Chapter- II: Review of Literature
All living organisms have to face the day to day challenges brought forth by the environment and adapt accordingly in order to survive. One of the major challenges faced, is combating the invading pathogens. All organisms in order to maintain their homeostasis combat these pathogens with the help of their immune system.

Rudimentary forms of immune system exists in bacteria too where restriction enzymes protect them from bacteriophage infections. Other primitive forms of immune system have been evidenced in plants to invertebrates, where antimicrobial peptides defensins, non organic molecules phytoalexins are involved in combating the microbial pathogens. In case of vertebrates the defense mechanisms are highly evolved and more complex. It is divided into two main branches, the Innate Immune system, a more primitive one and Adaptive Immune system, a highly evolved one.

The invading pathogen has to evade various layers of immune defense system in order to spread its infection and the first layer of defense is the surface barrier which firstly includes the physical barrier i.e. the skin, the exoskeleton in insects, mucus in the respiratory and gastrointestinal tract etc. Secondly the chemical barriers i.e. lysozyme present in the tears, phospholipase A2 in saliva, gastric acid in the stomach etc. and finally the biological barriers which include the microbial flora in the gastrointestinal tract that compete with the pathogens for food and space and also bring about changes in the microenvironment like pH levels etc. However, if the pathogen is able to breach these surface barriers and enter the system, it has to face the next layer of immune defense, the innate immune system. The innate immune system mounts an immediate but a non-specific immune response against the pathogen in order to limit its spread of infection and simultaneously thereafter it activates the adaptive immune system for a more pronounced and specific immune response to take over. The
adaptive immune system upon activation by innate immune system, kills the invading pathogen with the help of the humoral and cell mediated response that simultaneously maintain a memory for a rapid response to future exposure to the same pathogen.

The innate immune system though involved at the beginning of the invasion has an important role to play and has gained the limelight just a few years back with focus shifting from the adaptive immune system. The components of the innate immune system include various germ-line encoded receptors and cellular components that recognize the broad structural motifs present on these microbial pathogens. These pathogen associated molecular patterns (PAMPs) are recognized with the help of Pattern Recognition Receptors (PRRs) present on the surface of the antigen presenting cells (APCs) that include mast cells, monocytes, tissue macrophages, B cells (component of the humoral immunity) etc. These APCs after getting stimulated themselves activate the cell mediated immune system by presenting the microbial antigens to the T cells.

The adaptive immune system has the ability to distinguish subtle differences among antigens. It can be divided into humoral immunity and cell mediated immunity. Humoral immunity includes the B lymphocytes which upon activation proliferate into plasma cells which produce various immunoglobulins IgM, IgG, etc. against the antigen. On the other hand the cell mediated system is governed by T cells, which play a central role in the adaptive immune system, being further subdivided into T helper cells and T cytotoxic cells. The T helper cells further subdivide into TH1 and TH2 cells, each subset can be distinguished by the cytokines they secrete as well as with their functional difference against a particular pathogen.
2.1. Innate Immune System

The innate immune system, as the name suggests is an inborn system coded by the germline itself in contrast to its more sophisticated and complex counterpart, the adaptive immune system. It comprises of cells, various receptors, other factors, and defense mechanisms involved in limiting the infection caused by the pathogen. The immune response mounted is immediate and non specific. The various components of the innate immune system can be divided into the humoral factors, cellular components and pattern recognition receptors (PRRs). All these components work together as a team to firstly, recognize, kill and limit the infection via acute inflammation and secondly activate the adaptive immune system. Acute inflammation is characterized by five classical symptoms of redness, swelling, pain, heat and loss of tissue function. These symptoms are caused due to the increased permeability of the vascular endothelium allowing extravasation of immune cells (Takeuchi et al., 2010).

2.1.1. Humoral Factors

The humoral factors of the innate immune system include the complement system, cytokines & chemokines. The name complement was given by Paul Erhlich in the 1890s, describing a heat labile agent that helps the cellular components of the immune system in killing the pathogen. The complement system acts on the invading pathogens in three different ways depending on the difference in the pathogen surface molecules. In the first way a large number of activated complement proteins bind covalently to the pathogen surfaces known as the phenomena of opsonization, enabling phagocytosis. Secondly, certain fragments of the complement system proteins act as chemo-attractant for the influx of phagocytes at the site of complement activation. Thirdly, certain components of the complement system damage the membrane of various bacteria by forming pores on the
surface (Janeway CA et al., 2001). On the other hand, cytokines and chemokines are the effector molecules, playing the role of a communicating bridge between cells of both the innate as well as adaptive immune system. Cytokines can work either individually or in synergy with other cytokines to bring upon the desired effect or may induce a cascade where action of one cytokine induces the target cell to produce one or more cytokines to further induce other target cells. Cytokines on the basis of their actions can be subdivided into proinflammatory cytokines which include TNF-α, IL-1, IL-6, IL-12 that promote an inflammatory response, and anti-inflammatory cytokines which include IL-6, IL-10, that control the proinflammatory cytokine response. TNF-α is an important inflammatory cytokine and its primary role is in the regulation of immune cells, induce fever by acting on the hypothalamus along with IL-1 and IL-6, stimulate phagocytosis in macrophages etc. IL-12 plays a pivotal role in initiating an early cell mediated response. IL-10 is an anti-inflammatory cytokine that down regulates the expression of Th1 cytokines. Chemokines are a subset of lower molecular weight cytokines that act as recruiting agents for the phagocytes to get attracted towards the site of primary infection as well as affect leukocyte behavior which mainly include MCP-1, MIP-1, CCL5 etc. MIP-1 causes chemotactic activity for monocytes and it has been implicated in pathogenesis of several diseases characterized by monocyte infiltration.

2.1.2. Cellular Components
The two major immune cells that play a vital role in the innate immune system are the dendritic cells and the monocytes/macrophages and others being the Natural Killer (NK) cells, Neutrophils and NKT cells. Certain nonprofessional cells like the endothelial cells, epithelial cells and fibroblasts also play role in innate immunity but only to a limited extent. Dendritic cells and monocytes are also known as the
professional Antigen Presenting Cells (APCs) and act as links between the innate and adaptive immune system. They express an array of scavenger receptors like pattern recognition receptors (PRRs) which upon detection and in response to the pathogens release large number of effector molecules like cytokines and chemokines. Dendritic cells are potent T cell stimulators and can be divided into two subsets, myeloid dendritic cells (mDCs), and plasmacytoid dendritic cells (pDCs). The mDCs represent 0.5% of the peripheral blood mononuclear cell population and can be distinguished from monocytes with presence of CD11c and absence of CD14 and CD16 (Van Voorhis et al., 1983). The pDCs also represent 0.5% of PBMC population and have shown to be potent IFN-γ producing cells against viral infection (Cella et al., 2000).

Monocytes are derived from hematopoietic bone marrow cells. After this process, monocytes circulate in the bloodstream where they mature. Then they migrate in the tissue to differentiate into macrophages which are larger in size and have increased phagocytic abilities (Figure 4). It has been shown that monocytes can be divided into three subsets according to the presence or absence of CD14, CD16 receptors as well as on the basis of cytokine production. The three subsets include CD14+CD16+ cells, CD14+CD16− cells and CD14dimCD16+ cells. The CD14+CD16− monocytes represent the major portion of the monocyte population and primarily release IL-10 instead of TNF-α and IL-1 (Ancuta et al., 2003; Ziegler-Heitbrock et al., 1992; Weber et al., 2000; Giessmann et al., 2003). On the other hand the CD14−CD16+ cells release TNF-α and IL-1 and has been reported to be found in larger numbers in patients suffering with acute inflammation or infectious diseases (Fingerle-Rowson et al., 1998; Horelt et al., 2002; Grage-Griebenow et al., 2001). The role of CD14 dimCD16+ cells that express CD16 and very low levels of CD14 is still unknown and are found to be poorly phagocytic and do not produce IL-1 and TNF-α. (Skrzeczynska-Moncznik et al., 2008; Auffray et al., 2009).
2.2. Pattern Recognition Receptors

Structural motifs that are conserved amongst the pathogenic microbial species like lipopolysaccharide (LPS), flagellin, CpG motifs etc. and which activate the immune response are called as Pathogen associated molecular patterns (PAMPs). These PAMPs are recognized by germline receptors called as Pattern recognition receptors (PRRs). This family of receptors include transmembrane proteins such as C-type lectin receptors (CLRs) and Toll like receptors (TLRs) as well as cytoplasmic proteins like Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi et al., 2010). Upon sensing of PAMPs the PRRs up-regulate the transcription of genes involved in inflammatory responses which include proinflammatory cytokines, IFNs, chemokines and antimicrobial proteins. Moreover, recent evidence has revealed that PRRs also recognize endogenous molecules released from damaged cells called as Damage associated molecular patterns (DAMPs) (Yang et al., 2010).
2.2.1. C-type Lectin Receptors (CLRs)
CLRs are transmembrane receptors characterized by the presence of a carbohydrate-binding domain. They recognize carbohydrates on microorganisms such as viruses, bacteria and fungi. It has been reported that CLRs either stimulate the production of proinflammatory cytokines or inhibit TLR-mediated immune complexes. (Takeuchi et al., 2010).

2.2.2. Retinoic acid inducible gene (RIG)-I-like receptor
RLRs are cytoplasmic receptors and the subfamily consist of three RLRs - RIG-1, MDA5, LGP2 (Takeuchi et al., 2009; Yoneyama et al., 2008). Each RLR is composed of two N terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain and a C-terminal regulatory domain. The RLRs recognize genomic RNA of dsRNA viruses and dsRNA that is generated as the replication intermediate of ssRNA viruses. It has been observed that the expression of RLRs is highly increased in response to type I IFN stimulation or virus infection (Takeuchi et al., 2010).

2.2.3. NOD like Receptor (NLRs)
The NLRs consist of cytoplasmic pathogen sensors that are composed of a central nucleotide-binding domain (NOD) and C-terminal leucine rich repeats (LRRs) (Inohara et al 2005). In addition, the N-terminal portions of most NLRs comprise of protein binding motifs such CARDs (caspase recruitment domain), a pyrin domain and a baculovirus inhibitor repeat (BIR) domain. The NLRs can be divided into four subfamilies of receptors on basis of N-terminal domain. The NLRs NOD1 and NOD2 comprising of the CARD domain in addition to NOD domain and LRR domains recognize the structures of bacterial peptidoglycans, muramyl dipeptide (MDP).
2.2.4. Toll like Receptors (TLRs)

TLRs are transmembrane proteins that were first identified in Drosophila. The word “Toll” (meaning “great” in German) was coined by Prof. Volhard in 1985. Drosophila Toll receptor was first shown to play a role in establishing dorso-ventral polarity in the developing embryo (Hashimoto et al., 1988; Takeda et al., 2003). It was only later that its role in innate immunity was identified by Lemaitre et al. 1996. In 1997 human homologue to Drosophila Toll was identified by Prof. Janeway and Prof. Medzhitov (Medzhitov et al., 1997) later named as TLR4. A year later Beutler group showed that the major function of TLR4 was as a LPS sensing receptor (Poltorak et al., 1998). Since then 10 TLRs have been identified in humans each having their role in the innate immune response, recognizing different set of microbial motifs as shown in Table 3 and Figure 5, mostly done in the laboratory of Prof. Akira.

Table 3 – Different TLRs showing their cell location, on the type of cells they are present and ligand type.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Location</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Lipopeptides</td>
<td>Cell surface</td>
<td>Monocytes/macrophages, B cells</td>
</tr>
<tr>
<td>TLR2</td>
<td>Glycolipids</td>
<td>Cell surface</td>
<td>Monocytes/macrophages, dendritic cells</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double stranded RNA</td>
<td>Cell compartment</td>
<td>Dendritic cells, B cells</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, heat shock protein</td>
<td>Cell surface</td>
<td>Monocytes/macrophages, dendritic cells, B cells</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>Cell surface</td>
<td>Monocytes/macrophages, dendritic cells</td>
</tr>
<tr>
<td>TLR6</td>
<td>Lipopeptides</td>
<td>Cell surface</td>
<td>Monocytes/macrophages, B cells</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single stranded RNA</td>
<td>Cell compartment</td>
<td>Monocytes/macrophages, B cells, a subset of dendritic cells</td>
</tr>
<tr>
<td>TLR8</td>
<td>Small synthetic compounds, ssRNA</td>
<td>Cell compartment</td>
<td>Monocytes/macrophages, a subset of dendritic cells</td>
</tr>
<tr>
<td>TLR9</td>
<td>CG motifs</td>
<td>Cell compartment</td>
<td>Monocytes/macrophages, dendritic cells, B cells</td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>
2.3. Structure and Chromosomal Location

TLRs are Type I integral transmembrane glycoproteins with their cytoplasmic region showing high homology with Interleukin 1 receptors (IL-1Rs) as shown in Figure 6. The cytoplasmic domain of TLR is known as Toll/Interleukin 1 receptor (TIR) domain and it contains a central five stranded parallel β sheets surrounded by five α helices on each side. The extracellular domain of TLRs contains 19-25 tandem copies of leucine rich repeats (LRRs). Each repeat contains 24-29 amino acids and contains the leucine rich sequence XLXXLXLXX and another conserved sequence XΦXXΦXXXXFXXLX where ‘X’ represents any amino acid, and Φ’ a hydrophobic amino acid. The LRRs create a large horseshoe structure consisting of β sheets and α helices. (Bell et al., 2003).
The sub-cellular localization of TLRs is also different. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface whereas TLR3, TLR7, TLR8 and TLR9 are expressed in different cell compartments.

As per gene location is concerned TLR1 and TLR6 map very closely to 4p14 (Rock et al., 1998; Takeuchi et al., 1999), TLR2 and TLR3 to 4q32 and 4q35 respectively, TLR4 to 9q32-33 and TLR5 to 1q33.3 (Rock et al., 1998), TLR7 and TLR8 located at Xp22 in tandem and TLR9 at 3p21.3 (Chuang TH et al., 2000; Du X et al., 2000). In addition the 10 TLRs are divided into 5 subfamilies on basis of their genomic structure where TLR2 consists of two exons and the coding region residing in the second exon itself. The second family consists of TLR7, TLR8 and TLR9 consisting of two exons as well but coded by both the exons. The third

Figure 6- Comparison between structure of TLRs and TIRs. Source - Akira & Takeda, Nature Reviews, Immunology, 2004.
and fourth family consists of TLR4 and TLR5 with four and five exons respectively and fifth family contains TLR3 consisting of five exons and the coding region residing in exon 2 to 5 (Takeda et al., 2003).

2.4. TLR Signaling

The signaling cascade involves various components which finally leads to the production of myriad of proinflammatory cytokines and chemokines for a potent immune response. Upon ligand binding the TLRs form homo-dimers, except for TLR2 which forms a heterodimer with either TLR1 or TLR6 depending on the ligand, (Figure 5) and undergo conformational changes required for downstream signaling. The first adaptor molecule to be involved in the signaling cascade is MyD88 (Myeloid differentiation primary response protein 88) which has a C terminal TIR domain and a N terminal Death Domain (DD) separated by a short linker sequence (Akira S et al., 2004). It interacts with the TLRs through its TIR domain and is an essential adaptor molecule for all the TLRs except for TLR3. It then interacts through its death domain with the N terminal death domain of IRAK4 (IL-1Receptor-associated kinase 4), a serine/threonine kinase. Upon activation IRAK4 further activates other IRAK family members, IRAK1 and IRAK2 (Kawagoe et al., 2008; Takeuchi et al., 2010) which then dissociates from MyD88 and activate TNFR-associated factor 6 (TRAF6) by phosphorylation. TRAF6 then forms a complex with Ubc13 and Uev1A proteins which collectively form the E2 ubiquitin conjugating enzyme complex where TRAF6 serve as the ubiquitin ligase (E3). TRAF6 catalyzes the formation of Lysine 63 (K63) linked polyubiquitin chain on TRAF6 and an unconjugated free polyubiquitin chain (Xia ZP et al., 2009) The unconjugated free K63 polyubiquitin chain activates a complex composed of TAK1 (Transforming growth factor β-activated kinase), TAB1 (TAK1-binding protein1) and TAB2 and TAB3. This complex in turn phosphorylates IκB kinase (IKK)-β and MAP kinase kinase 6. Thereafter the IKK complex
composed of IKK-α and IKK-β and NF-κB essential modulator (NEMO) phosphorylates IκBa, a NF-κB inhibitor protein. Phosphorylated IκBa is degraded by the ubiquitin-proteasome system, thereby freeing NF-κB. NF-κB then translocates into the nucleus and activates the expression of proinflammatory cytokine genes (Takeuchi et al., 2010). In addition, another transcription factor AP1 also gets activated via the MAP kinase pathway, which also translocates into the nucleus targeting the cytokine genes. The signaling cascade is shown in Figure 7.

**Figure 7**- TLR signaling cascade. Source – Takeuchi & Akira, Cell, 2010.
Moreover, in addition to MyD88 another adaptor molecule involved in the signaling cascade of TLR2 and TLR4 is TIRAP (TIR domain-containing adaptor protein, also known as Mal (MyD88 adaptor like protein). Like MyD88 this adaptor molecule has a TIR domain but lacks a Death domain. In the signaling cascade of TLR2 and TLR4, TIRAP acts as an essential bridge between the TLR and MyD88 (Fitzgerald et al., 2001; Horng et al., 2001; Krutzik et al., 2003). Likewise another adaptor molecule TRIF (TIR domain containing adaptor inducing IFN-β) is involved in the signaling cascade of TLR3 majorly. TRIF interacts with TRAF6 and TRAF3 with its N terminal TRAF binding motifs. This signaling cascade with TRIF finally leads to the activation of IRF3 and IRF7 (IFN regulatory factor) which translocates to the nucleus, activating the expression of type I-IFNs (Takeuchi et al., 2010).

2.5. TLR and diseases
Since the discovery of TLRs more than a decade ago, its role in various diseases has been elucidated from cancers to inflammatory diseases which include, Tuberculosis, (Ben-Ali et al., 2004) severe Sepsis, (Barber et al., 2006) Asthma, (Waltraud et al., 2004) Crohn’s disease, (Torok et al., 2004) Staphylococcal infection, (Lorenz et al., 2000). TLR activation leads to NF-κB activation finally leading to the expression of cytokines and chemokines. This in turn leads to the recruitment of immune cells required for killing and clearing of the microbial pathogens. In the case of leprosy the heterodimer of TLR2/TLR1 is responsible for the identifying the triacylated lipoprotein of Mycobacterium leprae (Krutzik et al., 2003). Whereas in cancers it has been shown that the TLR pathway maybe subverted by the neoplastic process resulting in a cascade leading to enhanced tumor cell invasion and immune surveillance evasion (Killeen et al., 2006). Moreover, TLR activation may promote carcinogenesis by creating a proinflammatory environment that is conducive for tumor growth and chemo-resistance (Chen et al.,
2007). However, recognition of the pathogen by the TLRs in various infectious diseases is a very crucial step which further orchestrates the downstream events, as the pattern of cytokines being expressed influences the type of adaptive immune response will be employed to fight the invading pathogen (McInturff et al., 2005).

2.6. TLR and Malaria

The complex life cycle of malaria parasite, *Plasmodium* gives it an undue advantage of surviving in the host for a longer time by evading the immune system. However, it is during the blood stage of the life cycle is the parasite exposed to the immune system and the machinery recognizing it is TLR. The role of TLRs in malaria was recently elucidated as it was showed that Glycophosphatidylionsitol (GPI) of *Plasmodium falciparum* (Krishnagowda et al., 2005), host fibrinogen bound hemozoin (Barrera et al., 2011) and parasite histone-DNA complex (Gowda et al., 2011) act as ligands for TLR2, TLR4 and TLR9 respectively.

2.7. Polymorphism and TLR

Polymorphism, occurrence of two or more genetic variants of a particular trait in a population, is a phenomenon that has helped an individual to evolve, adapt and survive. Polymorphisms can either have a positive effect which leads to their selection or have a negative effect that eliminates them from the population be it under the influence of environment or disease pressure.

As observed in case of Sickle cell anemia, the heterozygotes and homozygous mutants have an advantage over the wild type as the deformed RBCs do not facilitate growth of the parasite, protecting them against malaria (Cooke et al., 2001; Weatherall et al., 2002). Similarly, it has been studied that \( \alpha^+ \) thalassaemia has a very strong protective effect
against malaria in both heterozygous as well as homozygous state (Allen et al., 1997).

Thus, genetic makeup of the individual has a very important role to play in its survival. Moreover, genetic polymorphisms in immune genes directly affect the immune response against a particular pathogen. One such immune gene that has gained importance in the recent years is Toll-like Receptors (TLRs).

A number of polymorphism studies have been carried on TLR2, TLR4, TLR9 and TIRAP/Mal. The polymorphism at residue positions Arg677Trp and Arg753Gln in TLR2 have been associated with tuberculosis making an individual susceptible to the disease (Ben-Ali et al., 2004). In addition, it has been studied that heterozygotes for residue position Arg753 alone are protected from developing late stage Lyme disease due to reduced signaling via TLR2/TLR1 (Schröder et al., 2005). Similarly, polymorphism at residue positions Asp299Gln and Thr399Ile in TLR4 have shown to confer risk for typhoid infection (Bhuvanendran et al., 2011), risk of different types of cancers (Kutikhin et al., 2011) including gastric cancer (Santini et al., 2008). Nucleotide positions -T1237C and -T1486C in TLR9 have also shown to be associated with Crohn’s disease (Torok et al., 2005), lung diseases (Pabst et al., 2011) and atopic eczema (Novak et al., 2007). In case of TIRAP, mutant at residue position 180L has shown to be protective against septic shock (Ferwerda et al., 2009). Moreover, heterozygous state of S180L in TIRAP has shown to be protective against pneumococcal disease, tuberculosis and bacteremia (Khor et al., 2007).

In addition mutations at these common polymorphic positions in TLR2, 4 and 9 have been associated with malaria (Mockenhaupt et al., 2006a; Mockenhaupt et al., 2006b). Moreover, the heterozygosity at residue position Ser180Leu in TIRAP (MAL), an adaptor molecule involved in the TLR signaling cascade, has shown to impart protection against malaria as published by Khor et al., 2007.