STRUCTURAL ANALYSIS OF MAMMALIAN TOLL-LIKE RECEPTOR SIGNALING NETWORK

by

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ABSTRACT

STRUCTURAL ANALYSIS OF TOLL-LIKE RECEPTOR SIGNALING NETWORK

Comprehensive maps of large-scale signaling networks are attractive for researchers interested in signal transduction mechanisms because the information they give can be used as a basis for mathematical interpretations about the topological structure. The objective in this study is to analyze the comprehensive TLR signaling map, which is a crucial part of the immune system, structurally; in order to enlighten its topological structure, i.e. the properties such as robustness, and the detection of the crosstalking molecules in sub-pathways of the large system. Ultimate goal is then to identify the key molecules as potential drug targets for the infectious diseases and PKC-ζ is suggested for the treatment of post-infection immunity diseases such as sepsis and atherosclerosis. Network topology is investigated via the analyses based on graph theory and linear pathways. The TLR network is found to be scale-free and it has small-world properties. According to the analysis based on graph theory, in- and out-degree distributions tend to fit the power law model ($P(k) \approx k^{-\gamma}$) with γ values of 2.12 and 2.14 and R^2 values of 0.96 and 0.92, respectively. The hubs for the in-degree connections are TLR2 ligand and IKK-α/IKK-β/IKK-γ complex (which is also a hub for the out-degree connection), whereas the hubs for the out-degree connections are found as TIR domain, TRAF6, Rac1/GTP complex, κB site/NF-κB (p65)/NF-κB (p50)/CBP complex, and MyD88. The network and phenotype diameters of TLR signaling network are calculated as 25 and 11, respectively with a mean path length of 8.98. Due to the small-world topology of the network, it can be concluded that the TLR network is highly robust, i.e. it can take alternative actions under perturbations. The critical signaling molecules, i.e. common species participating in most of the pathways leading to the phenotypes, are found to be: the complexes of Ubc13/Uev1A/TRAF6/TAB2/TAB3/TAK1/TAB1, TLR2/TLR2 ligand and Rac1/GTP.

ÖZET

TOLL-BENZERİ ALMACIN SİNYAL İLETİ AĞININ YAPISAL OLARAK İNCELENMESİ

Büyük ölçekli sinyal ileti ağyapılarının kapsamlı haritalarının içerdiği bilgiler, ağyapıların topolojik yapılarını matematiksel olarak yorumlamaya olanak sağlaması dolayısı ile sinyal iletimi konusunda araştırma yapanlar için çok çekici hale gelmiştir. Bu çalışmadaki amaç; bağışıklık sisteminde kilit rol oynayan Toll-benzeri almacın (TLR) kapsamlı haritasını yapısal olarak inceleyerek, çevresel ve genetik değişikliklere olan dayanıklılığı ve bu büyük sistemin içinde yer alan küçük sinyal ileti ağlarının birbirleriyle olan iletişimlerinde rol alan moleküllerin tespiti gibi topolojik özelliklere ışık tutmaktır. Nihai amaç ise tespit edilen bu moleküllerin, bulaşıcı hastalıkların tedavisinde kullanılan ilaçlar için hedef olup olamayacağının belirlenmesidir ve bu çalışmada PKC-ζ molekülü septik şok ve atheroskleroz gibi enfeksiyon sonrası oluşan hastalıklara karşı kullanılan ilaçlara hedef olarak gösterilmiştir. Ağyapının topolojisi, grafik kuramı ve doğrusal yolizi tekniklerine dayanarak incelenmiş ve yapısı ölçek-bağımsız ve küçük-dünya olarak tespit edilmiştir. Grafik kuramına dayalı incelemeye göre, iç ve dış-bağlanabilirlik dağılımı sırasıyla yüzde doksanaltı ve doksanikilik doğrulukla 2.12 ve 2.14 olarak bulunmuş ve kuvvet yasası modeline ($P(k) \approx k^{-\gamma}$) uygunluğu belirlenmiştir. İç-bağlanabilirlik merkezleri olarak TLR2 hücre dışı sinyal proteini ve IKK-α/IKK-β/IKK-γ birleşiği bulunmuştur. Sonuncu merkez aynı zamanda dış-bağlanabilirlik merkezidir ve bulunan diğer dış-bağlanabilirlik merkezleri; TIR alanı, TRAF6, Rac1/GTP birleşiği, κB bölgesi/NF-κB (p65)/NF-κB (p50)/CBP birleşiği ve MyD88'dir. TLR sinyal ileti ağyapısının çapı ve fenotip çapı sırasıyla 25 ve 11, ortalama yol uzunluğu ise 8.98 olarak bulunmuştur. Küçük-dünya özelliğinden dolayı TLR ağyapısının çevresel ve genetik değişikliklere karşı bir hayli dayanıklı olduğu sonucuna varılabilir. Fenotiplere giden yolizlerinin çoğunda görev alan moleküller: Ubc13/Uev1A/TRAF6/TAB2/TAB3/TAK1/TAB1, TLR2/TLR2 hücre dışı sinyal proteini ve Rac1/GTP birleşikleri olarak bulunmuştur.

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1. INTRODUCTION

Fossil record states that there are approximately 2.5 billion years of difference between the appearance of first sophisticated unicellular organisms (similar to bacteria) and multicellular organisms. One of the causes why multicellular organisms evolve slowly can be based upon the necessity of complex mechanisms, so called signal transduction (signaling) pathways, for the cells to communicate with each other (Alberts *et al.*, 2002a).

These communication mechanisms depend mostly on extracellular signal molecules (ligands), which are produced by cells to signal to their neighbors or cells further away. Communication mechanisms also depend on detailed systems of proteins contained by each cell for cell-specific responses. These proteins include cell-surface receptor proteins (binding the signal molecule) and a variety of intracellular signaling proteins (distributing the signal to relevant parts of the cell). Target proteins are activated at the end of each activated intracellular signaling pathway in order to change the behavior of the cell (Alberts *et al.*, 2002a). Signal transduction is essential for all multi-cellular functions in higher-level organisms and therefore signal transduction research capacity and resources are being ever-increased. As the number of large-scale signaling network reconstruction studies increases day by day, the field of the development and application of methods in order to identify the topological structures and quantitatively describe the properties (Papin *et al.*, 2005) of these large-scale networks by dynamic and structural analyses has extensively grown. The structural analyses of large-scale networks provide valuable insight into the mechanisms of the networks by defining function-structure relationships (Papin *et al.*, 2005) and they do not require the knowledge of the kinetic parameters (Papin *et al.*, 2005; Oda and Kitano, 2006).

Signaling molecules and their networks also play important roles in molecular diagnosis and therapy of diseases as almost all known diseases exhibit some kind of dysfunction at gene level and hence there has been a great deal of enthusiasm to identify novel drug targets based on the knowledge of key signal transduction components which have links to diseases. Understanding cellular signaling therefore is central for gaining

insight into the molecular mechanisms behind diseases as well as adaptation of living cells to changes in the environment.

A very important signaling network in mammalian cells is the toll-like receptor (TLR) signaling pathway, which has been evolutionarily well conserved in both invertebrates and vertebrates (Hoffmann and Reichhart, 2002; Roach *et al.*, 2005). It is the front-line subsystem against invasive microorganisms for both innate and adaptive immunity (Iwasaki and Medzhitov, 2004), i.e. alerts the immune system in the presence of microbial invasion and initiates the immune response (inflammation) against the pathogen (Goldstein, 2004). Due to the role of the TLR signaling pathway in the induction of strong inflammation; it can be involved in post-infection conditions such as atherosclerosis, leprosy, inflammatory bowel syndrome (IBD), lung airway hyperactivity in allergic asthma, and in sepsis. With the same point of view, TLRs can also be associated with autoimmune diseases such as systemic lupus erythematosus (SLE) or other immune unresponsive diseases like cancer (Bhattacharjee and Akira, 2005).

This thesis presents the topological analysis of Toll-like receptor signaling pathway by using the structural analysis techniques, namely linear pathway analysis and the analysis based on graph theory. In the last decade, methods derived from graph theory have been developed to understand the structure of the systems of molecular interactions (Jeong *et al.*, 2000; Bu *et al.*, 2003; Barabási and Oltvai, 2004; Klamt *et al.*, 2006). The graph theoretic analysis can detect the components that are well or poorly connected (Papin *et al.*, 2005) in the interaction network and thus it is a useful guide for experimental studies on protein interactions. The recent comprehensive TLR signaling network map (Oda and Kitano, 2006) has the overall bow-tie and small-world structures, those being characteristics of the robust evolvable systems (Kitano, 2004). In bow-tie structures, a variety of ligands bind to corresponding receptors leading to diverse phenotypes via activations of the intermediate signaling molecules in the network. In the present study, the topological structure of the signaling network was also captured as a system of linear paths connecting each ligand to a phenotype, while performing the pathway analysis. The investigation of the signaling network topology; in terms of the pathway and overall crosstalk and participation and specificity of molecules in signaling pathways belonging to

the TLR signaling network, were performed by analyzing their linear paths of which any network state can be defined as a unique, nonnegative, linear combination.

After introducing the goals of thesis and the methods applied for achieving these goals in the first chapter (Introduction), the following chapter (Theory) concentrates on giving theoretical base and insights about related phenomena such as cell signaling, reconstruction and mathematical (dynamic and structural) analysis of signaling pathways and lastly the TLR signaling pathway and the roles of sphingolipids in it. The third chapter focuses on how the TLR signaling network is reconstructed by Oda and Kitano (2006) and its properties, and then the computational analysis methods which are adopted and applied to the TLR signaling network in hand. The Results and Discussion chapters (Chapters 4 and 5) follow by informing the reader what the topological results of the analyses are and what they may imply. Lastly the thesis is concluded and the main points are summarized and some recommendations are given for further studies on this topic (Chapter 6).

2. THEORETICAL BACKGROUND

2.1. Cell Signaling

Signaling is the study of how cells communicate, and it has an important role on all aspects of biology, from development to disease (Downward, 2001). Like all living things, all cells must continually sense their environment and make decisions on the basis of the information they gather. Signaling can be studied at the level of the individual cell or the whole multicellular organism (Downward, 2001). For individual cells, signaling is crucial for taking decisions about division, specialization, death and metabolic control. For example, single cell organisms regulate their metabolic processes by sensing whether there is a toxic compound or nutrient nearby. In more specialized cells, signaling is central to immunity and the transmission of nerve impulses. At the level of whole multicellular organism, signaling controls growth, development and aspects of metabolism and behavior. Cells in multicellular organisms (e.g. human), must sense the presence of neighboring cells and hormones when making decisions of whether to proliferate, differentiate or die (Downward, 2001). It is reasonable to claim that comprehensive characterization of signaling pathways can lead eventually to define certain signaling molecules as drug targets for curing diseases in which those pathways are defective (Downward, 2001).

A simple intracellular signaling pathway is shown in Figure 2.1 (Alberts *et al*., 2002a). In general, a trans-membrane protein complex (embedded in the plasma membrane) works as a receptor in an intracellular signaling pathway. Extracellular signal molecule, i.e. the ligand, binds to the receptor and activates the signal transduction. A cascade of intracellular signaling proteins transmits the signal to the target proteins and finally initiates a response. The response may be the alteration of metabolism if the target protein is a metabolic enzyme. On the other hand, if the target protein functions as a transcriptional regulator, the response may be the change in gene expression. Another possibility is the transmission of the signal to a cytoskeletal protein, which alters the shape or location of the molecule/cell.

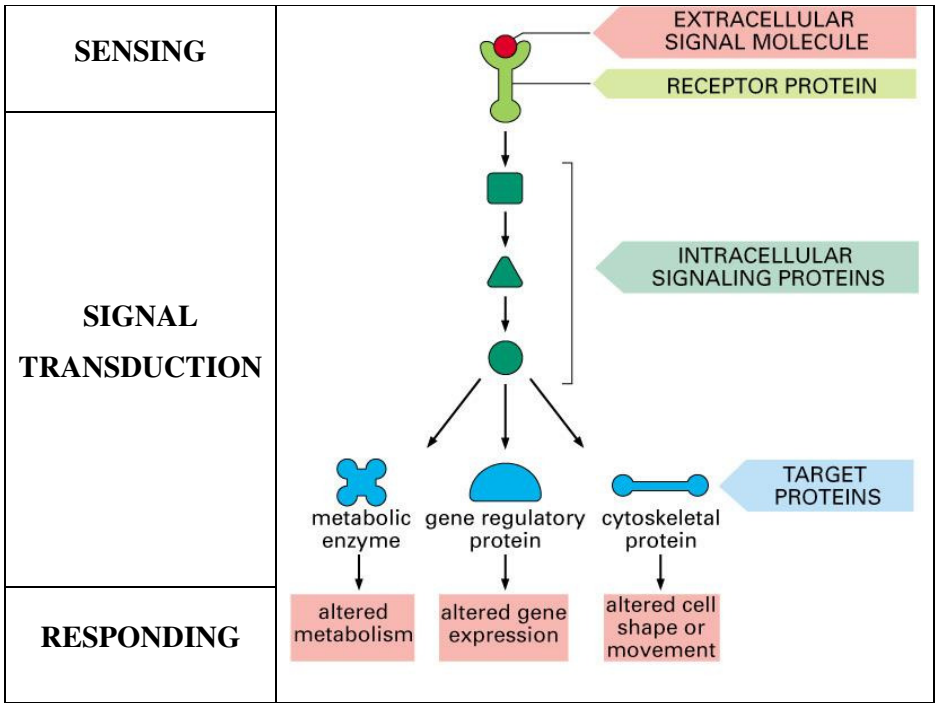


Figure 2.1. A simple intracellular signaling pathway (Alberts *et al.*, 2002a) and the phases of cell signaling

2.1.1. Ligand

An extracellular signal molecule is called the ligand, and different types and numbers of ligands take place in cell signaling. Yeast cells communicate with one another for mating by secreting several kinds of small peptides whereas in higher animals, cells communicate by means of hundreds of kinds of signal molecules including; proteins, small peptides, amino acids, nucleotides, steroids, retinoids, fatty acid derivatives, and even dissolved gases such as nitric oxide and carbon monoxide. Most of these molecules are secreted from the signal cell by exocytosis or released by diffusion through the plasma membrane. On the other hand, some ligands remain tightly bound to the surface of signal cell (Alberts *et al.*, 2002a).

2.1.2. Receptor and the Target Protein

A target cell responds to a signal, regardless of its nature, by means of a specific protein called the receptor; which binds the signal molecule and initiates a response in the target cell. There are two types of receptors, namely intracellular receptors (crossing the cell membrane) and cell-surface receptors (trans-membrane proteins on the target cell surface). In the case of the activation of an intracellular receptor, which is inside the target cell, the ligand needs to be sufficiently small and hydrophobic in order to diffuse across the plasma membrane of the target cell. On the contrary; the cell-surface receptor proteins bind the hydrophilic ligand with high affinity and once they are activated, they convert this extracellular event into one or more intracellular signals (cascade of intracellular signals) which in turn alter the behavior of the target cell (Alberts *et al*., 2002a). Toll-like receptors (TLRs) belong to the family of cell-surface receptors and malfunctions in TLR signaling may underlie infectious diseases such as AIDS. There are three known classes of cell-surface-receptor proteins: ion-channel linked, G-protein linked, and enzyme linked. Ion-channel linked receptors are involved in rapid synaptic signaling between electrically excitable cells in which ion permeability of the plasma membrane changes. G-protein linked receptors act indirectly to regulate the activity of target protein, called a G-protein. Enzyme linked receptors either functions directly as enzymes or they are associated with enzymes (Alberts *et al*., 2002a).

The target proteins in signaling differ in type (Figure 2.1) and their responses vary accordingly. The phenotype phenomenon is defined as the observable character of a cell or an organism by Alberts *et al.* (2002a) and the responses of target proteins are classified as phenotypes by Oda and Kitano (2006). Phenotypes taking place in a cell are important because the species specific to the pathways leading to the pathogenic phenotypes (e.g. tumorigenesis (Oda *et al.*, 2005a)), those causing diseases such as cancer, might be considered as potential drug targets.

2.1.3. Signal Transduction

The process of signal progression from sensing to responding requires the transfer of biological information. Signal transduction refers to any process by which a cell converts one kind of signal or stimulus into another, mostly involving ordered sequences of biochemical reactions inside the cell. In bacteria and other single-cell organisms; the variety of signal transduction processes which the cell is capable of, influences how many ways it can react and respond to its environment. In multicellular organisms; in addition to the ones involved in single-cell organisms, many different signal transduction processes are required for coordinating the behavior of individual cells to support the function of the organism as a whole. Then expectedly, as the organism gets more complex, the signal transduction processes that the organism possesses also become more complex through gene duplication and divergence (Alberts *et al*., 2002a).

2.1.4. Mechanisms of Cell Signaling

2.1.4.1. Cell Signaling Based on the Distance of Ligand Effect. Contact-dependent signaling takes place if a ligand binds to the surface of the signaling cell and affects only the cells which are in contact with it (Figure 2.2A). This type of signaling is important during development and in immune responses (Alberts *et al.*, 2002a). On the other hand, a ligand might be secreted to the extracellular space and either carried to the targets further away or act as local mediators affecting only on neighboring cells, which is designated as paracrine signaling (Figure 2.2B). Paracrine signals are not allowed to diffuse too far and are taken up via being either destroyed by extracellular enzymes or immobilized by the extracellular matrix of the neighboring target cells (Alberts *et al.*, 2002a). However, paracrine signaling is not sufficient for the coordination of the cellular behavior of a complex multicellular organism. In these organisms, specialized cells such as nerve cells (neurons) or hormones have evolved for communication of the cells, which are located in separate parts throughout the organism. Neurons use the synaptic signaling mechanism (Alberts *et al.*, 2002a), which takes place as follows: When neurons are activated; they send electrical impulses along their axons and as the impulses reach to the nerve terminals, a chemical signal (neurotransmitter) is secreted at the specialized cell junction (chemical synapse) which ensures that the neurotransmitter is delivered to the specific target cell

(Figure 2.2C). The other type of specialized cells, i.e. hormones, secretes the ligands into the bloodstream, through which they are carried to the target cells (Figure 2.2D). This type of signaling process is named as endocrine signaling (Alberts *et al.*, 2002a).

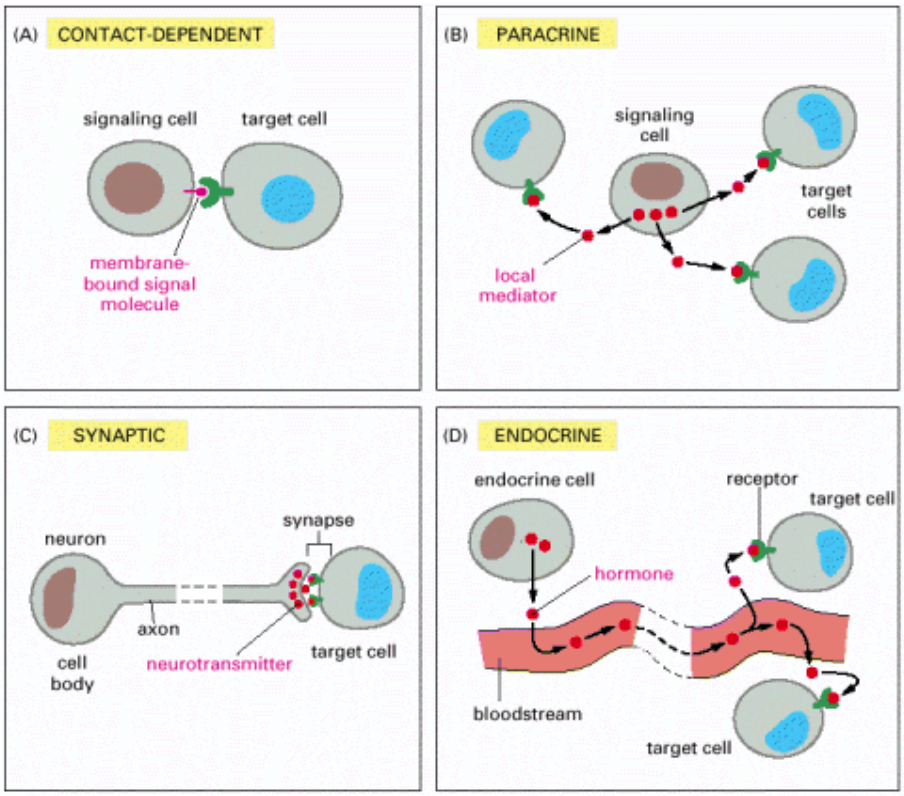


Figure 2.2. Forms of intercellular signaling (Alberts *et al.*, 2002a)

2.1.4.2. Cell Signaling Based on the Mechanism of Signal Transmission. In biological systems, signal transmission occurs mostly through two mechanisms: (i) protein-protein interactions and enzymatic reactions such as protein methylation, phosphorylation and dephosphorylation (post-translational modifications) or (ii) protein degradation or production of intracellular messengers (Bhalla and Iyengar, 1999).

There are four basic categories of signal transduction events (Figure 2.3). The classical case of a transduced signal relates a single input to a single output. However, some outputs require the concatenation of multiple inputs. Other signaling interactions may occur in which the transduction of a single input generates multiple outputs, a type of

signal pleiotropy. Lastly, complex signaling events arise when multiple inputs trigger interacting signaling cascades that result in multiple outputs (Papin and Palsson, 2004a).

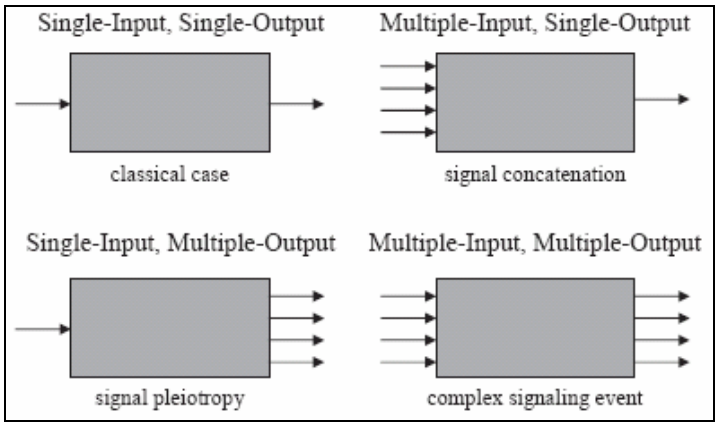


Figure 2.3. Classification of signal transduction input–output relationships (Papin and Palsson, 2004a)

2.2. Signaling Network Reconstruction

The reconstruction of large scale cellular signaling networks is not only enabled by the extensive studies on cell signaling mechanisms over the past 20 years, but also by the existence of high-throughput technologies. The cellular signaling networks, those being actively studied, focus on various target cell behaviors (phenotypes) including; transcriptional regulation (Papin *et al.*, 2005), mechanotransduction, cytoskeletal organization, organelle assembly and metabolism (Helmke and Schwartz, 2004). The descriptions of signaling mechanisms in ever-more detail by means of the recent genomic technologies have resulted in the frequent reconstructions of signaling networks, whose sizes keep increasing (Papin *et al.*, 2005). Some examples are the networks recently reconstructed by Papin and Palsson (2004a), Oda *et al.* (2005a), Oda *et al.* (2005b) and Oda and Kitano (2006). Such reconstructions will enable a systemic understanding of signaling network function, which is crucial for studying diseases as diverse as asthma and cancer (Finkel and Gutkind, 2003). However; properties, which arise from the whole-cell function, can be characterized on the condition that relationships between different cellular components are described mathematically in an integrated manner (Levchenko, 2003; Weng *et al.*, 1999).

Network reconstruction is defined as the process of integrating different data sources (i.e. incorporating the interconnectivity and functional relationships that are inferred from experimental data) to create a chemically accurate representation of the chemical events which underlie a biochemical reaction network (Papin *et al.*, 2005). Each of the genome annotation, biochemical experimentation, cell-physiology characterizations, expression arrays, and other similar sources provide different types of datum contributing to the reconstruction of a given cellular signaling network (Papin *et al.*, 2005).

Due to the fact that the comprehensive data regarding the interactions in a network usually lacks, signaling network reconstruction can be performed by focusing on three different approaches (Fig. 2.4), namely reconstruction of highly connected nodes (hubs), forming linear pathways and identifying signaling modules (Meyer and Teruel, 2003). The first approach involves comprehensively listing the compounds and reactions that are associated with a given protein, ion or metabolite (Papin *et al.*, 2005). The linear pathways, which are formed in the second approach to network reconstruction, connect signaling inputs to signaling outputs. For example, such a pathway might be delineation of all of the steps from the binding of a growth factor to its receptor through to the subsequent activation of a transcription factor that induces the expression of target genes (Papin *et al.*, 2005). The modules, which are identified in the third approach, historically consist of groups of compounds and proteins that function together under certain conditions. The detailed kinetic analyses, those being enabled by means of the modules, revealed the concentrations of various effector proteins and helped to understand processes such as feedback mechanisms (Papin *et al.*, 2005). For example, the EGF-receptor system has been extensively analyzed, and the effects of receptor internalization and autocrine signaling loops have been described in detail (Wiley *et al.*, 2003).

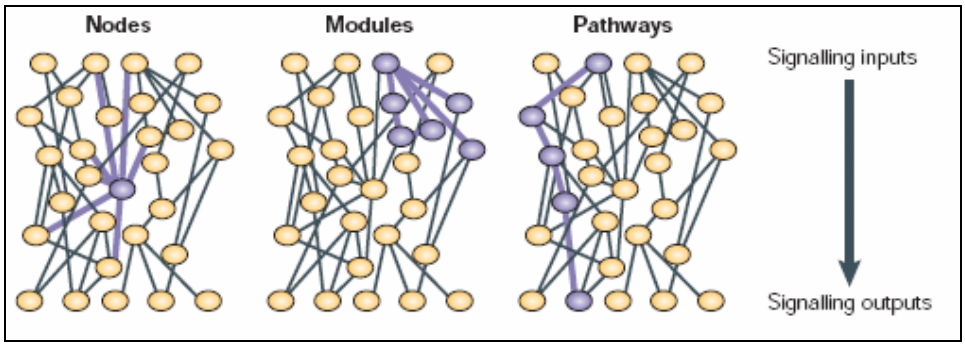
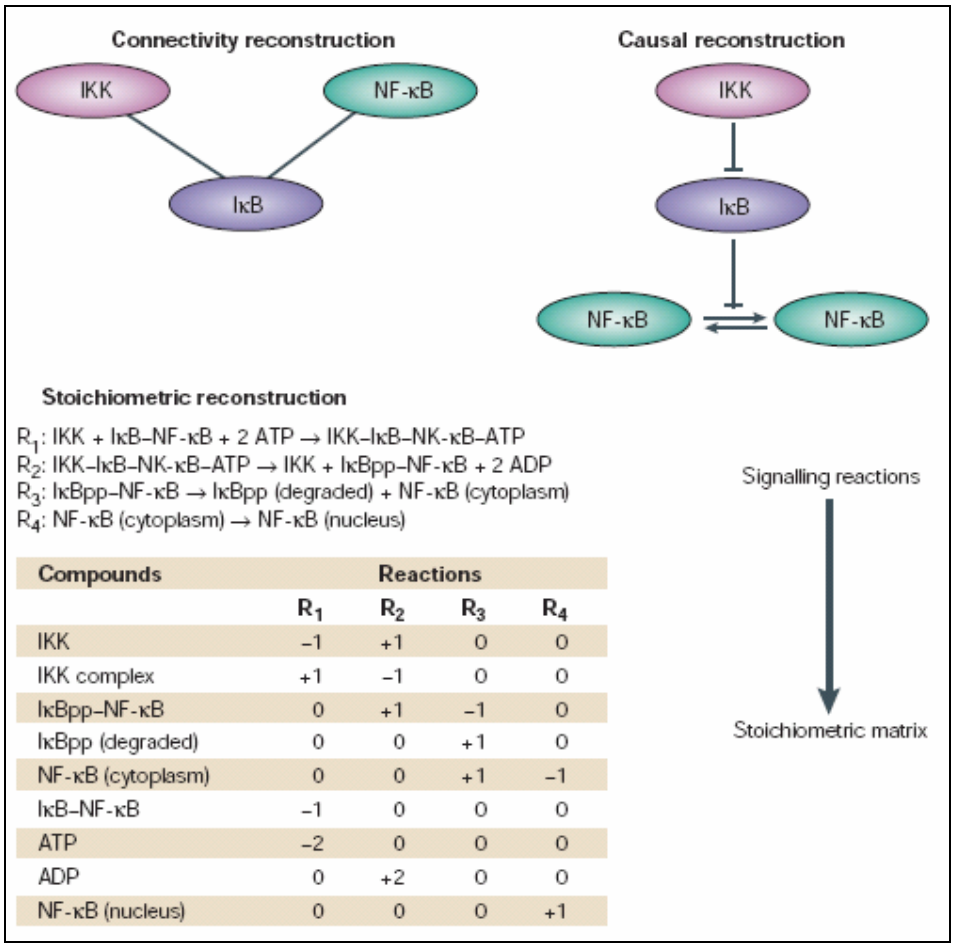


Figure 2.4. Approaches in reconstruction of signaling networks (Papin *et al.*, 2005)

Reactions amongst components in signaling networks are chemical transformations. There are three levels of resolution in reconstructions; namely connectivity, causal and stoichiometric reconstructions (Fig. 2.5) and these levels are classified according to the desired level of detail, which is a primary function of the amount of available data (Papin *et al.*, 2005). A connectivity reconstruction, embodying the first level of detail, lists the associations between network components as a simple connectivity (for example, nuclear factor (NF)-κB is functionally connected to IκB kinase (IKK)) or a more involved set of relationships, which shows the intermediates between signaling input and output (for example, NF-κB is functionally connected to IKK through the inhibitor of NF-κB (IκB)). The next level of detail in reconstruction, i.e. the causal reconstruction, describes cause-and-effect relationships and is often analyzed with differential equations (for example, IKK interacts with the IκB–NF-κB complex such that NF-κB is activated). The most detailed reconstruction, i.e. the stoichiometric reconstruction, involves kinetic relationships. As signaling reactions are chemical transformations, they can also be represented by a more mechanistic description such as a stoichiometric matrix. This representation accounts for all chemical events that occur in a given network. For example, one IKK complex binds to and phosphorylates one IκB–NF-κB complex with two ATP molecules, which leads to the degradation of IκB and the nuclear localization of NF-κB (R_1-R_4). This relationship can be written out as a series of stoichiometric equations and its accompanying matrix (Figure 2.5) (Papin *et al.*, 2005).



Compounds	Reactions			
	R_1	R_2	R_3	R_4
IKK	−1	+1	0	0
IKK complex	+1	−1	0	0
IκBpp–NF-κB	0	+1	−1	0
IκBpp (degraded)	0	0	+1	0
NF-κB (cytoplasm)	0	0	+1	−1
IκB–NF-κB	−1	0	0	0
ATP	−2	0	0	0
ADP	0	+2	0	0
NF-κB (nucleus)	0	0	0	+1

Figure 2.5. Three levels of resolution in reconstructions (Papin *et al.*, 2005)

Experimental techniques are continually being developed to identify the components and decipher the interactions in cellular signaling networks (Papin *et al.*, 2005). The existing biological knowledge for a given system is composed of several types of datum obtained by applying several experimentation methods (the big box at the bottom of Figure 2.6). Each datum type provides unique information that can be incorporated into a chemically accurate reconstruction (for example, a stoichiometric matrix). The first type of datum includes the identification of components and endpoints of a network (for example, genome sequencing or genome-wide location analysis). The second type of datum characterizes the interactions between network components (for example, yeast two-hybrid and immunoprecipitation data identify protein–protein interactions and protein complexes). The third datum type describes the network behavior of the integrated components (for example, perturbation analysis and cDNA arrays delineate how entire networks function

under various conditions). Each of these results provides unique types of datum that can be used to generate a cellular network reconstruction (for example, genome sequencing enables the annotation of the genes that are present in a given organism) (Papin *et al.*, 2005).

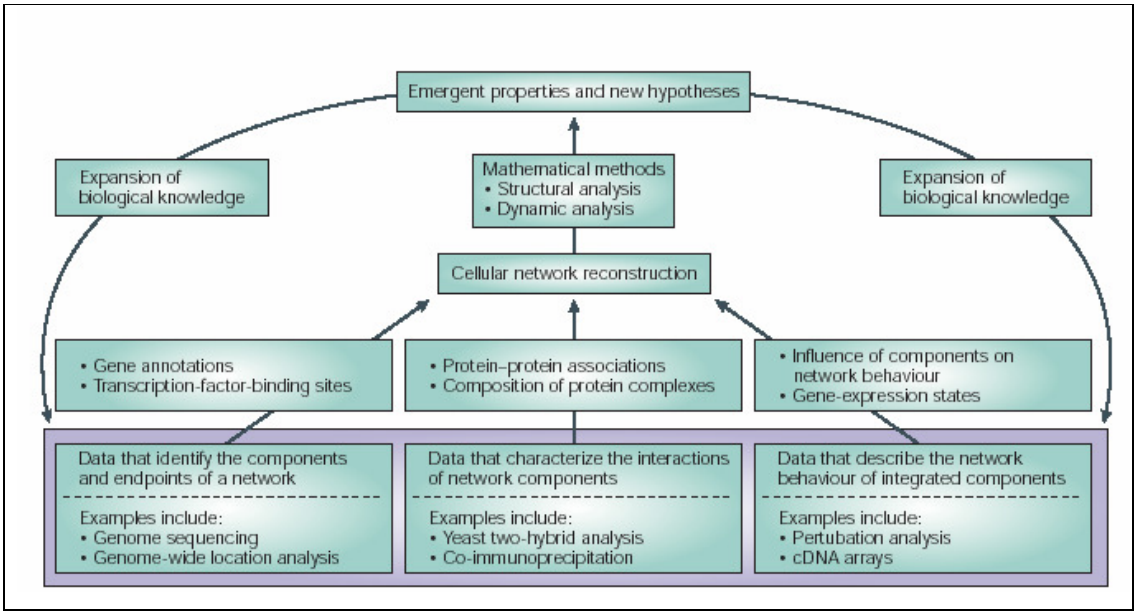


Figure 2.6. Integrative and iterative process of cellular signaling network reconstruction (Papin *et al.*, 2005)

With a network reconstruction, dynamic and structural analysis techniques can be used to describe emergent properties of the network, and generate new hypotheses. The present biological knowledge for the given system then expands and gets revised based on these characterizations. This process can be iterated to offer increasingly more accurate descriptions of a given biochemical network (Papin *et al.*, 2005).

2.3. Mathematical Analysis of Signaling Networks

Whole-network analyses are necessary to elucidate the global properties enlightening the complexity of the signaling systems. Network reconstructions allow the application of mathematical methods which can quantitatively describe the properties of signaling networks (Papin *et al.*, 2005). Large-scale signaling networks are complex and their

complexity necessitates the use of methods from systems sciences, which are quite mathematical, to understand the network properties of cellular signaling through dynamic and structural analyses. These analyses measure the time-variant and the time-invariant/topological properties of a network, respectively and the different results provided can be integrated to characterize the properties of reconstructed signaling networks (Papin *et al.*, 2005).

2.3.1. Dynamic Analysis of Signaling Networks

A dynamic analysis of a reconstructed signaling network can be carried out once the associated kinetic parameters are known. The timescales that are associated with signaling processes can be estimated, and crudely divided into two groups, namely signaling activities and signaling responses (Papin *et al.*, 2005). Signaling activities typically occur rapidly, such as most of the protein conformational changes, kinase/phosphatase reactions (Goodman *et al.*, 1998; Vuong *et al.*, 1991), and the physical movement of signaling compounds by diffusion or cytoskeleton-dependent mechanisms (Teruel *et al.*, 2000; Theurkauf *et al.*, 1994), all of which occur over a timeframe that ranges from fractions of a second to seconds. However, signaling responses can occur over a wider range of timescales. For example, signaling responses that are coupled with metabolic processes or intermediate phenotypes (Stryer, 1995; Neves *et al.*, 2002), and elements of chemotactic and mechanotransduction behavior (Stryer, 1995) can occur over a timeframe of fractions of a second whereas other signaling responses occur over a timescale that is an order of magnitude slower. For example, transcriptional events (Zubay, 1973; McAdams and Arkin, 1998), cellular growth (Alberts *et al.*, 2002a) and receptor internalization (Bomsztyk *et al.*, 1989; Chang *et al.*, 1996; Jullien *et al.*, 2002) require several minutes, or longer, in response to a signal. This timescale separation is a crucial consideration for dynamic network analyses and can lead to simplifications that enable more thorough analyses, which would otherwise be difficult (Papin *et al.*, 2005).

As numerical values for kinetic parameters are typically difficult to obtain (Bailey *et al.*, 2001), dynamic analyses are usually only carried out for causal and stoichiometric reconstructions (Figure 2.5) of smaller cellular signaling network reconstructions. These studies have analyzed complex network properties and the coupling of experimental data

with these mathematical analyses can enable the identification of previously unknown signaling mechanisms. An elegant study that shows the benefit of integrative experimental and mathematical analyses deciphered the importance of particular IκB isoforms in feedback loops that involved the NF-κB signaling module. Predictions were experimentally verified in knockout mouse models (Hoffman *et al.*, 2002). The WNT signaling module, which is important for development as well as oncogenesis, was recently represented using an extensive set of kinetic reactions (Lee *et al.*, 2003). Predictions were made for dynamic profiles of concentrations of β-catenin and other signaling mediators, and these matched experimental results. These dynamic analyses of signaling modules show the complex properties that can be studied once the reconstruction of only a limited number of reactions have been completed and experimental data are integrated with the model predictions (Papin *et al.*, 2005).

2.3.2. Structural Analysis of Signaling Networks

In large signaling networks, where a simple visual inspection is not possible and precise quantitative models are infeasible, structural analysis will be useful (Klamt *et al.*, 2006) and these networks can entirely undergo structural analysis, as this does not require an extensive knowledge of the parameters that have been determined from detailed experimentation (Papin *et al.*, 2005). Structural analyses of connectivity reconstructions (Figure 2.5) can generate hypotheses regarding the structure of the global network as well as the function of individual proteins (Figure 2.7). Recently published examples illustrate the analyses that have led to hypotheses concerning global, modular and individual protein function (Papin *et al.*, 2005). Those functions can be listed as: the scale-free nature (will be discussed in Section 2.3.2.2) of the yeast protein-protein interaction network (Jeong *et al.*, 2001), the partitioned groups of proteins associated with known signaling families such as the RAS-ERK/MAPK pathway by clustering analysis (Rives *et al.*, 2003), and the defined groups of protein-protein interactions having significant functions in the yeast proteome by spectral analysis (Bu *et al.*, 2003), respectively. Initial structural analyses of causal reconstructions (Figure 2.5) of signaling networks have also highlighted the value of these analyses in describing network properties (Schuster *et al.*, 2000). So far, stoichiometric analyses of signaling networks are limited, owing to a lack of corresponding reconstructions. However, the structural analysis of the network features of a

stoichiometric reconstruction (the JAK (Janus-activated kinase) – STAT (signal transducer and activator of transcription) signaling network), by Papin and Palsson (2004b), has led to descriptions of protein synthesis requirements and energy demands of signaling networks, as well as mathematical definitions of network properties such as crosstalk and pathway redundancy (Papin *et al.*, 2005).

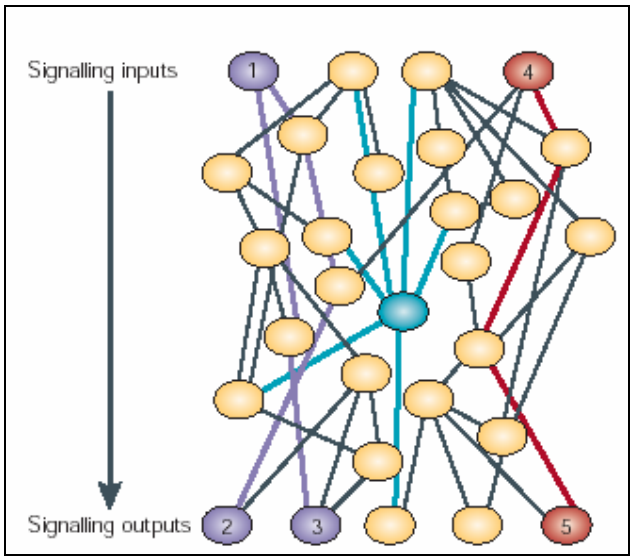


Figure 2.7. Structural analyses of signaling networks (Papin *et al.*, 2005)

Structural analyses can identify components that are well (hubs) or poorly connected and therefore of potential interest for drug targeting (Papin *et al.*, 2005; Nikolsky *et al.*, 2005). For example, the blue component is the most highly connected node in the schematic of a signaling network (Figure 2.7), and so drugs that inhibit the activity of this hypothetical component could have the broadest effect on the functions of the network. Structural analyses can also characterize which signaling inputs generate which signaling outputs. For example, in the schematic of signaling network (Figure 2.7) the signaling inputs 1 and 4 can generate signaling outputs 2, 3 and 5.

As a result, it can be considered that the logic behind signaling networks can be conceived better by constructing a comprehensive map of molecular interactions because it provides the data necessary to perform architectural analyses (will be discussed in Sections 2.3.2.1 and 2.3.2.2) even in the absence of kinetic parameters (Papin *et al.*, 2005; Oda and

Kitano, 2006). But this process is not direct as it seems because few methods have been proposed for the structural analysis of a given signaling network (Klamt *et al.*, 2006), whereas the reconstruction of signaling networks can be employed by using a large number of theoretical methods based on perturbation experiments (Styczynski and Stephanopoulos, 2005). Besides, structural analysis methods have been barely developed and applied to signaling and regulatory networks unlike the structural analysis of metabolic networks, which is a well-established field (Klamt *et al.*, 2006).

2.3.2.1. Pathway Analysis. Metabolic Pathway Analysis (MPA) enables the screening for a number of different flux distributions or even the computation of all theoretical possible pathways within the defined metabolic network (Förster, 2003). In pathway analysis, the topology of biological networks is investigated via fundamental routes (pathways) in the networks (Schuster *et al.*, 1999) and through this analysis, the structure of the network and the overall metabolic capabilities of the cell can be defined (Çakır, 2006). Thus, MPA is one of the main approaches for the flux analyses of metabolic networks (Papin *et al.*, 2003; Schilling *et al.*, 1999). There are three different approaches to find these routes. In the first approach, all possible linear paths from inputs to outputs are constructed (Seressiotis and Bailey, 1988; Mavrovouniotis *et al.*, 1990; Mavrovouniotis, 1992; Steffen *et al.*, 2002). A set of linearly independent basis vectors in flux space can be found using the second approach (Fell, 1990; Schilling and Palsson, 1998) and finally the third approach is to adopt the concept of 'elementary flux modes' (conically independent basis vectors), which was proposed and improved by Schuster and Hilgetag (1994) and Schuster *et al.* (1996). According to the last concept, any steady state flux distribution of a network can be defined in terms of non-negative linear combination of these fundamental routes, i.e. elementary flux modes (EFM) (Schuster *et al.*, 1996).

Although metabolic and signaling networks resemble each other; some differences such as the lack of mass flow conservation and continuity of the reaction flux in signaling networks, does not allow the adoption of elementary flux mode concept for pathway analysis of signaling networks, as it is in metabolic networks (Zevedei-Oancea and Schuster, 2005; Klamt *et al.*, 2006). Klamt *et al.* (2006) have been able to compute an equivalent concept in signaling networks to the elementary flux mode in metabolic networks, by determining the feedback cycles (i.e. whether an interaction is activation or

inhibition) and all the signaling paths between any pair of species. In this thesis, a similar approach is adopted in the pathway analysis but the main differences are the consideration of the feedback cycles after the results are obtained and finding not the elementary flux modes but the linear paths between inputs (ligands) and outputs (phenotypes) (linear pathway analysis). The calculation of elementary flux modes is not preferred due to the large size of the network in consideration (Toll-like Receptor (TLR) signaling network). MPA is computationally incapable when the number of reactions taking place in the cell (in metabolic networks) is high (Acuner and Uzun, 2006). For example, according to an elementary flux mode calculation, an increase in the number of reactions by 4-fold may increase the number of the resulting EFMs by 1000-fold (14 reactions and 8 EFMs vs. 58 reactions and 8726 EFMs) (Acuner and Uzun, 2006). Similarly in a large-scale signaling network, the number of interactions exceeds the level required for the application of MPA by yielding an abundance of fundamental routes that it is computationally unmanageable to adopt the approaches suggested above.

2.3.2.2. Analysis Based on Graph Theory. The study of networks in the form of mathematical graph theory is one of the fundamental foundations of discrete mathematics (Newman, 2003). The first true proof in the theory of networks was Euler's solution of the Königsberg bridge problem by using graph theory (for the first time in history) in 1735 (Newman, 2003). The emergence of new methods such as graph theory for enabling the structural analyses of networks, is due to the fact that human eye is inadequate to picture and gain an understanding of the structure of networks including hundreds of interactions. During the twentieth century, graph theory has significantly developed. In recent years, network researches have focused on large-scale statistical properties of graphs such as path lengths and degree distributions, rather than the analysis of single small graphs. Those properties are important for the characterization of the structure and behavior of networks (Newman, 2003).

In mathematical representation of protein interaction networks (graphs); proteins are the nodes (vertices), and a node is the fundamental unit of the network, whereas the connections (links) between proteins are edges and they represent the binary interactions of the nodes in the graph. Figure 2.8 is an example of a small network with six nodes and seven edges.

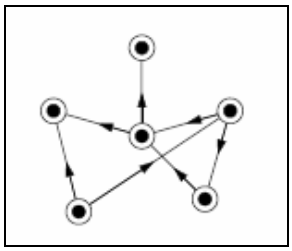


Figure 2.8. Vertices (nodes) and edges (links) in a small directed network (Newman, 2003)

Glossary of some terms related with the graph theory is presented below, in order for the concepts to be understood better.

Directed / Undirected: If an edge runs in only one direction (Newman, 2003), i.e. every edge is assigned an initial and a terminal vertex (Diestel, 2005), it is directed but if the edge runs in both directions it is undirected (Newman, 2003). A graph is then directed if all of the edges it includes are directed (Newman, 2003).

Degree (Connectivity): Degree is a characteristic of nodes, indicating how many links there are between a node and the other nodes in the network. In other words, degree of a node is the number of edges connected to it (Newman, 2003), i.e. the neighbors of the node (Diestel, 2005). It is denoted by "k" in undirected networks. In directed networks; there are two representations as "k_{in}" and "k_{out}" for incoming edges (interactions) to the node and outgoing edges (interactions) from it, respectively.

Hub: Hub is defined as a highly connected node (Barabási, 2002), i.e. a node with a high degree (number of neighbors). The hubs in the overall network (e.g. the blue component in Figure 2.7) are of special importance for drug targeting (Papin *et al.*, 2005; Nikolsky *et al.*, 2005) as they significantly affect the whole network through their crowded neighbors.

Path Length: Path length is a measure of distance in networks and the path length between two nodes specifies how many links are passed while traveling from one node to the other. Path length is a basic concept and there are many quantities calculated based on

it such as the shortest path length, geodesic path, mean path length and the diameter of the network.

Large-scale statistical properties such as network diameter, the shortest path length, the mean path length, clustering coefficient and degree distribution can be calculated by taking the maximum, minimum, average or probability distribution of the overall properties introduced above.

Shortest Path Length (SPL) (or Geodesic Path): SPL is the path with the smallest number of links between two selected nodes, i.e. the shortest path through the network from one node to another (Newman, 2003). The following two quantities are global properties of the network as all pairs of the nodes in the network are considered: *Mean path length (MPL) (or Characteristic path length)* is the average of the SPLs over all pairs of nodes (Watts and Strogatz, 1998), whereas the *network diameter* is the maximum SPL throughout the network (Newman, 2003).

Clustering Coefficient (C): Clustering coefficient is defined as the probability of two nodes to be neighbors, given that they are neighbors of the same node (Grabowski and Kosiński, 2007) and it is a measure of the cliquishness, i.e. tendency to associate with a specific group, of a network (Watts and Strogatz, 1998). Calculation of C for directed networks is explained in Section 3.2.2.2.

Degree Distribution (P(k)): The probability of a randomly chosen node having exactly k links is defined as degree distribution (Barabási, 2002; Newman, 2003). As being a probability, the value of $P(k)$ varies between zero and one. There are two different types of degree distributions, originating from different networks, namely Poisson and Power Law, those being specific to random and scale-free networks, respectively (Figure 2.9) (Barabási, 2002). The degree distribution of a random network follows a bell curve, i.e. Poisson distribution having a peak, implying that there are no hubs; whereas scale-free networks have a power law degree distribution, predicting that many nodes have a few links and they are connected to each other via a few major hubs (Barabási, 2002). A histogram following power law distribution does not have a peak; instead it is a

continuously decreasing curve from which it can be predicted that many proteins with small degrees coexist with a few proteins having large degrees (Figure 2.9).

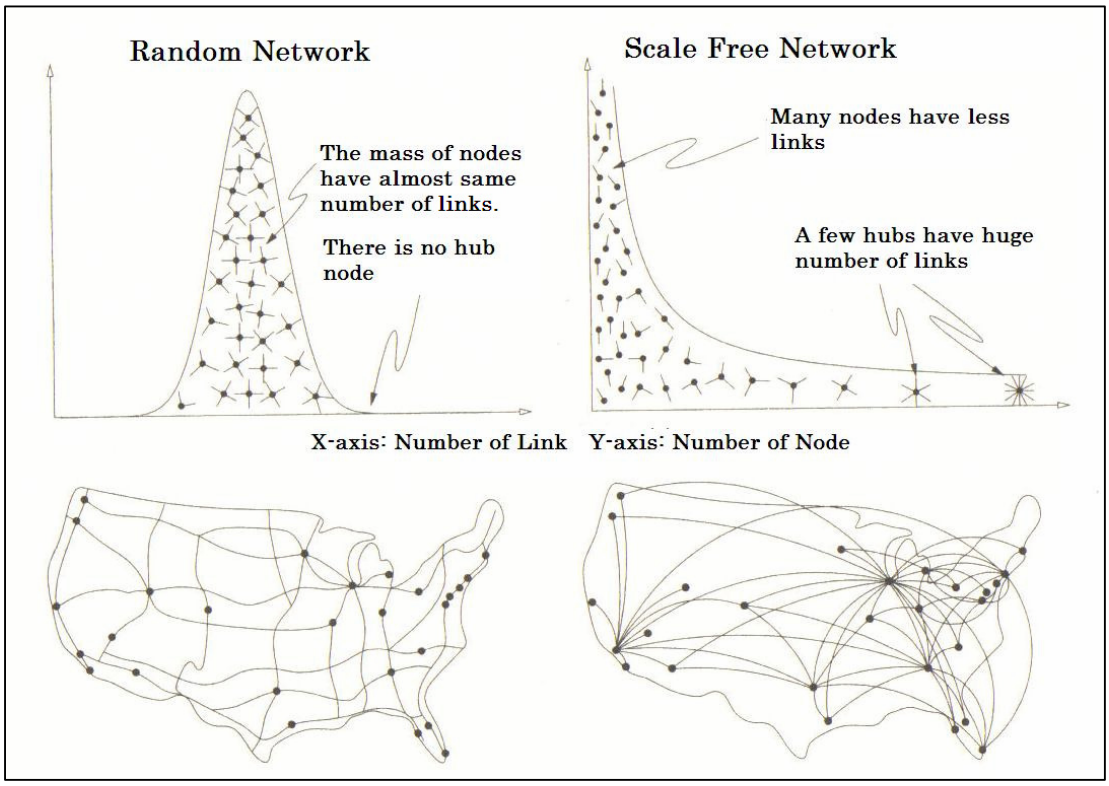


Figure 2.9. Random and scale-free networks (Barabási, 2002)

Degree Exponent: Degree exponent is a unique exponent by which each power law is characterized. The Poisson distribution follows $P(k) \sim e^{-\beta k}$ relation (implying that probability of finding a highly connected node decays exponentially for k values larger than the average k) and therefore when k vs. log(P(k)) is plotted, a line is obtained and its negative slope gives the degree exponent, β multiplied by k (βk). On the other hand, the power law degree distribution follows the relation: $P(k) \sim k^{-\gamma}$ where the parameters k and γ are the degree and the degree exponent, respectively. Degree exponent, being the negative slope of the straight line, can be directly found from the log-log plot of k vs. P(k). Barabási (2002) suggests that degree exponent, for most of the large systems, varies between two and three.

Until recently, complex networks have been modeled using the classical random network theory (Erdös and Renyi, 1965; Bollobas, 1985), which assumes that each pair of nodes in the network is connected randomly with probability P(k), leading to a statistically homogeneous network in which, despite the fundamental randomness of the model, most nodes have the same number of links (the average k value (<k>) at the peak). On the other hand, empirical studies on the structure of the World Wide Web (Albert *et al.*, 1999; Faloutsos *et al.*, 1999), social networks (Barabási and Albert, 1999) and scientific collaboration network (Barabási *et al.*, 2002) have reported serious deviations from this random structure, showing that these systems are described by scale-free networks (Barabási and Albert, 1999).

As the distinction between scale-free and exponential networks emerges as a result of simple dynamic principals (Amaral *et al.*, 2000; Dorogovtsev and Mendes, 2000), understanding the large-scale structure of cellular networks cannot only provide valuable and perhaps universal structural information, but could also lead to a better understanding of the dynamic processes that generated these networks. In this respect the emergence of power-law distribution is intimately linked to the growth of the network in which new nodes are preferentially attached to already established nodes (Barabasi and Albert, 1999), a property that is also thought to characterize the evolution of biological systems (Hartwell *et al.*, 1999).

2.4. Toll-like Receptor (TLR) Signaling

Toll-like receptor (TLR) signaling is a crucial part of immune system since TLRs are accepted as the key molecules that alert the immune system in the presence of microbial infections and they appear to be one of the most ancient, conserved components of the immune system of both invertebrates and vertebrates (Alberts *et al.*, 2002b).

Since mid-1800s, scientists have been trying to identify the agents causing infectious diseases (Tuberculosis, malaria, AIDS, etc.), i.e. pathogens. Pathogens can rapidly evolve in order to infect the host's cell with numerous methods and humans develop mechanisms to resist these infections. There are two main defenses, namely; innate immune responses

and adaptive immune responses. The first mechanism can take action immediately after the infection and the action does not depend on whether the host has exposed to the pathogen earlier or not. In addition to being the protector for the host during the first critical hours of infection, innate immune responses are also required to activate adaptive immune responses. Being the second defense mechanism, adaptive immune responses act slower but more powerfully and are highly specific for the pathogen (Alberts *et al*., 2002b).

Operation principle of the innate immune system can be defined as recognizing particular types of molecules which are not present in host but are common to many pathogens. These molecules are known as pathogen-associated immunostimulants and their detection in the host results in inflammatory response (pain, redness, heat and swelling at the site of infection) and in phagocytosis of the immunostimulants by phagocytes (Figure 2.10) (Alberts *et al*., 2002b).

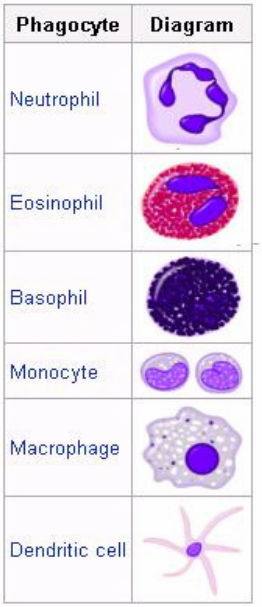


Figure 2.10. Phagocyte types

There are various pathogen-associated immunostimulant types, embodied by viruses, bacteria and protozoan pathogens, some of which are listed below (Alberts *et al*., 2002b; Gazzinelli and Denkers, 2006):

- Any peptide containing formylmethionine at the N-terminus, which is of bacterial origin,
- Cell-wall components and structural proteins in bacteria (Figure 2.11):
  - Peptidoglycan cell wall (TLR2 ligand),
  - Flagella of bacteria (TLR5 ligand),
  - Lipopolysaccharide (LPS) on Gram-negative bacteria (TLR4 ligand),
  - Lipoteichonic acids (lipopeptides) on Gram-positive bacteria (TLR2 ligand),
  - Short sequences in bacterial DNA such as "unmethylated CpG motif" (TLR9 ligand),

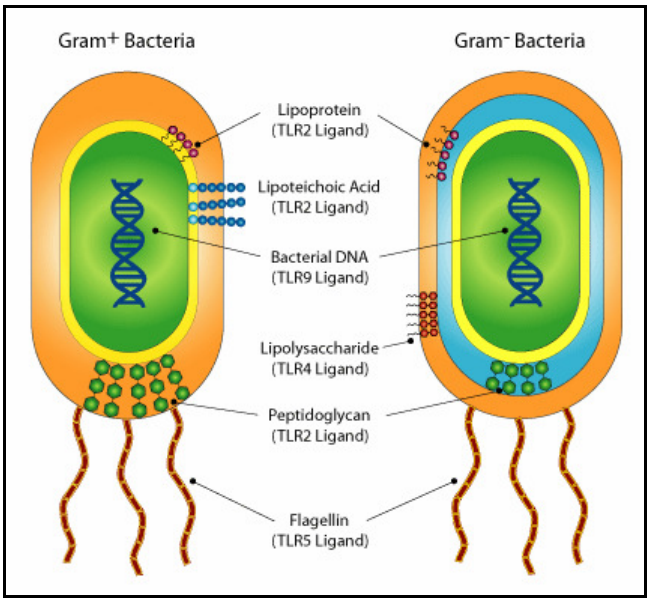


Figure 2.11. Some immunostimulants on bacteria

- Short sequences in viral DNA such as "unmethylated CpG motif" (TLR9 ligand),
- Double-stranded RNA of viruses (TLR3 ligand),
- Single-stranded RNA of viruses (TLR7 and TLR8 ligand),
- Zymosan, glycan and chitin in the cell walls of fungi,
- Glycosylphospatidylinositol (GPI) anchors in *Plasmodium* (parasite) (TLR2 and TLR4 ligand).

These immunostimulants occur on the pathogen surface in repeating patterns which are recognized by pattern recognition receptors (PRRs) including soluble receptors in the blood and membrane-bound receptors on the surface of host cells. Many of the mammalian cell-surface pattern recognition receptors are members of the Toll-like receptor (TLR) family (Alberts *et al*., 2002b) and they are capable of sensing organisms ranging from protozoa to bacteria, fungi to viruses (Bhattacharjee and Akira, 2005).

TLR proteins are encoded by *Toll* genes, which were first identified in the early 1980s by Nusslein-Volhard and Anderson while they were trying to identify the genes controlling the establishment of the dorso-ventral axis of the fruit fly *Drosophila melanogaster* embryo (Belvin and Anderson, 1996). In addition to the original function, Toll was also assigned an immune function in the control of the expression of antimicrobial peptides (Belvin and Anderson, 1996). Toll pathway was then analyzed for immunodeficiency phenotypes and it was concluded that Toll pathway controls the response to fungal and Gram-positive bacterial infections (Lemaitre *et al.*, 1996; Rutschmann *et al.*, 2002; Alberts *et al*., 2002b).

In mammalians, 11 TLRs have been identified and these TLRs form a receptor superfamily with the interleukin 1 receptors (TLR/IL-1R Superfamily) all of which have TIR (Toll/IL-1 receptor) domain, containing a conserved region of amino acids, in common (Akira, 2003; Oda and Kitano, 2006). TLR/IL-1R superfamily signaling (a compact form is in Figure 2.12) is mediated through proteins: MyD88, IRAKs, TAK1, TAB1, TAB2, TRAF6, etc (Oda and Kitano, 2006).

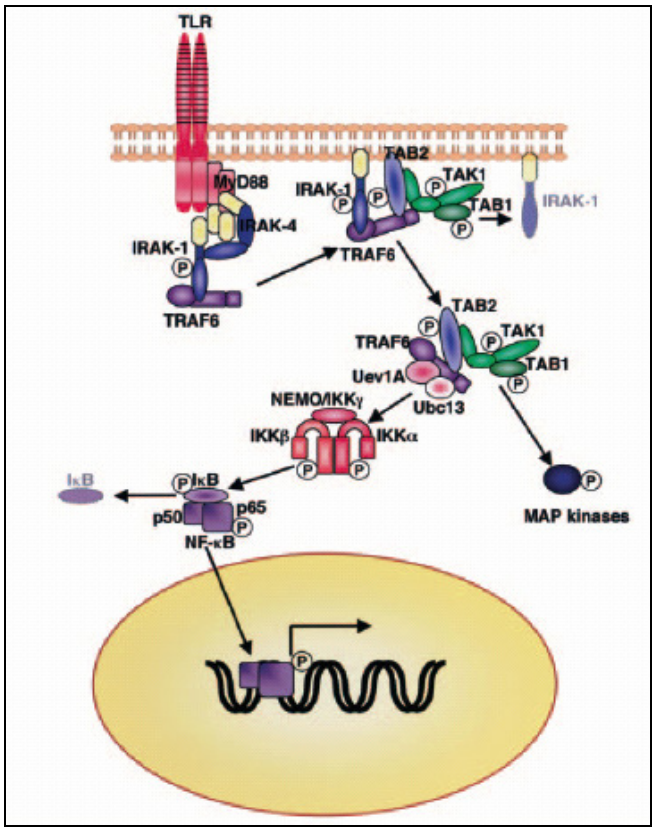


Figure 2.12. TLR/IL-1R superfamily (Akira, 2003)

2.5. The Role of Sphingolipids in Immunity

Sphingolipids, a major class of lipids in cell membranes, play various roles in biological processes. As bioactive and structural molecules, they have signaling activities and biophysical properties that are essential for regulating various cellular, tissue and systemic functions. Moreover, sphingolipids are receiving increasing attention as contributors to the pathogenesis of several human disorders, including, cancer, inflammation and neurological, immune and metabolic disorders (Zeidan and Hannun, 2007).

Inflammation is a local and systemic response to infectious, allergic or injurious agents that can lead to pathological consequences (e.g. tissue fibrosis and cellular transformation) when left uncontrolled. A growing body of literature supports a pleiotropic

role for S1P (Sphingosine-1-Phosphate) and C1P (Ceramide-1-Phosphate) in the inflammatory cascade (Chalfant and Spiegel, 2005).

An example of the proinflammatory activity of S1P and C1P is the promotion of macrophage survival and cytokine production. Whereas Sphingosine-1-Phosphate Receptors (S1PRs) are differentially involved in the various steps of inflammation, C1P might act by directly engaging intracellular effectors (such as cPLA2) in light of the fact that no C1P receptors have been identified yet. Therefore, based on the literature, one could envision Sphingosine kinase (SK), CK and S1PRs as potential targets for anti-inflammatory therapy (Zeidan and Hannun, 2007).

Gram-negative sepsis remains a leading cause of death in the surgical intensive care unit (Dunn, 1994). During this syndrome, an excessive inflammatory response occurs, leading to the development of multiple organ dysfunction syndrome and adult respiratory distress syndrome (Sharma and Kumar, 2003). Several different inflammatory cells are involved, but the macrophage is central to the regulation of the inflammatory process through production of a number of inflammatory mediators (Jarrar *et al.*, 1999).

Endotoxin, derived from the outer membrane of gram-negative bacteria, is in part responsible for the activation of the macrophage (Beutler and Poltorak, 2001). Activation of the macrophage by endotoxin requires binding to the Toll-4–like receptor (TLR4) complex. This receptor complex is composed of TLR4, CD14, and MD2 (Fujihara *et al.*, 2003). Assembly of this receptor complex appears to occur on microdomains, containing high concentrations of cholesterol and glycosphingolipids. These cholesterol-rich microdomains, known as lipid rafts (Triantafilou *et al.*, 2002), constitute a relatively large fraction of the plasma membrane (Pike, 2003) and they appear to function as platforms for signal transduction. Once the receptor complex is activated, a conformation change within TLR4 occurs, leading to intracellular phosphorylation and activation of the mitogen-activated protein kinases (MAPK), composed of extracellular signal-regulated kinase (ERK) 1/2, p38, and c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) (Guha and Mackman, 2001). The activation of these kinases is essential for the production and liberation of the various inflammatory mediators required for the subsequent host proinflammatory phenotype characteristic of sepsis.

3. MATERIALS AND METHODS

3.1. A Comprehensive Map of the Toll-like Receptor (TLR) Signaling Network

3.1.1. Reconstruction Process of the TLR Map

The map of TLR and IL-1R signaling networks (Figure 3.1) was constructed by Kanae Oda and Hiroaki Kitano, via assembling and interpreting multiple published articles (411 references) and including possible interactions verified by experiments (Oda and Kitano, 2006). Using these interactions, possible pathways in mammalians were incorporated via a modeling support software, CellDesigner ver.2.2 (http://celldesigner.org/) (Funahashi and Kitano, 2003). In order to be widely accessed and understood, the map should be constructed based on some standards and with a machine and human readable representation. This signaling network map is compatible with the Systems Biology Markup Language (SBML: http://sbml.org/) (Hucka *et al.*, 2003) and the symbols used for the representation of molecules and interactions are based on the process diagram to achieve a standard graphical notation system. The process diagram allows the representation of molecular interactions explicitly and it is based on the Systems Biology Graphical Notation (SBGN: http://sbgn.org/) (Kitano *et al.*, 2005) (Figure 3.2). For simplicity; all compounds, but proteins, genes, RNAs, and ions including lipids and carbohydrates, are assumed as 'simple molecule' (Oda and Kitano, 2006).

The map includes 444 reactions of different types namely; state, omitted and unknown transitions, associations, dissociations and transports. There are 652 species, i.e. entities taking part in the reactions, in the map and they are categorized as proteins, complexes (oligomers), RNAs, ions, degraded products, phenotypes, and genes (Oda and Kitano, 2006). Additionally; 28 more species, which are all pathogens such as bacteria, fungi, viruses, are included in the group of genes therefore making 680 species in total.

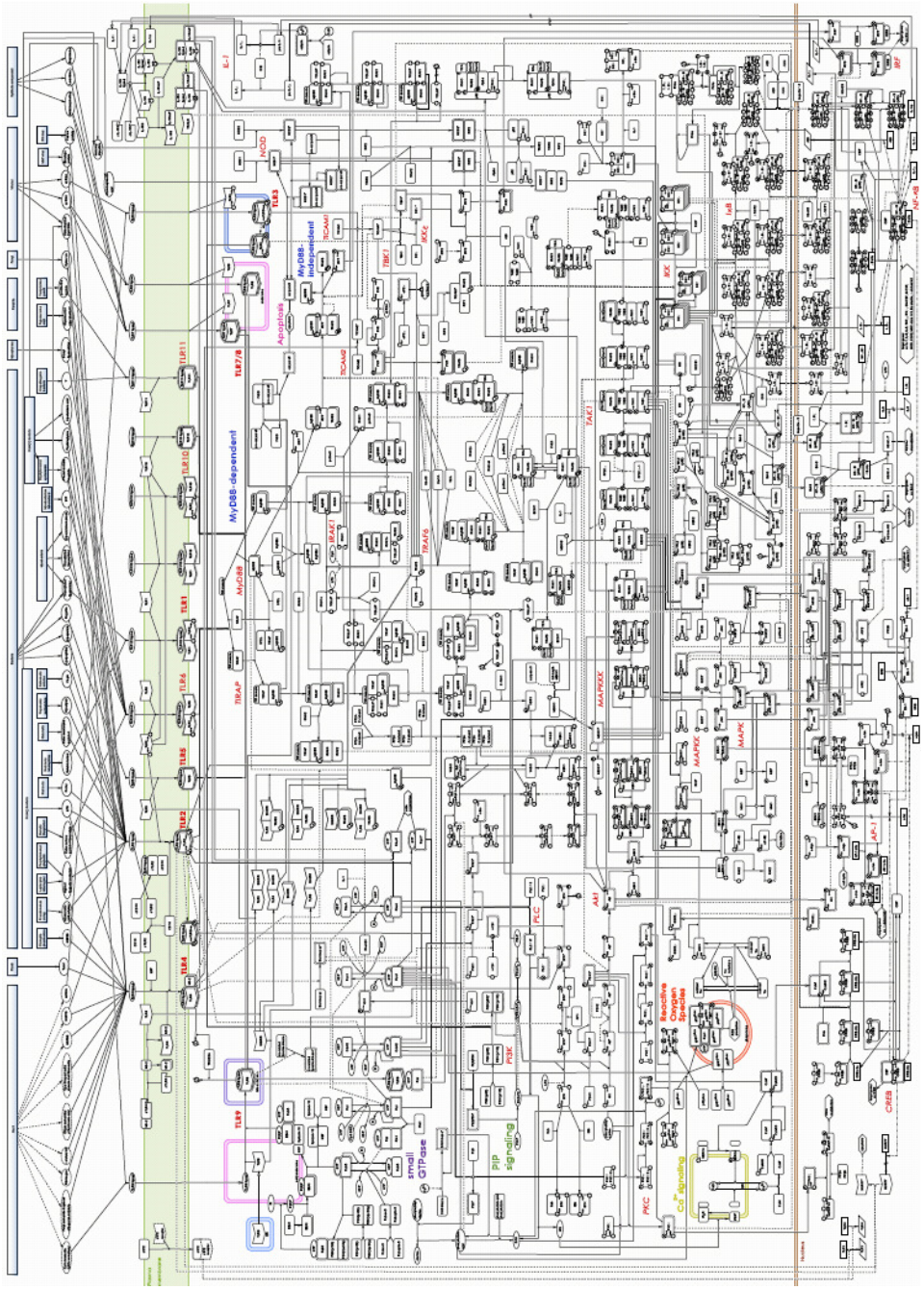


Figure 3.1. A comprehensive map of the TLR signaling network (Oda and Kitano, 2006)

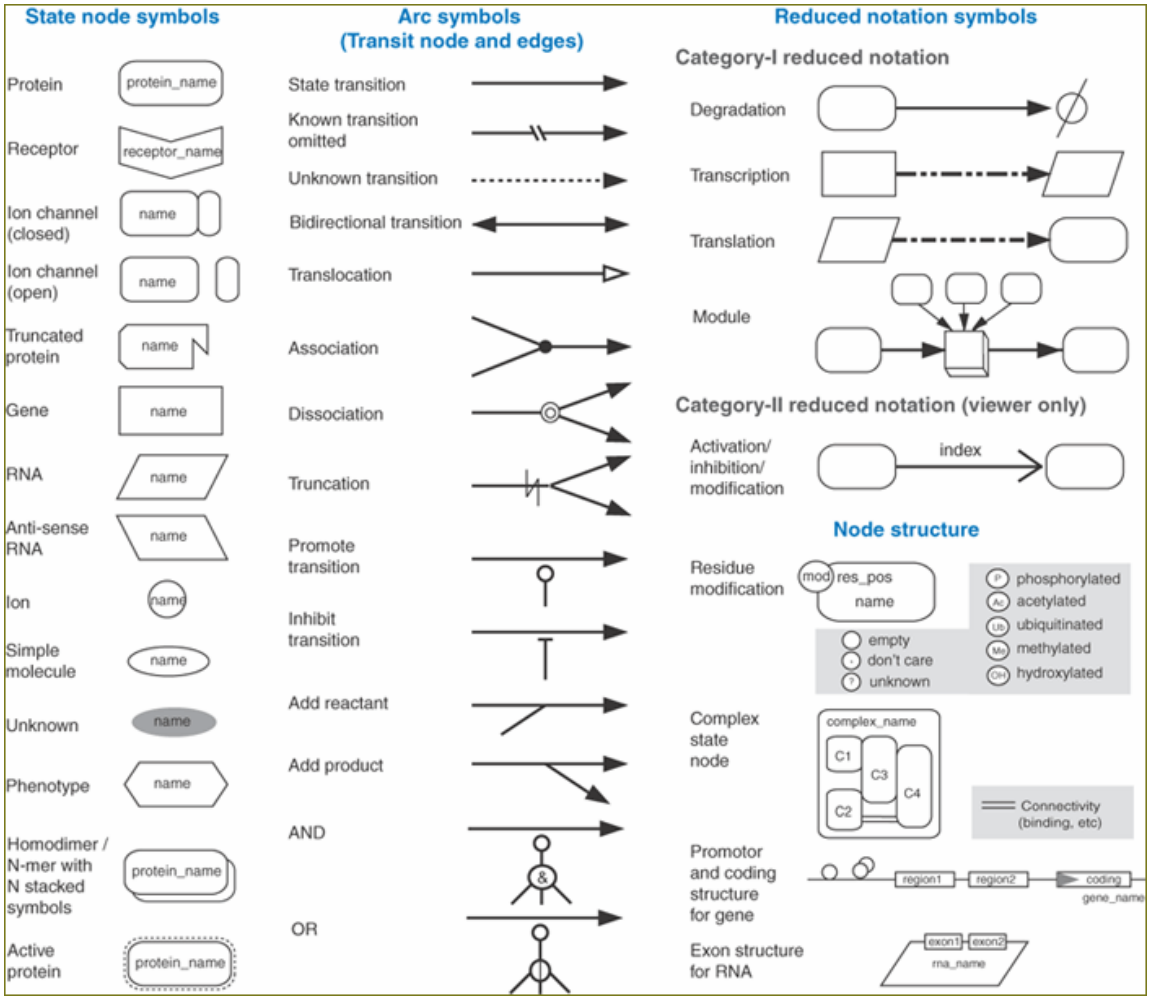


Figure 3.2. Set of symbols for representing biological networks with process diagrams (Kitano *et al.*, 2005)

While analyzing this map, pathways initiating from the ligands and leading to the phenotypes are of special importance (Section 2.1.2). Phenotypes are categorized as a distinct type of species in the TLR map of Oda and Kitano (2006) and are designated with a specific symbol (Figure 3.2), but ligands belong to the simple molecule and protein categories. TLR map embodies 14 ligands and 16 phenotypes (Table 3.1).

Table 3.1. Ligands and phenotypes in the TLR map [1]

Ligands	Phenotypes
TLR2	p. apoptosis
TLR3	p. actin organization
TLR4	p. ROS production
TLR5	p. protein synthesis
TLR7	p. translation
TLR8	n. target gene ex. Bcl-2
TLR9	n. cell cycle
TLR10	n. target genes ex. IFNα/β
TLR11	n. histone deacetylation
TLR1/2	n. apoptosis
TLR1/10	n. target genes ex. cyclin D1
TLR2/6	n. target genes ex. β-defensin 2
TLR2/10	n. transcription
IL-1 RI	n. RNA metabolism
	n. target genes ex. ICER
	n. target genes ex. IL-1, IL-8, IL-6, TNFα, IFNγ, GM-CSF, M-CSF, iNOS, COX2, MHC class I/II, E-selectin, β-defensin2

[1] whether a phenotype takes place in plasma or nucleus is denoted by an initial p or n in the phenotype name, respectively

3.1.2. Architectural Features of the TLR Map

The comprehensive map (Figure 3.1) is barely readable because of its complicated nature; therefore simplification is required in order to analyze the architecture of the global network. By only focusing on the flow of information and causal relationships, i.e. flows of activations and inhibitions, a simplified form of the map is created (Figure 3.3) (Oda and Kitano, 2006).

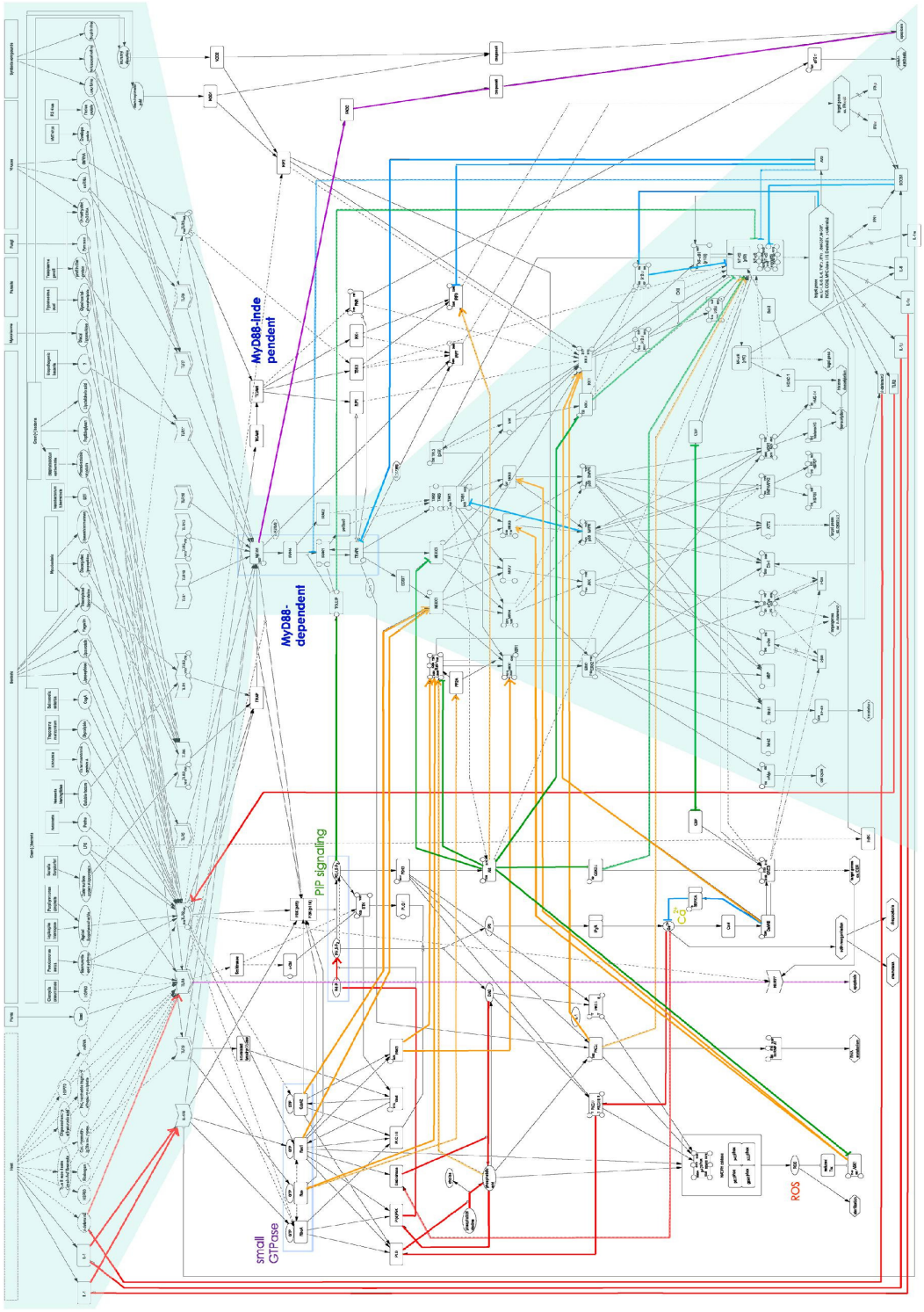


Figure 3.3. The architecture of the TLR signaling network (Oda and Kitano, 2006)

From the simplified map (Figure 3.3) it can be seen that TLR signaling pathways are approximately divided into four parts, namely MyD88 dependent and independent pathways, small GTPase module and PIP signaling (Oda and Kitano, 2006):

- Main system (Figure 3.4):
  - It includes MyD88-IRAK4-IRAK1-TRAF6 as a bow-tie core process (IRAK4 and IRAK1 are designated together as IRAK in Figure 3.4),
  - It activates nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) cascade, and as a result many target genes such as cytokines (essential for the innate immune response and the maturation and proliferation of the cell) are induced.

- Subsystems with a small GTPase module and phosphatidylinositol phosphate (PIP) signaling module:
  - They are collateral modules assisting the main bow-tie network,
  - The small GTPase module receives inputs from IL-1R, TLR9, TLR4, and TLR2,
  - The PIP signaling module receives inputs from IL-1R, small GTPase module, TLR2, TLR3, and MyD88,
  - Small GTPase and PI3 kinase (PI3K) activate NF-κB and MAPK (Arbibe *et al.*, 2000; Xu *et al.*, 2003; Sarkar *et al.*, 2004),
  - These subsytems have a crucial role in the battle against invaders and they cooperate to exclude pathogens by actin reorganization (leading to chemotaxis and phagocytosis) and to kill them by producing reactive oxygen species (ROS).

- MyD88-independent pathway (Figure 3.4):
  - It is limited to and stimulated by TLR3 and TLR4, through TICAM1 and TICAM2 (TICAM1 and TICAM2 are also known as TRIF and TRAM, respectively (Bhattacharjee and Akira, 2005)), i.e. TLR adaptor molecules, (Yamamoto *et al.*, 2003),

- It activates NF-κB and the interferon-regulatory factor (IRF) family, which in turn induces cytokines (e.g. IL-1, IFN–γ, TNF–α, etc.) and type I interferon (IFN–α, IFN–β),
- The induction of IL-1 activates autocrinely MyD88-dependent pathways and the two subsystems, resulting in the activation of the whole system.

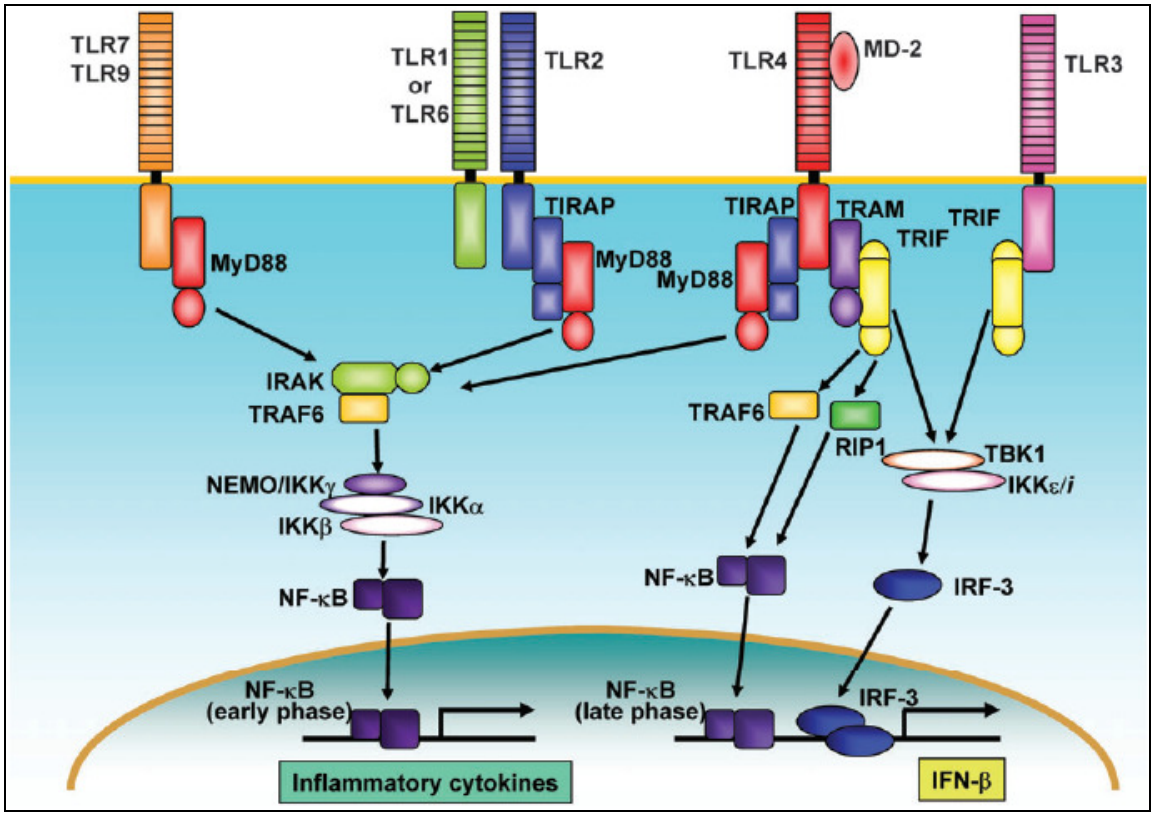


Figure 3.4. MyD88-dependent and -independent pathways (Takeda and Akira, 2005)

As it was mentioned, one distinctive characteristic of the main system (central subsystem) and in fact of the signaling network itself is having a bow-tie (hour-glass) structure (Beutler, 2004), where MyD88 is the essential core (Oda and Kitano, 2006). Systems having this type of structure are regarded as robust (Csete and Doyle, 2004) and robustness is claimed to be a requirement for evolvable systems since this feature enables a system to be stable against perturbations (Kitano, 2004). Although EGFR signaling network also has a bow-tie structure (Oda *et al.*, 2005), TLR signaling network differs

from it having supplementary pathways which assist the downstream behaviors of the main bow-tie network.

3.1.3. Feedback and Feedforward Controls

There are multiple system controls in the TLR system. In total, seven positive feedback and seven negative feedback loops are identified (shown in red and blue in Figure 3.3, respectively). Among positive feedback loops, the four loops (Nos. 1–4 in Table 3.2) play roles in the regulation from the output to the input, and one (No. 5) is in the bow-tie lower wing. Six negative feedback loops are classified as follows: two (Nos. 8 and 9) are in the bow-tie lower wing, two (Nos. 10 and 11) are from the output to the lower wing, one (No. 12) is from the output to the bow-tie core process, and the last one (No. 13) is from the output to the input. The remaining two positive (Nos. 6 and 7) and one negative (No. 14) feedback loops exist in the subsystems involved in the regulation of the concentration of the cytosol calcium. There are conflicting feedback loops. For example, the feedback from IL-1a and IL-1b to IL-1RI (Nos. 1 and 2, respectively) provides positive feedbacks, whereas the feedback from interleukin 1 receptor antagonist (IL-1ra) to IL-1RI (No. 13) provides a negative feedback. The map predicts balance of activation between IL-1 and IL-1ra affects proinflammatory response of the system. A recent paper reports that this is actually the case (Matsuki et al, 2006). In addition to these feedback controls, there is a possible negative feedforward control (shown in purple). MyD88 also mediates apoptosis via a Fas-associated death domain–caspase-8-dependent pathway, and TLR4 and TLR2 can induce apoptosis through an orphan nuclear receptor Nur77 by a caspase-independent pathway, although its precise mechanism is unclear (Kim et al, 2003). Thus, the TLR system induces the activation of the immunity to survive, while it prepares cell death at the same time. At a cell-level view, this mechanism could be considered as a negative feedforward control (Table 3.2) (Oda and Kitano, 2006).

Table 3.2. Feedback and feedforward controls in the TLRs system (Oda and Kitano, 2006)

	No.	Origin		Destination		Note
Feedback						
Positive	1	IL-1α	Transcriptional target of NF-κB	IL-1RI	Activates NF-κB via the MyD88-dependent pathway	
	2	IL-1β	Transcriptional target of NF-κB	IL-1RI	Activates NF-κB via the MyD88-dependent pathway	
	3	TLR2	Transcriptional target of NF-κB	MyD88, PI3K, small GTPase	Activates NF-κB	
	4	β-Defensin2	Transcriptional target of NF-κB	TLR4	Activates NF-κB via the MyD88-dependent and -independent pathway	Controversial
	5	IKKβ	Activated by NIK	TPL2(p58)	Activates NIK	Through the process yet identified
	6	PLD	Activated by PKC alpha, beta II, and cytosol Ca^{2+}	PI4,5-P2	Material of IP3 which increases cytosol Ca^{2+} via IP3R	
	7	DAG kinase	Activated by cytosol Ca^{2+}	PI4,5-P2	Material of IP3 which increases cytosol Ca^{2+} via IP3R	Through the process yet identified
Negative	8	NF-κB1(p105)	Activated by IKKβ	TPL2(p58)	Activates IKKβ via NIK	
	9	p38αMAPK	Activated by MKK3	TAK1	Activates MKK3	
	10	IκBα	Transcriptional target of NF-κB	NF-κB	Transcriptional factor of A20	
	11	A20	Transcriptional target of NF-κB	NF-κB	Transcriptional factor of A20	
	12	A20	Transcriptional target of NF-κB	TRAF6	Activates NF-κB via the MyD88-dependent pathway	
	13	IL-1ra	Transcriptional target of NF-κB	IL-1RI	Activates NF-κB via the MyD88-dependent pathway	
	14	CaMKII	Activated by cytosol Ca^{2+}	SERCA	Decreases cytosol Ca^{2+}	
Feedforward						
Negative	15	TLR2		NUR77	Causes apoptosis	
	16	TLR4		NUR77	Causes apoptosis	
	17	MyD88		FADD	Causes apoptosis via the activation of caspase-8	
	18	Src kinases	Activates BTK	BTK	Inhibited by Src kinases via c-Cbl	Through the process yet identified

3.1.4. Regulations Between Main System and the Subsystems

While describing the bow-tie structure, it was mentioned that unlike the EGFR signaling network, TLR signaling network has supplementary pathways assisting the downstream behavior of the central bow-tie subsystem. This difference stems from the several crosstalk regulations between the main bow-tie pathway and two subsystems. Positive and negative regulations are shown in orange and green in Figure 3.3, respectively. There are 13 positive and seven negative crosstalk regulations, all of which go towards the bow-tie lower wing. For example, small GTPases and ROS can stimulate MAPK cascade by nine ways, and v-akt murine thymoma viral oncogene homolog (Akt) can inhibit both MAPK cascade and NF-κB activation through five different mechanisms. From these facts, Oda and Kitano suggest that regulations from other systems concentrate in the bow-tie lower wing (Oda and Kitano, 2006).

3.2. Systematic Analysis of the Network Topology

While analyzing the topology of a signaling network; it is important to study the protein-protein interactions, which are accepted as the building blocks of biological networks, and therefore the necessity to transform the reactions into interactions (connections between molecules) emerges.

In the TLR signaling network map presented by Oda and Kitano (2006), the species are classified as reactants, products, or modifiers taking place in a reaction. For the sake of simplicity, the different states of the same species (such as phosphorylated, acetylated, hydroxylated, etc.) or species transported from one place to another, are designated differently. The reactions in the map are of the types mentioned in Section 3.1.1 and also include the interactions such as the catalyses, inhibitions, and transcriptional activations. Since all of the reactions at hand are directed; the interactions obtained are also directed. Being directed implies that the interactions do not need to be reversible (i.e. the adjacency matrix, formation of which is explained in Section 3.2.2.1, is not necessarily symmetric), for example if a reaction is directed from A to B (where A is the reactant and B is the modifier, or A is the reactant/modifier and B is the product, or both of them are reactants

or modifiers in the reaction form), a signal coming to species A is conducted to B but not vice versa unless otherwise stated.

In this thesis, two different methods of topological analysis were adopted; namely, linear pathway analysis and analysis based on graph theory. These methods utilize different data as inputs; therefore the network at hand should be processed before analyzing, in order to obtain appropriate inputs. The pairwise interactions are used (with or without some adjustments) as the starting point in both of the analyses. The interactions with adjustments are the inputs for the linear pathway analysis, which will be explained in the following section. The other method of analysis based on the graph theory, requires an input in the form of an adjacency matrix, the construction of which will be explained in Section 3.2.2.1.

3.2.1. Linear Pathway Analysis

3.2.1.1. Transformation of TLR Signaling Network Map into an Interaction List. Under the assumption that reactants, modifiers, and products participating a reaction interact with and within each other, all pairwise directed interactions between proteins (or other types of species) in this network were obtained. The direct transformation of a reaction into a set of interactions was performed as follows:

Reaction: $A + B \xrightarrow{C+D} E + F$
Species: A, B (reactants); C, D (modifiers); E and F (products)
Interactions: A-B, C-D, A-C, A-D, B-C, B-D, C-E, C-F, D-E, D-F

When these interactions are given as inputs to the NetSearch algorithm (Steffen *et al.*, 2002) and the linear paths from the ligands (e.g. B) to a specific phenotype (e.g. E) are asked for, many of the resulting pathways come out to be redundant. This is due to the fact that, these binary interactions do not include the dependence of a reaction on all of the reactants. In other words, in order for the reaction above to take place, both of the species A and B are necessary but when NetSearch algorithm encounters B, it can build a path passing through B and C to produce E by using the interactions: B-C and C-E. To avoid that sort of misleading paths, the species belonging to the same group in the reactions

(reactants, modifiers, and products) are assumed to be interacting in an additionally formed complex species. This pre-processing method is applied to the previous example:

Reaction: $A + B \xrightarrow{C+D} E + F$
Species: A, B (reactants), AB (reactant complex); C, D (modifiers), CD (modifier complex); ABCD (reactant + modifier complex), E and F (products)
Interactions: A-B, A-AB, B-AB, C-D, C-CD, D-CD, AB-CD, AB-ABCD, CD-ABCD, ABCD-E, ABCD-F

Using this approach, when NetSearch algorithm encounters B, it builds a path passing through B, AB, and ABCD to produce E by using the interactions B-AB, AB-ABCD, and ABCD-E. In fact; A-B, C-D and AB-CD interactions, i.e. the interactions among the group of species belonging to the reactants or modifiers, are also a cause for redundancy. For example if the algorithm encounters A in the search of pathways leading to E; one of the resulting pathway will be A-AB, AB-ABCD, and ABCD-E similar to the previous case, but some others will be A-B, B-AB, AB-ABCD and ABCD-E or A-B, B-AB, AB-CD, CD-ABCD and ABCD-E. The last two results involve extra and unnecessary steps to the target and to avoid this type of behavior; the interactions A-B, C-D and AB-CD can be ommited.

3.2.1.2. Algorithm. The TLR signaling map is converted to a binary interaction list, as explained in the previous section, in order to obtain the appropriate format for one of the inputs required for the NetSearch algorithm (Steffen *et al.*, 2002). The required inputs for this program, operating in MS-DOS screen, are in the form of text files (with .doc or .txt extensions) and these inputs are the separate files including the list of all of the species, binary interactions between them, the ligands and the phenotypes (or any type of species which is the target cell in the interested pathways). After these inputs are supplied, this algorithm can give all linear paths starting with the ligand(s) and ending with the phenotype(s) on the condition that the maximum path length between ligands and phenotypes is constrained by the user because otherwise infinitely many results can be found. Once the linear paths between ligands and different phenotypes are obtained, the percentage of participation of each molecule can be detected. These participation percentages may reveal the molecules which are special to certain signaling pathways, or

which have common roles in different signaling pathways. In order to perform participation percentage calculations; the linear paths, found by NetSearch algorithm (Steffen *et al.*, 2002), should be converted to the original form by substituting the complex species with their contents.

3.2.2. Analysis Based on Graph Theory

3.2.2.1. Transformation of TLR Signaling Network Map into an Adjacency Matrix. The data obtained from the reactions, i.e. pairwise interactions (according to the initial schema explained in Section 3.2.1.1), are converted to a binary (made up of zeros and ones) square matrix (S). The conversion process is based on the pairwise interactions, i.e. if the i^{th} species interacts with the j^{th} species; then S(*i,j*) is one, else S(*i,j*) is zero (Arga *et al.*, 2007). This binary and sparse (the number of zeros are considerably more than the number of ones so that the matrix uses low memory) matrix (S), is called adjacency matrix and since the interacting species comprise the rows and columns, it has a dimension equal to the number of species (680×680) in the network. After obtaining the required input, that is the adjacency matrix, it is used in the algorithm created by Arga *et al.* (2007), and implemented in MATLAB 7.0 (MathWorks, Inc.) in order to find the graph properties of the network such as the network diameter, the mean path length, the connectivities of the nodes and the number of hubs. In addition to these properties, clustering coefficient is calculated by using the adjacency matrix.

3.2.2.2. Algorithm. In the algorithm used, the successive powers of the adjacency matrix are found by multiplying the matrix by itself and the element of the n^{th} power of the adjacency matrix ($S^n(i,j)$) gives the number of paths with path length of *n* from molecule *i* to molecule *j* (Arga *et al.*, 2007). It is important to note that, it is not always possible to find a path between two nodes (molecules) since the TLR network is directed. After finding the power matrices, shortest path lengths (SPL) can be obtained. Shortest path lengths are defined as the minimum number of interactions between directly connected nodes and can be calculated by determining the minimum value of *n* when $S^n(i,j)$ is equal to a nonzero value. The mean path length and the network diameter, which are the statistical properties, are then calculated by taking the average (Watts and Strogatz, 1998)

and maximum (Newman, 2003) of the shortest nonzero path lengths in the network, respectively.

Another statistical approach, providing an insight about the robustness of the model, is adopted for the topological analysis of the network. This approach is based on the probability of a node to have a certain number of degree (k), P(k) (Section 2.3.2.2). P(k) is obtained by counting all of the nodes having the degree of k, and then dividing the number by the total number of nodes, N and repeating this procedure for every different k until the P(k) distribution is obtained. In order to apply this method, the in-degree (k_{in}) and the out-degree (k_{out}) of each node in the directed network should be found by summing up each of the columns and rows, where the species reside, respectively. The undirected degree, k, for each node is the sum of k_{in} and k_{out}. However, if we sum up the degrees of all nodes, the result will be twice the real total connectivity in the network because each connection is counted twice as being in and out for different nodes. So, the total connectivity is calculated either dividing the total connectivity by two, or summing up only k_{in} or k_{out} (Figure 3.5). There is another quantity used in the networks named average degree, $\langle k \rangle$ and it is defined as the average number of edges per node (Equation 3.1) (Watts and Strogatz, 1998).

$$\langle k \rangle = \frac{1}{N} \sum_{i=1}^{N} k_i \tag{3.1}$$

where N is the total number of nodes in a network and k_i's are the individual degrees of each node .

Clustering coefficient, which is another structural property of a network, is calculated by slightly modifying the following equation (Valverde *et al.*, 2002):

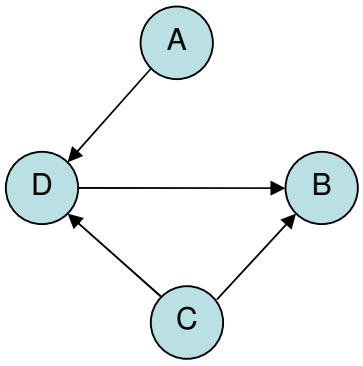
$$C = \left\langle \frac{2}{k_i (k_i - 1)} \sum_{j=1}^{N} \xi_{ij} \left[\sum_{k \in \Gamma_i} \xi_{jk} \right] \right\rangle \tag{3.2}$$

In the equation above; k_i is the undirected degree of i^{th} species ($k_{i,in} + k_{i,out}$), ξ_{ij} is the $S(i,j)^{th}$ element of adjacency matrix, $\langle\ \rangle$ means averaging all over the nodes and $k \in \Gamma_i$ means that ξ_{jk} is counted only if the k^{th} species is in interaction with the i^{th} species. However, Equation 3.2 is valid for undirected networks and needs to be modified since the TLR network is directed. According to Grabowski and Kosiński (2007) clustering coefficient for directed networks is:

$$C = \left\langle \frac{E_i}{k_i(k_i - 1)} \right\rangle \tag{3.3}$$

In the equation above; k_i definition is identical to the one in Equation 3.2 and E_i is the number of connections between neighbors of the i^{th} species which corresponds to the summation terms in Equation 3.2. Therefore the clustering coefficient calculated for directed networks by using Equation 3.3 is half of the one calculated for undirected networks by using Equation 3.2.

Since the network analyzed is a large-scale one, it is better to exemplify this method by a small-scale network (Figure 3.5) to perceive the concepts of adjacency matrix and shortest path length better.



S	A	B	C	D
A	0	0	0	1
B	0	0	0	0
C	0	1	0	1
D	0	1	0	0

$\rightarrow k_{in}$	$k_{out} \rightarrow$	$\rightarrow k \rightarrow$
0	1	1
2	0	2
0	2	2
2	1	3

Σk_{in}	Σk_{out}	Σk	$<k>$
4	4	8	1

Figure 3.5. A small-scale example of a directed network, its adjacency matrix and degrees

From Figure 3.5, the construction of an adjacency matrix (S) using the directed interactions between the molecules of A, B, C, and D can be observed. The in- and out-degrees are also calculated by the summation of columns and rows, respectively. The next step is to take the second and third powers of S, in order to find the number of paths with a path length of two and three, respectively.

S^2	A	B	C	D
A	0	1	0	0
B	0	0	0	0
C	0	1	0	0
D	0	0	0	0

S^3	A	B	C	D
A	0	0	0	0
B	0	0	0	0
C	0	0	0	0
D	0	0	0	0

Figure 3.6. The successive powers of the adjacency matrix

There is only one two steps long path, from A to B and from C to B and no three step long path (Figure 3.6). After obtaining this group of matrices, composed of the powers of S, the algorithm constructs the SPL matrix by searching each of the power matrix in order to find the minimum nonzero n for a pair of molecules (i,j) (Figure 3.7).

SPL	A	B	C	D
A	0	2	0	1
B	0	0	0	0
C	0	1	0	1
D	0	1	0	0

Figure 3.7. The shortest path length (SPL) matrix

This result implies that the minimum number of steps necessary to reach B starting from A is two, and one step is enough to reach from A to D, C to B, C to D, and D to B. For this small-scale example, mean path length and network diameter are 1.2 and 2, respectively.

Clustering coefficient for this directed small-scale example (Figure 3.5) is calculated by using Equation 3.3 as the following:

	A	B	C	D
k_i	1	2	2	3
E_i	0	1	1	1
C_i	0	1/2	1/2	1/6

Figure 3.8. The clustering coefficient calculation steps

E_i values are found based on the neighborhoods of species as explained above. For example, A has only D as a neighbor and therefore there are no interactions between its neighbors. B has two neighbors, namely C and D and they have one interaction and similarly C's neighbors are B and D having only one interaction. Lastly, D has three neighbors, namely A, B and C among which only B and C are interacting. Average clustering coefficient for this small-scale system is found as 0.29, by summing all C_i and dividing by four.

4. STRUCTURAL ANALYSIS OF TLR SIGNALING NETWORK

In the previous section; the reconstruction process and architectural properties of the comprehensive TLR signaling network, formed by Oda and Kitano (2006), have been elucidated. Next, the methods which enable the systematic topological analysis of this network have been presented in detail with the necessary algorithm descriptions. This section focuses on the results obtained from the applied methods and their interpretation.

4.1. Linear Pathway Analysis

The field of structural analysis has mainly focused on metabolic networks and many methodologies are developed and applied in this area, while analyses of the signaling and regulatory networks are not widely encountered (Klamt *et al.*, 2006). But as the number of reconstructed signaling and regulatory networks increases day by day, the necessity and curiosity of structurally analyzing these networks emerges. For this purpose, either the methods used for the structural analysis of metabolic networks can be adapted, or new methods can be introduced. If the former approach is going to be applied, the existing methods should be modified as the properties and the available data are network type specific. For example, mass flow conservation and continuity of the reaction flux lack in signaling networks and these differences prevent the adoption of elementary flux mode concept as the way it is applied in metabolic networks (Zevedei-Oancea and Schuster, 2005; Klamt *et al.*, 2006). In addition to these facts; the large size of the Toll-like Receptor (TLR) signaling network yields an abundance of fundamental routes, making the structural analysis methods such as metabolic pathway analysis computationally unmanageable. Therefore, the linear pathway analysis is performed for the first part of structural analysis.

4.1.1. Transformation of TLR Signaling Network Map into an Interaction List

The interactions are found from the map of the Toll-like Receptor (TLR) signaling network as explained in Section 3.2.2.1. Two different approaches are followed during the calculations, (i) treating the reactants, modifiers and products of a reaction as separate species interacting with each other (this approach is also applied in the analysis based on graph theory, where the number of species and interactions are equal to the number of nodes and edges, respectively); and (ii) combining the reactants and modifiers taking place in a reaction and treating them as one complex species which is in interaction with each of the products. The numbers of interactions found in each of the cases are 1292 and 1410, respectively while the number of species has increased to 1058 from 680 (Table 4.1). The increase in the numbers is due to the introduction of complex species to the system.

Table 4.1. Numerical results obtained by transformation of reactions into interactions

Approach	Number of Species	Number of Interactions
(i)	680	1292
(ii)	1058	1410

4.1.2. Phenotype Diameter

The diameter of the directed TLR signaling network, resulting from the analysis based on the graph theory, will be given in Section 4.2. However, a different quantity can be found through the application of linear pathway analysis concerning the efficiency of the network. Based on the fact that phenotypes are the changes (responses) occurring once the signal is transmitted to the target cell, it is important to specify the maximum number of steps required in a network for the signal transduction to reach any of the phenotypes, i.e. the phenotype diameter. This quantity is so important that it is defined as the network diameter (Durmuş Tekir, 2007). The phenotype diameter in directed TLR signaling network is found as 11 (Table 4.2) and two different pathways having this length exist, namely the paths from TLR2 ligand and TLR4 ligand to the phenotype which is the transcription of target genes ex. β-defensin 2.

Table 4.2. Shortest path lengths of the phenotypes in the TLR network

Phenotype	Shortest Path Length
p. apoptosis	5
p. actin organization	4
p. ROS production	6
p. protein synthesis	6
p. translation	9
n. target gene ex. Bcl-2	10
n. cell cycle	10
n. target genes ex. IFNα/β	7
n. histone deacetylation	10
n. apoptosis	4
n. target genes ex. cyclin D1	8
n. target genes ex. β-defensin 2	11
n. transcription	10
n. RNA metabolism	8
n. target genes ex. ICER	7
n. target genes ex. IL-1, IL-8, IL-6, TNFα, IFNγ, GM-CSF, M-CSF, iNOS, COX2, MHC class I/II, E-selectin, β-defensin 2	7

4.1.3. Participation of Molecules

In order to examine and to be able to compare the pathways starting from any of the ligands and leading to a specified phenotype; the maximum number of steps (set by the user in NetSearch) is manipulated for each phenotype in such a way that they yield linear paths with approximately the same order of magnitude (10^3) (with the exception of protein synthesis phenotype, for which only two different pathways can be found regardless of the limitation to the maximum number of steps). After obtaining the 16 group of pathways leading to different phenotypes, the percentages of all 680 species in the network are found in each group by counting the corresponding species and dividing the number of times it was encountered to the total number of paths in that group. Then, the species taking place in all of the pathways (100% participation) leading to a phenotype are determined (Table 4.3). For verification, a phenotype is chosen (ROS production) and the process explained above is repeated under the limitation of three different maximum number of steps (48, 50 and 57) and different number of pathways are obtained (1871, 2218 and 6453). Although the percentages of species differ in three cases, the species with 100% participation are identical (Table 4.3).

Table 4.3. Species taking place in 100% of the pathways leading to the phenotypes[1]

Phenotype	**Species**
n. target gene ex. Bcl-2	NF-κB (p50)/Bcl-3 complex
n. cell cycle	c-Myc ERK1 ERK2
n. target genes ex. IFNα/β	ISRE
n. histone deacetylation	κB site κB site/NF-κB (p50) complex HDAC-1 HDAC-1* NF-κB (p50)
n. target genes ex. IL-1, IL-8, IL-6, TNFα, IFNγ, GM-CSF, M-CSF, iNOS, COX2, MHC class I/II, E-selectin, β-defensin 2	κB site CBP κB site/NF-κB (p65)/ NF-κB (p50)/CBP complex NF-κB (p50)/NF-κB (p65) complex
n. apoptosis	TLR4 ligand/TLR4/ MD-2 complex TLR2/TLR2 ligand complex NUR77 NUR77*
n. target genes ex. cyclin D1	Ubc13/Uev1A/ TRAF6/TAB2 TAB3/ TAK1/TAB1 complex JNK p38α MAPK p38β 2MAPK ATF2
n. target genes ex. β-defensin 2	c-Jun JNK AP-1 site
n. transcription	MSK1 ERK1 ERK2 p38α MAPK p38β 2MAPK
n. RNA metabolism	PKCζ PDK1* Phosphatidic acid Phosphatidyl serine hnRNP A1
n. target genes ex. ICER	CBP CBP/CREB/ CRE site complex CRE site/CREB complex
p. apoptosis	caspase8 FADD/pro-caspase8 complex TIR domain/MyD88 complex
p. protein synthesis	PKR TICAM1 TICAM1* TICAM2* TLR3/TLR3 ligand complex dsRNA eIF2α

Table 4.3. Species taking place in 100% of the pathways leading to the phenotypes[1]- continued

p. ROS production	$p40^{phox}$ $p47^{phox}$ $p67^{phox}$ $gp91^{phox}/p22^{phox}/ p47^{phox}$/Rac1/ GTP/$p40^{phox}/ p67^{phox}$ complex $p22^{phox}/gp91^{phox}$ complex Rac1/GTP complex Phosphatidic acid
p. translation	eIF-4E ERK1 ERK2 Mnk1 p38α MAPK p38β 2MAPK
p. actin organization	IL-1RI ligand/IL-1RI/ IL-1RAcP complex

* means that the protein binds to other molecules and often makes a conformational change (Oda and Kitano, 2006)

[1] whether a phenotype takes place in nucleus or plasma is denoted by initial n or p in the phenotype name, respectively

Table 4.3 only presents full participation of the species to the pathways; however participation percentages higher than 70% (Table A.1) can also be assumed significant. This is due to the fact that although the percentages differ as the limitation of maximum number of steps is changed; the participation percentages of species above 70% do not decrease beneath it. MyD88 is of special importance, being the core protein of the bow-tie structured TLR network and an out-degree hub (Table 4.7). Although it participates 100% of the pathways leading to only one phenotype (p. apotosis) (Table 4.3), it takes important roles in pathways leading to five other phenotypes (Table 4.4).

Table 4.4. MyD88 participation ($\geq 70\%$) in the pathways leading to the phenotypes

Phenotype	**MyD88 Percentage (%)**
n. RNA metabolism	91
n. target genes ex. cyclin D1	79
n. transcription	76
p. translation	71

4.1.4. Pathway Crosstalk

In this aspect of analysis, sharing of identical signaling molecules in different signaling pathways is used as a measure of crosstalk (Schwartz and Baron, 1999). According to the linear pathway analysis; the critical signaling molecules in pathway crosstalk, i.e. those participating in most of the pathways leading to the phenotypes, are found to be the complexes of Ubc13/Uev1A/TRAF6/TAB2/TAB3/TAK1/TAB1 (Ubiquitin-conjugating enzyme/Ubiquitin-conjugating enzyme variant/TNF Receptor Associated Factor 6/TAK1-binding protein 2/TAK1-binding protein 3/TGFβ-activated kinase 1/TAK1-binding protein 1), TLR2/TLR2 ligand, and Rac1/GTP (Ras-related C3 botulinum toxin substrate 1/Guanosine-5'-triphosphate) with the percentages of 75%, 75% and 87.5%, respectively.

The common species having a role in the crosstalk of pathways leading to different phenotypes throughout the network can be determined by observing the identical species in Table 4.3 (Table 4.5).

Table 4.5. Common species taking place in 100% of the pathways leading to the phenotypes

Phenotype	Species
n. histone deacetylation n. target genes ex. IL-1, IL-8, etc.	κB site
n. target genes ex. ICER n. target genes ex. IL-1, IL-8, etc.	CBP
n. cell cycle n. transcription p. translation	ERK1 ERK2
n. target genes ex. cyclin D1 n. target genes ex. β-defensin 2	JNK
n. target genes ex. cyclin D1 n. transcription p. translation	p38α MAPK p38β 2MAPK
n. RNA metabolism p. ROS production	phosphatidic acid

4.1.5. Molecules Specific to Phenotypes

On the contrary to Section 4.1.4, the species can be classified based on their uniqueness in the pathways leading to a certain phenotype. The path-specific species, i.e. the species functioning only in the paths leading to a specified phenotype, are of special importance for drug targeting. This is due to the fact that; these species can be inhibited (deleted) if they are detected to have an enhancing role in a disease, without considering the rest of the network as the path-specific species do not contribute to other alternative pathways leading to beneficial or vital phenotypes.

Table 4.6. Species taking place in 100% of the pathways leading only to the relevant phenotype

n. apoptosis	NUR77 NUR77*
n. cell cycle	c-Myc
n. histone deacetylation	HDAC-1 HDAC-1*
n. RNA metabolism	hnRNP A1 PKCζ
n. target gene ex. Bcl-2	NF-κB (p50)/Bcl-3 complex
n. target genes ex. cyclin D1	ATF2
n. target genes ex. β-defensin 2	c-Jun AP-1 site
n. transcription	MSK1
p. apoptosis	Caspase8 FADD/pro-caspase8 complex
p. protein synthesis	TICAM1 TICAM1* TICAM2* PKR eIF2α TLR3/TLR3 ligand complex dsRNA
p. ROS production	$p40^{phox}$ $p67^{phox}$ $p47^{phox}$ $gp91^{phox}/p22^{phox}/ p47^{phox}$/Rac1/ GTP/$p40^{phox}/ p67^{phox}$ complex $p22^{phox}/gp91^{phox}$ complex
p. translation	Mnk1 eIF-4E

Four phenotypes out of 16, namely transcription of target genes ex. ICER, IFNα/β, IL-1 etc., and actin organization, do not have specific species in their pathways (Table 4.5) and this is another indication of high cross-talk between pathways.

4.2. Analysis Based on Graph Theory

All of the large-scale statistical properties described in Section 2.3.2.2 can be calculated, once the interaction graph is formed by using the interaction data supplied by the reconstructed network. In this thesis, a similar approach to Klamt *et al.* (2006) is adopted in representing the interaction network, i.e. the formation of interaction graph. But the main difference is that Klamt *et al.* (2006) stored the directed graph in an $m \times q$ incidence matrix, in which columns are the interactions and rows are the species, similar to the stoichiometric matrices of metabolic reaction networks; whereas an $n \times n$ adjacency matrix, in which both columns and the rows are the species (Section 3.2.2.1), is formed for storing the directed interaction graph in this thesis. As far as the memory requirement is concerned; by using an adjacency matrix, the structure of a graph can be stored more efficiently than by an incidence matrix (Klamt *et al.*, 2006) as the adjacency matrix is sparse (Section 3.2.2.1).

The shortest path length (SPL) matrix is obtained according to the algorithm of Arga *et al.* (2007) explained in Section 3.2.3.2, and the diameter of the directed network is found to be 25 with a mean path length (MPL) of 8.98. Clustering coefficient is calculated as 0.009, by using Equation 3.2. Watts and Strogatz (1998) use the characteristic (mean) path length (MP*L*) and the clustering coefficient (*C*), definitions and calculation steps of which are given in Sections 2.3.2.2 and 3.2.2.2, respectively, in quantification of the structure of a network. Characteristic path length is a global property whereas clustering coefficient is a local one. Small-world networks are defined as networks having a few long-range edges, i.e. short-cuts, which cause a drop in the characteristic (mean) path length. Therefore; calculated MP*L* value becomes as small as the characteristic path length of the random network ($\mathrm{MP}\,L \geq L_{random}$) whereas C is significantly larger than C_{random} ($C >> C_{random}$). They found that these random properties used in the comparison, tend to approach the following quantities: $L_{random} \approx \ln(n)/\ln(k)$ and $C_{random} \approx k/n << 1$, where n is the number

of vertices (=680 in the TLR network) and k is the average number of edges per vertex, i.e. the average connectivity (= 1.86). L_{random} and C_{random} are obtained based on the definitions above as 10.52 and 0.003, respectively.

TLR network can be said to show small-world phenomenon (Watts and Strogatz, 1998), which is a general feature of many complex biological networks, as having a mean path length similar to the characteristic path length of the random network and a clustering coefficient three times the random counterpart.

In order to determine the structure of the network (random or scale-free), the degree distribution of the network is also found. As explained in Section 3.2.3.2, the probabilities of the nodes having a certain number of connectivity (degree), k, gives the degree distribution. In this directed TLR network, there are two degree distributions, namely in- and out-degree distribution (Figure 4.1). The shapes of the degree distributions give a clue about the network structure (Figure 2.9). Another indicator of the structure is the line fitted to the plots of k vs. log [P(k)], and log (k) vs. log [P(k)], which are drawn using the in-degree (k_{in}) data (Figure 4.2) and the out-degree (k_{out}) data (Figure4.3) obtained. Depending on whether the line fits the former plot or the latter best, the structure is determined to be random or scale-free, respectively.

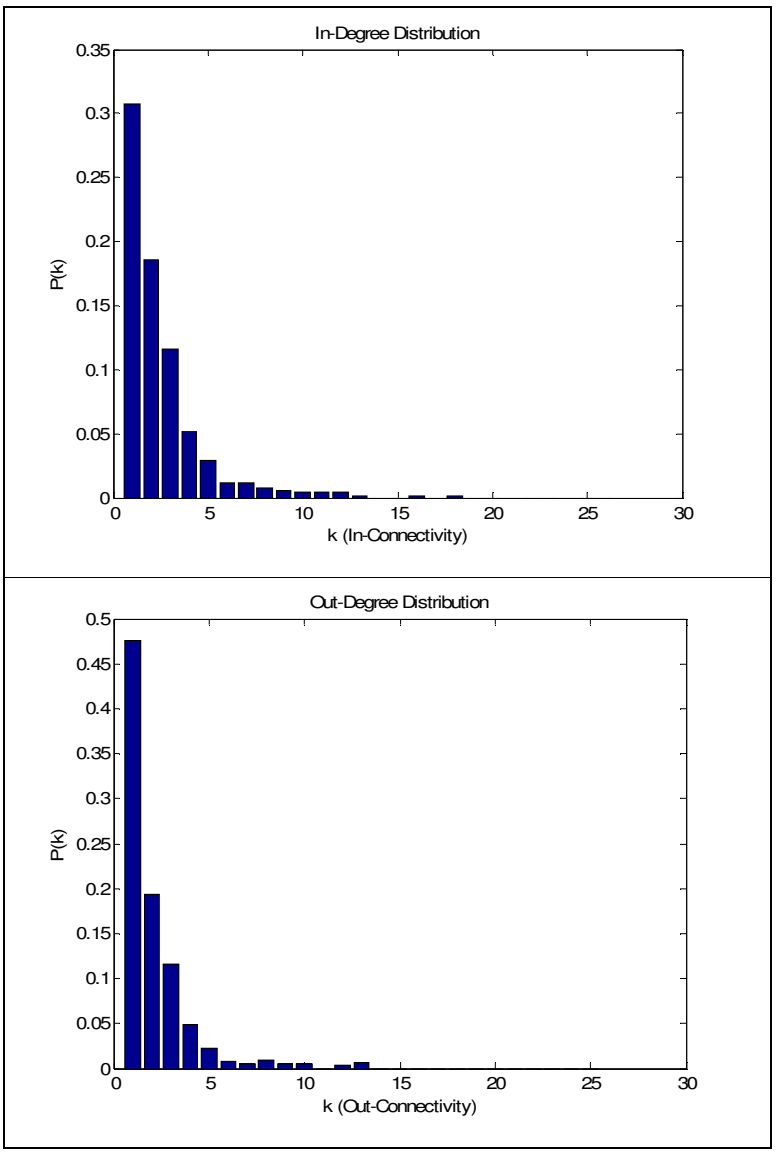


Figure 4.1. In- and out-degree distributions

From both of these distributions, it can be seen that there are many nodes with few connectivities and a few highly connected nodes (hubs). This is the first sign of the scale-free network topology. Hubs are also believed to make the network, in which they exist, look like a small world by dominating the structure (Barabási, 2002). Hubs in the overall network are of special importance for drug targeting (Papin *et al.*, 2005; Nikolsky *et al.*, 2005).

The hubs for in-degree distribution have 16 and 18 connectivities, whereas the hubs for out-degree distribution have 12 and 13 connectivities. The former hubs are: TLR2 ligand and IKK-α/IKK-β/IKK-γ complex (which is also a hub for the out-degree connection), whereas the latter hubs are found as TIR domain, TRAF6, Rac1/GTP complex, κB site/NF-κB (p65)/NF-κB (p50)/CBP complex, and MyD88 (Table 4.7).

Table 4.7. Hubs for in- and out-degree connections (numbers in parenthesis stand for the number of connectivity)

In-degree Hubs	Out-degree Hubs
TLR2 ligand (16)	MyD88 (12)
IKK-α/IKK-β/IKK-γ complex (18)	IKK-α/IKK-β/IKK-γ complex (12)
	TRAF6 (13)
	TIR domain (13)
	Rac1/GTP complex (13)
	κB site/NF-κB (p65)/NF-κB (p50)/CBP complex (13)

Networks such as the Internet, the World Wide Web (Albert *et al.*, 1999), the cell and the social networks share the scale-free topology as the TLR network. This topology is assumed to be an indicator of high resilience to errors, i.e. robustness (Barabási, 2002). Although any mutations in these highly connected signaling molecules (hubs) will drastically lengthen the paths from any species to the phenotypes; failures in the scale-free networks can affect small nodes and large hubs equally. Since the number of small nodes is greater than the hubs, they are more likely to break apart. The impact of failures on small nodes is not fatal for the whole network and therefore, topological robustness is due to the uneven structure of scale-free networks (Barabási, 2002).

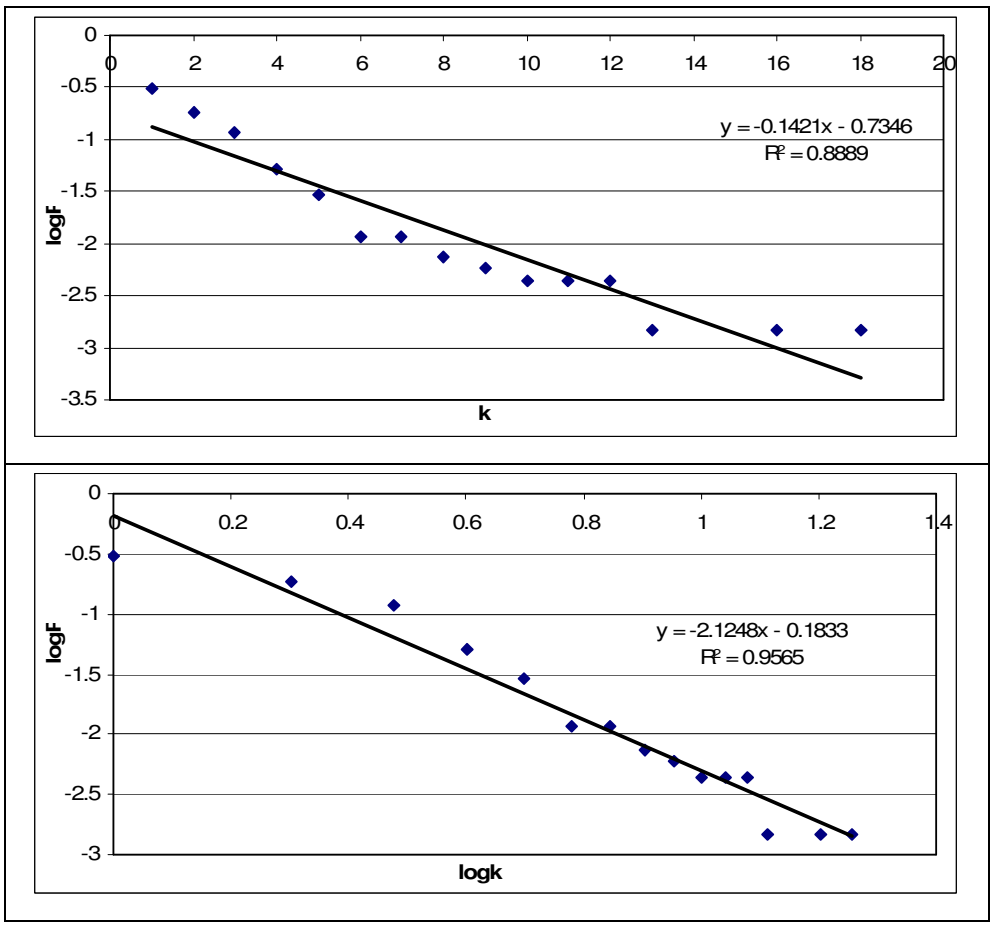


Figure 4.2. Logarithmic plots of in-degree distribution

Figure 4.2 proves that the in-degree distribution is scale-free because the line in the second plot fits a power law model with the in-degree exponent, γ_{in}=2.12 and R^2=0.96.

Out-degree distribution has similar properties with the previous in-degree distribution. Therefore; it is also scale-free, γ_{out} =2.14, and R^2=0.92 (Figure 4.3). These two unique degree exponents, both being between two and three, agree with the consistent pattern suggested by Barabási (2002) for large networks. Several researchers in literature have investigated directed networks, and obtained power law models for both outgoing and incoming edges (Albert *et al.*, 1999; Jeong *et al.* 2000; Krapivsky *et al.*, 2001; Tadic´, 2001a, 2001b; Grabowski and Kosiński, 2007).

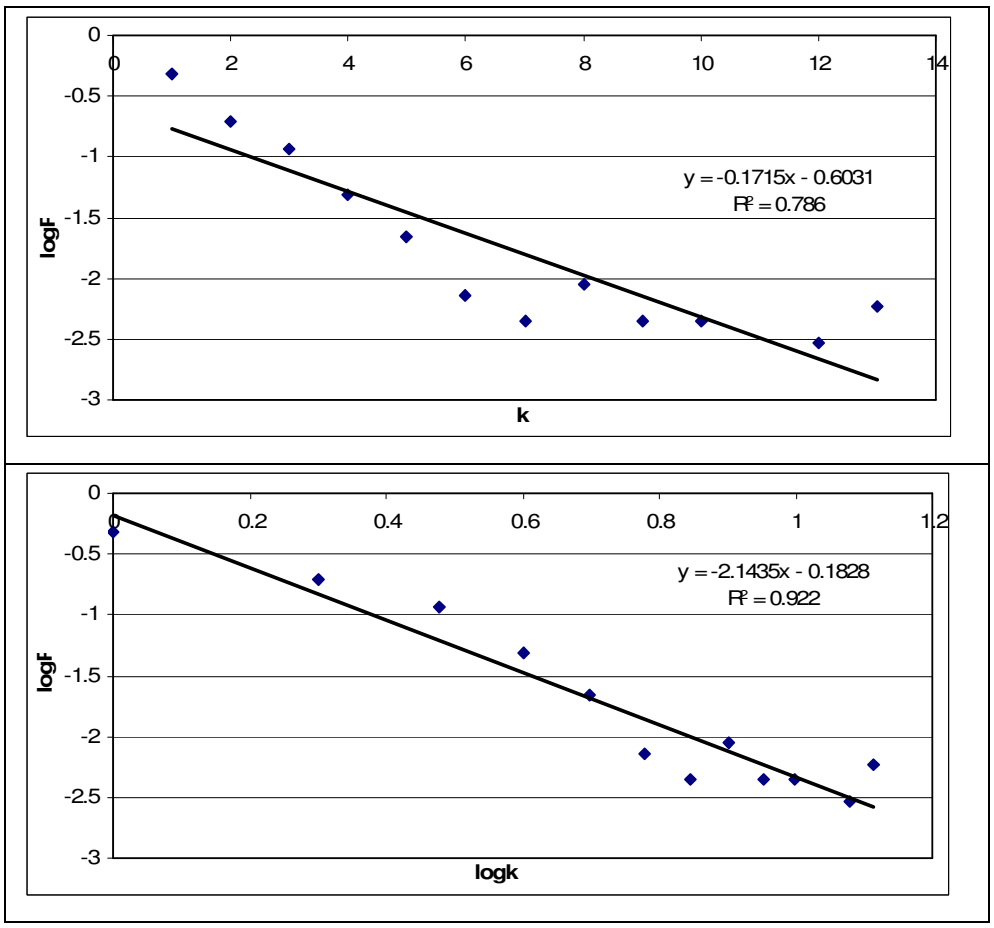


Figure 4.3. Logarithmic plots of out-degree distribution

5. DISCUSSION

5.1. Important Molecules and Their Functions in the TLR Network

As explained previously, activation of Toll family receptors (TLRs and IL-1R) results in the transcriptional induction of a number of genes involved in the host defense such as antimicrobial molecules or cytokines. These genes have binding sites for the transcription factors NF-κB and AP-1 in their promoter, in common (Bilak *et al.*, 2003). TIR domain receptors are associated with an intracytoplasmic plurimolecular platform in which the MyD88 factor plays a central role. MyD88 is composed of an amino-terminal death domain (DD) and a carboxy-terminal TIR domain, and it interacts in a ligand-dependent manner with the receptors through these domains. TIR domain in MyD88 interacts with the TIR domain of receptors, whereas the DD domain with the DD domain of the serine-therionine kinase IRAK (IL-1R associated kinase) (Akira *et al.*, 2001; Michel *et al.*, 2001; Medzhitov, 2001). TIR domain/MyD88 complex is found to be an out-degree hub (Table 4.7) in addition to its 100% role in the pathways leading to the phenotype of apoptosis taking place in plasma. Interaction of IRAK with MyD88 triggers autophosphorylation of the kinase and this affects the interaction with the receptor complex and allows IRAK to interact with TRAF6, which has a role in downstream signaling with the receptors of the TNFα family. TRAF6 associates with a dimeric ubiquitin-conjugating enzyme complex composed of Ubc13 and Uev1A. This complex mediates activation of the TAK1 kinase, which then phosphorylates IκB kinase and MKK6 kinase, leading to the activation of NF-κB and AP-1 sites. The resulting complex of Ubc13/Uev1A/TRAF6/TAB2/TAB3/TAK1/TAB1 participates 100% of the pathways leading to the phenotype of target gene ex. cyclin D1 and 84.4% of the pathways leading to the phenotype of target gene ex. IL-1, IL-8 etc. (Table A.1) whereas NF-κB site participates 100%. Ubc13/Uev1A/TRAF6/TAB2/TAB3/TAK1/TAB1 complex is also found to be the species through which the pathways leading to many of the phenotypes (75%) crosstalk (Section 4.1.4).

The transcription factor NF-κB, which is critical for inducible expression of many genes involved in immunity and inflammation (Ghosh *et al.*, 1998), exists in the cytosol of resting cells bound to inhibitory IκB proteins. Stimulation with specific inducers, such as TNFα or LPS, activates an IκB kinase (IKK) complex that phosphorylates IκB, triggering its degradation by the proteasome (Karin and Ben-Neriah, 2000) and allowing free NF-κB to translocate to the nucleus and activate gene expression. The critical role of histone acetylases in regulating the transcriptional activity of NF-κB has focused attention on acetylation in the overall context of NF-κB activation. Transcriptionally inactive NF-κB in resting cells consists of homodimers of either p65 or p50 complexed with the histone deacetylase HDAC-1. Only the p50/HDAC-1 complexes bind to DNA and suppress NF-κB dependent gene expression in unstimulated cells (Zhong *et al.*, 2002). Appropriate stimulation causes nuclear localization of NF-κB complexes containing phosphorylated p65 that associates with CBP and displaces the p50/HDAC-1 complexes. It has been shown that p65 interacts with distinct histone deacetylase (HDAC) isoforms to negatively regulate gene expression (Ashburner *et al.*, 2001; Chen *et al.*, 2001; Ito *et al.*, 2000; Lee *et al.*, 2000). κB site, NF-κB (p50) and HDAC-1 participate 100% of the pathways leading to the phenotype histone deacetylation whereas κB site, NF-κB (p50), NF-κB (p65) and CBP not only take place in all of the pathways leading to the phenotype of target genes ex. IL-1, IL-8 etc. (Table 4.3) but also their complex is found to be an out-degree hub (Table 4.7). It can then be said that pathways leading to these two phenotypes crosstalk through the κB site (Table 4.5) and the ones leading to the phenotypes of target genes ex. ICER and IL-1, IL-8, etc. have CBP in common (Table 4.5).

Although p50 does not contain a transactivation domain and cannot assist histone acetyltransferase (HAT) activity, it can interact with other nuclear proteins to promote gene transcription (Cha-Molstad *et al.*, 2000; Fujita *et al.*, 1993; Heissmeyer *et al.*, 1999). For example, p50 interacts with Bcl-3, which helps HATs to induce expression of kB-regulated genes (Dechend *et al.*, 1999).

MAP kinases are critical mediators of signal transduction in mammalian cells (Cobb and Goldsmith, 1995; Ip and Davis, 1998; Kyriakis and Avruch, 2001; Lewis *et al.*, 1998; Schaeffer and Weber, 1999). There are three major groups of mammalian MAP kinases,

namely the extracellular signal related protein kinases (ERK), the p38 MAP kinases and the c-Jun NH_2-terminal kinases (JNK). The ERK1 and ERK2 protein kinases are major targets of the Ras signaling pathway and they are involved in proliferation, tumorigenesis and differentiation. The pathways leading to cell cycle, transcription and translation phenotypes, in which ERK1 and ERK2 participate 100%, crosstalk through these two species (Table 4.5). The p38 MAP kinases and JNK group are activated by inflammatory cytokines and by the exposure of cells to environmental stress (Davis, 2000). They are activated by three MAP kinase kinases namely, MKK3, MKK6 and MKK4. The first two activate only the p38 MAP kinases whereas the last one activates both the p38 MAP kinases and the JNK. In TLR network; MKK4, JNK and p38 MAP kinase group (α and β) participate to the pathways leading to the phenotype of target gene ex. cyclin D1, and ERK1 ERK2 and p38 MAP kinase group participate to the pathways leading to the phenotypes of transcription and translation with MKK3-MKK4-MKK6 and MKK3-MKK6, respectively. As p38 MAP kinase group is activated by all three of the MAP kinase kinases; it takes place in all of the the pathways leading to all of the three phenotypes stated (Table 4.5). It is established that JNK is required for the normal regulation of AP-1 transcription activity and this is mediated, in part, by the phosphorylation of the transcription factors ATF2, c-Jun, JunB and JunD (Davis, 2000). JNK and ATF2 take place in the pathways leading to the phenotype target gene ex. cyclin D1 whereas c-Jun, JNK and AP-1 site participate together to the pathways leading to the phenotype target gene ex. β-defensin 2 (Table 4.3). JNK is then common in the pathways leading to both phenotypes (Table 4.5).

Pathogenic leukocytes play critical roles in the innate immune response to pathogens. An important component of this response is the ability of leukocytes to generate reactive oxygen species (ROS) via a membrane associated NADPH oxidase (Babior, 1999; Lambeth, 2000). Historically, the involvement of a GTP binding protein in the NADPH oxidase regulation was suspected (Gabig *et al.*, 1987; Doussiere *et al.*, 1988; Seifert *et al.*, 1986) and it was simultaneously shown that either Rac1 or Rac2 GTPase was required for oxidase activity (Abo *et al.*, 1991; Knaus *et al.*, 1991). Additionally, phosphotidic acid (PA) is considered to involve in the activation of NADPH oxidase (Park, 1996) (Table 4.3). In the unstimulated neutrophil, Rac exists as a complex with GDP dissociation inhibitor (GDI) in the cytosol (Chuang *et al.*, 1993) and when the phagocyte is activated by

chemokines or phagocytic particles, Rac gets activated and dissociates from GDI to become membrane associated in its GTP-bound form (Quinn *et al.*, 1993). Activation of the neutrophil also results in phosphorylation on multiple sites of cytosolic $p47^{phox}$, which exists in a complex with a third cytosolic component, $p67^{phox}$, followed by the translocation of the $p47^{phox}$/$p67^{phox}$ complex to the membrane (Dusi *et al.*, 1992; Rotrosen and Leto, 1990). The translocation of $p47^{phox}$, $p67^{phox}$ and Rac to the plasma membrane is finalized by the formation of an active complex with integral flavocytochrome b_{558} (cyt *b*), which possesses an NADPH binding site, FAD, 2 hemes and 2 subunits, namely $gp91^{phox}$ and $p22^{phox}$ (Parkos *et al.*, 1988). An additional cytosolic component, termed $p40^{phox}$ (Wientjes *et al.*, 1996), may play a role in regulating the response of the system to phosphatidylinositol-3-phosphate (PI3-P) *in vivo* (Ellson *et al.*, 2001). And it is found that; the complex of $gp91^{phox}$/$p22^{phox}$/$p47^{phox}$/Rac1/GTP/$p40^{phox}$ /67^{phox} participates to 100% of the pathways leading to the phenotype of ROS production (Table 4.3) and also is specific to this phenotype (Table 4.6) in the TLR network. Additionally, Rac1/GTP complex is both found as an out-degree (Table 4.7) and the species through which the pathways leading to most of the phenotypes (87.5%) crosstalk (Section 4.1.4).

In immune cells, apoptosis is a key phenomenon in what is referred to as activation-induced cell death. It has been shown that this type of macrophage death can occur in the septic mouse model and that TLR2 or TLR4 signaling is required in this process (Kim *et al.*, 2003). Also it can be concluded that NUR77 is involved in the macrophage death because NUR77 expression correlates with cell death and cell death is reduced significantly in NUR77-deficient macrophages (Kim *et al.*, 2003). In TLR signaling network; TLR4, TLR2 and Nur77 take place in 100% of the pathways leading to the phenotype of apoptosis in nucleus (Table 4.3) and NUR77 is specific to this phenotype (Table 4.6).

TLR2 recognizes a wide spectrum of microbial components, i.e. ligands (Takeda and Akira, 2005). These include: lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, GPI (glycosylphosphatidylinositol) anchors from *Trypanosoma cruzi*, a phenol-soluble modulin from Staphylococcus epidermis, zymosan from fungi and glycolipids from *Treponema maltophilum* (Takeda *et al.*, 2003). In addition, TLR2

reportedly recognizes LPS preparations from non-enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pyroli* (Hirchfeld *et al*., 2001; Wertz *et al.*, 2001; Smith *et al.*, 2003). Thus, it is a highly interacting species and found as an in-degree hub (Table 4.7) and a species through which the pathways leading to many phenotypes (75%) crosstalk.

5.2. Function of Sphingolipids in TLR Signaling

The TLRs are thought to interact directly with the microbial ligands but in addition, co-receptors may greatly enhance the TLR-dependent responses (Fischer *et al.*, 2007). For example, lipopolysaccharide (LPS) binds directly to TLR4 at higher body temperatures, but mostly, the LPS/LBP complex is presented to TLR4 via CD14 and MD2 (Wright *et al*., 1990; Shimazu *et al*., 1999; Fitzgerald *et al*., 2004; Saitoh *et al*., 2004). The broad 'pattern recognition' by TLRs is essential for the tissue response to a wide range of invasive microbes (Section 2.4). It does not explain the ability of mucosal TLRs to discriminate pathogens from commensals (harmless organisms benefiting from human), however (Svanborg *et al*., 2001; Bergsten *et al*., 2004; Ruby *et al*., 2004; Fischer *et al*., 2006); for this, alternative, pathogenspecific co-receptors appear to be required (Fischer *et al*., 2006).

Sphingolipids are interesting potential TLR co-receptors at mucosal sites (Svanborg *et al*., 2001). Pathogens manipulate host cell sphingolipids to enhance colonization, tissue attack or invasion. The glycosphingolipids are used as receptors for adhesins, toxins or viruses, each recognizing a specific oligosaccharide domain. For example, uropathogenic *Escherichia coli* use P fimbriae for binding to glycosphingolipids on uroepithelial cells, but this interaction also initiates the inflammatory host response. Our previous studies have demonstrated that this host response requires the presence of TLR4 in the target cells, besides the glycosphingolipids with the appropriate binding epitope. The inflammatory response is lacking in cells and mice genetically deficient in TLR4 even though the glycosphingolipid receptors are present and attachment is intact. It has also been shown that ceramide, the lipid part of sphingolipids, is released when uroepithelial cells are challenged with P-fimbriated *E. coli* (Fischer *et al.*, 2007).

Free ceramide is then phosphorylated and downstream signaling involves serine-threonine kinases and ceramide-activated kinase (CAK) or protein kinase C (PKC-ζ) and thus the released ceramide can induce an inflammatory response (Kolesnick and Golde, 1994; Hannun and Obeid, 1995; Hedlund *et al*., 1996). Together these findings suggest that ceramide may activate TLR4 and, hence, act as a signaling intermediate between the glycosphingolipid receptor and TLR4.

5.3. Post-infection Immunity Diseases

TLRs and IL-1R have crucial roles in the innate immunity, being responsible for sensing the pathogens invading the host (e.g. human) for the first time and initiating the proper downstream signal to produce reactive oxygen species (ROS), inflammatory cytokines (such as IL-1, IL-6, TNF-α, etc.), interferon and chemokines for protection. Once these molecules are up-regulated, antigen-specific adaptive immune response can also be developed (Bhattacharjee and Akira, 2005) for protection against re-infection. The lack of recognition mechanism of the pathogens will cause serious diseases and probably death, as well as the excessive activation of the pro-inflammatory mediators such as IFNγ (interferon-γ) and TNF (tumour-necrosis factor) (Gazzinelli and Denkers, 2006). Multiple inflammatory diseases such as cancer, atherosclerosis and sepsis (Gazzinelli and Denkers, 2006) can be involved in the latter case. Atherosclerosis is considered a chronic inflammatory disease, in the progression of which TLR4 and MyD88-mediated signaling are proved to have essential roles (Bhattacharjee and Akira, 2005). It is also found that protozoa can activate the TLR-MyD88 signaling pathway to induce pathological changes in the host, such as the rodent malaria caused by *Plasmodium berghei* (Adachi *et al.*, 2001). Therefore, negative regulation of MyD88 dependent TLR signaling pathways is vital for the hosts and if the disease has occurred, drugs targeting to block this pathway can succeed (Bhattacharjee and Akira, 2005).

TLR4 is known to have a crucial role in lipid-mediated pro-inflammatory signaling and LPS (lipopolysaccharide, also known as bacterial endotoxin) is one of the ligands activating it to produce inflammatory cytokines (Cuschieri *et al.*, 2006). A specific antagonist for TLR4 signaling pathway can reduce the harmful effects of inflammation (Bhattacharjee and Akira, 2005). This antagonist may either act on MyD88, deficiency of

which in mice stops the production of inflammatory cytokines such as TNF-α in response to ligands (Takeda and Akira, 2005); or on the mechanism of the TLR4 complex assembly which takes place on the lipid rafts, i.e. sphingolipid-structured microdomains.

When bacterial endotoxin occurs in the host, the following signal transduction mechanism takes place. Activation of the macrophage by endotoxin requires LPS to bind to the acute-phase protein LPS-binding protein (LBP). Once bound to LBP, LPS binds to the LPS recognition receptor, CD14 (Heumann *et al.*, 2003), which is a GPI (glycosylphosphatidylinositol)-anchored protein that does not have a cytoplasmic domain (Pugin *et al.*, 1998; Schutt, 1999). This protein is contained within a glycolipid-enriched microdomain in the plasma membrane, termed the lipid raft (Triantafilou *et al.*, 2002). Following complex binding of LPS-LPB to CD14, assembly of the TLR4 complex, composed of CD14, TLR4, myeloid differentiation protein 2, and heat shock protein (HSP)70, occurs on the lipid raft (Jiang *et al.*, 2000; Triantafilou and Triantafilou, 2002). Assembly and activation of this complex result in the membrane translocation of the intracellular adaptor protein MyD88, followed sequentially by the intracellular activation of IL-1 associated kinase-1 (IRAK-1) and MAPK (Hazeki *et al.*, 2003; Cushieri *et al.*, 2004a) leading ultimately to the production of pro-inflammatory cytokines.

MyD88, as being the core protein of bow-tie structure and being involved with high percentages in the pathways leading to five different phenotypes (Tables 4.3 and 4.4), is crucial for innate immunity and targeting it for treatment may cause other problems for the host. The atypical PKC, PKCζ, is essential toward the LPS-induced lipid raft mobilization of TLR4 within macrophages, TLR4-signaling, and TNF-α production and therefore the modulation of PKC-ζ activity during Gram-negative infections may limit associated inflammatory-induced morbidity (Cushieri *et al.*, 2004b). Additionally, Oda and Kitano (2006) have shown that PKCζ can stimulate MAPK cascade (Figure 3.3) and specifically the complex of IKK-α/IKK-β/IKK-γ. The inhibition of the complex of IKK-α/IKK-β/IKK-γ results in the down-regulation of the complex κB site/NF-κB (p65)/NF-κB (p50)/CBP, which is a species participating in 100% of the pathways leading to the phenotype of cytokine production (i.e. target genes ex. IL-1, IL-8, IL-6, TNFα, IFNγ, GM-CSF, M-CSF, iNOS, COX2, MHC class I/II, E-selectin, β-defensin 2).

6. CONCLUSIONS AND RECOMMENDATIONS

Comprehensive structural analysis of a large-scale directed signaling network of Toll-like receptors, which is recently constructed by Kitano and Oda (2006), has been performed via linear pathway analysis and the analysis based on graph theory. These analyses enlightened the structural features of the network with the assistance of topological properties. On one hand, linear pathway analysis yielded the participation percentages of molecules in the pathways leading to the phenotypes, the molecules having a role in the crosstalk of pathways, the molecules those being specific to the pathways targeting certain phenotypes, and species that can be identified as potential drug targets. On the other hand, graph theoretic approach shaped and quantified properties such as the degree distributions and the hubs, the diameter and the mean path length with the clustering coefficient.

6.1. Conclusions

Investigation of the molecules involved in the linear paths from the ligands to the phenotypes shows that many of the pathways in TLR network are crosstalking with each other via the complexes Rac1/GTP, Ubc13/Uev1A/TRAF6/TAB2/TAB3/TAK1/TAB1 and TLR2/TLR2 ligand with the percentages of 87.5%, 75% and 75%, respectively. Additionally; a high crosstalk is observed between the pathways leading to transcription of target genes ex. ICER, IFNα/β, IL-1 etc., and actin organization, as these pathways do not have any specific species.

The modulation of the atypical PKC, PKC-ζ, activity during Gram-negative bacterial infections may limit associated inflammatory-induced morbidity as it is essential toward the LPS-induced lipid raft mobilization of TLR4. Additionally, it can stimulate MAPK cascade and specifically the complex of IKK-α/IKK-β/IKK-γ, activation of which up-regulates the complex κB site/NF-κB (p65)/NF-κB (p50)/CBP, which is a species participating in 100% of the pathways leading to the phenotype of cytokine production (i.e. target genes ex. IL-1, IL-8, IL-6, TNFα, IFNγ, GM-CSF, M-CSF, iNOS, COX2, MHC

class I/II, E-selectin, β-defensin 2). Thus inhibition of PKC-ζ can be suggested as a drug mechanism for treating post-infection immunity diseases such as septick shock and atherosclerosis.

The directed interaction graph of TLR signaling network with 680 nodes (species) and 1292 edges (interactions between the species) (with 1058 nodes and 1410 edges when some species are assumed to constitute complex species), shows a small-world phenomenon having a mean path length of 8.98, a clustering coefficient of 0.009, and network and phenotype diameters of 25 and 11, respectively.

The in-degree and out-degree distributions of the nodes follow power law ($P(k) \approx k^{-\gamma}$) with γ_{in}=2.12 and γ_{out} =2.14 due to the scale-free nature of the TLR signaling network.

There are two in-degree and six out-degree hubs (Table 4.5) in the scale-free TLR network and therefore it is assumed to be robust.

The TLR2 ligand and the complex of Rac1/GTP are found to function as both hubs and species through which the network cross-talks, by graph theoretical and linear pathway analyses, respectively.

6.2. Recommendations

This study aims to clarify the unknown topological features of the TLR signaling network. The results, which may be considered as starting points for further researches, indicate the key points (crucial signaling molecules) in the signal transduction mechanisms.

Graph theory, by which the hubs in the TLR signaling network are identified, is a useful guide for experimental studies on protein interactions because the hubs are good targets for further experimental studies.

Linear pathway analysis also has an important role in revealing the important points affecting the structure of large-scale signaling mechanisms. Drug targets identified by this analysis are worth to take into consideration in further studies.

Other structural analysis techniques, such as Flux Balance Analysis or Metabolic Pathway Analysis, as well as dynamic analysis techniques can be applied as a further step for analyzing the network thoroughly and in order to enlighten the time-variant properties of the signaling network, as long as the required kinetic data are available.

Alternatively, most probable paths leading to phenotypes can be identified by using z-score values and correlation coefficient can be found in a micro-scale analysis of the network to elaborate on the effects of species on the phenotypes.

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APPENDIX A: ADDITIONAL DATA

Table A.1. Species taking place in most of the pathways (≥ 70%) leading to the phenotypes

Phenotype	Species	%
n. target gene ex. Bcl-2	NF-κB (p50)/Bcl-3 complex	100
	kappa B site	83.45233
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	76.90046
	Complex_br_(_kappa_ B site/NF-_kappa_ B_br_ (p65)/_br_NF-_kappa_ B_br_(p50)/CBP)	76.22848
	Complex_br_(IKK_alpha/IKK_beta/_br_IKK_gamma)	71.86056
	Akt	71.69257
n. cell cycle	c-Myc	100
	ERK1 ERK2	100
	ERK1_br_ERK2	100
	MKK1	100
	Complex_br_(Rac1/GTP)	100
	MKK1	94.64286
	TPL2_br_(p58)	82.51488
	Complex_br_(TRIP6/RIP2/_br_NOD1*)	73.73512
	MEKK1*	73.06548
	RKIP	73.06548
n. target genes ex. IFNα/β	ISRE	100
	Complex_br_(ISRE/IRF3)	99.24966
	IRF3	99.24966
	Akt	99.24966
	Akt	88.94952
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	87.44884
	TBK1*	73.80628
n. histone deacetylation	κB site	100
	κB site/NF-κB (p50) complex	100
	HDAC-1	100
	HDAC-1*	100
	NF-κB (p50)	100
	Complex_br_(_kappa_ B site/NF-_kappa_ B_br_ (p65)/_br_NF-_kappa_ B_br_(p50)/CBP)	88.742
	Akt	78.76333
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	77.61194
	Complex_br_(NF-_kappa_ B_br_(p50)/NF-_kappa_ B_br_ (p65))	75.99147
	CBP	75.99147
n. target genes ex. ICER	CBP	100
	CBP/CREB/ CRE site complex	100
	CRE site/CREB complex	100
	Complex_br_(_kappa_ B site/NF-_kappa_ B_br_ (p65)/_br_NF-_kappa_ B_br_(p50)/CBP)	84.1714
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	81.19808
	Akt	74.81417

Table A.1. Species taking place in most of the pathways (≥ 70%) leading to the phenotypes-continued

n. target genes ex. IL-1, IL-8, IL-6, TNFα, IFNγ, GM-CSF, M-CSF, iNOS, COX2, etc.	κB site	100
	CBP	100
	κB site/NF-κB (p65)/ NF-κB (p50)/CBP complex	100
	NF-κB (p50)/NF-κB (p65) complex	100
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	84.38426
	Akt	78.88278
n. apoptosis	TLR4 ligand/TLR4/ MD-2 complex	100
	TLR2/TLR2 ligand complex	100
	NUR77	100
	NUR77*	100
	Complex_br_(CBP/CREB/_br_CRE site)	99.81061
	Complex_br_(CRE site/CREB)	99.81061
	NUR77	99.81061
	CBP	99.81061
	Complex_br_(_kappa_ B site/NF-_kappa_ B_br_ (p65)/_br_NF-_kappa_ B_br_(p50)/CBP)	84.84848
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	75.56818
	Akt	75
n. target genes ex. cyclin D1	Ubc13/Uev1A/ TRAF6/TAB2 TAB3/ TAK1/TAB1 complex	100
	JNK	100
	p38α MAPK	100
	p38β 2MAPK	100
	ATF2	100
	Complex_br_(IRAK1/TRAF6/_br_MEKK3/TIFA/_br_Ubc13/Uev1A)	100
	Complex_br_(IRAK1/TRAF6/_br_TIFA/Ubc13/_br_Uev1A)	93.6796
	MKK4	91.55194
	MyD88	79.84981
	IRAK4	79.16145
	Complex_br_(IRAK1/TRAF6/_br_TIFA/Ubc13/_br_Uev1A)	78.66083
	Uev1A	78.34793
	Ubc13	76.03254
	TRAF6	76.03254
	pellino1	73.02879
	IRAK-M	72.65332
	IRAK2c	72.65332
	IRAK2d	72.65332
	pellino2	72.65332
	TIFA	72.65332
	TIR domain	72.65332
n. target genes ex. β-defensin 2	c-Jun	100
	JNK	100
	AP-1 site	100
	Complex_br_(c-Jun/c-Fos)	85.03695
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	76.16995

Table A.1. Species taking place in most of the pathways (≥ 70%) leading to the phenotypes-continued

n. RNA metabolism	PKCζ	100
	PDK1*	100
	Phosphatidic acid	100
	Phosphatidyl serine	100
	hnRNP A1	100
	Complex_br_(Rac1/GTP)	100
	PI4,5-P_sub_2	100
	Complex_br_(GTP/Ras)	100
	PI3,4,5-P_sub_3	99.95693
	Complex_br_(Cdc42/GTP)	99.39707
	Complex_br_(PI3K (p110)/PI3K(p85)*)	98.57881
	Complex_br_(TLR2/TLR2 ligand)	97.84668
	Complex_br_(PI3K (p110)/PI3K (p85))	96.72696
	MyD88	94.31525
	Complex_br_(TLR3/TLR3 ligand)	90.78381
	Complex_br_(IL-1RI_br_ligand/IL-1RI/_br_IL-1RAcP)	90.78381
	Complex_br_(Rac1*/GTP)	90.78381
	Complex_br_(IL-1RI_br_ligand/IL-1RI/_br_IL-1RAcP)	90.78381
	PDK1	90.78381
	PDK1*	90.78381
	IL-1RI	81.95521
	IL-1RI_br_ligand	81.65375
	IL-1RAcP	81.65375
	Complex_br_(IL-1RI_br_ligand/IL-1RI)	80.74935
	IL-1RII	80.74935
	IL-1ra	80.74935
	sIL-1RI	80.74935
	sIL-1RII	80.74935
	Complex_br_(TLR2/TLR2 ligand)	80.74935
	kappa B site	80.74935
	Complex_br_(NF-_kappa_ B_br_(p50)/NF-_kappa_ B_br_ (p65))	80.74935
	Complex_br_(_kappa_ B site/NF-_kappa_ B_br_ (p65)/_br_NF-_kappa_ B_br_(p50)/CBP)	79.24203
	CBP	77.64858
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	77.64858

Table A.1. Species taking place in most of the pathways (≥ 70%) leading to the phenotypes-continued

n. transcription	MSK1	100
	ERK1 ERK2	100
	p38α MAPK	100
	p38β 2MAPK	100
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	100
	Complex_br_(IRAK1/TRAF6/_br_TIFA/Ubc13/_br_Uev1A)	100
	MKK4	91.42857
	Complex_br_(IRAK1/TRAF6/_br_MEKK3/TIFA/_br_Ubc13/Uev1A)	79.72789
	MKK3	79.18367
	MKK6	79.18367
	IRAK4	79.18367
	MyD88	79.18367
	Uev1A	75.64626
	Ubc13	75.64626
p. protein synthesis	PKR	100
	TICAM1	100
	TICAM1*	100
	TICAM2*	100
	TLR3/TLR3 ligand complex	100
	dsRNA	100
	eIF2α	100
p. translation	eIF-4E	100
	ERK1 ERK2	100
	Mnk1	100
	p38α MAPK	100
	p38β 2MAPK	100
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	100
	Complex_br_(IRAK1/TRAF6/_br_TIFA/Ubc13/_br_Uev1A)	100
	MyD88	86.53702
	IRAK4	77.33732
	MKK4	71.27898
	Complex_br_(IRAK1/TRAF6/_br_MEKK3/TIFA/_br_Ubc13/Uev1A)	70.68063
	MKK3	70.30666
	MKK6	70.30666

Table A.1. Species taking place in most of the pathways (≥ 70%) leading to the phenotypes-continued

p. apoptosis	caspase8	100
	FADD/pro-caspase8 complex	100
	TIR domain/MyD88 complex	100
	pro-caspase8	99.91929
	FADD	99.91929
	Complex_br_(TIR domain/TIRAP/_br_MyD88)	99.91929
	MyD88	99.91929
	Complex_br_(TLR8/TLR8 ligand)	99.1929
	Complex_br_(TLR9/TLR9 ligand)	99.1929
	Complex_br_(TLR7/TLR7 ligand)	99.1929
	TIR domain	99.03148
	Complex_br_(TLR11/TLR11 ligand)	98.70864
	Complex_br_(TLR5/TLR5 ligand)	98.70864
	Complex_br_(TLR10/TLR10 ligand)	98.70864
	Complex_br_(TLR2/TLR10/_br_TLR2/10 ligand)	98.70864
	Complex_br_(TLR1/TLR10/_br_TLR1/10 ligand)	98.70864
	Complex_br_(IL-1RI_br_ligand/IL-1RI/_br_IL-1RAcP)	97.25585
	Complex_br_(IL-1RI_br_ligand/IL-1RI)	97.25585
	IL-1RI_br_ligand	97.25585
	sIL-1RII	97.17514
	sIL-1RI	97.17514
	IL-1ra	97.17514
	IL-1RII	97.17514
	IL-1RAcP	97.17514
	IL-1RI	97.17514
	CBP	95.2381
	Complex_br_(_kappa_ B site/NF-_kappa_ B_br_ (p65)/_br_NF-_kappa_ B_br_(p50)/CBP)	95.2381
	Complex_br_(NF-_kappa_ B_br_(p50)/NF-_kappa_ B_br_ (p65))	95.2381
	kappa B site	95.2381
	Akt	85.23002
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	72.31638

Table A.1. Species taking place in most of the pathways (≥ 70%) leading to the phenotypes-continued

p. actin organization	IL-1RI ligand/IL-1RI/ IL-1RAcP complex	100
	IL-1RI	98.38085
	IL-1RI_br_ligand	98.38085
	IL-1RAcP	98.38085
	Complex_br_(IL-1RI_br_ligand/IL-1RI)	98.38085
	IL-1RII	98.38085
	IL-1ra	98.38085
	sIL-1RI	98.38085
	sIL-1RII	98.38085
	kappa B site	96.51531
	Complex_br_(NF-_kappa_ B_br_(p50)/NF-_kappa_ B_br_ (p65))	96.51531
	Complex_br_(_kappa_ B site/NF-_kappa_ B_br_ (p65)/_br_NF-_kappa_ B_br_(p50)/CBP)	96.51531
	CBP	96.51531
	Akt	84.61809
	Complex_br_(TLR2/TLR2 ligand)	83.49173
	Complex_br_(Rac1/GTP)	79.37346
	Complex_br_(Ubc13/Uev1A/_br_TRAF6/TAB2_br_TAB3/_br_TAK1/TAB1)	74.26962
p. ROS production	$p40^{phox}$	100
	$p47^{phox}$	100
	$p67^{phox}$	100
	$gp91^{phox}/p22^{phox}/ p47^{phox}$/Rac1/ GTP/$p40^{phox}/ p67^{phox}$ complex	100
	$p22^{phox}/gp91^{phox}$ complex	100
	Rac1/GTP complex	100
	Phosphatidic acid	100
	PI4,5-P_sub_2	96.79316
	Complex_br_(GTP/Ras)	93.42598
	Complex_br_(TLR2/TLR2 ligand)	88.56227
	PI3,4,5-P_sub_3	85.8899
	GTP	85.14164
	DAG	84.28648
	Complex_br_(Cdc42/GTP)	82.78995
	PKC_zeta	78.94174
	phosphatidyl_br_serine	77.28487
	p47_super_phox	77.28487
	PKC_alpha_ *_br_PKC_beta_ II	77.28487
	PKC_delta_ *	77.28487
	BTK*	75.62801
	Vav1	72.90219
	Complex_br_(RhoA/GTP)	72.7953
	Src kinases*	70.39017
	BTK*	70.39017
	BTK*	70.39017