-FADD interaction, and thereby with the DISC structure. That is, they impose a steric clash if they occur simultaneously (**Figure 5.5c**). Dickens et al. proposed a model for CASP8 tandem DED oligomer/chain and their interaction with the FADD [198]. In their model, DED of FADD interacts with DED1 of CASP8, instead of DED2 (however the previously identified interface regions involve DED2 of CASP8 (helices 2 & 5) and FADD-DED (helices 1 & 4)) and CASP8 tandem DEDs form a chain such that DED2 of first CASP8 monomer interacts with DED1 of the second monomer. Unfortunately, I couldn't obtain the same chain structure with PRISM and therefore cannot check whether this proposed chain structure is plausible with the entire DISC assembly. (With our CASP8-DED dimer model, I cannot form a filament/chain)

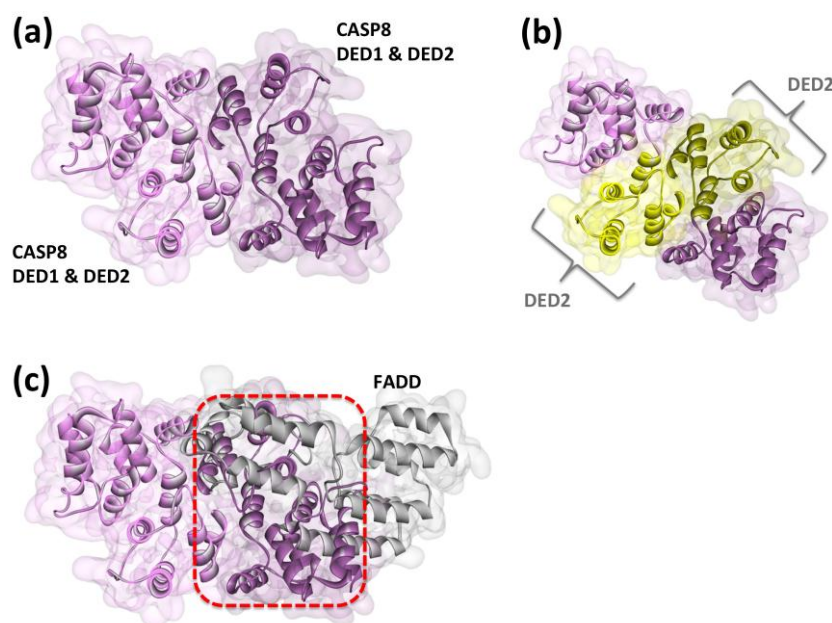


Figure 5.5: Dimer of tandem DEDs of CASP8. (a) I found possible dimer architecture between DEDs of two CASP8 molecules. (b) DED2 of each CASP8 monomer (highlighted with yellow) are in contact. (c) This tandem DED-dimer structure is mutually exclusive with CASP8-FADD interaction since they have almost completely overlapping binding site. Red dashed rectangle shows the steric clash.

Death fold domains, namely DD, DED, CARD (Caspase recruiting domain), and PYD (Pyrin domain) [208] form either ring or helical structures. Ring formation is observed in DISC [202] and inflammasome; and helical transformation is observed in Myddosome [90] and PIDDosome [211]. In all of the helical assemblies and ring structures, there are three types of interfaces/interactions required to stabilize the helix (type-I, type-II, and type-III) [90, 202, 211]. The interface between DED1 and DED2 in the tandem DED is type-I interface, which I found by superimposition with 3mopEB. I speculate that they might need at least 2 other interfaces (type-II and type-III) in order to stably assemble into a complex. Although I couldn't obtain a dimer structure for the tandem DEDs with type-II or type-III interfaces by PRISM, I built a dimer structure by structural superimposition with these interfaces (**Figure 5.6a**). I superimposed the structural alignments of 3mopEB (type-I) and 3mopEA (type-II) with the tandem DED structure. As shown in **Figure 5.6a**, there are 2 residues, Asp15-

Glu36 and Asp33-Gln35, involved in interfaces on each tandem DED in the dimer. In this dimer structure, both type-II and type-III interfaces are observed (**Figure 5.6b**). I can also build a trimer of tandem DEDs by structurally aligning two dimers (**Figure 5.6c**) (but not a tetramer, due to steric clash). The trimer structure of tandem DEDs of CASP8 is feasible with the DISC assembly: each FADD molecule in DISC organization recruits three CASP8 molecules (**Figure 5.7**). This result supports the previous reports of observed higher stoichiometries of FADD to CASP8 in DISC [198, 209]. This structure may clarify how CASP8 gets activated when it is recruited to DISC and initiate apoptosis.

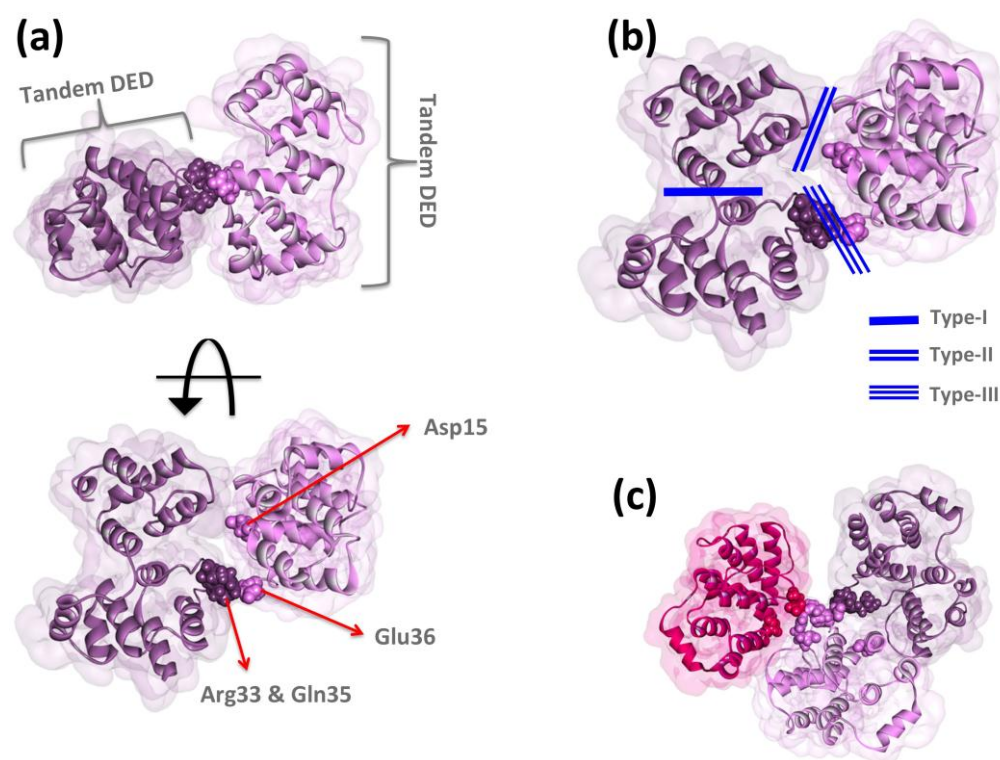


Figure 5.6: Dimer and trimer structures of CASP8 tandem DED. (a) CASP8 tandem DED dimer structure obtained by superimposition with 3mopEA (type-II) and 3mopEB (type-I) (Not a PRISM prediction). Arg33 and Gln35 of one CASP8 are in contact with Asp15 and Glu36 of the other. (b) In this dimer structure, all type-I, -II, and -III interfaces, that are observed in helical and ring structures formed by death fold superfamily members [90, 202, 211], are present. (c) Tandem DED trimer can also be built by structural alignment of the dimers.

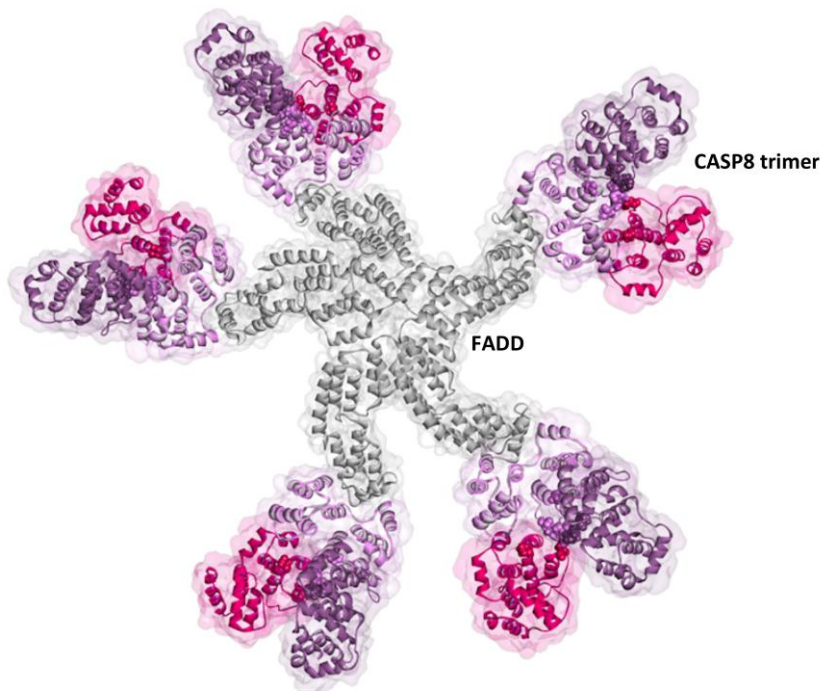


Figure 5.7: CASP8 tandem DED trimer structure that is obtained by superimposition is plausible with the DISC structure.

5.1.3. CASP8-RIPK1

RIPK1 (receptor (TNFRSF)-interacting serine-threonine kinase 1, also known as RIP1) is another component of the DISC complex [212]. Normally it does not interact directly with CASP8; instead it uses FADD as a bridging adaptor. RIPK1 is a mediator of cellular responses to inflammation and stress [213] and programmed necrosis, so called necroptosis. FADD binds CASP8 through its DED and uses its DD to bind Fas and RIPK1 (Figure 5.8a). DD of RIPK1 does not have a resolved structure and I built its model.

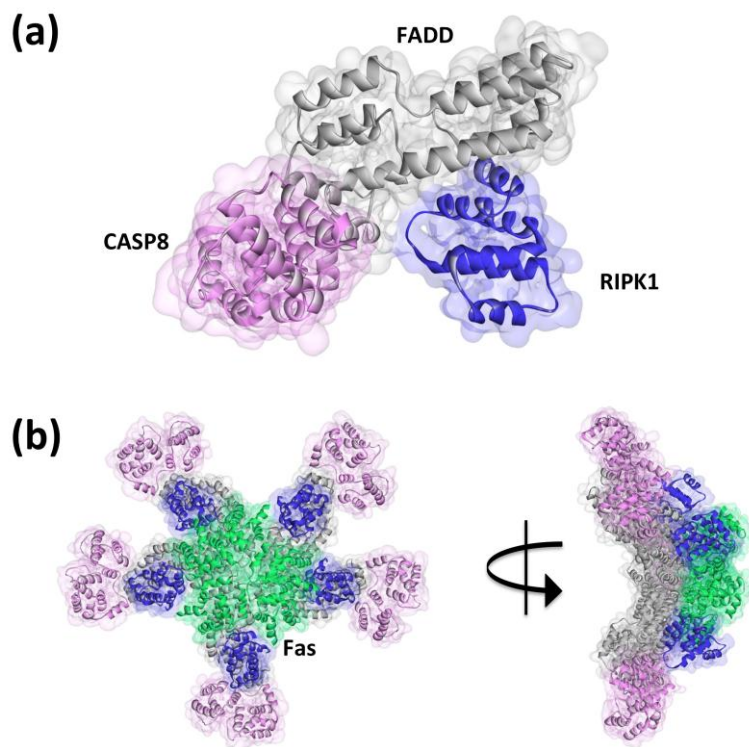


Figure 5.8: FADD-RIPK1 interaction complex. (a) FADD serves as a bridge between CASP8 and RIPK1: FADD interacts with CASP8 through DED and with RIPK1 through DD. Fas receptor and FADD nucleates the formation of a helical DISC oligomer. Fas-FADD helical assembly has a resolved structure (3oq9.pdb). Although it is not included in this crystal structure, complete DISC also involves CASP8 and RIPK1 proteins. As expected, CASP8-FADD interaction model that is compatible with DISC formation. (b) The entire DISC complex with Fas receptor (green), FADD (gray), CASP8 (pink) and RIPK1 (blue). FADD-RIPK1 interaction model also feasible with DISC oligomer.

Our FADD-RIPK1 interaction model is compatible with the DISC structure (**Figure 5.8b**). Although only part of the DISC complex has a crystal structure (only the Fas-FADD interaction through DDs), it includes CASP8 and RIPK1 in the large oligomeric complex. The architectures that I present may complement the DISC structure and represent the entire DISC assembly (**Figure 5.8b**).

5.1.4. CASP8-BCL2

Since apoptosis plays important roles in establishing cellular homeostasis, it is a highly regulated process. It has several negative regulators and BCL2 is one of them. BCL2 is an anti-apoptotic protein, inhibiting cytochrome-c release from the mitochondria [214]. It is a substrate for caspases and gets cleaved by these enzymes at its Asp31 and Asp34 [215]. Prevention of its cleavage inhibits apoptosis [214]. CASP8 and CASP3 inhibitors compromise the cleavage of BCL2 at both sites [215].

The structure of CASP8-BCL2 complex that I obtained by PRISM has both Asp31 and Asp34 at the interface (**Figure 5.9**). This interaction architecture does not interfere with CASP8-homodimer. This is expected, since CASP8 needs to dimerize in order to get activated and cleave downstream proteins, such as BCL2. If BCL2 interaction overlapped with the CASP8-dimerization interface, how would monomeric (non-functional) CASP8 cleave BCL2? Our model demonstrates that anti-apoptotic role of BCL2 is not attributed to interfering with CASP8 dimerization.

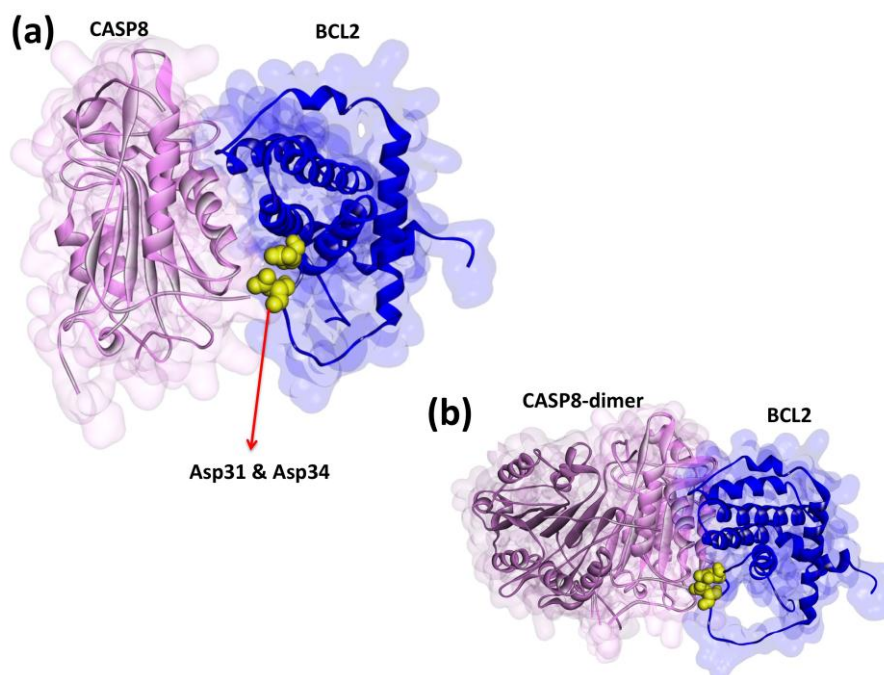


Figure 5.9: The architecture of CASP8-BCL2 interaction. BCL2 is a substrate for CASP8 and it gets cleaved at residues Asp31 and Asp34 by CASP8 [215]. These residues, highlighted by yellow spheres, are found at the interface of our CASP8-BCL2 model. As shown in the lower right corner, this interaction does not interfere with CASP8 homodimerization.

5.1.5. CASP8-cIAP1

The cellular inhibitors of apoptosis (cIAPs) are E3 ubiquitin ligases and regulators of caspases. They possess one or three BIR (Baculoviral IAP repeats) domains [216]. cIAP1 has three BIR domains. When cIAP1 interacts with CASP8, it blocks apoptosis, but the inhibition mechanism is unclear.

Structures of cIAP1 interaction complexes with CASP9 (3d9t.pdb) (**Figure 5.10a**) and SMAC (second mitochondria-derived activator of caspases) (3d9u.pdb) (**Figure 5.10b**) are available in PDB [217] and their interfaces are almost identical, implying that their interactions with cIAP1 are mutually exclusive. When SMAC binds to cIAP1 and occupies the interface, caspases can no longer bind to cIAP1. SMAC liberates CASP8 from cIAP1; thus it is pro-apoptotic. Similar to these interactions, the XIAP-

SMAC (1g3f.pdb) crystal structure also has the same interface β -sheet (**Figure 5.10c**). This region is part of the BIR domains and the interaction site is common for all cIAPs.

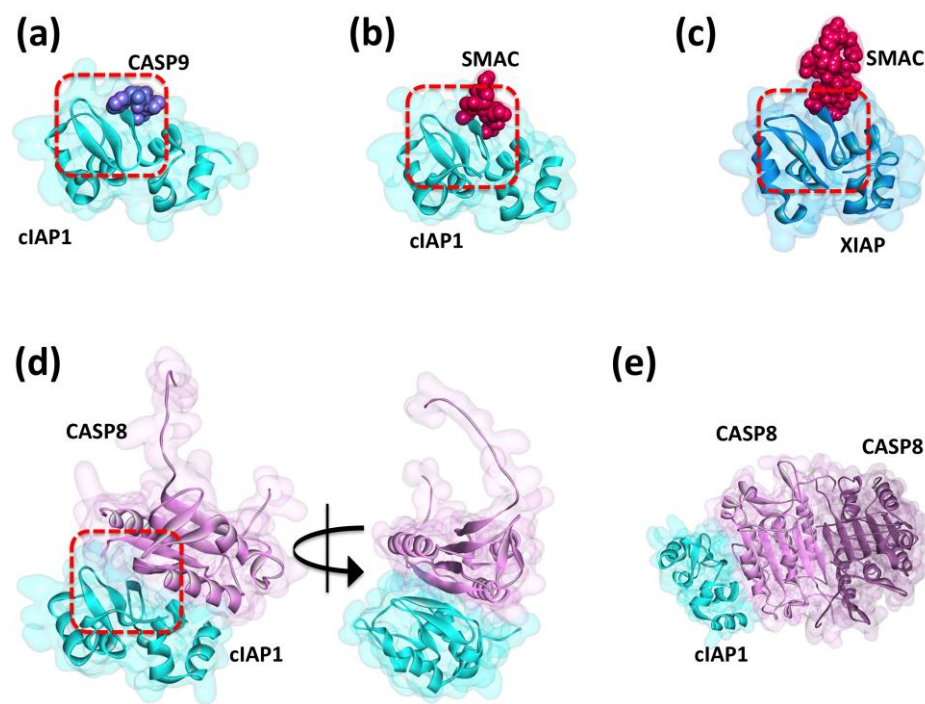


Figure 5.10: The structures of cIAP interactions. Resolved structures of (a) cIAP1-CASP9 (3d9t_AB.pdb), (b) cIAP1-SMAC (3d9u_AB.pdb), and (c) XIAP-SMAC (1g3f_AB.pdb) have common β -sheet of cIAP1 and XIAP at the interface, as shown with the dashed purple squares. (d) Our cIAP1-CASP8 interaction model also has the same region at the interface. This interaction imposes an extended β -sheet at the interface. (e) cIAP1 interaction does not prevent CASP8 dimerization.

The cIAP1-CASP8 architecture that I obtained shows that the catalytic domain of CASP8 binds to BIR3 domain of cIAP1 in a very similar fashion to that of the cIAP1-CASP9 interaction (**Figure 5.10d**), which was expected. The interface of the cIAP1-CASP8 complex also presents a β -sheet extension. This interaction does not interfere with CASP8-dimerization (**Figure 5.10e**), which raises the question of how cIAP1 inhibits CASP8 function. It seems that cIAP1 does not have to interfere with CASP8 dimerization in order to suppress apoptosis since other cIAPs, such as XIAP prevent homodimerization of CASP9 [218], but not CASP3 [219] and CASP7 [216]). Similarly, cIAP1 may not abolish CASP8-dimerization. Furthermore, XIAP competes

with substrates to bind to caspases [216]. This may hold for cIAP1 as well. **Figure 5.2** demonstrates that cIAP1 and BCL2 bind to overlapping sites on CASP8. Thus their interactions with CASP8 are mutually exclusive. If cIAP1 binds to CASP8, BCL2 (CASP8 substrate) cannot bind and gets cleaved. Another mechanism for CASP8 inhibition by cIAP1 may be its ubiquitination by cIAP1. cIAPs are E3 Ub-ligases and may add K48 ubiquitin chain to caspases and target them for degradation.

5.1.6. CASP8-cIAP2

CASP8-cIAP2 architecture that I obtained is similar to CASP8-cIAP1 interaction. It also allows CASP8 homodimerization (**Figure 5.11**) but not via a β -sheet extension. cIAP2 does not have resolved 3D structures with any caspase. Therefore, I cannot compare them with our model.

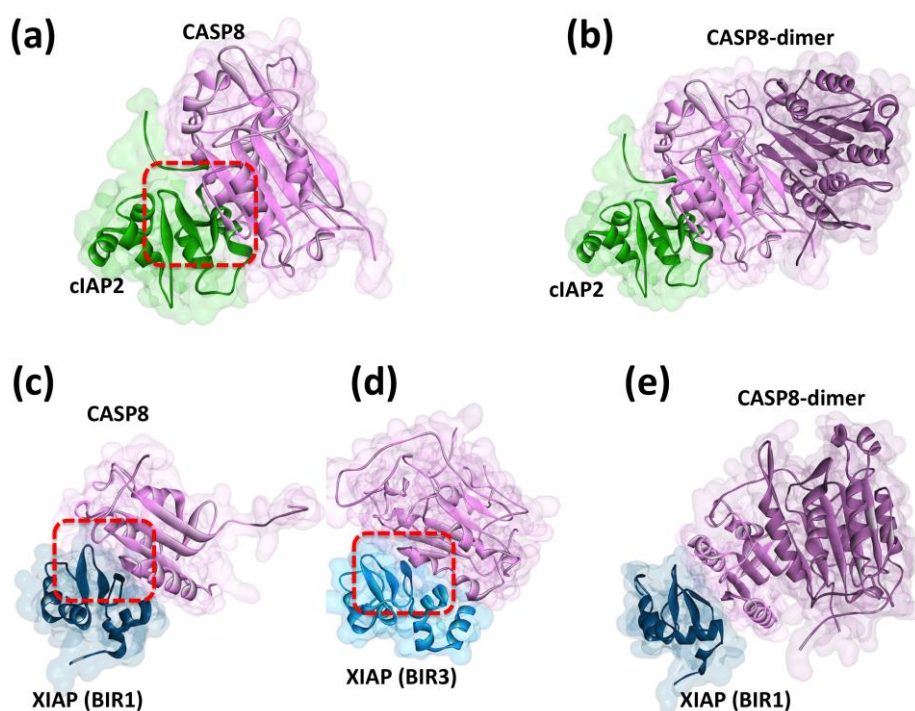


Figure 5.11: cIAP2 and XIAP interactions with CASP8. (a) cIAP2-CASP8 interaction complex has similar interface region (dashed purple square) to cIAP1-CASP8 shown in Figure 4d. (b) This interaction does not inhibit CASP8-dimerization. (c) Both BIR1 and (d) BIR3 of XIAP can bind to CASP8 according to PRISM results. Both of them have similar interface architectures. (e) XIAP-CASP8 interaction also does not hinder CASP8-dimer formation.

5.1.7. CASP8-XIAP

XIAP has three BIR domains: it uses BIR2 to interact with CASP3 and CASP7; and BIR3 to associate with CASP9. The structures of XIAP complexes with SMAC (1g3f.pdb –BIR3 of XIAP) [220], CASP3 (1i3o.pdb –BIR2 of XIAP) [219], CASP7 (1i4o [216], 1i51 [221], 1kmc –BIR2 of XIAP), and CASP9 (1nw9.pdb –BIR3 of XIAP) [218] are available. However, the ways XIAP binds to these caspases differ. This raises the question of whether these crystal structures reflect physiological forms/complexes.

I found possible interactions of CASP8 with both BIR1 and BIR3 domains of XIAP (**Figure 5.11c & d**). The architecture that I obtained by PRISM (**Figure 5.11c**) is very similar to CASP8-cIAP1 and CASP9-cIAP1 structures. The interface consists of β -sheet extension and does not interfere with CASP-dimerization (**Figure 5.11e**).

5.1.8. CASP8-p35

Baculoviruses have the p35 protein that inhibits caspases. The CASP8-p35 complex has a resolved structure in PDB (1i4eAB), as shown in **Figure 5.12**, but PRISM could not find this complex by using its own structure as a template. In the resolved structure, it has been shown that CASP8 is bound to p35 through a covalent thioester bond [222]. However, the structures that PRISM generates are linked through non-covalent bonds. This may be the reason why PRISM couldn't model the interaction.

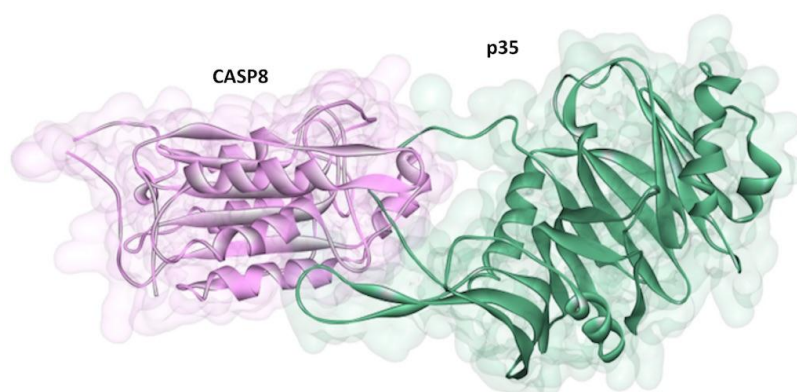


Figure 5.12: CASP8-p35 interaction complex has a crystal structure (1i4e_AB.pdb). I couldn't find this architecture by PRISM.

5.1.9. Effects of CASP8 mutations on its interactions

Structural details of protein-protein interactions provide insights into how proteins associate with each other and how inhibitors regulate function. They help to reveal the mechanisms of oncogenic mutations, sometimes through effects on the interactions. Generally, disease-causing mutations either disrupt the overall structure of the proteins by lowering their stability or destabilizing (or overstabilizing) their interactions [157]. Although core mutations are extremely important, interface mutations may have more drastic effects on the physico-chemical properties than non-interface mutations [156]. Oncogenic mutations are often enriched in interfaces [223].

I obtained CASP8 mutations that are clinically observed in cancer patients from cBioPortal [155] and mapped them on the structures. If the mutations fall on interface residues or their first neighbors, I created mutant CASP8 structures *in silico* and re-ran PRISM with the mutant structures. There are 20 nonsense and frameshift mutations that lead to the loss of the C14 peptidase domain of CASP8 (Table A.7). They abolish all its interactions, except FADD, which associates with DED of CASP8.

I observed that interface residues (and their first neighbors) of CASP8 in its interactions with cIAP1, cIAP2, XIAP, and BCL2 are not mutated frequently in cancers (Table A.8). There are only 4 mutations on these interfaces, 3 of which abrogate some of these interactions since their binding energy increase significantly. This result may be expected because CASP8 interactions with these proteins prevent apoptosis and thus contribute to resistance to cell death in cancer. It is logical that oncogenic mutations do not abolish these interactions. The interface residues of CASP8 in cFLIP interaction and CASP8 homodimerization are frequently mutated, but do not abolish these interactions (Table A.8). cFLIP interactions with mutant CASP8 still has interaction energy below -100 energy units. Since the interfaces are extended in the CASP8-cFLIP and CASP8-CASP8, that is, there are more contacting residues, a single point mutation may not be enough to destabilize the interaction. The CASP8-FADD interface has eight mutations and five of them prevent the interaction, which again contributes to resistance to cell death since without CASP8-FADD interaction, CASP8 cannot be recruited to DISC and initiate apoptosis. The number of

hydrogen-bonds and salt bridges may be altered due to disease-causing mutations [166]. However, most of the mutations that I analyzed by PDBePISA server [224] did not change the number of H-bonds and salt bridges. Only the P394Q and P394L mutations of CASP8 increased the number of H-bonds from 16 to 18 in CASP8-homodimer and E396G mutation decreased it to 14. The alterations in intermolecular bonds either weaken or enhance the interactions.

In conclusion, the architectures of CASP8 interactions shed light on how CASP8 gets activated upon recruitment to DISC, how it is regulated by other proteins, and how its clinically observed oncogenic mutations affect the interactions.

5.2. TRAF3 Interactions

TRAF family of proteins has important roles in the pathways of immune system. TRAF6 and TRAF3 are E3 ubiquitin ligases and take part in TLR signaling [66]. However, they lead to opposing outcomes in this pathway. While TRAF6 enables signaling through the conventional downstream path, which activates NF- κ B and results in inflammation, TRAF3 signals through IRFs to produce IFNs and IL-10 (an anti-inflammatory cytokine). As shown in **Figure 3.13**, TRAF6- and TRAF3-dependent parallel pathways of TLRs are competitive. Besides TLR pathway, TRAF3 is also important in TNFR signaling.

I modeled all binary interactions of TRAF3, which execute its function by forming homo- or hetero-trimers [66, 153]. Therefore, I considered only the interaction models that are compatible with its trimer structures. **Figure 5.13** displays the binary interactions of trimeric TRAF3 protein. The straight lines that converge to the same site on TRAF3 bind to same/overlapping interfaces on TRAF3 and have steric clashes. Therefore, they cannot co-exist. For example, I found that NIK (NF- κ B inducing kinase) and some surface receptors such as LTBR, BAFR, and CD40 (TNFR family members) bind to overlapping interfaces on TRAF3. Normally in unstimulated cells (when receptor is not bound to its ligand), NIK is bound to TRAF3 and ubiquitylated by cIAPs (cellular inhibitor of apoptosis). This Ub chain attached to NIK constitutively targets NIK for degradation in order to keep its abundance below a

certain threshold for its function (**Figure 5.14a**) [225]. On the other hand, if receptor is ligated with its ligand, NIK is not degraded: it accumulates. Accumulation of NIK leads to activation of alternative NF- κ B pathway [225]. Our structural model reveals how/why NIK is not degraded and accumulates since cytoplasmic part of the receptor interacts with TRAF3 and prevents TRAF3-NIK interaction (**Figure 5.14b**). Since receptor is already bound to TRAF3, NIK cannot associate with TRAF3 (because they bind to overlapping interfaces on TRAF3). If NIK cannot bind to TRAF3, it cannot be ubiquitinated by cIAPs and targeted for degradation. Structural details of interactions once again shed light on how/why distinct downstream outcomes are observed under different conditions - stimulated or unstimulated cell in our case here.

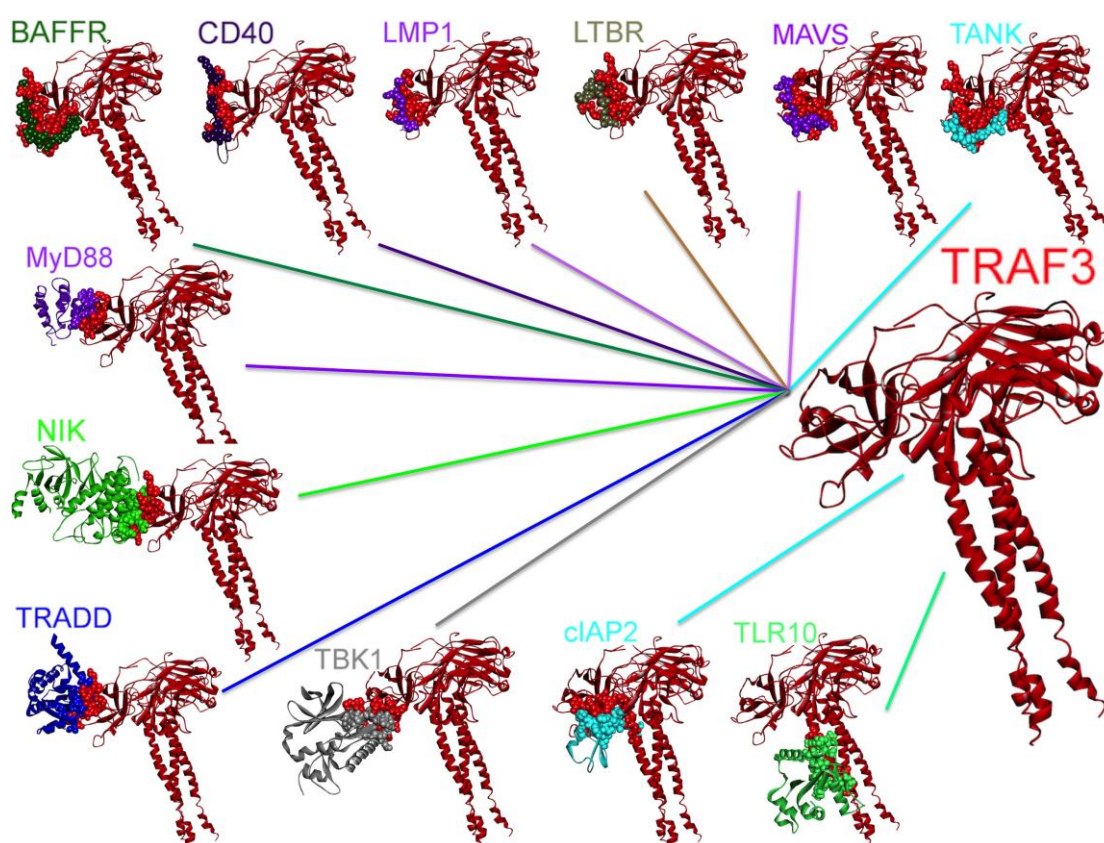


Figure 5.13: TRAF3 interactions with its partners. For simplicity, only one monomer's interactions in TRAF3 homo-trimer are shown. Spheres show the interface residues. The straight lines mark the locations of the interfaces of the respective interactions on TRAF3. If the straight lines converge on the same site on TRAF3, this means that those proteins bind to same/overlapping interfaces on TRAF3, thus those interactions are mutually exclusive.

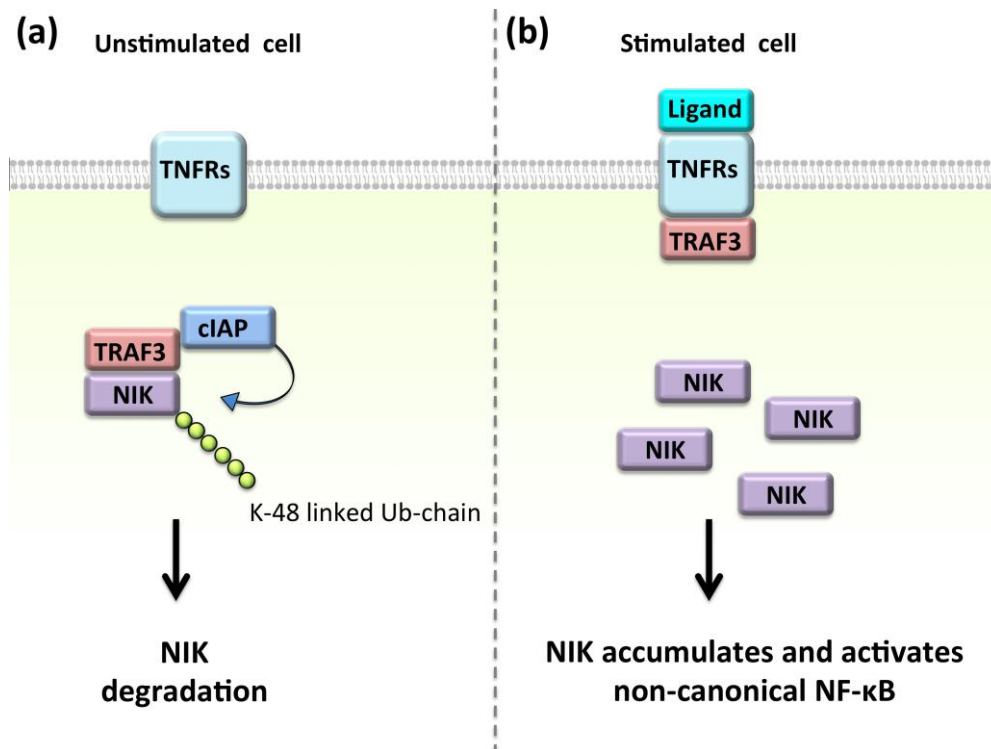


Figure 5.14: Our structural models reveal why NIK is degraded in unstimulated cells but not in stimulated cells. (a) In the unstimulated cell, NIK is bound to TRAF3 gets ubiquitinated by cIAPs, and gets degraded. **(b)** When TNFRs recognize their ligands they recruit TRAF3 to their cytoplasmic domain. Since TNFRs and NIK bind to the same site on TRAF3, TNFR-TRAF3 interaction prevents NIK-TRAF3 interaction and NIK ubiquitination. NIK accumulates and activates non-canonical NF-κB.

Without structural information, what I knew was that TRAF3 inhibits canonical NF-κB through TRAF6 in TLR pathway and non-canonical NF-κB through NIK in TNFR pathway. However atomic details of TRAF3 interactions corrected this information: TRAF3 does not inhibit TRAF6, instead, it competes with TRAF6 to bind to MyD88 (**Figure 5.15**). That's why TRAF3 restricts TRAF6-dependent NF-κB activation. Similarly, TRAF3 does not block NIK: TNFRs and NIK compete to bind to TRAF3 (**Figure 5.15**).

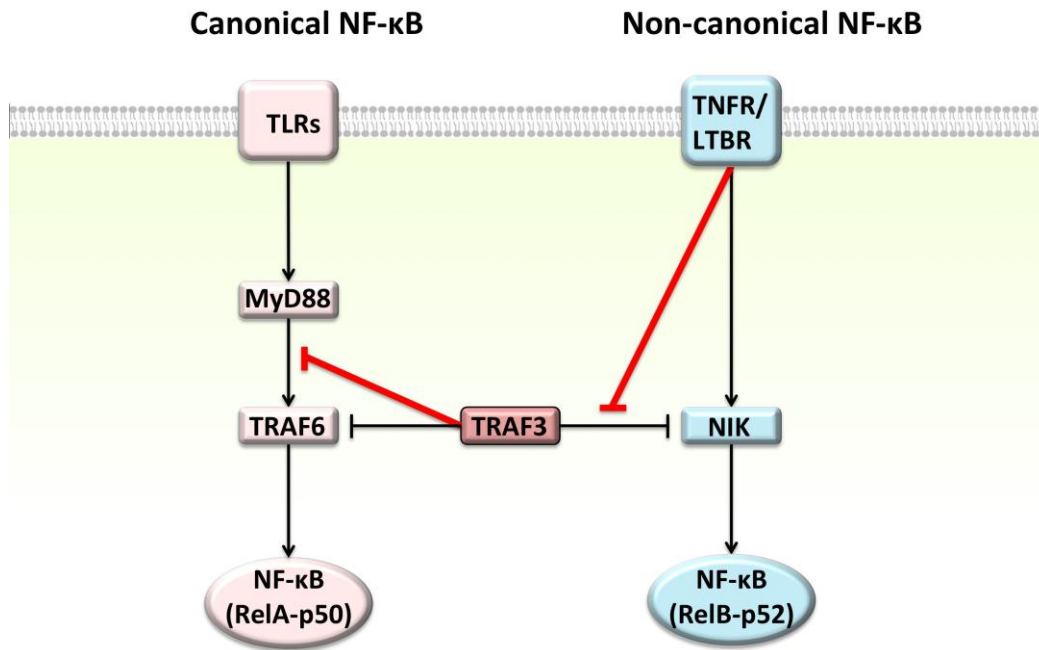


Figure 5.15: Structural details of TRAF3 interactions revealed how TRAF3 inhibits both canonical and non-canonical NF-κB pathways. Black lines show what was known before and red lines show what is revealed by structural details of our models. In TLR signaling, TRAF3 does not inhibit TRAF6 itself, but MyD88-TRAF6 interaction. In TNFR signaling, TRAF3 does not block NIK, but TRAF3-TNFR interaction prevents TRAF3-NIK. (Courtesy Drs. C. VanWaes and Z. Chen)

5.3. RIPK1 Interactions

Another important scaffolding protein in TLR pathway is RIPK1 (receptor (TNFRSF)-interacting serine-threonine kinase 1, also known as RIP1), which is a mediator of cellular responses to inflammation and stress [213] and programmed necrosis, so called necroptosis. **Figure 5.16** shows the binary interactions of RIPK1. I found that RIPK1 acts as a bridge between CASP8 and FADD (**Figure 5.17**). This trimeric complex is named as “Ripoptosome”, which is a death-inducing complex assembled independent of death receptors and their ligands and stimulate either apoptosis or necrosis [226]. XIAP was found to prevent spontaneous assembly of Ripoptosome complex [226-228]. In our model (**Figure 5.16**), XIAP and FADD bind to same site on

RIPK1. So, if XIAP occupies the binding site on RIPK1, it blocks the formation of Ripoptosome core complex (CASP8-RIPK1-FADD). Inhibition of cIAPs by SMAC mimetics results in RIPK1-dependent cell death [213].

Moreover, CASP8 and EGFR bind to same site on RIPK1, preventing ripoptosome assembly (**Figure 5.16**). CASP8 and EGFR have competing functions. A recent study suggests that EGF treatment to Glioblastoma cells that express both wild type EGFR and oncogenic mutant EGFRvIII results in association of RIPK1 with FADD [213]. Normally, when EGFR recognizes its ligand EGF, it forms dimers. Maybe formation of EGFR dimers prevents EGFR-RIPK1 interaction; promoting ripoptosome formation and cell death.

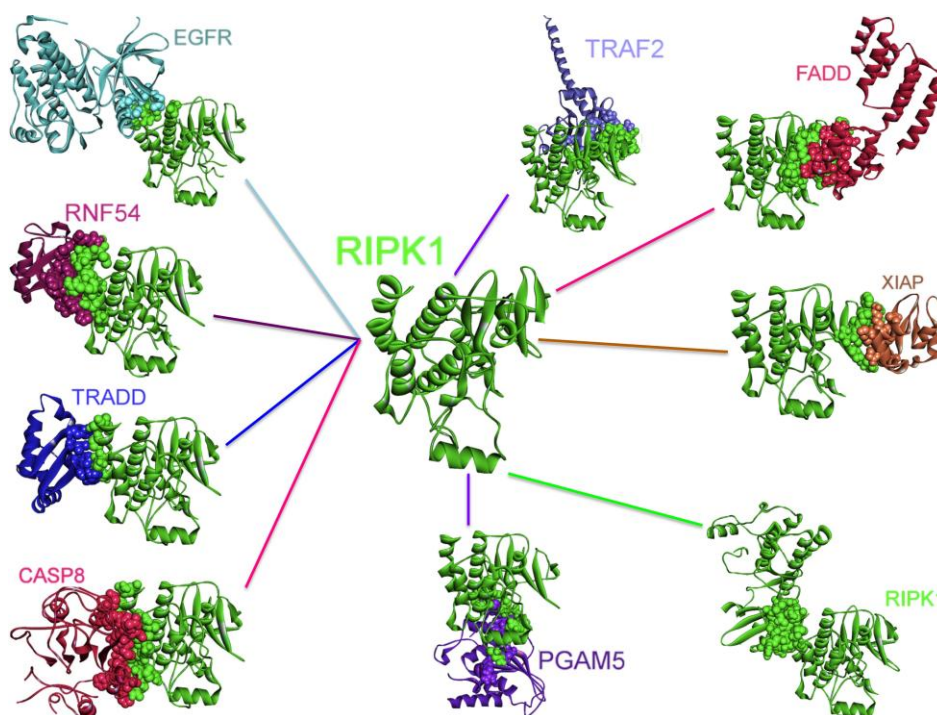


Figure 5.16: RIPK1 interactions with its partners. Spheres show the interface residues. The straight lines mark the locations of the interfaces of the respective interactions on RIPK1. If the straight lines converge on the same site on RIPK1, this means that those proteins bind to same/overlapping interfaces on RIPK1, thus those interactions are mutually exclusive.

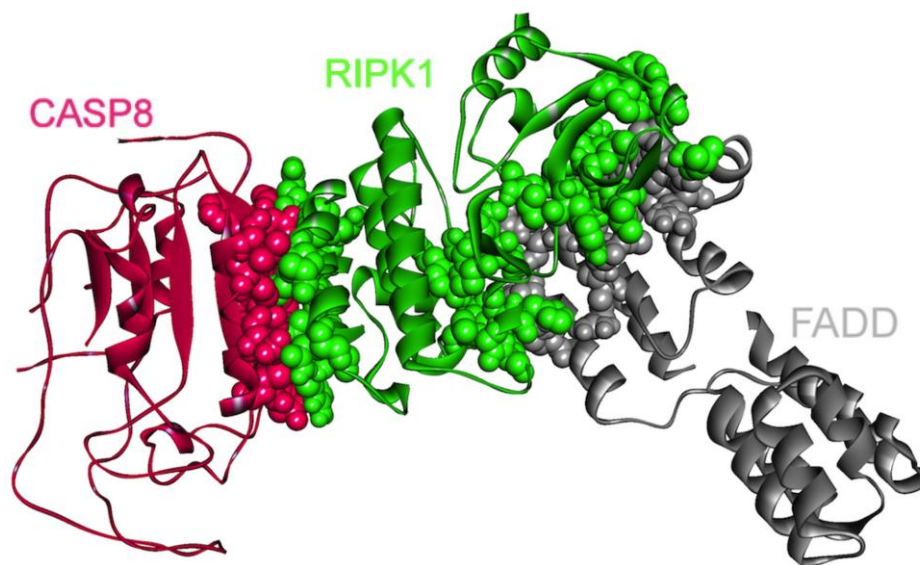


Figure 5.17: Core components of Ripoptosome complex. RIPK1 acts as a bridge between CASP8 and FADD. Spheres show the interface residues. PDB_ID of CASP8 is 2k7z:A, RIPK1 is 4ith:A, and FADD is 2gf5A.

In conclusion, the findings in this chapter illuminate how CASP8 gets activated when it is recruited to DISC complex; how its mutations help to resist cell death in cancer; the inhibition mechanisms of TRAF3 in classical and non-classical NF- κ B pathways; the crosstalk of TLR pathway with other signaling cascades, such as TNFRs.

Chapter 6

CYTOKINES AND THEIR ROLES IN CANCER

Cytokines and lymphoreticular infiltrates comprise the majority of the tumor microenvironment and mediate the dialog between tumor and normal tissues. They are one of the most significant components in cancer-inflammation crosstalk. Our aim in this chapter is to provide an overview of cytokines in cancer from a structural standpoint. Below, I continue with a broad description of cytokines and their roles in cancer development. I follow by providing structural case studies of major cytokines, showing the usefulness of structures in characterizing cytokine function and regulation, and understanding mechanisms of mutations and inhibitors/antagonists in cytokine signaling.

Cytokines execute their function by binding to their receptors on target cells and activating downstream pathways. Pathways merge and diverge at several points with more than one parallel downstream path, as in the case of TNF- α , IL-1 β and IL-6 in **Figure 2.6**. A cytokine can also bind to different receptors with varying affinities. Differences in receptors' concentration, orthosteric or allosteric mutations that alter the affinity of the cytokine to the receptors, all can lead to distinct consequences. Structural details can capture differences in affinity and efficacy. Further, not only alterations in downstream pathways, but also variations in cytokine concentrations due to upstream changes can impact cellular outcomes. Activation of oncogenes, such as Ras, results in inflammatory cytokine expression [109, 111]. Constitutive activation of NF- κ B, the major pathway that regulates immune inflammatory responses [107] and produces pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 [104, 108], leads to chronic inflammation, which promotes cancer [47, 58, 61]. Inhibition of NF- κ B in tumor cells switches inflammation-induced tumor growth to regression and has been suggested as an effective therapy [107]. LPS of gram-negative bacteria activates the TLR pathway, which stimulates the NF- κ B family of transcription factors [47]. LPS causes metastatic tumor growth and NF- κ B is responsible for this action [107]. Thus, upstream pathways control the cytokine concentrations in cells and have vital roles in cancer development. Below, I provide cytokine case studies from a structural standpoint to understand their roles in cancer.

In the cytokine case studies described below, I used PRISM algorithm to model missing structures of protein-protein complexes, such as TNF- α -TNFR1 and IL10-IL10RB complexes. Computational tools help enrich the available structural data for protein interactions and analyze the effects of mutations on the interactions and pathways.

6.1. Case Studies

6.1.1. IL-1 β

IL-1 β can activate its downstream target cells by forming a signaling complex with two membrane-bound receptors: IL-1 receptor type I (IL1R1) and IL-1 receptor accessory protein (IL-1RAP/IL1RACp). Due to its crucial mediating inflammatory response, IL-1 β signaling is strictly regulated through a ‘decoy’ receptor IL-1RII (IL-1 receptor type II, IL1R2) and a receptor antagonist IL-1RA [229]. Although IL1R2 does not have an intracellular TIR domain, which is necessary for signaling [230], the extracellular regions of IL1R1 and IL1R2 are homologous, permitting efficient binding of the IL-1 β ligand to both receptors [229] essentially through the same residues (**Figure 6.1**). IL-1 β signaling can be blocked by the decoy receptor IL1R2 either by preventing the interaction of IL-1 β with IL1R1 through competitive binding [231, 232] or by decreasing the concentration of IL1RAP, which is an essential member of the signaling complex, by forming a non-signaling complex with IL-1 β and IL1R2 [233, 234] (**Figure 6.1**).

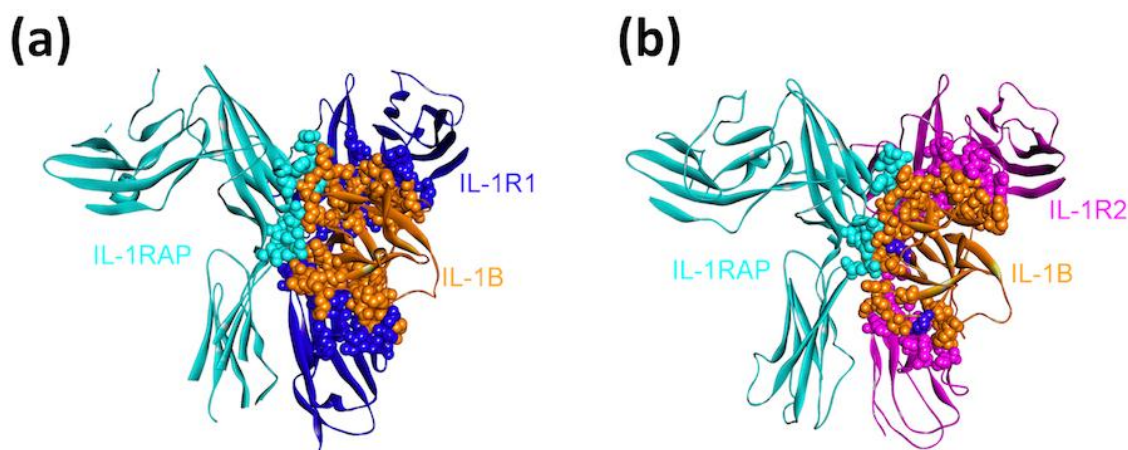


Figure 6.1: Structure of IL-1 β , IL-1R1, IL-1R2, and IL-1RAP complex. (a) IL-1 β & IL-1R1 complex (PDB Code_Chains: 4dep_DEF). (b) IL-1 β & IL-1R2 complex (PDB Code_Chains: 3o4o_ABC). Atoms of interface residues are represented with balls. There are many common residues on IL-1 β that binds to IL-1R1 and IL-1R2. Purple residues

(Gln48 and Glu111) are computational hot spots and are specific to IL-1R2 and IL1RAP binding, respectively [103].

IL1R2, the decoy receptor, is upregulated in pancreatic and ovarian cancer [235, 236]. The immune system induces apoptosis; however, this decoy receptor can protect pancreatic cancers [237] by blocking IL-1 signaling. Targeting IL1R2 is considered effective for inhibiting tumor angiogenesis [236] as IL-1 is essential in tumor angiogenesis and invasiveness [119]. While inhibiting the IL-1 β and IL1R2 interaction is a therapeutic aim, it is challenging since the extracellular domains of IL1R1 and IL1R2 are homologous and IL-1 β uses essentially the same residues for binding. When the structures of the signaling and non-signaling complexes of IL-1 β (PDB Codes: 4dep and 3o4o, respectively) are compared, a few -though crucial- differences can be observed. Computational hot spot residues on IL-1 β specific to IL1R2 and IL1RAP binding include Gln48 and Glu111, respectively, which may be specifically targeted by drugs with the aim of inhibiting these interactions (**Figure 6.1b**). Other residues specific to IL-1 β – IL1R2 binding consist of Ser13, Ser21, Tyr24, Lys27, Asp35 and Asn129; whereas Lys109 is specific for IL-1 β – IL1RAP binding.

6.1.2. TNF- α

TNF- α , a pro-inflammatory cytokine, plays a fundamental role in inflammation and host defense [238-240]. TNF- α is found both as soluble and membrane-bound forms. Its overexpression is seen in several chronic inflammatory diseases such as Crohn's disease and ulcerative colitis and also in cancer [104, 239, 241]. Therefore, there are many studies on treatment of such diseases with anti-TNF therapies, including anti-TNF antibodies, soluble TNF receptors (TNFRs), and small molecule inhibitors [240-242].

TNF- α signals through two receptors: TNFR1 (also known as p55 or TNFRSF1A) and TNFR2 (also known as p75 or TNFRSF1B), which stimulate two distinct intracellular pathways as can be seen in **Figure 2.6**. TNFR1 is ubiquitously expressed in almost all cells, but TNFR2 expression is restricted to certain cell types, such as T-lymphocytes, endothelial cells, oligodendrocytes and some neuron subtypes [239]. The extracellular parts of these two receptors are similar but their cytoplasmic parts are different. TNFR1

possesses a death domain that allows interaction with other death domain containing proteins like TRADD (TNFR1-associated death domain) and FADD, whereas TNFR2 does not [239]. This variance in their cytoplasmic parts leads to interaction with different proteins and distinct interaction partners will result in diverse cellular outcomes.

TNF- α mostly signals through TNFR1, possibly because it is ubiquitously expressed. TNFR1 activation induces apoptosis via TRADD and FADD and inflammation. On the other hand, TNFR2 recruits TRAF2 and TRAF1 and activates NF- κ B and expression of pro-survival genes [239]. Since TNFR2 does not have a death domain, it cannot interact with death domain containing adaptors. TNFR1 and TNFR2 downstream pathways also crosstalk (**Figure 2.6**) and they have certain overlapping functions.

The 3D structure of TNF- α -TNFR2 complex is available in the PDB (PDB Code_Chains: 1alq_AR) [243], but the TNF- α -TNFR1 structure has not yet been solved. I modeled this interaction by PRISM [36], using the template interface 1tnrAR, which is the interface between TNF- β -TNFR1 [244]. As shown in **Figure 6.2**, interfaces of the interactions of TNF- α with its receptors almost fully overlap. Therefore, these two interactions are competitive; either TRADD is recruited to the receptor, or TRAF2.

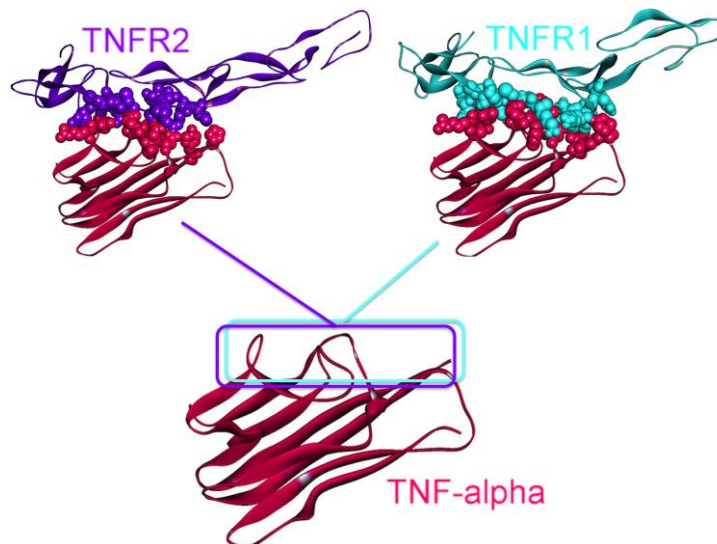


Figure 6.2: TNFR1 and TNFR2 bind to the same site on TNF- α , thus they are mutually exclusive. Structure of TNF- α and TNFR2 complex is available in PDB (PDB Code_Chains: 1alq_AR), but I modeled TNF- α and TNFR1 interaction by PRISM. Atoms of interface residues are represented with balls. Solid lines and rectangles indicate the

position of interfaces on TNF- α . Since the rectangles overlap almost completely, these two interactions cannot co-exist [103].

Some TNF- α mutants/variants (PDB Codes: 2e7a and 2zpx), in which six receptor-binding residues are substituted, specifically bind to TNFR1, but not to TNFR2 and inhibit its signal transduction [238, 240]. I note that even though the authors did not observe interactions between these mutant TNF- α 's and TNFR2, PRISM suggested this interaction, with even more favorable energy than the interaction with TNFR1. This difference may stem from the fact that crystal structures typically do not reflect the physiological conditions. This may explain why PRISM still finds this interaction with the mutant while the interaction did not take place *in vitro*. In addition, some antagonist proteins 2L (poxviral protein) [245] and CTLD (C-type lectin-like domain) [241] (PDB Codes: 3it8 and 3l9j, respectively), also bind to TNF- α at the interface between TNF- α and the receptors, thus interfering with its receptor-binding and signaling (see **Figure 6.3** and **6.4**). Thus, structural details of interactions can provide insight into the mechanisms of mutant proteins or antagonists blocking or altering downstream pathways. Also, these structures of the complexes with the antagonists can assist pharmaceutical efforts to develop anti-TNF therapeutics for treatment of autoimmune and chronic inflammatory diseases, such as Crohn's disease.

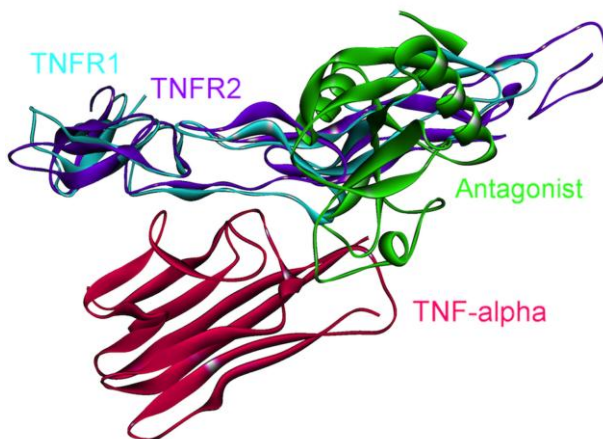


Figure 6.3: Receptors and antagonist bind to same site on TNF- α . Therefore, if antagonist occupies the interface on TNF- α , TNFRs can no longer bind there and thus TNF signaling is blocked. Complex structure of TNF- α and antagonist is available in PDB (PDB Code_Chains: 3l9j_CT) [103].

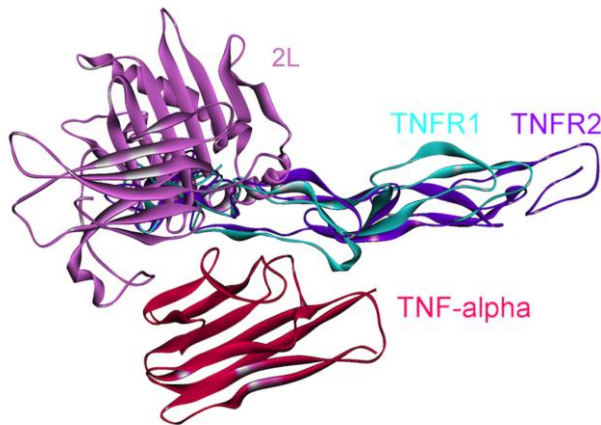


Figure 6.4: Receptors and poxvirus 2L protein bind to same site on TNF- α . Therefore 2L protein inhibits association of TNF- α with its receptors, and thus its signaling. Complex structure of TNF- α and 2L is available in PDB (PDB Code_Chains: 3it8_AD) [103].

Function and signaling of TNF- α does not depend on only its interactions with the receptors; its higher order oligomerization modes also play essential roles in TNF- α signaling. TNF- α performs its functions by forming homo-trimers with a 3-fold symmetry (PDB Code_Chains: 1alq_ABC), each TNF- α monomer interacting with a TNFR1 or TNFR2 (see **Figure 6.5**, parts A and B) [243]. I obtained trimeric TNF- α -TNFR1 by superimposition of monomeric interaction predicted by PRISM [36] and the available structure of the trimeric TNF- α -TNFR2 complex (PDB Code_Chains: 1alq_ABC). Moreover, panel C in **Figure 6.5** displays a small molecule inhibitor binding to TNF- α and preventing its homo-trimerization, although this inhibitor still allows dimerization (PDB Code_Chains: 2az5_ABA). This molecule not only blocks TNF- α trimerization; it also blocks its signaling [242]. Thus, higher order oligomerization of TNF- α may be as important as its interactions with receptors in TNF- α signaling. Structural pathways can capture the oligomerization modes of proteins and their altered interactions due to a mutation, inhibitor or antagonist binding, which is not the case in pathway diagrams.

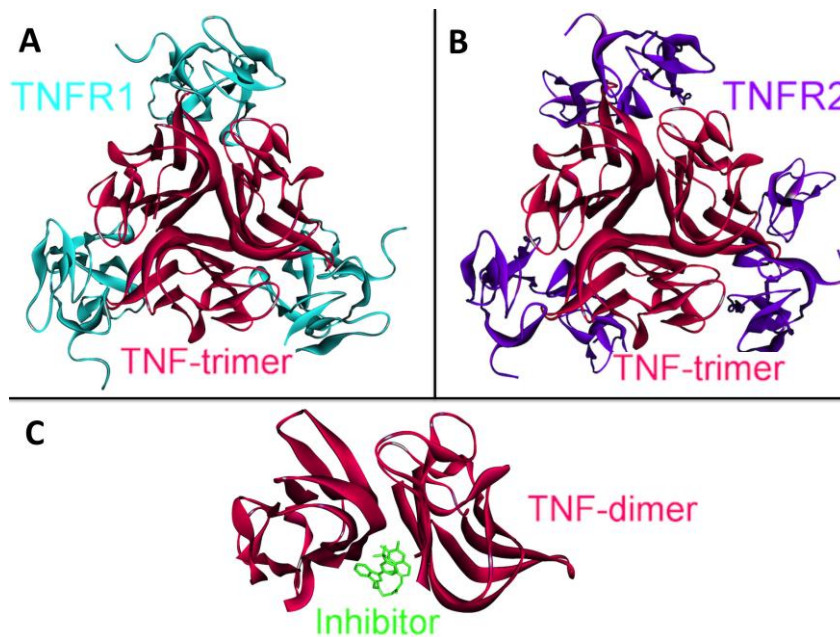


Figure 6.5: Structure of TNF- α homotrimer and homodimer. TNF- α should form homo-trimers in order to execute its function. TNF- α homotrimer has 3-fold symmetry (PDB Code_Chains: 1alq_ABC). (A) Trimeric TNF- α and TNFR2 complex is available in PDB (PDB Code_Chains: 1alq_ABCRST). (B) I obtained trimeric TNF- α and TNFR1 complex by superimposition of monomeric interaction predicted by PRISM with the available trimeric structure (PDB Code_Chains: 1alq_ABC). (C) A small molecule inhibitor binds to TNF- α and prevents homo-trimerization, although it allows dimerization (PDB Code_Chains: 2az5_ABA) [103].

6.1.3. IL-6

Intracellular IL-6 signaling initiates through the association of IL-6 – IL-6R (IL6RA, CD126) complex with IL6RB (gp130, CD130) protein (**Figure 6.6**) and induction of IL6RB dimerization [246] resulting in a hexameric complex capable of signaling [247]. A soluble form of IL-6R (sIL-6R) was also found in body fluids [248, 249] and its affinity to bind to IL-6 (similar to membrane bound IL-6R) leads to the IL-6 trans-signaling process so that the IL-6 – sIL-6R complex can stimulate IL6RB expressing cells, although they lack membrane bound IL-6R [250-252]. Experiments showed that IL-6 signaling can act both as pro- and anti-inflammatory/regenerative depending on whether IL-6 trans-signaling or classical signaling takes place, respectively [253-257].

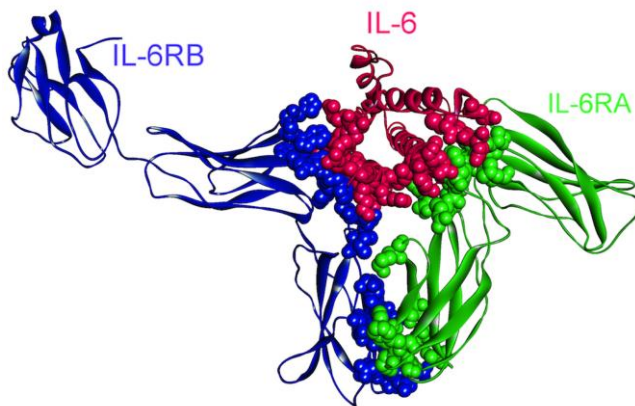


Figure 6.6: Structure of IL-6, IL-6RA, and IL-6RB complex (PDB Code_Chains: 1p9m_ABC). Atoms of interface residues are represented with balls [103].

Initiated IL-6 signaling can activate JAK/STAT [246], Ras-Raf [258] and PI3K/AKT signaling pathways (Figure 2.6) [259, 260]. IL-6 has anti-apoptotic and pro-tumorigenic activities regulated by these different pathways [261]. Due to being one of the most ubiquitously deregulated cytokines in cancer, IL-6 is shown to modulate growth and differentiation in several malignant tumor cells [262, 263] and its overexpression with its receptors (IL-6R and sIL-6R) has been found in a wide range of cancers [261]. IL-6 – JAK – STAT signaling path has been found to be important for tumorigenesis in various tumor models such as ovarian, lung, bladder, breast and colon cancers [261].

Targeting IL-6 signaling pathway in cancer therapy is supported by a numerous preclinical and translational observations. The pathological role of IL-6 in cancer has also been validated with successful treatment of certain diseases with drugs inhibiting IL-6 signaling [261]. Currently, several monoclonal antibodies (mAbs) and mAb mixtures against IL-6 and IL-6R (in preclinical models) are being developed with encouraging results in cancer cell lines and animal models [264-267]. Additionally, small molecule compounds for the inhibition of IL-6 or downstream proteins are also developed and being evaluated in preclinical and clinical cancer models [259].

There are 14 oncogenic mutations (from the COSMIC database [112, 268]) that correspond to residues on the binding interfaces of IL-6 – IL-6R – IL6RB hexameric complex (Figure

6.6) and these mutations are related mostly to colon cancer (7 mutations), and also endometrial (2), lung (1), liver (2), breast (1) and stomach (1) cancers (see **Table A.9**). Based on the fact that IL-6 and its receptors are overexpressed in various cancer types, these mutations are speculated not to be loss-of-function mutations, with the complex being turned on constitutively.

6.1.4. TGF- β

The anti-inflammatory cytokine TGF- β superfamily regulates key processes such as development, and its deregulation is involved in the pathogenesis of various diseases including cancer [269]. Being a growth inhibitor, TGF- β functions in normal tissue homeostasis. It has three isoforms (TGF- β 1, TGF- β 2 and TGF- β 3) [270]. Active ligands TGF- β 1 and 3 bind to the transmembrane TGF- β type II receptor (T β RII, TGFBR2) with high affinity and selectivity, subsequently activating and recruiting the TGF- β type I receptor (T β RI, TGFBR1, ALK5) into the complex [271]. Smad signaling, the main downstream signaling pathway is then initiated via the phosphorylation of receptor-associated Smads (Smad 2 and 3) by the activated T β RI. The activated Smad 2/3 and Smad 4 then form a stable complex (**Figure 2.6**), which translocates into the nucleus [271].

Under pathological conditions such as cancer progression, intracellular signaling networks of tumor cells are disrupted by tumor suppressor loss and oncogenic mutations through inhibition of the TGF- β homeostatic function [271]. TGF- β signaling has been shown to have two opposing roles in cancer: tumor suppression and tumor promotion. The tumor suppressing effect of TGF- β signaling is observed in the early stages of tumorigenesis, whereas tumor progressing effects are emphasized once the tumor is developed, when carcinoma cells gain oncogenic mutations in order to resist the growth inhibition mediated by TGF- β ; under these circumstances TGF- β expression increases in the tumors [271, 272]. TGFBR1, TGFBR2, SMAD4 and SMAD2 are the most commonly mutated genes in TGF- β signaling pathway particularly in colon, pancreas and gastric cancers [272]. The mutations are loss of function, implying that the disruption of TGF- β signaling has roles in those types of cancers. On the other hand, TGF- β pathway mutations are not common in breast and skin cancers [272].

Considering the dual role of TGF- β pathway in cancer, an ideal therapy would simultaneously suppress TGF- β 's tumor-promoting activity and reactivate the tumor-suppressing function of the pathway [271]. There are many TGF- β signaling antagonists under development because blocking TGF- β and its pathway offers therapeutic opportunities. Currently, there are pre-clinical and clinical data related to four major classes of these antagonists: receptor kinase inhibitors, TGF- β antibodies (ligand traps), peptide aptamers and antisense oligonucleotides [271].

The 3D structures of TGF- β 1-TGFBR1-TGFBR2 and TGF- β 3-TGFBR1-TGFBR2 complexes are available in the PDB (PDB Code_Chains: 3kfd_BJF and 2pjy_ACB, respectively). When the structures of these complexes are aligned, it is observed that the ternary complexes are in good agreement, supporting the similarity of signaling mechanism through isoforms of TGF- β (**Figure 6.7**). There are many small molecule receptor kinase inhibitor structures in the PDB (PDB_Codes: 1py5, 1rw8, 1vjy, 2wot, 2wou, 2x7o, 3faa, 3gxl, 3hmm, 3kcf and 3tzm), targeting the protein kinase domain of TGFBR1 as it is involved in its interactions with the downstream proteins. Small molecule oral drugs targeting receptor kinases have been developed widely in the last few years [271].

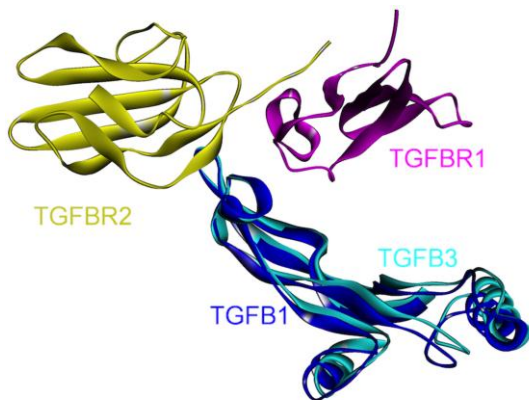


Figure 6.7: Superimposed structures of TGF β 1 and TGF β 3, in complex with TGFBR1 and TGFBR2 (PDB Code_Chains: 3kfd_BJF and 2pjy_ABC, respectively) [103].

6.1.5. IFN- γ

IFN- γ is a homodimeric cytokine, recognized by two separate surface-receptors, IFNGR1 (IFN- γ R α) and IFNGR2 (IFN- γ R β) (**Figure 2.6**) [273, 274]. Abnormal IFN- γ levels are associated with many autoimmune and auto-inflammatory diseases, liver and breast cancers [275]. In the signaling cascade, IFN- γ dimer first binds to two IFNGR1s and forms a symmetric intermediate complex (**Figure 6.8**), which subsequently recruits two chains of IFNGR2, generating an active signaling complex with a 1:2:2 stoichiometry [273]. Thus, a homodimer of IFN- γ binds to a heterotetramer of IFNGR1-IFNGR2s. Crystal structure of the complex between IFN- γ homodimer and two IFNGR1s is in the PDB (PDB_Codes: 1fg9), but the structure of the IFN- γ -IFNGR2 is currently unavailable.

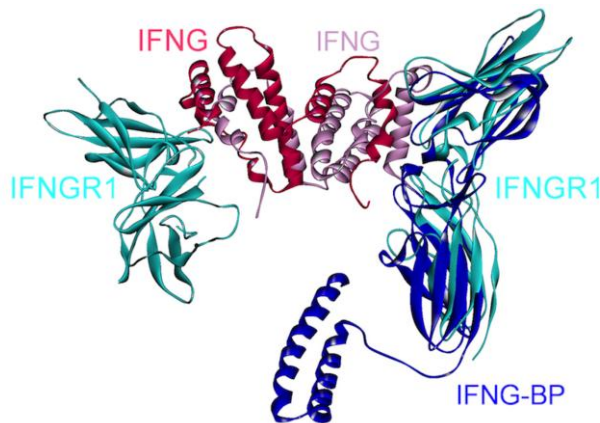


Figure 6.8: Superimposed structure of IFN- γ , IFNGR1 and viral protein IFN- γ BP. A homodimer of IFN- γ interacts with two IFNGR1s (PDB Code_Chains: 1fg9_ABCD). A soluble viral protein IFN- γ BP, which has a similar 3D structure with the extracellular part of IFNGR1, competes to bind to IFN- γ with IFNGR1 since they bind to overlapping interfaces on IFN- γ (superimposed structures of PDB Code_Chains: 1fg9_ABCD and 3bes_LR) [103].

IFN- γ is an anti-viral cytokine, but some viruses, such as Ectromelia virus (ECTV) evolved a mechanism to suppress IFN- γ signaling, preventing initiation of an anti-viral host response [275]. The genome of ECTV encodes an immuno-modulatory protein, IFN- γ BP (IFN- γ binding protein), which displays high structural similarity to the extracellular domain of IFNGR1. As can be seen in **Figure 6.8**, IFN- γ BP competes with IFNGR1 to

bind to IFN- γ , since they have overlapping interfaces on IFN- γ . This soluble viral protein mimics the receptor and disrupts its association with IFN- γ . Moreover, IFN- γ BP needs to form tetramers in order to antagonize the IFN- γ pathway [275]. The reason for this requirement is currently unknown. One possibility is that monomeric interactions in the tetramer provide an optimal conformation for IFN- γ association; another may relate to higher specificity. This example demonstrates another case in which higher order oligomerization plays critical roles in signaling. Viral strategies to neutralize cytokines that are catastrophic for viral replication or action have inspired scientists to imitate viral proteins and design antagonists, which are generally composed of an antibody fused with the extracellular portion of the surface-receptor [275]. Structural details of the interactions between cytokines and such viral products serve as guidelines to achieve the aim of antagonizing the cytokines. A selective peptide inhibitor against IFN- γ and IL-10 was recently developed, employing a similar method in which the inhibitor comprises a conserved amino acid sequence of cytokine receptors [276]. In addition, inhibitors of IFN- γ have been shown to be effective in inflammatory bowel disease treatment [275, 277], which is a chronic inflammatory disease and predisposes individuals to colorectal cancer [278].

6.1.6. IL-10

IL-10 is an important immunomodulatory cytokine that suppresses expression of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF- α , by inhibiting NF- κ B transcription factors [113]. IL-10 also blocks the development of regulatory T cells (T_{reg}S) and Myeloid-derived suppressor cells (MDSCs), which are inhibitors of anti-tumor immunity [102, 109, 279]. In the presence of IL-10, tumor-specific immunity can be activated, but suppression of IL-10 signaling contributes to tumor progression by inhibiting anti-tumor immunity and allowing excessive inflammation that promotes cancer. IL-10 has two receptors – IL-10RA and IL-10RB – both of which are required for IL-10 signaling [280, 281].

I constructed the IL-10 centered human structural protein-protein interaction network with first and second-degree protein neighbors. This network is composed of 49 proteins and 70 interactions between them (**Figure 6.9**). Among these 70 interactions, the structures of only two (IL-10 - IL-10RA and APOE - LRP1) are deposited in the PDB in a complex

form. By using PRISM, I predicted the structures of the PPI interfaces and 40 additional interactions were structurally modeled (**Figure 6.9**). As a result, the available structural data increased from 2 to 42.

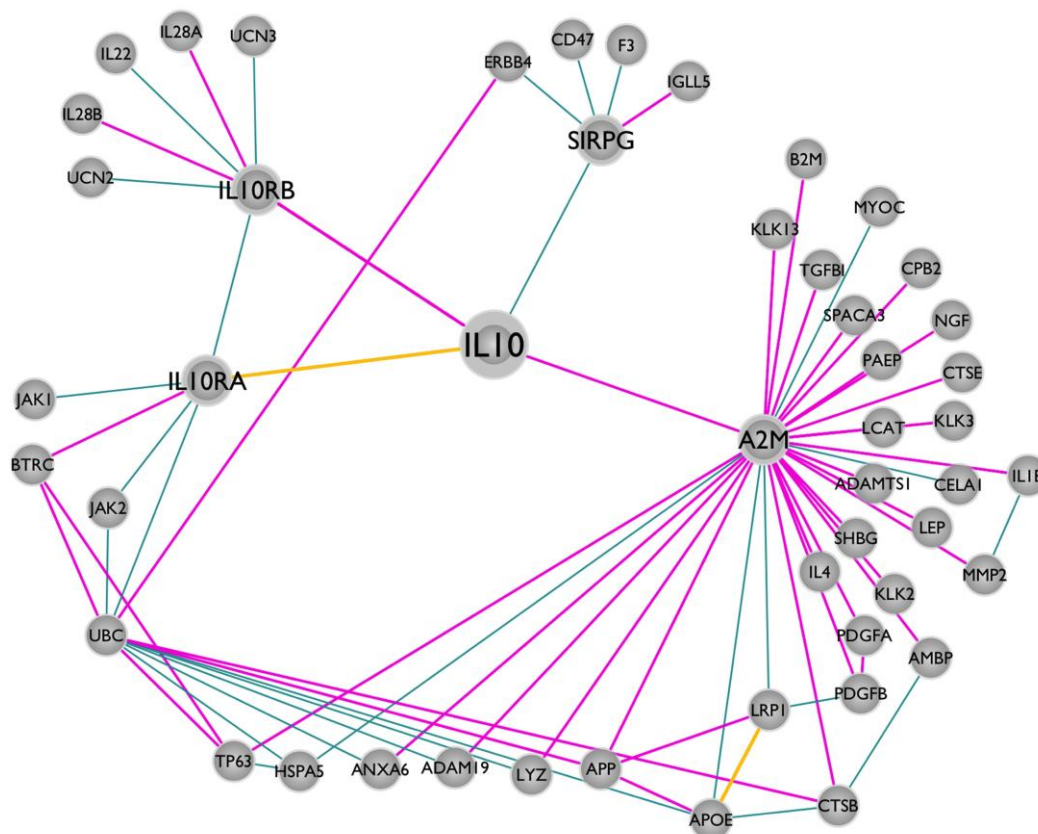


Figure 6.9: The PPI network of IL-10. There are 49 proteins and 70 interactions in this network and only 2 of the interactions have structural data in a complex form in the PDB (edges highlighted in yellow). I modeled the interfaces for 40 additional interactions. Thus there are 42 interactions with interface models (edges highlighted in pink). The remaining 28 edges (out of 70) could not be modeled and are shown in cyan [282].

The structure of the complex between IL-10 with IL-10RA is available in the PDB (PDB_ID: 1y6k_LR). However, the IL10-IL10RB interaction has not yet been solved. I modeled this interaction by PRISM. IL-10 needs to dimerize and bind to both of its receptors simultaneously in order to transduce the signal [283]. According to our model, IL-10 can interact with both receptors at the same time, without a steric clash (see **Figure**

6.10). This complex structure elucidates the mechanisms of some cancer mutations, such as E41* nonsense mutation on IL-10RB, which is a clinically observed mutation in adenocarcinoma with 2% frequency [155]. This mutation abolishes IL10-IL10RB interaction [284] and thus prevents IL-10 signaling. Prevention of IL-10 signaling allows excessive inflammation and promotes development of T_{regs} and MDSCs, which inhibit anti-tumor immunity and permit tumor growth [279].

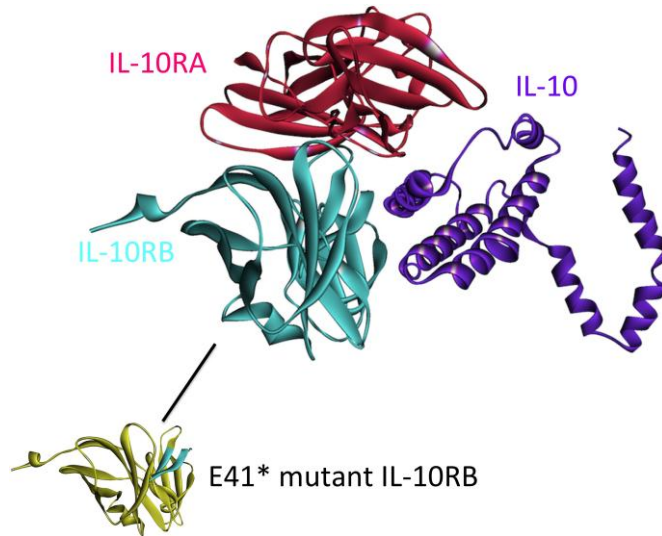


Figure 6.10: Structure of IL-10, IL-10RA and IL-10RB complex, obtained by superimposition of IL10-IL10RA and IL10-IL10RB complexes. Structure of IL10-IL10RB is available in PDB (PDB Code_Chains: 1y6k_LR) but interaction of IL-10 (PDB Code_Chain: 2ilk_A) with IL-10RB (PDB Code_Chain: 3lqm_A) was modeled by PRISM [284]. IL-10 needs to bind to both IL-10RA and IL-10RB simultaneously to propagate the signal downstream. Yellow region on IL-10RB below, would be gone in E41* mutant form of IL-10RB and this mutation abolishes the interaction of IL10-IL10RB since the majority of the interface would be missing [103].

6.1.6.1. Reconstruction of the Structural PPI Network of IL-10 Centered Signaling

I used the String server [285] for selecting the first and second-degree neighbors of IL-10. Only interactions with experimental evidence and confidence score larger than 0.4 (the default confidence value) were considered. There were 4 first-degree and 45 second-degree neighbor proteins of IL-10. Overall, I had 50 proteins comprising the IL-10 centered protein-protein interaction network. 39 of these 50 proteins have structural data in the PDB

(corresponding to 958 PDB chains) and I could build models for the remaining 10. IGHV3-6's sequence information could not be found, so this protein is not included in our analysis. I reduced the redundancy of similar interface architectures for each protein, using TM (template modeling)-align [286]. PDB structures having TM-scores larger than 0.5 and RMSD under 2.5Å were classified. Then, a representative PDB structure was assigned for each similar structure group and I ended up with 127 representative structures for 39 target proteins. The final IL-10 centered network is composed of 49 proteins and 70 interactions.

In conclusion, cytokines play important roles in cancer initiation, progression, angiogenesis, metastasis and immunotherapy. Cytokines are major players in cancer-related inflammation. Cytokines and lymphoreticular infiltrates comprise the majority of the tumor microenvironment and mediate a dialog between tumor and normal tissues. To survive, evade cancer-specific immunity and tolerate chemotherapy, tumors change the relative cytokine concentrations in the microenvironment. Different cytokine cocktails trigger different responses: while some combinations contribute to tumor initiation or promotion, others are used to activate anti-tumor immunity in cancer immunotherapy. In this study, I focused on cytokines and their roles in cancer from a structural standpoint. Exploiting the crystal or modeled structures of PPIs, I illustrated that (1) some parallel pathways are mutually exclusive, like TNFR1- and TNFR2-dependent paths in TNF- α signaling, whereas others can co-exist, such as IL10 downstream paths, and that (2) oncogenic mutations that fall on the interface of protein complexes, such as E41* nonsense mutation on IL-10RB, may abolish the interaction and block the subsequent downstream signaling. I further showed that (3) oligomerization modes of proteins can affect function; and that (4) mimicking the natural antagonists of cytokines such as 2L in TNF- α signaling and viral protein IFN- γ BP in IFN- γ signaling, can be an efficient method to develop novel therapeutic inhibitors of cytokines. Collectively, all aspects of cytokine signaling including their upstream and downstream pathways and crosstalk with other pathways, their interactions with receptors, oligomerization modes, sequence variants, mutations, all have a role in malignant transformation. Atomic details of cytokine pathways and interactions can help to better understand the mechanisms of how and why cytokines induce carcinogenesis. I conclude by emphasizing that structures of interactions are essential for a complete in-depth picture of cancer.

Chapter 7

METHODS

This chapter presents the methods used in this study. I explain how I build the structures of PPIs, how I obtained the structures of monomeric proteins and how I assessed the effects of mutation on the interactions.

7.1. Modeling Protein-Protein Interactions and Construction of the Structural TLR Network

I obtained the structures of binary protein interaction complexes by PRISM (PRotein Interactions by Structural Matching) [36, 49]. PRISM is a knowledge-based algorithm. It employs prior interface (template) data of known crystal/NMR structures of protein complexes and predicts structures of novel complexes that the target proteins can potentially form. It searches for template interface motifs on the surface of the target proteins, taking into account geometrical complementarities and hotspot conservation. Its inputs are structures of unbound proteins and template interface dataset; and the outputs are structural models of the bound PPI complexes. The inputs are not limited to monomers, i.e. multimeric proteins can also serve as inputs. In order to deduce the similarity between target and the templates, structural alignment is performed. **Figure 7.1** shows the basic flow of PRISM. After structural alignment, there comes a filtering step, where a combination of structural and evolutionary thresholds i.e. RMSD and number of matching hotspots are applied. At the last step, output models are scored and the flexibly refined to relieve possible steric clashes by FiberDock [287]. FiberDock assigns a binding energy score by taking van der Waals interactions, partial electrostatics, hydrogen and disulfide bonds into account [287]. An energy score below -10 indicates a favorable interaction [18, 36, 288]. For a pair of target proteins, PRISM may generate more than one model. Therefore, it is possible to build numerous distinct oligomeric complexes. However, I think that the most stable complex is the one that is supported by the experiments. In order to determine which model is more stable and physiologically relevant, I crosscheck the interface residues of our models with available mutational/biochemical data in the literature. In addition, oligomerization modes of proteins are also taken into consideration.

For instance, TRAF3 and TRAF6 proteins perform their functions by forming homo- or hetero-trimers.

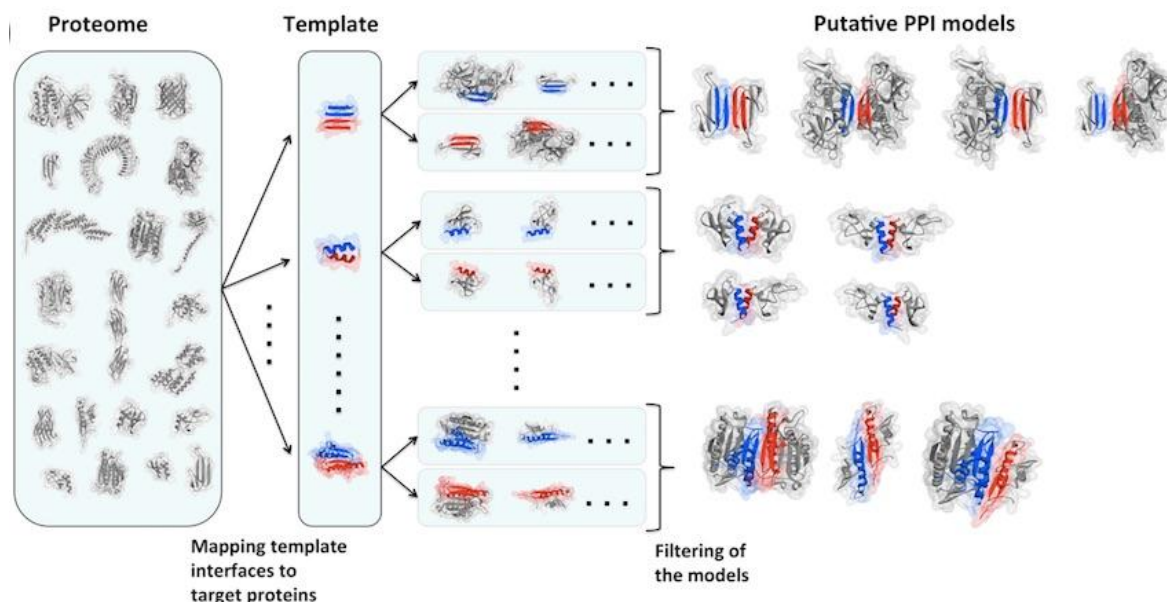


Figure 7.1: The rationale behind PRISM. Interface-based PPI prediction approaches. The interface architectures that are extracted from co-crystallized protein complexes are aligned to query protein structures: local alignment is performed, instead of global alignment. If both faces of the interface have a match, putative PPIs can be constructed. With this strategy, the whole interactome can be built with lower computational cost compared to template-free docking [289].

7.2. Modeling of proteins with unknown structures

PRISM requires 3D structures of target proteins in order to model the complex structures of the targets. If the structure of a given target protein is missing from the PDB, I model it by exploiting the I-TASSER server [139]. TIR domain structures of BCAP, SIGIRR, and ST2 have not been resolved yet. I built model of BCAP TIR domain (10-144 residues). The template structures used to build the BCAP TIR domain structure are 3h16A (Crystal structure of a bacteria TIR domain, PdTIR from *Paracoccus denitrificans*), 3ub2A (TIR domain of Mal/TIRAP), 4lqcA (The crystal structures of the Brucella protein TcpB and the TLR adaptor protein TIRAP show structural differences in microbial TIR mimicry), and 4c6sA (Crystal structure of the TIR domain from the *Arabidopsis thaliana* disease

resistance protein RRS1). Sequence identities, coverage, and normalized Z-scores between the query proteins and the templates are given in **Table A.10**.

The SIGIRR TIR domain structure was also missing in the PDB. I built its model (163-310 residues) and the template used is 1t3gA (TIR domain of human IL-1RAPL).

I also modeled the ST2 TIR domain (375-558 residues) and the templates are 1t3gA, 3j0aA (Homology model of human TLR5 fitted into an electron microscopy single particle reconstruction), and 1fyvA (TIR domain of human TLR1).

I built model of TLR4 TIR domain. The templates are 1fyvA, 3j0aA, and 1fyxA (TLR2 TIR domain).

Structures of two DED domains of CASP8 are not available in PDB. I modeled these tandem DEDs together (residues 2-177). 2f1sA, 2bbrA, and 3cl3A are used as a template structure. 2f1sA and 2bbrA are structures of tandem DEDs of MC159 (vFLIP) and 3cl3A is structure of ks-vFLIP (produced by the Kaposi's sarcoma herpes virus). The model has 0.85 RMSD over 159 residues with 2bbrA and 2f1sA; and 1.16 RMSD over 157 residues with 3cl3A.

To date, the DD of RIPK1 does not have a resolved structure. I built its model (residues 584-668) and the template structures used in this process are 4o6xA (DD of Ankyrin-3), 2of5H (DD of CRADD), 4d8oA (DD of Ankyrin-2), 3ezqB (DD of FADD). The model has 1.06 Å RMSD over 76 residues with the template structures.

7.3. Mapping clinically observed mutations onto the interfaces of negative regulators and *in silico* mutagenesis

I obtained clinically observed mutation data from the cBioPortal for Cancer Genomics (The Cancer Genome Atlas, TCGA) [[155](#)]. The residue numbers in the pdb files are corrected according to the FASTA sequences. I mapped the mutations on to the structures of negative regulators and performed *in silico* mutagenesis for the ones that fall onto the interface residues or the first neighbors of interface residues. *In silico* mutagenesis is performed by either FoldX plugin [[290](#)] of YASARA [[291](#)] followed by energy

minimization or the “MutateAminoAcid” function of the Discovery Studio molecular visualizer (Discovery Studio 3.5, Accelrys Inc., San Diego, CA, USA), I identified interface residues and hotspots by the HotRegion database, which uses conservation, accessible surface area and pair potentials [292]. I re-ran PRISM with the mutant protein structures to check whether the mutation abolishes the interaction. If interactions of the mutant structures have binding energy score higher than that of the wild type, then I conclude that this mutation is destabilizing. I calculated the number of hydrogen-bonds and salt-bridges in complexes with both wild type and mutant structures by utilizing the PDBePISA webserver [224].

Chapter 8

CONCLUSION

The main focus of this dissertation is the construction of structural networks of TLR and other cancer-related pathways to shed light on their roles in cancer-inflammation crosstalk. Although inflammation is required for proper host defense, excess inflammation contributes to cancer. TLR pathway is one of the key pathways that trigger inflammation. While overactivation of TLR pathway leads to constitutive inflammation, its malfunction makes individuals more susceptible to infections. So, which conditions makes TLR pathway to contribute to malignancy rather than being involved in immune defense mechanism? What goes wrong in TLR pathway, that transforms the host defense strategies to be beneficial for cancer? I offered a solution to this problem by constructing structural network of TLR pathway. In order to comprehend TLR pathway in detail and in which conditions it is out of control and resulting in excess inflammation, structural knowledge of PPIs – how proteins interact – is vital.

Computational tools help enrich the available structural data for PPIs and analyze the effects of mutations on the interactions and pathways. I utilized PRISM algorithm to model the structures of PPIs and performed *in silico* mutagenesis analysis to predict the outcomes of mutations.

Overall, I observed that, proteins with opposing functions tend to bind to same/overlapping interfaces on the target protein. Structural details clarify why opposing functions are not activated simultaneously because many of them are mutually exclusive due to steric clashes.

Here, I provide the structural basis for the assembly of key multimeric signalosomes, regulation of TLR signaling, and mutation mechanisms. I propose a model for TLR clustering upon activation, and found that almost all parallel pathways of TLRs are competitive, restricting the classical NF- κ B pathway. Our models show that negative regulators tend to interfere with the assembly of key signaling complexes, compete with proteins to bind to their partners, and modify ubiquitination states and target them for

proteosomal degradation. Several oncogenic mutations on interfaces tend to disrupt interactions in TLR pathway, liberating the conventional downstream paths and constitutive activation of NF- κ B. Better understanding of TLR signaling and regulation holds promise for novel treatment strategies for autoimmune diseases and cancer.

In addition to TLR pathway, I also modeled interactions of other cancer-related pathways, such as CASP8, TRAF3 and cytokine pathways. The PPI architectures that I obtained reveal the activation mechanism of CASP8 to trigger apoptosis and how its mutations contribute to “resisting cell death” in cancer. They also illuminate how/why distinct downstream outcomes are observed under different conditions, i.e. stimulated or unstimulated cell (TRAF3-NIK or TRAF3-TNFR interactions). They explain the inhibition mechanisms of classical and non-classical NF- κ B pathways by TRAF3 and the crosstalk of TLR pathway with other signaling cascades, such as TNFRs and Fas receptor.

The architectures that I present may help in drug discovery efforts to develop better anti-inflammatory and anti-cancer therapeutics for treatment of autoimmune and chronic inflammatory diseases and cancer in the future. Diseases often arise from formation or inhibition of certain PPIs. Therefore, instead of knocking-out a particular disease-causing protein as a whole and thereby inhibiting all of its interactions, targeting its particular interface to block a specific interaction is a better strategy to develop effective therapies. To this end, availability of the PPI structures has utmost importance. I conclude by emphasizing that structures of PPIs are crucial for having a complete in-depth picture of the cell; understanding the pathogenesis mechanisms of diseases; and development of better drugs.

APPENDIX

Supplementary Tables:

Table A.1: PRISM predictions used to construct structural TLR network.

PPI	Protein1	Protein2	Template Interface	Interaction Energy
TLR4-TLR4 (BB)	Model	Model	2g37AB	-31.45
TLR4-TLR4 (FF)	Model	Model	1z41AB	-17.68
TLR4-TLR4 (FF2)	Model	Model	1ufoCD	-22.98
TLR4-Mal (interface1)	Model	3ub2A	1c0gSA	-15.41
TLR4-Mal (interface2)	Model	4lqdA	1pzmAB	-25.4
Mal-Mal	3ub2A	3ub2A	3urrAB	-14.46
Mal-MyD88	4lqdA	2js7A	1mo1AB	-27.07
MyD88-MyD88 (BB1)	2z5vA	2z5vA	3lyhAB	-22.52
MyD88-MyD88 (BB2)	2js7A	2js7A	1a96AB	-16.8
MyD88-MyD88 (FF1)	2js7A	2js7A	1bjfAB	-35.74
MyD88-MyD88 (FF2)	2z5vA	2z5vA	1gylAB	-30.96
TLR4-TRAM (interface1)	Model	2m1wA	3gmgAB	-15.94
TLR4-TRAM (interface2)	Model	2m1wA	3o2qDE	-15.24
TRAM-TRAM	2m1wA	2m1wA	1pzmAB	-24.63
TRAM-TRIF	2m1wA	2m1xA	3urrAB	-19.11
MyD88-TRAF6	3mop	1lb5A	3lfrAB	-11.63
MyD88-TRAF3	3mopABCDEF	1fl1A	2oh1CD	-16.77
MyD88-FADD	3mopA	2gf5A	1mu4AB	-18.33

Table A.2: Details of interactions of negative regulators of TLRs: namely the target and template structures, interaction energies obtained by PRISM and the references that show the physical interactions of the given protein pairs.

PPI	Protein1	Protein2	Template Interface	Interaction Energy	Reference
BCAP-TLR4	Model	Model	3dahAB	-46.7	
BCAP-Mal	Model	4lqdA	3urrAB	-57.21	[87]
BCAP-MyD88	Model	4eo7A	3f1rAB	-39.95	[87, 163]
BCAP-TRAM	Model	2m1wA	3bdvAB	-27.05	
BCAP-TRIF	Model	2m1xA	3dhxAB	-29.97	[165]
SIGIRR-TLR4	Model	Model	3imoBD	-22.7	[99, 167-169]
SIGIRR-Mal	Model	4fz5A	1unhBE	-36.06	
SIGIRR-MyD88	Model	4domA	3f13AB	-27.84	[168, 169]
SIGIRR-TRAM	Model	2m1wA	1pzmAB	-11.38	
SIGIRR-TRIF	Model	2m1xA	1ntcAB	-21.38	[172, 173]
ST2L-TLR4	Model	Model	2bq1EF	-16.67	[169]
ST2L-Mal	Model	3ub2A	3urrAB	-40.66	[162]
ST2L-MyD88	Model	4eo7A	2oh1CD	-18.47	[162, 168, 169]
ST2L-TRAM	Model	2m1wA	3sf8AB	-43.48	
ST2L-TRIF	Model	2m1xA	3grzAB	-22.39	
TRAF6-p62	2k0bX	3hctA	3jygAB	-12.09	[193]
p62-CYLD	1q02A	1whmA	2yvzAB	-20.64	[187, 189, 190]
TRAF6-CYLD	1whlA	3hcuA	2ekyBD	-21.28	[188]
TRAF6-A20	3hcsA	3zjgB	1f5qAB	-32.08	[182]
A20 dimer	2vfjB	2vfjC	3dkbCF	-48.57	[180]
TRAF3-DUBA	3tmp	1flIA	2a6aAB	-16.29	[196]

Table A.3: Interface residues on the negative regulators of TLRs

PPI	Protein1	Protein2	Template Interface	Interface Residues on Protein1	Interface Residues on Protein2
BCAP-TLR4	Model	Model	3dahAB	H44, L46, G47, P48, E49, A50, S51, S53, A54, L57, Q76, H77, F78, K80, P81, A82, L83, P85, L86, L87, Q88, R89	W686, E690, V692, K693, N694, E696, E697, G698, R709, D798, V800, L801, R803, H804, W807, R811
BCAP-Mal	Model	4lqdA	3urrAB	E20, E21, Q24, Y25, Q27, T28, L29, L31, S32, Q35, I41, L42, P129, E130, V133, A134	L152, K158, M161, L162, L165, E167, P188, P189, E190, R192, F193, M194
BCAP-MyD88	Model	4eo7A	3f1rAB	A54, L57, S58, L61, S62, R64, R89, A90, F91, H92, P94, H95	S209, E210, E213, K214, F235, K238, F239, K241, S242, L243, S244
BCAP-TRAM	Model	2m1wA	3bdvAB	F91, H92, P93, P94, R96, V97, V98, L111, D116, A118, H119, W120, K138, A139, E142, D143	E87, D88, T90, D91, L94, R95, V96, N98, L99, Q101, D102, D103, I111, E114, L176, N177, E197, E198, F202
BCAP-TRIF	Model	2m1xA	3dhxAB	P17, D18, E20, E21, W22, Q24, Y25, Q27, T28, L31, S32, Q35, Q39, K40, I41, T43, R45	R463, L464, L466, H467, N470, M473, M474, S475, L477, G481, P483, D502, T503, S505, L506, S508
SIGIRR-TLR4	Model	Model	3imoBD	E209, L233, S234, R235, A236, C238, S239, S241, F242, R243, E244, P268, A269, A272, L275, Q278	D685, N689, K693, N694, E697, V699, V800, L801, R803, H804, W807, R808, R811
SIGIRR-Mal	Model	4fz5A	1unhBE	T260, R274, R277, Q278, R280, H281, V283, T284, L285, L286, L287, R289, D298, F299	L152, P155, W156, K158, Y159, L162, T166, A185, A186, P188, P189, E190, R192
SIGIRR-MyD88	Model	4domA	3f13AB	A208, E209, P210, S211, A212, S234, R235, A236, C238, S239, F242, R243, E244, A272, L275	R218, P245, G246, A247, H248, Q249, K250, R251, L252, I253, I267, F270, I271, T272, L295, P296
SIGIRR-TRAM	Model	2m1wA	1pzmAB	Q264, R265, R266, D267, H270, L273, R274, R277, L285, L286, L287, R289, P290, G291	E87, D88, D89, T90, D91, E92, R95, F112, A113, E114, M115, H117, H121, L122, Q123, N177
SIGIRR-TRIF	Model	2m1xA	1ntcAB	W237, C238, S241, F242, E244, C247, L250, E251, P271, A272, L275, L276, Q278, H279, R280, H281	A402, E429, N459, F460, D461, R463, L464, L466, H467, Q468, N470, S500, D502, S505, L506
ST2L-TLR4	Model	Model	2bq1EF	L376, Y377, R385, Y387, E398, H402, D497, E410, N411, G414, T416, L417, C418, I419, G421, R422, D423, M424, L425, P426, K440	S681, Q682, E684, D685, W686, N689, E690, L691, K693, N694, Q738, H739, I768, L770, Q771, K772, V773, E774, L777, W796, D798, S799, V800, L801, R803, W807
ST2L-Mal	Model	3ub2A	3urrAB	R385, Y387, S389, E398, H399, H402, Q403, I404, D407, E410, N411, I419, G421, R422, D515, N519, K520, R521, S522	L152, Q153, P155, K158, Y159, L162, L165, T166, P188, P189, E190, R192, F193, M194
ST2L-MyD88	Model	4eo7A	2oh1CD	R396, E481, M482, E483, A484, L485, S486, E487, E494, Q497, T509, I510, K511, R513, E514, H516, I517, K526	L228, Q229, K231, D234, T237, L241, K262, E263, F264, P265, S266, I267, R269, F270
ST2L-TRAM	Model	2m1wA	3sf8AB	F458, E461, Q462, E463, V464, H467, M490, L491, Q492, A495, L496, S499, L503	D91, L94, R95, N98, L99, P175, L176, N177, P179, L180, P181, R184, E197, E198
ST2L-TRIF	Model	2m1xA	3grzAB	P384, R385, Y387, K388, S389, A394, S395, E398, V401, H402, R422, L425, P426	A402, V433, H434, S458, N459, F460, D461, R463, L466, H467, N470, Q471, Q498, S500, D502,

					L506
TRAF6-p62	2k0bX	3hctA	3jygAB	E65, S66, E69, C70, P71, L74, H87, V107	P387, A390, P392, R393, I395, E396, N421, Y422
p62-CYLD	1q02A	1whmA	2yvzAB	A390, D391, L394, I395, L398, L402, E409, G410, G411, T414, L417, Q418, T419	S223, S225, P229, L230, N300, I303, P304, E305, S306, S307, G308, P309
TRAF6-CYLD	1whlA	3hcuA	2ekyBD	P62, P63, E65, S66, P71, H87, K104, C105, P106, V107, D108, N109	V127, P130, R147, Q185, L186, F187, Q188, S207, G206, P209, S210
TRAF6-A20	3hcsA	3zjgB	1f5qAB	Q54, D57, P71, I72, C73, L74, M75, C93, D100, A101, G102, K104, C105, P106, V107, D108, N109, P135	K20, R24, T42, R45, Y46, Q241, Y244, D331, N334, P336, K337, E338, I339,
A20 dimer	2vfjB	2vfjC	3dkbCF	P7, L12, S13, N14, M15, R16, V19, R22, E23, D119, D344, L348, H351	P7, L12, S13, N14, M15, R16, V19, R22, E23, D119, D344, L348, H351, E352
TRAF3-DUBA	3tmp	1flIA	2a6aAB	H470, R505, R506, L508, G509, D510, F537, V538, A539, T541, V542, N545, G546	M191, D192, T195, Q198, W202, K205, Y301, T303, G304, T305, S306

Table A.4: The list of clinically observed mutations on negative regulators of TLRs, which interface they fall onto and which interactions that they abolish. Energy score below -10 indicates favorable interaction. If the difference of energy scores between mutant and wild type is positive, then the mutation is destabilizing.

Cancer Study	Mutation	Mutation Type	Protein	Interface of PPI	Disrupted PPI	Energy Score	Difference of Energy Scores
Melanoma (TCGA)	G48S	Missense	BCAP	BCAP-TLR4	None	-40.33	6.37
NCI-60	E50K	Missense	BCAP	BCAP-TLR4	BCAP-TLR4	-8.9	37.8
Melanoma (Broad)	P82L	Missense	BCAP	BCAP-TLR4	BCAP-TLR4	-7.7	39
Melanoma (Yale)	D113H	Missense	BCAP	BCAP-TLR4, BCAP-TRAM	None	-13.0 -16.97	33.7 10.08
Breast (TCGA pub)	E21K	Missense	BCAP	BCAP-Mal, BCAP-TRIF	BCAP-TRIF	-25.16 -9.26	32.05 20.71
Lung squ (TCGA)	L27Q	Missense	BCAP	BCAP-Mal, BCAP-TRIF	BCAP-TRIF	-51.4 -1.18	5.81 28.79
Uterine (TCGA)	V134M	Missense	BCAP	BCAP-Mal	None	-44.24	12.97
Head & neck (Broad)	A136D	Missense	BCAP	BCAP-Mal	BCAP-Mal	None	None
Melanoma (Broad)	L60F	Missense	BCAP	BCAP-MyD88	None	-41.88	-1.93
Colorectal (Genentech)	C66Y	Missense	BCAP	BCAP-MyD88	BCAP-MyD88	12.12	52.07
Stomach (TCGA pub)	K139fs	FS del	BCAP	BCAP-TRAM	BCAP-TRAM	-4.87	22.18
Head & neck (TCGA)	S39N	Missense	BCAP	BCAP-TRIF	None	-11.92	18.05
pRCC (TCGA)	L282M	Missense	SIGIRR	SIGIRR-Mal, SIGIRR-TRIF	SIGIRR-TRIF	-33.01 -9.84	3.05 11.54
Cervical (TCGA)	S297F	Missense	SIGIRR	SIGIRR-Mal	None	-22.88	11.18
Breast (TCGA)	Y377N	Missense	ST2	ST2-TLR4	None	-11.99	4.68
Melanoma (TCGA)	P384Q	Missense	ST2	ST2-TLR4	ST2-TLR4	-5.45	11.22
Melanoma (TCGA)	P406L	Missense	ST2	ST2-TLR4	None	-12.45	4.22
Melanoma (Yale)	E410K	Missense	ST2	ST2-TLR4	None	-14.96	1.71
Lung adeno (TCGA pub)	L425Q	Missense	ST2	ST2-TLR4	ST2-TLR4	2.06	18.73
Melanoma (TCGA), Melanoma (Yale)	R439Q	Missense	ST2	ST2-TLR4	None	-19.3	-2.63
Stomach (TCGA)	E226K	Missense	CYLD	CYLD-p62	None	-20.38	0.26
Melanoma (TCGA)	P229S	Missense	CYLD	CYLD-p62	None	-18.34	2.3
Head & neck (TCGA)	N300S	Missense	CYLD	CYLD-p62	None	-15.82	4.82
Uterine (TCGA)	I303V	Missense	CYLD	CYLD-p62	CYLD-p62	None	None
Lung adeno (TCGA)	A305S	Missense	CYLD	CYLD-p62	None	-16.7	3.94
ccRCC (TCGA)	P130T	Missense	CYLD	CYLD-TRAF6	None	-10.35	10.93
Uterine (TCGA)	L186R	Missense	CYLD	CYLD-TRAF6	None	-13.92	7.36

Table A.5: Nonsense or frame-shift mutations on negative regulators. Although these mutations do not fall onto the interface residues, they lead to loss of the whole domain of the proteins that are covered in the PPI complexes modeled in this study.

Cancer Study	Mutation	Mutation Type	Protein	Disrupted PPI
Stomach (TCGA pub)	G9*	Nonsense	BCAP	BCAP-TLR4, BCAP-Mal, BCAP-MyD88, BCAP-TRAM, BCAP-TRIF
Prostate (Broad/Cornell 2012)	P2fs	FS del	SIGIRR	SIGIRR-TLR4, SIGIRR-Mal, SIGIRR-MyD88, SIGIRR-TRAM, SIGIRR-TRIF
Bladder (TCGA)	Q111*	Nonsense	SIGIRR	SIGIRR-TLR4, SIGIRR-Mal, SIGIRR-MyD88, SIGIRR-TRAM, SIGIRR-TRIF
Esophagus (Broad)	E30*	Nonsense	ST2	ST2-TLR4, ST2-Mal, ST2-MyD88, ST2-TRAM, ST2-TRIF
Lung adeno (TCGA pub)	Y50fs	FS del	ST2	ST2-TLR4, ST2-Mal, ST2-MyD88, ST2-TRAM, ST2-TRIF
Lung adeno (TCGA pub)	S125*	Nonsense	ST2	ST2-TLR4, ST2-Mal, ST2-MyD88, ST2-TRAM, ST2-TRIF
Stomach (TCGA pub)	G228fs	FS ins	ST2	ST2-TLR4, ST2-Mal, ST2-MyD88, ST2-TRAM, ST2-TRIF
NCI-60	A248fs	FS del	ST2	ST2-TLR4, ST2-Mal, ST2-MyD88, ST2-TRAM, ST2-TRIF
chRCC (TCGA)	M285fs	FS ins	ST2	ST2-TLR4, ST2-Mal, ST2-MyD88, ST2-TRAM, ST2-TRIF
Stomach (Pfizer UHK)	L350fs	FS del	ST2	ST2-TLR4, ST2-Mal, ST2-MyD88, ST2-TRAM, ST2-TRIF
Melanoma (Broad)	W361*	Nonsense	ST2	ST2-TLR4, ST2-Mal, ST2-MyD88, ST2-TRAM, ST2-TRIF
Colorectal (TCGA pub)	G47*	Nonsense	CYLD	CYLD-p62, CYLD-TRAF6
Colorectal (TCGA)	G47*	Nonsense	CYLD	CYLD-p62
CCLE	S159fs	FS del	CYLD	CYLD-p62
Head & neck (TCGA)	G179fs	FS del	CYLD	CYLD-p62
Head & neck (TCGA pub)	G179fs	FS del	CYLD	CYLD-p62
NCI-60	E245*	Nonsense	CYLD	CYLD-p62

Table A.6: The details of CASP8 interactions obtained by PRISM. Energy score below -10 indicates favorable interaction.

PPI	Protein1	Protein2	Template	Interaction Energy
CASP8-cFLIP	3h11A	3h11B	3h11AB	-85.99
CASP8-P35	1i4eA	1i4eB	1i4eAB	PRISM couldn't find
CASP8-CASP8	2funB	2funB	2nn3CD	-242.2
CASP8-cIAP1	3d9tB	1f9eA	1ncvAB	-19.91
CASP8-cIAP2	1qduA	2uvlA	3p01BC	-24.6
CASP8-XIAP	2popB	1qduA	1ijxBE	-28.79
CASP8-FADD	2gf5A	Model	3t75AB	-19.59
FADD-RIPK1	2gf5A	Model	2gmyBC	-42.78
CASP8-BCL2	1gjhA	2funB	3mvpAB	-19.72
CASP8-MALT1	2k7zA	3uo8B	1ufoEF	-30.88

Table A.7: Nonsense and frameshift mutations on CASP8 that abolish all of its interactions, except FADD.

Cancer Study	Mutation	Mutation Type
CCLE, Pancreas (TCGA), Uterine (TCGA), Uterine (TCGA pub)	E36*	Nonsense
Lung adeno (TCGA), Lung adeno (TCGA pub)	E56*	Nonsense
Bladder (BGI), Breast (TCGA)	R68*	Nonsense
CCLE, Head & neck (TCGA)	R68*	Nonsense
Liver (TCGA)	I76fs	FS ins
Head & neck (TCGA)	Y78fs	FS ins
Head & neck (Broad)	E89*	Nonsense
Breast (TCGA), Head & neck (TCGA)	Q97*	Nonsense
Head & neck (TCGA)	I98fs	FS del
Cervical (TCGA), Lung squ (TCGA)	S109*	Nonsense
Uterine (TCGA)	E111del	IF del
CCLE	F120del	IF del
CCLE	IF142del	IF del
CCLE, Melanoma (TCGA)	I144fs	FS del
Stomach (Pfizer UHK)	G153*	Nonsense
Stomach (TCGA pub)	V163fs	FS del
ACC (TCGA)	C164fs	FS del
CCLE	I174fs	FS del
Head & neck (TCGA)	Y178del	IF del
Breast (Sanger)	E195*	Nonsense

Table A.8: The effect of missense mutations of CASP8 that fall onto the interface residues or their first neighbors on the interactions. Energy score below -10 indicates favorable interaction.

Cancer Study	Mutation	Mutation Type	Interface of PPI	Disrupted PPI	Energy Score
Bladder (BGI), Uterine CS, Uterine (TCGA)	R68Q	Missense	CASP8-FADD	None	-11.33
CCLE	R68P	Missense	CASP8-FADD	CASP8-FADD	-2.59
Stomach (TCGA)	G94D	Missense	CASP8-FADD	CASP8-FADD	27.78
Head & neck, Melanoma (TCGA)	S99F	Missense	CASP8-FADD	CASP8-FADD	11.62
Liver (AMC)	R102W	Missense	CASP8-FADD	None	-17.4
Stomach (TCGA)	R102M	Missense	CASP8-FADD	CASP8-FADD	-6.18
Colorectal (Genentech)	M104I	Missense	CASP8-FADD	CASP8-FADD	25.97
Head & neck (Broad)	L105H	Missense	CASP8-FADD	None	-11.54
Uterine (TCGA)	C236Y	Missense	CASP8-cIAP1, CASP8-cIAP2, CASP8-XIAP	CASP8-cIAP1	14.65 -21.99 -19.17
Breast (TCGA)	L277F	Missense	CASP8-BCL2	CASP8-BCL2	No results
Head & neck (TCGA pub)	D303V	Missense	CASP8-cIAP1, CASP8-cIAP2, CASP8-XIAP	CASP8-cIAP1, CASP8-XIAP	15.46 -32.04 -3.86
Head & neck (TCGA pub)	D303G	Missense	CASP8-cIAP1, CASP8-cIAP2, CASP8-XIAP	None	-15.87 -12.6 -17.46
Liver (AMC)	P394Q	Missense	CASP8-CASP8, CASP8-cFLIP	None	-222.16 -102.95
Stomach (TCGA pub)	P394L	Missense	CASP8-CASP8, CASP8-cFLIP	None	-253.62 -102.42
Liver (TCGA)	E396G	Missense	CASP8-CASP8, CASP8-cFLIP	None	-245.56 -100.89
CCLE	A397T	Missense	CASP8-CASP8, CASP8-cFLIP	None	-235.58 -100.39
Breast (BCCRC)	G418A	Missense	CASP8-BCL2	CASP8-BCL2	24.3
CCLE	S424T	Missense	CASP8-BCL2	CASP8-BCL2	14.72
Stomach (Pfizer UHK)	L440I	Missense	CASP8-CASP8, CASP8-cFLIP	None	-238.1 -99.29
Head & neck (TCGA)	T441I	Missense	CASP8-CASP8, CASP8-cFLIP	None	-248.06 -94.02
Colorectal (Genentech)	I442T	Missense	CASP8-CASP8, CASP8-cFLIP	None	-233.81 -96.7
Uterine (TCGA)	T444P	Missense	CASP8-CASP8, CASP8-cFLIP	None	-244.03 -104.47
CCLE	V446A	Missense	CASP8-CASP8, CASP8-cFLIP	None	-238.34 -103.16

Stomach (TCGA)	M463I	Missense	CASP8-CASP8, CASP8-cFLIP	None	-242.46 -103.17
CCLE	P464L	Missense	CASP8-CASP8, CASP8-cFLIP	None	-243.6 -84.65
CCLE	L474R	Missense	CASP8-cFLIP	None	-105.66

Table A.9: The clinically observed oncogenic mutations that fall into IL6-IL6RA-IL6RB interfaces.

Mutation	Mutation Type	Tissue Type	Protein	Interface of PPI
186_Y190delYSTVY	Deletion	Liver	IL-6RB	IL6-IL6RB
193delN	Deletion	Liver	IL-6RB	IL6-IL6RB
I239N	Missense	Stomach	IL-6RB	IL6RB-IL6RA
S279C	Missense	Breast	IL-6RB	IL6RB-IL6RA
R52Q	Missense	Large intestine	IL-6	IL6-IL6RB
R58Q	Missense	Large intestine	IL-6	IL6-IL6RA
L61L	Missense	Large intestine	IL-6	IL6-IL6RA
D62N	Missense	Large intestine	IL-6	IL6-IL6RB
R207W	Missense	Endometrium	IL-6	IL6-IL6RA
R210Q	Missense	Large intestine	IL-6	IL6-IL6RA
R250I	Missense	Endometrium	IL-6RA	IL6-IL6RA
H280Y	Missense	Large intestine	IL-6RA	IL6RB-IL6RA
E297Q	Missense	Lung	IL-6RA	IL6-IL6RA
D281N	Missense	Large intestine	IL-6RA	IL6RB-IL6RA

Table A.10: Sequence identities, coverage, and normalized Z-scores between the query proteins and the template structures. The normalized Z-score greater than 1 implies a good alignment [139].

Query protein	Template protein	PDB_ID of template	Sequence identity of query and template	Coverage	Normalized Z-score
BCAP	PdTIR from <i>Paracoccus denitrificans</i>	3h16A	0.22	0.93	1.23
BCAP	TIR domain of Mal/TIRAP	3ub2A	0.19	0.83	1.19
BCAP	Brucella protein TcpB TIR domain	4lqcA	0.21	0.86	1.13
BCAP	TIR domain from the Arabidopsis Thaliana disease resistance protein RRS1	4c6sA	0.22	0.95	1.06
SIGIRR	TIR domain of human IL-1RAPL	1t3gA	0.32	0.96	2.72
ST2L	TIR domain of human IL-1RAPL	1t3gA	0.35	0.89	2.53
ST2L	Homology model of human Toll-like receptor 5 fitted into an electron microscopy single particle reconstruction	3j0aA	0.22	0.87	3.5
ST2L	TIR domain of human TLR1	1fyvA	0.23	0.87	2.63
TLR4	TIR domain of human TLR1	1fyvA	0,39	0,97	2,76
TLR4	Homology model of human Toll-like receptor 5 fitted into an electron microscopy single particle reconstruction	3j0aA	0.29	0.99	3.68
TLR4	TLR2 TIR domain	1fyxA	0.4	0.97	2.77

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VITA

Emine Güven Maıorov was born in Karaman, Turkey, on January 19, 1985. She received the B.Sc. Degree in Molecular Biology and Genetics from Boğaziçi University in 2009 and M.Sc. Degree in Chemical and Biological Engineering from Koç University in 2011. She received her Ph.D. Degree from Koç University in Chemical and Biological Engineering in 2015. From September 2009 to August 2015 she worked as a teaching and research assistant at Koç University. She studied as a guest researcher in National Cancer Institute (NCI), NIH.

Her research mainly focuses on structural modeling of protein-protein interfaces, their integration to protein-protein interaction networks, systems biology approaches to understand the crosstalk between cancer and inflammation. She has published articles in prestigious journals such as Scientific Reports, Biophysical Journal, BMC Genomics, Seminars in Cancer Biology, Cancers, and Current Opinion in Structural Biology.

She will continue her academic career as a postdoctoral associate at NCI, where she will be focusing on cancer and inflammation.

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EDUCATION

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(Expected) **PhD, Chemical and Biological Engineering**
Koc University, Computational Systems Biology Group, Istanbul, Turkey
Thesis title: "Understanding the relation of cancer and inflammation by constructing structural Toll-like Receptor pathway." *Supervisors: Professor Ozlem Keskin & Professor Attila Gursoy*

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ACADEMIC & PROFESSIONAL EXPERIENCE

09/2009-06/2015 Research and Teaching Assistant, **Koc University**, Istanbul, Turkey

07/2014-09/2014 Guest Researcher, **National Cancer Institute**, Cancer and Inflammation Program, Leidos Biomedical Research Inc., Frederick National Laboratory for Cancer Research. *Supervisor: Professor Ruth Nussinov*

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- 06/2008-09/2008 Guest Researcher, **Harvard Medical School**, Department of Cell Biology.
Supervisor: Professor Robin Reed.
- 07/2007-09/2007 Guest Researcher, **Harvard Medical School**, Department of Cell Biology.
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- 01/2007-06/2009 Intern, **Bogazici University**, Department of Molecular Biology and Genetics.
Supervisor: Professor Esra Battaloglu.

PUBLICATIONS

1. **Güven-Maiorov E***, Muratcioglu S*, Keskin O, Gursoy A. Advances in template-based protein docking by utilizing interfaces towards completing structural interactome. (Submitted to *Current Opinion in Structural Biology*). (* **Equally contributed**)
2. **Güven-Maiorov E**, Keskin O, Gursoy A, Tsai CJ, VanWaes C, Chen Z, Nussinov R. The architecture of TIR domain signalosome in Toll-like receptor Signaling Pathway. (Under minor revision in *Scientific Reports*).
3. **Güven-Maiorov E**, Keskin O, Gursoy A, Nussinov R. Structural view of negative regulation of Toll-like receptor- mediated inflammation. (Under minor revision in *Biophysical Journal*).
4. **Güven-Maiorov E**, Acuner Ozbabacan SE, Keskin O, Gursoy A, Nussinov R (2014) Structural Pathways of Cytokines May Illuminate Their Roles in Regulation of Cancer Development and Immunotherapy. *Cancers* 6: 663-683.
5. **Güven-Maiorov E***, Acuner Ozbabacan SE*, Engin HB*, Kuzu G*, Muratcioglu S*, et al. (2014) The Structural Network of Interleukin-10 and Its Implications in Inflammation and Cancer. *BMC Genomics* 15 Suppl 4: S2. (* **Equally contributed**)
6. **Güven-Maiorov E**, Keskin O, Gursoy A, Nussinov R (2013) The structural network of inflammation and cancer: Merits and challenges. *Semin Cancer Biol* 23: 243-251.
7. **Güven-Maiorov E**, Keskin O, Ng OH, Ozbek U, Gursoy A (2013) Identification of interconnected markers for T-cell acute lymphoblastic leukemia. *Biomed Res Int* 2013: 210253.

IN PREPERATION

1. **Güven-Maiorov E**, Keskin O, Gursoy A, VanWaes C, Chen Z, Nussinov R. Structural insights into the CASP8 interactions, mutations, and their roles in tumorigenesis.
2. **Güven-Maiorov E**, Keskin O, Gursoy A, Muftuoglu M. Modifying Base-excision repair for therapeutic benefit of neurodegenerative diseases.

TEACHING EXPERIENCE

- | | |
|-------------|---|
| Spring 2015 | Drug Design (CHBI 550) |
| Fall 2014 | Thermodynamics (CHBI 503) |
| Spring 2014 | Drug Design (CHBI 550) |
| Fall 2013 | General Biology-I Laboratory (MBGE 101) |
| Spring 2013 | Drug Design (CHBI-550) |
| Fall 2012 | Biochemistry (MEDI 201) |

Spring 2012	Biochemistry (MEDI 201)
Fall 2011	Introduction to Cellular and Molecular Biology Laboratory (MBGE 202)
Spring 2011	Cell and Developmental Biology (MBGE 204)
Fall 2010	Introduction to Cellular and Molecular Biology Laboratory (MBGE202)
Spring 2010	General Biology-I Laboratory (MBGE 102)
Fall 2009	Introduction to Physics (PHYS 101)

POSTERS

1. ISMB' 2015: "A structural view into signaling and regulation of Toll-like receptor pathway and its implications in inflammation and cancer crosstalk", Emine Guven-Maiorov, Ruth Nussinov, Ozlem Keskin, Attila Gursoy, Dublin, Ireland 2015
2. Cell Symposia: Cancer, Inflammation and Immunity'2015: "Structural Toll-like Receptor pathway may illuminate its roles in inflammation and cancer crosstalk", Emine Guven-Maiorov, Ruth Nussinov, Ozlem Keskin, Attila Gursoy, Sitges, Spain 2015
3. ISMB' 14: "Structural Toll-like Receptor pathway may illuminate its roles in inflammation and cancer crosstalk", Emine Guven-Maiorov, Ruth Nussinov, Ozlem Keskin, Attila Gursoy, Boston, USA 2014
4. Biophysical Society Thematic Meeting on Modeling Biomolecular Systems, Interactions, Dynamics, and Allostery: "Structural Toll-like Receptor pathway may illuminate its roles in inflammation and cancer crosstalk", Emine Guven-Maiorov, Ruth Nussinov, Ozlem Keskin, Attila Gursoy, Istanbul, Turkey 2014
5. International Symposium on Integrating Spectroscopic and Theoretical Methods to Analyze Molecular Machines: "Template-based Assembly of TIR-Domain Signalosome in Toll-like Receptor Pathway", Emine Guven-Maiorov, Ruth Nussinov, Ozlem Keskin, Attila Gursoy, Schloss Ringberg in Bavaria, Germany 2014
6. ISMB' 11: "Identification of Interconnected Markers for T-cell Acute Lymphoblastic Leukemia", Emine Guven-Maiorov, Ozlem Keskin, Attila Gursoy, Vienna, Austria, 2011
7. HIBIT'11: "Revealing Temporal and Functional Relations in Breast Cancer Expression Profile Using Biclustering Based Analysis", Emine Guven-Maiorov, Omer An, Ozlem Keskin, Attila Gursoy, Turkey, 2011
8. HIBIT'10: "Attacking Interface and Interaction Networks", Billur Engin, Emine Guven-Maiorov, Omer An, Ozlem Keskin, Attila Gursoy, Turkey, 2010

SKILLS & COMPETENCES

Language: English (Fluent), Russian (Beginner), Turkish (Native)

Programming: Python, Matlab, Unix/Linux

Protein Visualization Tools: Discovery Studio, Molsoft, VMD, Chimera, Yasara, Pymol

Protein Docking Tools: PRISM, Flexpepdock, Cluspro

Virtual Screening Tools:	Autodock, AutodockVina, Molegro, FRED (OpenEye), GLIDE (Schrödinger), GOLD, CLC drug discovery workbench
Microarray Analysis Tools:	Expander, Pinnacle-Z
Network Visualization Tools:	Cytoscape
Simulation Tools:	NAMD
Fields of Expertise:	Protein-protein interactions, docking, homology modeling, protein structure and function, structure-based drug design (computer-aided drug design), microarray data analysis
Laboratory techniques:	PCR, RT-PCR, cloning, electrophoresis (agarose gel, PAGE), DNA/RNA extraction, protein purification, Western blot, cell-culture, micro-injection, siRNA transfection, IP, nuclear extract preparation, xCELLigence (real-time monitoring of cells without incorporation of labels), dissection of dorsal root ganglia from mice, in vitro systems for coupling transcription-to-splicing in RNAP-II transcripts (invented in Reed lab)

WORKSHOPS & CERTIFICATES

1. Drug Design Workshop by Kumlesh K. Dev (Supported by Erasmus Program) (2014), Koc University, Istanbul, Turkey
2. Computational Drug Discovery Workshop by Schrödinger (2012), Yeditepe University, Istanbul, Turkey
3. Corporate Relations and Resource Development by Richard Haass (2010), Koc University, The Evolving World Order
4. EMBO Young Scientists Forum in Turkey (2008), Bogazici University, Istanbul, Turkey

GRANTS & AWARDS

1. ISMB Travel Fellowship, 2015
2. Merit Scholarship, Koc University, Turkey, 2009-2015
3. Merit Scholarship, Bogazici University, Turkey, 2003-2009
4. Turkish Prime Ministry Undergraduate Fellowship, 2003-2009
5. Istanbul Municipality Undergraduate Fellowship, 2003-2009
6. IKEV Undergraduate Fellowship, 2003-2009

MEMBERSHIPS

1. ISCB (International Society for Computational Biology)
2. IEEE (Institute of Electrical and Electronics Engineers)
3. TEMA (Turkey Erosion Control, Reforestration & Protection of Nature Foundation)
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