INTRODUCTION
AND
REVIEW OF LITERATURE
INTRODUCTION

When the earth was formed about 4.6 billion years ago, it was lifeless and inhospitable place. According to evidences, first form of life appeared on the earth around 3.5 billion years ago and has subsequently taken many forms, all of which continue to evolve, and as a consequence of evolution now earth is a place where a large number of organisms of diverse species live together(1,2). In order to survive and propagate these species interact with each other in various ways and establish different ecological relationships like mutualism, commensalisms and parasitism. Parasitism is an intimate relationship between two organisms in which one (the parasite or pathogen) lives on, off or at the expense of the other (host). Pathogens negatively influence host fitness, and subsequently hosts develop defense machinery against them, i.e. a performing immune system, in order to reduce fitness cost induced by the pathogens. The ability of the host to resist infection or damage caused by the pathogen is known as Immunity. The word “immunity” (L: immunis – free of) was used in the context of being free of the burden of taxes or military conscription and later on it was adopted to designate this naturally acquired protection against diseases such as measles or smallpox.

HISTORY OF IMMUNOLOGY

In ancient times, people were not much aware about the pathogens and the existence of an immune system, which fights against pathogen. People believed that getting disease is a result of punishment or curse given by God in response to their evil deeds or sin. In 1798 we begin to understand immunity, when the English physician Edward Jenner (1749-1823) published a report that people could be protected from deadly smallpox by sticking them with a needle dipped in the pus from a cowpox boil. The great French biologist and chemist Louis Pasteur (1822-1895) theorized that such immunization protects people against disease by exposing them to a version of a microbe that is harmless but is enough like the disease-causing organism, or pathogen, that the immune system learns to fight it. Modern vaccines against diseases such as measles, polio, and
chicken pox are based on this principle. In 1882 a German scientist Robert Koch shook the medical world (and trumped Pasteur) by being the first to isolate the microbe that caused the human disease of tuberculosis.

In 1888 Emile Roux and Alexandre Yersin isolated a soluble toxin from cultures of diphtheria. The bacterium itself is only found in the throat but its destructive effects are found throughout the body. Clearly to us the bacteria must be sending out an invisible factor, most likely chemical in nature, to cause the body wide destruction. This idea was the hypothesis of Roux and Yersin. They filtered diphtheria cultures to remove the bacteria and then used the remaining fluid filtrate (we call supernatant) to inject into healthy animals. As expected the animals showed diphtheria lesions but without any obvious presence of bacteria. Next on the podium were Emil von Behring and Shibasaburo Kitasato who took serum from animals infected with diphtheria and injected it into healthy animals. When these animals were later inoculated with diphtheria they were found to be resistant to infection. We now know this method of conferring infection resistance as “passive immunity”. This first demonstration of defense against infection was revealed and described as mediated by “antitoxin”. It was clear to Behring and Kitasato that the antitoxin was specific only for diphtheria; it did not confer any defense against other forms of infection. We now know this antitoxin to be antibodies produced specifically against the diphtheria microbe. Rudolf Kraus in 1897 first visualized the reaction of antitoxins to bacteria by simply adding serum from infected animals to a culture of the bacteria and seeing a cloudy precipitate develop as the antibodies bound together the bacteria.

In the late nineteenth century; a scientific debate was waged between the German physician Paul Ehrlich (1854-1915) and the Russian zoologist Elie Metchnikoff (1845-1916). Ehrlich and his followers believed that proteins in the blood, called antibodies, eliminated pathogens by sticking to them; this phenomenon became known as humoral immunity. Metchnikoff and his students, on the other hand, noted that certain white blood cells could engulf and digest foreign materials: this cellular immunity, they claimed, was
the real way the body fought infection. Modern immunologists have shown that both the humoral and cellular responses play a role in fighting disease. They have also identified many of the actors and processes that form the immune response.

**IMMUNE SYSTEM**

The selective pressure imposed by infectious pathogens has driven multi-cellular organisms to develop immune defense mechanisms known as Immune system which protects the host by destroying the invading microbes or neutralizing the factors responsible for their virulence (3). The immune system is a complex and highly developed system, yet its mission is simple: to seek and kill invaders but its presence and proper functioning is essential for a person to lead a healthy life. If a person born with a severely defective immune system, are prone to get infection from virus, bacterium, fungus or parasite and also hyperactivation of the immune system leads to autoimmune disease.

The need for defense against microbial invasion possesses two fundamental problems for the host immune system. First, there are many would-be pathogens. How does the host recognize and destroy all of them? Second, how does the host discriminate between the constituents of the external world and the constituents of ‘self”? In vertebrates, the immune response hinge upon the functional integration between two arms of the immune system: the innate immune system, which is rapid but antigen non-specific and adaptive immune system, which is antigen specific but elicited later. In different ways, each system has solved both fundamental problems.

**Innate immune system**

The innate immune system is an evolutionarily older defense strategy; it was developed even before the separation of invertebrates and vertebrates. The innate immune system is what we are born with and it is rapid but nonspecific; it is genetically based and we pass
it on to our offspring. Immunity offered by the innate immune system is called innate, or natural, immunity and it includes two parts. One part, called humoral innate immunity, involves a variety of substances like complement proteins and defensins which are found in the humors, or body fluids. These substances interfere with the growth of pathogens or clump them together so that they can be eliminated from the body. The other part, called cellular innate immunity, is carried out by cells called phagocytes that ingest and degrade, or “eat” pathogens. All together innate Immunity was formerly thought to be a rapid and non-specific immune response characterized by engulfment and digestion of microorganisms and foreign substances by phagocytic cells and act as antigen presenter to adaptive immune cells. Later on with the advent of Toll Like Receptors (TLRs), a kind of Pattern recognition receptors (PRRs), redefined the role of the innate immune system as a system having considerable specificity in discriminating between Pathogens from self and it is essential for efficient functioning of the innate as well as for adaptive immune system.

**Pattern Recognition Receptors**

In order to protect against infection, one of the first things the body must do is the detection of the presence of microorganisms. The body initially does this by recognizing molecules unique to groups of related microorganisms and is not associated with human cells. These unique microbial molecules are called pathogen-associated molecular patterns or PAMPs. To recognize PAMPs, various body cells have a variety of germline-encoded receptors called pattern-recognition receptors or PRRs capable of binding specifically to conserved portions of these molecules (4). Cells that typically have pattern recognition receptors include macrophages, dendritic cells, endothelial cells, mucosal epithelial cells, and lymphocytes. Many pattern-recognition receptors are located on the surface of these cells where they can interact with PAMPs on the surface of microbes. Others PRRs are found within the phagolysosomes of phagocytes where they can interact
with PAMPs located within microbes that have been phagocytosed. Some PRRs are found in the cytosol of the cell.

There are two functionally different major classes of pattern-recognition receptors: endocytic pattern-recognition receptors and signaling pattern-recognition receptors (5).

**a. Endocytic Pattern-Recognition Receptors**

Endocytic pattern-recognition receptors are found on the surface of phagocytes and promote the attachment of microorganisms to phagocytes leading to their subsequent engulfment and destruction. They include:

- **mannose receptors**: These receptors found on the surface of phagocytes bind mannose-rich glycans, the short carbohydrate chains with the sugar mannose or fructose as the terminal sugar that are commonly found in microbial glycoproteins and glycolipids but are rare in those of humans. C-type lectins found on the surface of phagocytes are mannose receptors.

- **scavenger receptors**: Scavenger receptors found on the surface of phagocytic cells bind to bacterial cell wall components such as LPS, peptidoglycan and teichoic acids etc. There are also scavenger receptors for certain components of other types of microorganisms, as well as for stressed, infected, or injured cells. Scavenger receptors include CD-36, CD-68, and SRB-1.

- **opsonin receptors**: These are soluble molecules produced as a Acute phase proteins like mannose-binding protein, C-reactive protein (CRP), Complement pathway proteins like C3b and C4b, Surfactant proteins in the alveoli of the lungs, such as SP-A and SP-D. These soluble molecules binds to microbes and help in phagocytosis. One portion of the opsonin binds to a PAMP on the microbial surface and another portion binds to a specific receptor on the phagocytic cell.

- **N-formyl Met receptors**: FPR and FPRL1 are N-formyl Met receptors on neutrophils and macrophages binds to N-formyl Methionine, which is an
amino acid seen only in prokaryotes and promotes the motility and the chemotaxis of these phagocytes. It also promotes phagocytosis.

b. Signaling Pattern-Recognition Receptors

Signaling pattern-recognition receptors bind a number of microbial molecules: LPS, peptidoglycan, teichoic acids, flagellin, pilin, unmethylated cytosine-guanine dinucleotide or CpG sequences from bacterial and viral genomes; lipoteichoic acid, glycolipids, and zymosan from fungi; double-stranded viral RNA, and certain single-stranded viral RNAs. Binding of microbial PAMPs to their PRRs initiates signaling which promotes the synthesis and secretion of intracellular regulatory molecules such as cytokines which are crucial for initiating innate immunity and adaptive immunity.

- **NODs (nucleotide-binding oligomerization domain):** NOD proteins, including NOD-1 and NOD-2, are cytosolic proteins that allow intracellular recognition of peptidoglycan components like muramyl dipeptide NAG-NAM-gamma-D-glutamyl-meso diaminopimelic acid and muramyl dipeptide NAG-NAM-L-alanyl-isoglutamine respectively. Binding of the muramyl dipeptides to NOD-1 or NOD-2 leads to the activation of genes coding for inflammatory cytokines such as IL-1, TNF-α, IL-8, and IL-12.

- **CARD-containing proteins:** CARD (caspase activating and recruitment domain)-containing proteins, such as RIG-1 (retinoic acid-inducible gene-1) and MDA-5 (melanoma differentiation-associated gene-5), are cytoplasmic sensors that bind viral RNA molecules produced in viral-infected cells and trigger the synthesis of cytokines called interferons that block viral replication within infected host cells in a manner similar to the endosomal TLRs.

- **Toll-Like Receptors (TLRs):** An array of signaling pattern-recognition receptors known as toll-like receptors (TLRs) are found on the surface of a variety of
defense cells and other cells. These TLRs play a major role in the induction of innate immunity and contribute to the induction of adaptive immunity.

**Adaptive immune system**

In addition to the innate defense mechanisms, jawed vertebrates (gnathostomes) have evolved an adaptive immune system mediated primarily by lymphocytes. By virtue of rearrangeable *immunoglobulin (Ig)* V, D, and J gene segments, the jawed vertebrates generate a lymphocyte receptor repertoire of sufficient diversity to recognize the antigenic component of any potential pathogen or toxin. All jawed vertebrates, beginning with cartilaginous fish, rearrange their V(D)J gene segments to assemble complete genes for the antigen receptors expressed by T and B lymphocytes. Antigen-mediated triggering of T and B cells initiates specific cell-mediated and humoral immune responses (6). T lymphocytes are primarily responsible for cell-mediated immunity, and B lymphocytes are responsible for humoral immunity, but they work together and with other types of cells to mediate effective adaptive immunity. Along with the natural killer cells, these specialized lymphoid cells are derived from committed progenitors in hematopoietic tissues, which then undergo unique V(D)J rearrangements of their antigen receptors to become clonally diverse lymphocytes. Newly formed T and B lymphocytes bearing autoreactive receptors can be eliminated by self-antigen contact in the thymus and bone marrow, respectively. The surviving T and B cells then migrate via the bloodstream to peripheral lymphoid tissues, where, following antigen recognition, they undergo clonal expansion and differentiation into effector T lymphocytes or antibody producing plasma cells or otherwise become memory cells that await re-exposure to their specific antigens. Generation of memory cell is a special characteristic of the adaptive immune system. A subsequent exposure to that same antigen results in activation of these memory cells which results in more rapid response with high amount of production of antibodies and it lasts for longer period of time.
**Toll- Like Receptors (TLRs)**

**History**

Toll is a German word in English it means fantastic, mad or amazing. In scientific context, Nusslein-Volhard and Anderson first used the word Toll to name a gene that they discovered in a genetic screen of Drosophila, the phenotype of which they thought to be Toll (7,8). In 1989, Charles A. Janeway Jr. predicted that the innate immune response initiates the adaptive immune response through pattern-recognition receptors that recognize microbial products, now called pathogen-associated molecular patterns (PAMPs)(9). The above prediction enforced immunologist to reorganize the thoughts about innate immune system which was ignored due to their much focus on the adaptive immune response and the generation of diversity in antibody repertoires. In 1996, Lemaitre and coworkers demonstrated that the Toll receptor, previously known for its essential role during Drosophila embryonic development, is required for antifungal defense in Drosophila (10). This finding stimulated the identification of the homologous mammalian Toll receptors and the demonstration of their importance in mammalian innate immunity. Subsequently, the progress of genome projects led to the identification of approximately 10 receptors in vertebrates that were structurally related to drosophila Toll, now collectively referred as Toll-like receptors (TLRs) (11).

Toll-like receptors are type I transmembrane receptors that sense molecular patterns associated with a broad range of pathogens including bacteria, viruses, fungi and protozoa. Upon activation TLRs mediate initial responses in innate immunity and are required for the development of the adaptive immune response. The first TLR identified was TLR1 (12), which was initially thought to have developmental functions because of its Drosophila homologues known for such functions (13). After the finding of Drosophila toll involved in anti-fungal resistance, Medzhitov et al discovered the second mammalian TLR (TLR4) and implied its role in adaptive immunity, (14). The importance of TLRs in adaptive immunity has been largely confirmed more recently (15). Now,
altogether thirteen TLRs have been described, of which some are not expressed either in mice (human TLR10) or in human (mouse TLR11-13) (16).

**Expression of TLRs**

Primarily, professional antigen presenting cells (APCs), macrophages and DCs, express most of the TLRs. TLRs expression differs among DCs subsets (17). Dendritic cells contain two different subsets, myeloid dendritic cell (MDC) and plasmacytoid dendritic cell (PDC) in human’s blood (18). MDCs express TLR 1, 2, 4, 5 and 8, and PDCs exclusively express TLR7 and TLR9 (19). Although, it is also reported that TLR7 is also expressed in MDC (20). Expression of TLR1, 2, 4 and 5 is observed in immature dendritic cells but decreases as the dendritic cells mature (21). TLR3 is only expressed in mature dendritic cells (22). Mast cells express TLR2, 4, 6 and 8 but not TLR5 (23, 24). Human Neutrophils expressed TLR1, 2, 4, 5, 6, 7, 8, 9, and 10-all the TLRs except TLR3 (25). Another intervention suggested that human NK cells also express TLRs (TLR2) (26). In addition to the innate immune cells some TLRs are also expressed by the lymphocytes like B-cells and T-cells.

**Regulation of TLR Expression**

Expression of TLRs is modulated by a variety of factors such as microbial invasion, microbial components, and cytokines. Infection by *Mycobacterium avium* augments TLR2 mRNA expression and decreased TLR4 mRNA expression in macrophages (27) and leads to increased TLR2 promoter activity accompanied by chromatin remodeling (28, 29). Nontypeable *H. influenzae* activates NF-kB through TLR2 and induces expression of TLR2 in epithelial cells in an autocrine manner (30, 31). Infection of mice with *E. coli* induces expression of TLR2 mRNA in γδ ±T cells, which is thought to represent a more primitive, early line of cellular defense, preprogrammed to recognize a limited set of antigens (32). Viral infection also induces expression of the TLR1, TLR2, TLR3, and TLR7 mRNAs in macrophages. Increased TLR expression is suppressed by
treatment with anti-IFN-α/β antibody, indicating that IFN-α/β mediates virus-induced activation of innate immunity via modulation of TLR expression (33). LPS enhances expression of TLR2 in macrophages and adipocytes (34, 35). In contrast, LPS stimulation of mouse macrophages causes a reduction in surface expression of the TLR4/MD-2 complex, and this may be one mechanism underlying the phenomenon of LPS tolerance (36, 37).

In addition to the pathogen and their components cytokines can also regulate the TLR expression. For instance, Colony-stimulating factor1 can down regulate TLR9 expression in macrophages and strongly suppresses CpG DNA-induced production of inflammatory cytokines (38). Macrophage migration inhibitory factor (MIF) is an important cytokine that mediates inflammation and sepsis (39). MIF-deficient mice are defective in their responses to LPS. Recently, this defect was shown to be the result of decreased expression of TLR4 (40). Expression of the Tlr2 gene in macrophages is induced by LPS and inflammatory cytokines such as IL-2, IL-15, IL-1β, IFN-γ, and TNF-α (41). IL-15, a cytokine that promotes extrathymic development and survival of T cells, especially CD8C T cells and NK cells, induces expression of the Tlr2 gene in T cell lines through the activation of Stat5 (42).

**Recognition by TLRs**

Each TLR recognizes a particular pathogen associated molecular patterns (PAMPs) or ligands, TLR can recognize its ligand although in monomer condition but for its efficient activation and recognition it forms homodimer and heterodimer (43). TLRs 2, 4 and 5 form homodimers whereas TLR1 and TLR6 forms heterodimer with TLR2 while recognizing their ligands. These TLRs detect a wide variety of PAMPs displayed on a variety of micro-organisms. Table-1 summarizes the known TLRs and their respective ligands.
Table 1: Table shows the cell type specific expression of different Toll-like receptors, their ligands and their regulation by various agents.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Ligand</th>
<th>Cell type</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>#TLR1</td>
<td>Hu, M</td>
<td>Tri-acyl lipopeptides (bacteria and mycobacteria), soluble factors</td>
<td>Ubiquitous</td>
<td>PHA down-regulates expression on T-cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Neisseria meningitides)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>Hu, M</td>
<td>Lipoproteins/ lipopeptides (a variety of pathogens), Peptidoglycan</td>
<td>Neutrophils, Dendritic cells and monocytes</td>
<td>Induced by LPS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Gram-positive bacteria), Porins, Zymosans,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycoinositolphospholipids (Trypanosoma cruzi), HSP70(Host), Glycolipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Treponema maltophilum), LPS(Leptospira) and Porphyromonas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>Hu, M</td>
<td>Double- stranded RNA(Virus), polynosine-polycytidylic acid (poly</td>
<td>Dendritic cells, natural killer cells</td>
<td>Induced by differentiation, reduced upon maturation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I:C))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>Hu, M</td>
<td>LPS(Gram-negative bacteria), Taxol (Plants), Fusion protein(RSV),</td>
<td>Macrophages, dendritic cells, epithelial</td>
<td>Enhanced by inflammatory cytokines and bacterial products, down-regulated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Envelope proteins(MMTV), HSP60(Clamydiapneumoniae), HSP60 and HSP70(Host), Oligosaccharides of</td>
<td>cells</td>
<td>by anti-inflammatory cytokines</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(host), Polysaccharides of heparan sulfate (host), Fibrinogen(host)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR5</td>
<td>Hu, M</td>
<td>Flagellin(bacteria)</td>
<td>Monocytes, immature dendritic cells, epithelial cells, NK cells, T cells</td>
<td>No alterations observed</td>
</tr>
<tr>
<td>#TLR6</td>
<td>Hu, M</td>
<td>Di-acyl lipopeptides (Mycoplasma)</td>
<td>B cells, monocytes, NK cells</td>
<td>No alterations observed</td>
</tr>
<tr>
<td>TLR7</td>
<td>Hu, M</td>
<td>Imidazoquinolines, Single stranded RNA and Bropirimine(synthetic</td>
<td>B cells, plasmacytoid precursors of DC</td>
<td>Highly inducible by IL-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>compounds)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR8</td>
<td>Hu, M</td>
<td>Imidazoquinoline</td>
<td>Monocytes, Natural killer cells, T cells</td>
<td>Highly inducible by IFN-γ, LPS</td>
</tr>
<tr>
<td>TLR9</td>
<td>Hu, M</td>
<td>CpG DNA (bacteria)</td>
<td>pDC precursors, B cells, Macrophages,</td>
<td>Inducible by IFN-γ, LPS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neutrophils, natural killer cells, microglial cells</td>
<td></td>
</tr>
<tr>
<td>TLR10</td>
<td>Hu</td>
<td>Not known</td>
<td>B cells, plasmacytoid precursors of DC</td>
<td>No alterations</td>
</tr>
<tr>
<td>TLR11</td>
<td>M</td>
<td>Profilin (T.gondii)</td>
<td>Urinary Tract, Macrophages</td>
<td>No alterations</td>
</tr>
<tr>
<td>TLR12</td>
<td>M</td>
<td>Not known</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR13</td>
<td>M</td>
<td>Not known</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note- # indicates in association with TLR2
Figure—Toll-Like Receptors located on membrane or endosome recognize their respective ligands and elicit MyD88-dependent or MyD88-independent signaling pathways.
**Structure of Toll like Receptor**

The sequence of Toll determined in 1988 revealed a tripartite structure with an N-terminal region containing tandem arrays of a short leucine-rich repeat (LRR) termed as ectodomain, a sequence likely to form a single transmembrane helix and a C-terminal domain significantly related to that of the vertebrate interleukin-1-receptors (IL-1R) and therefore it is known as Toll-interleukin receptor (TIR) domain (44,45).

**Ectodomain** - It has Leucine rich repeats of approximately 24 amino acids length that are characterized by a conserved pattern of hydrophobic residues. Each LRR folds into a secondary structure consisting of a short parallel \( \beta \)-sheet, a turn and a more variable region. The conserved hydrophobic residues form the core of this secondary structure. The blocks of repeats form a curved, solenoidal structure with the short parallel \( \beta \)-sheets forming the inner convex surface of the structure. Specific molecular recognition is often achieved by interactions mediated through the side chains of variable residues protruding from the short parallel \( \beta \)-strands, contributed by each LRR that form the inner concave surface of the solenoid. These side chains point out of the structure and can be viewed as a combinatorial code that has evolved to bind specific ligands.

**Transmembrane domain** - Like other type I receptors, the ectodomains of the TLRs are connected to the cytoplasmic TIRs by a single transmembrane \( \alpha \)-helix. Although there is no striking pattern of sequence conservation in these segments (other than hydrophobicity), the transmembrane and juxtamembrane sequence are likely to play critical roles in receptor activation.

**TIR domain** - The Toll/IL-1 Receptor (TIR) domain was first characterized due to homology between the intracellular regions of the mammalian IL-1 receptor (IL-1R) and the Drosophila protein Toll. The domain consists of three 'boxes' of conserved residues set in a core sequence ranging from 135 to 160 amino acids. Intervening residues may
vary, as sequence conservation between domains is only 20-30%. Two interfaces are responsible for mediating TIR domain interactions, which include receptor/adaptor oligomerization and association between receptors and adaptors. TIR domain interactions between receptors and adaptors play a key role in activating cellular signal transduction pathways in response to various pathogens (46).

**Figure**- Structure of Toll like Receptors showing ectodomain having Leucine rich repeats (LRRs), Transmembrane domain and Intracellular TIR-domain.
**TLR Signaling**

The hallmark of the TLR signaling is that upon ligation of cognate ligand, TLRs activate the transcription factors NF-κB and AP1, leading to the production of inflammatory cytokines such as tumor necrosis factor (TNF)-α and up-regulation of the costimulatory molecules CD80 and CD86 on dendritic cells (DCs) (47). The pathway is very similar to that of IL-1R, since the cytosolic TIR domain is in common. TLR signaling pathway has been classified into two groups depending on the involvement of an adaptor protein. There are four major adapters- the myeloid differentiation primary response gene 88 (MyD88), the TIR domain-containing adapter protein (TIRAP)/MyD88-adaptor-like (MAL), the TIR domain-containing adapter inducing Interferon-β (TRIF) and the TRIF-related adapter molecule (TRAM). While TLR1, TLR2, TLR6, TLR7 and TLR9 signal through a MyD88-dependent pathway, TLR3 signals through a MyD88-independent TRIF-mediated pathway; TLR4 signals through both MyD88-dependent and – independent pathways (48).

(a) **MyD88-dependent pathway**: MyD88 contains both a TIR domain and a death domain. On activation of TLRs it get associated with TIR domain of the TLR, its association recruits members of the IL-1 receptor associated kinase (IRAK) family through death domain homophilic interactions. IRAK1 and IRAK4 are serine – threonine kinases involved in the phosphorylation and activation of tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6). After phosphorylation by IRAKs, TRAF6 forms a complex with Ubc13 and Uev1A. Collectively these proteins form a ubiquitin conjugating enzyme (E2) for which TRAF6 serve as the ubiquitin ligase (E3). TRAF6 activates a MAPK kinase kinase (MAPKKK) called transforming growth factor β-activated kinase (TAK-1). Activated TAK-1 phosphorylates MKK3 and MKK6, the kinase upstream of p38 MAPKs and JNK. In addition TAK-1 can activate IkBα kinase complex (IKK), which consist of IKKα, IKKβ and IKKγ. The phosphorylation of IkBα leads to its degradation and release of NF-kB and the activation of NF-kB dependent genes, such as TNF-α, IL-1 and IL-6. In addition to JNK and p38 MAPKs, TLRs activate ERK1 and ERK2 MAPKs. The mechanism of ERK activation relies on another family known as Tpl2 (49). An alternative player in the MyD88–dependent pathway is the
adaptor molecule TIRAP or MAL. TIRAP can dimerize with MyD88 for initiation of downstream signals from TLR4 and TLR2 but not for other TLRs.

(b) *MyD88 independent pathway*: TLR3 and TLR4 even in absence of MyD88 are capable of inducing certain signaling pathway, which is mediated by another adaptor molecule known as TRIF also known as TICAM-1 (50). MyD88 independent TLR4 signaling also involves TRAM in addition to TRIF for the induction of interferon (IFN)-α and IFN-β genes. In case of TLR3 signaling TRIF is used as the only adaptor molecule. TRIF activation by TLR3 or activation of TRIF/TRAM by TLR4 signal leads to the activation of IRF3 (Interferon-regulatory factor 3) (51). IRF3 is constitutively expressed in various cells and upon activation of its C-terminal domain the formation of IRF3 dimers is induced, allowing translocation to the nucleus, where it activates Type I IFN gene transcription. Binding of IFNβ to the type I IFN receptor results in activation of the transcription factor STAT-1 (signal transducer and activator of transcription-1).

**Figure-** This figure shows a simplified scheme of MyD88-dependent and MyD88 independent TLR signaling. The activation of the TIR domain results in sequential recruitment of the adapter molecule MyD88, IRAK and TRAF-6, leading to the activation of NF-κB-inducing kinase (NIK). NIK activates IκKα and IκKβ resulting in IκB phosphorylation, followed by ubiquitination and degradation. The freed NF-κB translocates to nucleus and transactivates Gene expression of such cytokines as IL-1β, IL-6, IL8, IL-12 and costimulatory molecules CD80 and CD86. In the MyD88-independent pathway, the adapters like TIRAP/Mal and TRIF can activate either NIK-dependent NF-
kB or IKKe/IKKi-dependent TBK-1 and IRF-3, respectively. The transcription factors translocate to nucleus and activate gene transcription.

**MODULATION OF IMMUNE RESPONSES BY TLRs**

*Regulation of Innate Immune response by TLRs*

TLR is an essential component of the innate immune system as it provides specificity to it and mediates various innate response required for the elimination of the pathogens. As TLR is a kind of Pattern recognition receptors (PRRs) it has the ability to recognize a particular pattern associated with the pathogens i.e. Pathogen associated molecular patterns (PAMPs) and get activated which results in many anti microbial and anti parasitic response. Phagocytosis of the foreign particle or pathogens is one of the major response among various innate responses are regulated by the TLRs. Different TLRs promote phagocytosis to varying degrees, TLR9 being the strongest and TLR3 being the weakest inducer of this process (52). TLR also induce the maturation of phagosome (53). TLRs allow generation of superoxide, Nitric oxide (NO) radicals, β-defensins and NADPH oxidase, which are required for the killing of the intracellular or extracellular pathogens, as a function of innate immune response. TLR2 and TLR4 on cognate interaction with their ligands induce inducible nitric oxide synthase (iNOS), which leads to production of NO radicals (54). TLRs, increased p47phox and gp91phox expression and enhanced superoxide anions release (55). TLR4 activation with LPS induces expression of mouse β-defensins-2,-3, and-6 (56). Interferon (IFN) α and β are made in response to virus infections by infected host cells. IFNα and IFNβ is secreted and binds to membrane receptors on nearby cells; binding activates second messengers that inhibit virus replication in those cells. TLRs like TLR3 and TLR4 on activation induce IFNα and IFNβ synthesis as an antiviral response (57). NK cells are activated by IFNα, IFNβ, and IL-12 to kill virus-infected cells and by IL-12 and TNF-α to produce high levels of IFNγ (58). TLRs on activation induce many inflammatory cytokines like IL-1, IL-6 and TNF-α (59, 60), which signal hypothalamus to increase body temperature (fever), liver to release acute phase proteins and bone marrow to release more neutrophils, which helps in the
clearance of pathogens. TLRs activation allows secretion of various chemokines like MIP-1α, MIP-1β, IL-8, RANTES and IP-10 etc. (61), which recruit phagocytes at the site of infection and facilitate the eradication of the pathogens. Complement which is also a component of innate system and help in innate immune response is also regulated by TLRs, as stimulation of TLR4 with Lipopolysaccharides (LPS) lead to increase in Complement factor 3 (C3) expressions. (62). Apoptosis of the cell infected with a pathogen is also a way of innate defence by which APCs like Macrophages and Dendritic cells etc. infected with invasive bacteria, limit the spread of the pathogens by localizing cell death at the site of pathogen invasion. TLR2 confers lipoprotein-induced apoptosis of macrophages, indicating the possible involvement of TLRs in infection induced cell death (63).

**Figure**- Toll like receptors mediate or promote various innate immune responses such as phagocytosis, lysosomal degradation of pathogens, generation of Reactive oxygen intermediate(ROI), Nitric oxide(NO) radicals and secretion of cytokines and chemokines like TNFα, IL-1, IL-6, IFN-α/β and MIP-1 etc.
A major event of adaptive immune response is activation of naïve T-cells. The efficient activation of T-cell is mediated by two signals, which they get from antigen presenting cells (APCs) like dendritic cells and macrophages. First signal is the recognition of the processed antigen associated with MHC molecule by the T-cell receptor (TCR) and second signal is in the form of costimulation by various costimulatory molecules present on the APCs. Immature dendritic cells residing in the periphery have a high capacity for endocytosis, which facilitate pathogens uptake. Upon activation by the different TLR agonist it undergo maturation process which involves modulation of expression of many costimulatory molecules such as CD80, CD86 and CD40 etc. and also increase in MHC molecules expression (64,65). In maturation process DCs lose the ability to phagocytose but gain the ability to migrate into lymph nodes, where they interact with T-cells to initiate adaptive immune response. TLR activation also enhances MHC peptide up loading via increasing Tap1 expression (66). Increase in expression of MHC molecules, costimulatory molecules and peptide loading to MHC molecule finally help in the elicitation of both primary and secondary signal that lead to efficient T-cell response and generation of effector and Memory T-cells. Activation of the TLRs lead to secretion of many cytokines like TNFα, IL-12 and IL-10 which affects the maturation of the APCs leading to differential T-Cell activation. TLR4 and TLR9 on activation induce production of IL-12 thereby skewing Th cell differentiation towards the Th1 type, whereas TLR2 on activation with porphyromonas gingivalis induce Th2 response (67). Combined activation by TLR7 and TLR3 ligands induce MyD88-dependent and -independent signaling together allowing a more rapid and more sustained bone marrow-derived DC (BMDC) activation with regard to the secretion of proinflammatory cytokines, like IL-6 and IL-12p70, and the expression of costimulatory molecules like CD40, CD70, and CD86. Furthermore, in the presence of combined TLR ligand-stimulated DCs, CD4 (+) and CD8 (+) T cells were insensitive toward the inhibitory effects of regulatory T cells. Most importantly, peptide-loaded BMDCs stimulated by TLR ligand combinations resulted in a marked increase of CTL effector functions in wild-type mice in vivo (68). Initiation of adaptive immune responses is also controlled by regulatory T cells (Treg cells), which act
to prevent activation of auto reactive T cells (69). TLR Microbial induction of the Toll pathway blocked the suppressive effect of CD4+CD25+ Treg cells, allowing activation of pathogen-specific adaptive immune responses. This block of suppressor activity was dependent in part on interleukin-6, which was induced by TLRs upon recognition of microbial products, (70) suggesting that TLRs regulate the adaptive immune response not only in the form of costimulation via APCs but also by soluble factors like cytokines and it acts as regulator of regulators.

TLRs regulate the T-cell response not only by being present on the APCs but also on the adaptive immune cells like T-cells and B-cells. Murine T cells express TLR1, TLR2, TLR6, TLR7 and TLR9. Pam3Cys, which is agonist for TLR1/2, co stimulates antigen-activated T cells, permits an increased cell proliferation and survival, associated with a sustained CD25 expression and an enhanced expression of Bcl-xL anti-apoptotic protein. In addition, we show that costimulation with Pam3CSK4 up-regulates IFN-gamma production but also granzyme B secretion and cytotoxic activity of antigen-activated T cells, indicating that TLR2 engagement enhances the major effector functions of CD8 T cells (71). Both human CD4+CD25+ Treg and CD4+CD25- T cells express TLR5 at levels comparable to those on monocytes and dendritic cells. Costimulation of effector T cells with anti-CD3 and flagellin resulted in enhanced proliferation and production of IL-2, at levels equivalent to those achieved by costimulation with CD28. In contrast, costimulation with flagellin did not break the hyporesponsiveness of CD4+CD25+ Treg cells, but rather, potently increased their suppressive capacity and enhanced expression of FOXP3 (72). These observations suggest that, in addition to their APC-mediated indirect effects, TLR ligands have the capacity to directly regulate T cell responses and modulate the suppressive activity of Treg cells. TLR2 ligand Pam3Cys, but not LPS (TLR4) or CpG (TLR9), directly acts on purified Tregs in a MyD88-dependent fashion. Moreover, when combined with TCR stimulation, TLR2 triggering augmented Treg proliferation in vitro and in vivo and resulted in a temporal loss of the suppressive Treg phenotype in vitro by directly affecting Tregs (73). Adaptive immune response mediated by the B-cells is also not untouched by TLRs. TLR9 signals B cell activation, proliferation and IgM production. Interaction of TLR9 with its ligand i.e. CpG DNA plays a key role in systemic lupus erythematosus and rheumatoid arthritis, two autoimmune disorders.
characterized by dysregulated production of DNA-reactive IgG. CpG DNA initiates germline C (gamma) 1, C (gamma) 2, and C (gamma) 3-gene transcription by activating B cells through a TLR9 which results in up-regulation of activation-induced cytidine deaminase, a key element of the B cell class switch-inducing machinery (74).

**Figure**- Toll like receptors elicits or promotes various adaptive immune responses such as antigen processing and presenting to the T-cell, changes the expression of co-stimulatory molecules on antigen presenting cells (APC) which affect T-cell activation. Secrete certain cytokines like IL-10 and IL-12 which affect the differentiation of naïve T-cells to Th1 or Th2 type of T-cells, TLR activation also induce the secretion of IL-6 which regulate the functioning of T-regulatory cells.

Above information suggest that involvement of the type of TLRs during process of pathogen recognition dictate the Th cell to attain a particular lineage like Th1 or Th2. TLRs as a component of Innate system it regulates various essential response against pathogen as well as it also act as a guiding star for the Adaptive immune system. All together, TLRs act as a connecting link between Innate and Adaptive immune system.
**Leishmaniasis**

The Leishmaniases is a group of diseases with a spectrum of clinical manifestations ranging from self-healing cutaneous ulcers to severe visceral disease and even death. All forms of Leishmaniases are caused by 20 species pathogenic for humans belonging to the genus *Leishmania*, a protozoa transmitted by the bite of a tiny 2 to 3 millimetre-long insect vector, the *phlebotomine sandfly*.

**History**

In 1901 William Leishman first demonstrated the protozoan parasite in the spleen of patients suffering from a malaria-like illness, and Charles Donovan described them as a new organism in 1903. Later on Ronald Ross established the link with the disease and named the organism *Leishmania donovani*.

**Geographical Distribution**

Human leishmaniasis is distributed worldwide, but mainly in the tropical and subtropical countries, with a total of 350 million people at risk. The disease has a prevalence of 12 million cases and an approximate incidence of 0.5 million cases of VL and 1.5 million cases of cutaneous leishmaniasis (CL) are expected to occur annually. Of the 0.5 million cases of VL, 90% are in five countries: Bangladesh, Brazil, India, Nepal and Sudan. Whereas, CL cases are predominant in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria. The geographical distribution of leishmaniasis is limited by the distribution of the sand fly, its susceptibility to cold climates, and its tendency to take blood from humans or animals and its capacity to support the internal development of specific species of Leishmania (http://www.who.int/tdr/disease/leish/diseaseinfo.htm).
Figure: Showing geographical distribution of Leishmaniasis in world

Epidemiology

*Leishmania*-endemic has expanded significantly, accompanied by a sharp increase in the number of recorded cases of the disease. The geographic spread is due to factors related mostly to development. Like many other tropical diseases, leishmaniasis is related to economic development and man made environmental changes, which increase exposure to the sand fly vector.

The severity of the disease increases with increasing prevalence of *Leishmania-HIV* co-infection and other immunosuppressive conditions. In the Mediterranean basin 1.5-9% of AIDS patients develop visceral leishmaniasis and 25-70% of the adult VL cases are related to HIV infection. De la Loma reported the first case of VL-HIV co-infection in 1985 (http://www.who.int/tdr/disease/leish/diseaseinfo.htm).

Sand fly: *The vector or carrier of Leishmania parasite*

Humans are infected via the bite of sandflies (subfamily *phlebotominae*) - tiny sand-coloured blood-feeding flies that breed in forest areas, caves, or the burrows of small rodents. Wild and domesticated animals and humans themselves can act as a reservoir of
infection. Out of 700 species of phlebotomine sandflies (Diptera, Psychodidae), 70 are known vectors of Leishmania. Only female sand flies feed on blood and are host to the parasite life cycle. Old World forms of *Leishmania* are transmitted by sandflies of the genus *Phlebotomus*, while New World forms mainly by flies of the genus *Lutzomyia*.

Classification of the Vector (Lewis *et al.*, 1977; Lewis *et al.*, 1982)

- **Phylum**: Athropoda
- **Class**: Hexapoda
- **Family**: Psychodidea
- **Genus**: Phlebotomus

*Leishmania*-Causative agent of Leishmaniasis

*Leishmania* are protozoa belonging to the order Kinetoplastida and the family Trypanosomatidae. *Leishmania* has a unique organelle called kinetoplast, which appears to be special part of the mitochondrion and is rich in DNA. Two types of DNA molecules, maxicircles, which encode mainly certain important mitochondrial enzymes and minicircles, which serve a function in the process of RNA editing, have been found in the kinetoplast. When giemsa stained, the kinetoplast is reddish purple and darker than the nucleus, contrasting the purple cytoplasm. Leishmania is dimorphic parasites which present as two principal morphological stages: the intracellular amastigote, within the mononuclear phagocytic system of the mammalian host, and the flagellated promastigote within the intestinal tract of the insect vector and in culture medium. The amastigote stage is a round or oval body about 2-6 µm in diameter, containing a nucleus, a kinetoplast and an internal flagellum seen clearly in electron micrographs. The amastigotes multiply within the parasitophorous vacuoles of macrophages. The promastigote stage has a long and slender body (about 15-30 µm by 2-3 µm), with a central nucleus, a kinetoplast and a long free anterior flagellum.
Classification of Leishmania (Lainson *et al*., 1987; Lainson *et al*., 1988)

**Sub-Kingdom:** Protozoa  
**Phylum:** Sarcomastigophora  
**Class:** Zoomastigophora  
**Order:** Kinetoplastida  
**Sub-Order:** Trypano-somatina  
**Family:** Trypanosomatidae  
**Genus:** Leishmania

*Life cycle of Leishmania and metacyclogenesis*

*Leishmania* species are digenetic organisms shuttling between a flagellated promastigote in the gut of the sand fly and an intracellular amastigote in the mammalian host. Promastigote attach to mononuclear phagocytes via receptor-mediated mechanisms and once inside the macrophage, the promastigotes undergo significant biochemical and metabolic changes, which results in the obligatory intracellular form of the parasite, the amastigote.
**Figure: Life cycle of Leishmania.** The life-cycle starts by the bite of parasitized female, as the sandfly feeds, promastigote forms of the leishmanial parasite enter the human host via the proboscis. The promastigote forms of the parasite are ingested by macrophage where they transform to amastigote forms and increase in number until the cell eventually bursts and infects other phagocytic cells and continue the cycle. The infected host is bitten by another female sandfly and the life cycle continues.

*In Sand fly*

The sand fly vector of genus *Phlebotomus* (old world) or *Lutzomyia* (new world) becomes infected when feeding on the blood of an infected individual or an animal reservoir (Fig.1). The *Leishmania* parasites live in the macrophages as round, non-motile amastigotes (3-7 μm in diameter). The fly ingests the macrophages during the blood meal and the amastigotes are released into the stomach of insect (75). Almost immediately the amastigotes transform into the motile, elongated (10-20 μm), flagellate promastigote form. The promastigotes then migrate to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission (76). Sand fly saliva selectively inhibits parasite killing by macrophages and nitric oxide production (77). The major surface glycoconjugate lipophosphoglycan (LPG) constitutes a dense glycocalyx that covers the entire surface of the parasite including the flagellum. Immature organisms, termed procyclins, express shorter LPG molecules but mature metacyclics bear the capping at the terminal β- galactose residues with α-arabinose and elongation by increasing the numbers of repeating disachharides unit by two to three folds. This mature metacyclic form of the organism is released from the midgut and migrates to the proboscis.

*In mammalian host*

When the sand fly next feeds on a mammalian host, it transfers the metacyclic *Leishmania* promastigotes to the host along with the saliva (78). Once in the host, the promastigotes are taken up by the macrophages where they rapidly revert to the amastigote form (79), survive and multiply inside the macrophages, eventually leading to the lysis of the macrophages. The released amastigotes are taken up by additional
macrophages and so the cycle continues. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow.

Types of Leishmaniasis in human

The leishmaniasis has been classified into different classes on the basis of the basic syndromes of the disease. Different species of Leishmania appear identical and are generally distinguished by clinical and geographic characteristics. Modern speciation by isozyme pattern, monoclonal antibodies, DNA hybridization, DNA restriction endonuclease fragment analysis, and chromosomal karyotyping is continuing to delineate new species, particularly in the new world, and to demonstrate the capacity of different species to cause similar clinical syndromes. There are four major syndromes-visceral leishmaniasis (kala azar), cutaneous leishmaniasis, monocutaneous leishmaniasis (espundia) and diffuse cutaneous leishmaniasis.

Visceral leishmaniasis (kala azar)

*L. donovani* causes kala azar, a disease that may be endemic, epidemic or may be sporadic. African kala azar is found in the eastern half of Africa. Indian kala azar has an age and sex distribution similar to African kala azar. The manifestations appear generally in 3 months. Fever, typically nocturnal and occasionally double-quotidian, is almost universal and is accompanied by tachycardia without sign of toxaemia. Diarrhoea and cough are frequent. Non-tender splenomegaly becomes dramatic by the third month. The liver enlarges conspicuously. Hypoalbuminaemia and polyclonal-hypergammaglobulinaemia (IgG and IgM) are constant features. Circulating immune complexes are frequently present. Immune-complex glomerulonephritis and interstitial nephritis have been described. Edema cachexia, and hyperpigmentation (kala azar means “black fever”) are late manifestations. After successful treatment, 3 to 10 per cent of cases develop post kala azar dermal leishmaniasis (PKDL) wart like nodules over the face and extensor surface of the limbs. In the Indian disease, PKDL appears after a latent period of 1 to 2 yr and may last for years (80).

Cutaneous leishmaniasis

This form of leishmaniasis is caused by a number of species in both the old and the new world. The disease is characterized by single or multiple localized lesions on exposed
areas of skin that typically ulcerate. \textit{L. tropica} and \textit{L. major} cause old world whereas \textit{L. mexicana} and \textit{L. brasiliensis} cause new world cutaneous leishmaniasis. The incubation period ranges from 2 to 6 wk. The initial lesions are often multiple and located to lower extremities. Regional lymphoadenopathy and satellite lesions are common.

\textit{Mucocutaneous leishmaniasis}

Mucocutaneous leishmaniasis and/or espundia, is caused primarily by \textit{L. brasiliensis} which typically produces several lesions on the lower extremities that undergo extensive ulcerations. After months to year, metastatic lesions appear in the nasopharynx. Nasal obstruction and epistaxis are frequent presenting symptoms (81).

\textit{Diffuse cutaneous leishmaniasis:}

It is characterized by widespread papules or nodules in the skin all over the body; does not heal spontaneously and is difficult to treat. In Africa (Kenya, Ethiopia), it is caused mainly by \textit{L.aethiopica}, and in Central America and northern South America, it is caused mainly by \textit{L.amazonensis}.

\textit{Animal model in Leishmania research}

Animals are the best model for the characterization of the disease and its impact on to the host. In case of Leishmaniasis study, Hamster and mouse are the two well-studied and suitable models for studying the infection and chemotherapy. But still monkey model is used for the vaccine trial. Although VL and CL can be studied in animal model, there is no animal model available for studying the PKDL (post kala azar dermal leishmaniasis).

\textit{Hamster model}

The Syrian or golden hamster, \textit{Mesocricetus auratus}, is commonly used to study the course of leishmania infection and the pathology of VL as disease course closely resembles human disease. Hamsters are used for histo-pathological studies, drug efficiency studies and vaccine studies despite the lack fine immuno-chemicals that limit the mechanistic exploration of immune response to \textit{Leishmania} infection. There are also limitations in using Hamsters as it requires greater quantity of drug for testing and more difficult to handle.
Mouse models

Different *Leishmania* species cause clinically distinct diseases and the severity of the disease caused by any given parasite can vary markedly between individual hosts (82). Till date, two host systems have been classified for studying *Leishmania* infection on the basis of susceptibility and resistance of the host. This observation extends to the murine *L. major* model where the strain of inbred mouse determines the outcome of infection, C57BL/6 mice being uniformly resistant and BALB/c consistently susceptible (83). It is well documented that Th1 immune response is the key event to prevent *Leishmania* infection. Activated Th1 cells induce IFN-γ that in turns activates the macrophages and kill the parasites. C57BL/6 mice mount early Th1 immune response and prevent the further growth of the parasite causes self-healing phenotype (84,85) whereas susceptible BALB/c strain mounts early Th2 response and results in non healing lesion and exaggeration of disease (84,86,87). Respective resistance and susceptibility of C57BL/6 and BALB/c strains depend not only on the Th1 and Th2 type of immune response of CD4+ T cells but also on the genetic background of the host. Initially it was shown that resistance or susceptibility of the recombinant strains of mouse was dictated by the haplotype of the host (88). Congenic mouse of a particular haplotype with either susceptible or resistant background could not correlate the susceptibility or resistance with the haplotype of the strain for the *Leishmania* parasites. It suggests that susceptibility or resistance of the host may be partly regulated by the haplotype with some other factors. Factors for susceptibility or resistance could be segregated by repetitive backcrossing of resistant B10.D2 and susceptible BALB/c strains. Loci on chromosomes 6, 7, 10, 11, 15, and 16 were associated with resistance, demonstrating the multigenic nature of this phenotype (89). Moreover, F1 progeny of BALB/c and C57BL/6 mice were shown to intermediary phenotypes for *Leishmania* infection suggested the contribution of genes either in susceptibility or resistance of the host. Bone marrow macrophages derived under influence of granulocytes macrophage-colony stimulating factor (GM-CSF) or IL-3 or monocytes-colony stimulating factor (M-CSF) further increase the respective resistance and susceptibility of these macrophages to *Leishmania* infection (90). These observations suggest the critical role of myeloid cells in
the resistance or susceptibility to \textit{Leishmania}. Resistance or susceptibility of myeloid cells to \textit{Leishmania} needs to be characterized further.

\textit{Virulence factors of Leishmania}

Virulence of a pathogen is its ability to cause disease or cause damage to the host. Every virulent pathogen possesses or secretes certain molecules which are essential for their virulence and they are referred as Virulence factors. \textit{Leishmania} also express certain virulence factors which helps them to establish infection to the host. \textit{Leishmania} promastigotes are covered with a dense surface glycocalyx, composed largely of molecules attached by glycosylphosphatidylinositol (GPI) anchor (91). These GPI anchored molecules include proteins such as the parasite surface protease gp63 and proteophosphogycans (PPGs). The most abundant constituent is a large GPI-anchored phosphoglycan called lipophosphoglycan (LPG) (92, 93). LPG and gp63 account for the virulence of the parasite. LPG has been implicated in many steps required for the establishment of macrophage infection and for the survival in insect vector (94, 95). LPG does not play a role in the amastigotes stage; however, amastigotes continue to make structurally related glycoconjugates (96, 97). On the other hand, gp63 also helps the parasite to enter in the host cells and for its survival. As an endoproteinase with a broad substrate spectrum, gp63 has the potential to degrade immunoglobulins, complement factors, and lysosomal proteins (98). Its proteolytic activity at pH 4 bears apparent relevance to the survival of amastigotes in the acidic environment of macrophage phagolysosomes (99).

\textit{Interaction of Leishmania with the host macrophage}

Leishmaniasis is an excellent example of a complex host-parasite interaction. Macrophages serve as a primary host for the Leishmania parasite. Leishmania while interacting with the host escape host mediated Innate as well as adaptive immune responses, in order to survive.
Evasion of host innate immune response

First restriction which every pathogen faces is the innate immune response initiated by the host against them. Leishmania also faces this restriction but escape from these responses as a result of their survival strategy. The very first step for several intracellular pathogens in establishing an infection in mammals is to get into its host cells and so does *Leishmania*. *Leishmania* has a dimorphic life cycle: as flagellated extracellular promastigotes in the sandfly vector and as aflagellate obligatorily intracellular amastigotes in host macrophages. After inoculation by the sandfly, the promastigotes are get into the phagocytes like neutrophils (100) and macrophages (101). Another route of entry into the macrophages is like a Trozan horse where apoptotic neutrophils carrying *Leishmania* are phagocytosed by macrophages (102). It remains unknown whether TLRs play any role in *Leishmania* internalization by mammalian host cells. In order to get internalize *Leishmania* promastigotes bind to some of the surface molecules like complement receptor 1 and 3 (CR1&3) and C3b of macrophage before they are internalized. CR1 and CR3 play major roles in both processes, and might act in concert to facilitate parasite binding and uptake. The interaction of the parasite with CRs occurs in three ways [1] in the presence of serum by activating the complement component C3 and binding through the C3bi fragment of complement to CR3; [2] through the serum-independent binding of the surface protease gp63 to CR3; and [3] through the binding of parasite lipophosphoglycan to the lectin-like site on CR3 and to CR1. Engagement of the CRs does not trigger the respiratory burst and, in fact, opsonization by complement improves parasite survival. In addition to complement receptors Fc gamma receptors are also involved in phagocytosis of Leishmania (103, 104).
Leishmania uses different strategies for internalization into its primary host macrophages such as by activating complement system and by using apoptotic neutrophils as a Trojan horse.

After internalization of organisms into phagosomes, secondary lysosomes are fused to form the complete parasitophorous vacuole or phagolysosome. Parasitophorous vacuole (PV) is an acidic compartment, which is rich in microbicidal peptides and hydrolytic enzymes helps in killing of the pathogen as a function of innate immune response. Leishmania escape from the above response in two ways either by avoiding the fusion of the phagosomes with the secondary lysosomes or by resisting the deleterious effect of microbicidal peptides and hydrolytic enzymes in parasitophorous vacuole. LPG of Leishmania promastigotes inhibits the fusion of the phagosomes with the secondary lysosomes. As LPG inhibits the generation of respiratory burst (105), during internalization Leishmania gets time to transform from its metacyclic forms to intracellular amastigotes. The amastigotes are more resistant to the enzymes and the acidic pH of the phagolysosomes. gp63 expressed by Leishmania also helps in protecting
the parasite from intraphagolysosomal cytolysis and degradation. *Leishmania* also inhibits generation of Nitric oxide (NO) which is required for their killing by inhibiting the induction of inducible nitric oxide synthase (iNOS) (also called NOS2) as a strategy to evade innate immune response (106, 107).

In addition to repressing the microbicidal activities of the host macrophage, *Leishmania* inhibits the ability of the host cell to display parasite antigens to other components of the immune system (108). Some studies have shown that *L. donovani* inhibits antigen presentation by repressing major histocompatibility complex (MHC) class II gene expression, both basal and particularly following stimulation with IFN-γ (109-111). Antigen presentation may be inhibited in this case by interfering with the loading of antigens onto MHC class II molecules (112, 113) or by sequestration of the MHC class II molecule and/or antigens within the phagolysosome (114, 115).

**Evasion of host adaptive immune response**

Leishmania for their survival not only inhibit the innate immune response but also they modulate the responses mediated by adaptive immune cells like T-cells and B-cells. Protective immunity against murine leishmaniasis is generally accepted to depend on the ability of the host to mount an IL-12-driven CD4+ Th1-type response, resulting in IFN-γ production. Since T cells play a major role in generating specific and memory T cell response in intracellular parasitic infections, T cells effector function has been characterized most extensively in *Leishmania* infection. It is reported that non-healing BALB/c mice infected with *L. major* contained transcript of IL-4 in their draining lymph node cells, in marked contrast to C57BL/6 mice that expressed transcript for IFN-γ but not IL-4 (116). This finding was confirmed by a kinetic analysis demonstrating the sustained expression of IL-4 mRNA in infected BALB/c mice with significant elevation of serum IgE levels that did not occur in C57BL/6 (117). Experimental data suggested the role of *Leishmania* specific CD4+ T cells to passively transfer the resistance or exacerbation of disease in immunodeficient or sublethally irradiated naïve hosts, correlating with their production of Th1 or Th2 cytokines (118-121). Some investigations suggested that the protective or non-protective immune response against *Leishmania* depends on the type of leishmanial antigen recognized by the T cells (122). Such finding
suggested that different antigens might drive Th1 or Th2 responses in susceptible BALB/c mice. It has been well documented that T cells can differentiate either in Th1 or Th2 type of effector cells and this plasticity of differentiation depends chiefly on the priming during differentiation (123). The finding was further substantiated using cells from transgenic mice expressing a single TCR (124,125). IL-4 was shown to induce Th2 whereas IL-12 induced Th1 differentiation (126-129). During early infection with *L. major* both resistant and susceptible host showed mixed response in CD4+ cell population consisting of IL-2, IL-4 and IL-13 that peaked at 4 days (130). IFN-γ transcripts were variable in different strains of mice. Strikingly, IL-4 production in infected mice was similar to fully developed Th2 clones in all the strains of mice analyzed. Administration of anti-CD4 antibody (131,132) and anti-IL-4 antibody(133, 134) was shown to heal the infection suggesting that CD4+ population, which induces the IL-4 in early infection, plays critical role in disease progression. The observation gained further support from the susceptibility of IL-4 transgenic mice in resistant background (135) and from the resistance of IL-4 gene deficient mice (136) to *L. major* infection. These findings suggested the role of IL-4 in disease progression. However, the role of IL-4 as a susceptibility factor has come under suspicion due to several observations. Firstly, IL-4 induction in *Leishmania* infection was shown to be dependent on other T cell factors like IL-2. Administration of anti-IL-2 or anti- IL-2 receptor antibody ameliorated the *L. major* infection (137) suggesting IL-2 as a susceptibility factor for leishmaniasis. This was further confirmed by a report showing IL-2 induced IL-4 production in CD4+ T cells (138). Secondly, the IL-4-deficient mice raised from BALB/c embryonic stem cells remained susceptible to *L. major* infection (139). Thirdly, a report demonstrated a dual role of IL-4 in *L. major* infection where depending on the phase of response and the antigen-presenting cells, IL-4 promoted Th1 response (140). These results finally shifted focus from IL-4 to IL-10 as a susceptibility factor.

IL-10 is another Th2 kind of cytokine having anti-inflammatory role (141) and its role has been also extensively explored in case of leishmaniasis. Administration of anti-IL-10 antibody during *L. major* infection further reduced the susceptibility of IL-4 receptor gene deficient mice (142). It has been shown that IL-10 dictates the susceptibility to *L. donovani* infection (143,144) and is required for higher parasite persistence in both
resistant C57BL/6 and susceptible BALB/c mice (145,146). Administration of anti-IL10 receptor antibody was shown to cure the *Leishmania* infection (146,147). Consistent with these findings, another report (148) suggested the role of IL-10 by using IL-10 gene deficient mice of both BALB/c and C57BL/6. These mice were resistant to *L. donovani* infection (148) and were producing more IL-12 and IFN-γ suggesting that IL-10 is the critical factor for disease progression. It has also been shown that co-administration of IL-10 plasmid with low dose of *L. major* inoculums, known to induce protective Th1 phenotype, promoted the disease in BALB/c mice (149) further confirming the disease progressive role of IL-10 (150).

Like IL-4 and IL-10 also the role of IL-12, produced by macrophages, and IFN-γ, produced by T- cells, has been studied in cases of leishmaniasis and are considered as the potential candidate cytokines based on their known ability to influence Th1 development (151-154). Addition of IFN-γ to parasite inoculum decreased substantially the amount of IL-4 recovered after *in vitro* stimulation of lymph node cells isolated 3 days later from BALB/c mice (155). IFN-γ Neutralization of IFN-γ at the time of infection by anti IFN-γ antibody made resistant mice to susceptible (156). IFN-γ gene disrupted mice of resistant background failed to clear the parasite and also demonstrated the default Th2 development of CD4+ T cells (157). IFN-γ receptor deficient mice of resistant background were also susceptible for leishmaniasis (158). The role of IL-12 in Th1 development has clearly been demonstrated *in vitro* by using transgenic TCR CD 4+ T cells (159-162). As noted previously, however, *Leishmania* promastigotes evade IL-12 induction during invasion of macrophages from both resistant and susceptible host (163). Neutralization of IL-12 during infection makes resistant mice susceptible for *Leishmania* infection (164). Another report suggested that reconstitution of IL-12p40-/- mice with exogenous IL-12 initiate the Th1 response and protect the mice (165). Moreover, IL-12 gene deficient mice of resistant background were susceptible for *L. donovani* infection (166). These observations suggested the critical and decisive role of endogenous IL-12 in leishmaniasis (167). The capacity of exogenous IL-12 to heal infected BALB/c mice correlated with powerful effect of IL-12 in suppressing IL-4 transcription and protein production. Further, the lack of Th1 immune response in BALB/c mice was due to unresponsiveness of these T cells for IL-12 (168) possibly through the inhibitory effect of
IL-4 on IL-12 receptor and responsiveness (169). Thus, Leishmania skew the CD4+ T-cells response towards Th2 type to establish infection by promoting secretion of IL-4, IL-10 and TGF-β and inhibits the secretion of IL-12 and IFN-γ. Due to dominancy of Th2 cytokines in *Leishmania* infection B-cell mediated class switching is also modulated by the parasite towards IgG1 rather than IgG2 (170).

**Leishmania and Toll like receptors**

Substantial studies demonstrated that different receptors mediate the uptake and phagocytosis of Leishmania sp. by macrophages, although the initial signaling events are unknown. Studies demonstrating the role of TLRs in the recognition of Plasmodium, Toxoplasma and Trypanosoma enforced several groups to initiate research into the role of TLRs in Leishmaniasis (171-176). The first study evaluating TLRs and the MyD88-dependent pathway in Leishmania infection was performed by Hawn et al. in 2002 (177). This study evaluated that Leishmania major infection induce IL-1α expression and it is mediated through MyD88 pathway as its expression decreased in MyD88 mice. In 2003, Muraille et al. demonstrated the importance of MyD88 in ability of the C57BL/6 mice to exhibit resistance against Leishmania infection. C57BL/6 mice become susceptible to *Leishmania* parasite in absence of MyD88 due to increased level of IL-4 and decreased level of IFN-γ and IL-12p40 (178). First report which suggested the direct interaction of *Leishmania* with the TLRs was from Becker et.al. who showed that the lipophosphoglycan (LPG) of Leishmania promastigotes interact with NK cell-expressed TLR2 (179). Furthermore, silencing of TLR2, TLR3, IRAK-1 and MyD88 expression by RNA interference was also performed to address the role of these components in antileishmanial defense. This study revealed that both TLR2 and TLR3 are involved in the production of NO and TNF-α by macrophages in response to *L donovani* promastigotes (180). TLR2-mediated responses are dependent on Galβ1,4Manα-PO4 containing phosphoglycans, whereas TLR3-mediated responses are independent of these glycoconjugates. TLR3 also plays a role in the leishmanicidal activity of the IFN-γ-primed macrophages (180). Also impairment in resistance to *L. major* was reported in mice deficient in TLR4. Interestingly in this study, compared to wild type controls, the growth/survival of parasites in the cutaneous lesions was drastically increased in mice
from a resistant background carrying an homozygous mutation of the *tlr 4* gene (TLR4 e/e) as soon as one day after inoculation of *L. major*. Later during infection an enhanced arginase activity was observed in mutant mice. This enzyme leads to the production of compounds essential for parasites proliferation in macrophages and its increase in mutant mice indicates that TLR4 signaling could enhance the microbicidal activity of macrophages harboring parasites (181). Results from studies comparing TLR4 deficient mice with TLR4 and IL-12β2 double deficient mice suggested an IL-12 independent role of TLR4 in anti-Leishmania immunity (182). The IL-12-dependent NK cell IFN-γ response was severely compromised in TLR9-deficient mice as well. In studies with *L. infantum* infection, Schleicher et al showed that in mature DC (mDC)-depleted mice, the IFN-γ response was abolished due to low IL-12 productions that can be rescued by CpG and IL-12. Thus, these data provided the first evidence that TLR9, mDC and IL-12 are functionally linked to the activation of NK cells in a model of VL (183). The requirement of TLR9 signaling for activation of NK cells by *L. major* was also recently reported (184).

The above evidences suggest that TLRs are involved in *Leishmania* infection and these are required for initiating the anti leishmanial immune response by the host. As most of the above studies which have been performed till date are involved in explaining the role of few TLRs like TLR2, TLR4 and TLR9, overlooks the role of other TLRs and also they have been studied in genetically resistant C57BL/6 mice not in susceptible BALB/c mice leaves so many questions, which are still required to be answered to understand the exact role of different TLRs in leishmaniasis. In our study, we have tried to explain the above concern.