CHAPTER 2

Review of literature

2. Review of literature

Infectious pathogens/antigens are the major forces for the selection procedure in human history **(Admetlla, 2008)**. Human migration in different parts of the world results in the exposure of the immune response genes to different local infections and thereby gets modified as per the demand of the environment. Thus, the pressure that has been exerted by the local pathogens causes the positive selection of some genetic markers in the population for developing protection against the pathogens **(Admetlla, 2008)**. The immune system comprised of two parts, innate and adaptive immunity. Innate Immunity plays a vital role in the recognition of the diverse set of foreign pathogens via some receptors and generates immune responses **(Schroder and Schumann**, **2005)**. They recognize it through some molecular receptors and send some signals through which different cell types release different cytokines by which they counteract the pathogens. Various families of molecular markers are there for the recognition of the pathogens of which TLRs or Toll like Receptors are the most important. TLRs are group-1 membrane glycoproteins that are conserved from ***C. elegans*** to human (**Kwai and Akira, 2010**). They are also known as the pattern recognition receptors (PRRs) or Danger associated molecular pattern (DAMPs). These receptors mainly act in innate immunity. There are mainly ten types of TLRs present in the human and mouse of which some are represented as pseudogenes due to the evolutionary constraints (**Barrio, 2009**).

Table 1: Toll like receptors and their ligands

Receptor	Ligands	Adapter(s)	Location
TLR 1	multiple triacyl lipopeptides	MyD88/MAL	cell surface
TLR 2	Multiple glycolipids, multiple lipoproteins, lipoteichoic acid	MyD88/MAL	cell surface
TLR 3	double-stranded RNA, poly I:C	TRIF	cell compartment
TLR 4	Polysaccharide, fibrinogen, Various opioid drugs	MyD88/MAL/TRIF/TRAM	cell surface
TLR 5	Bacterial flagellin, Profilin	MyD88	cell surface
TLR 6	multiple diacyl lipopeptides	MyD88/MAL	cell surface
TLR 7	single-stranded RNA	MyD88	cell compartment
TLR 8	small synthetic compounds; single-stranded Viral RNA, phagocytized bacterial RNA	MyD88	cell compartment
TLR 9	unmethylated CpG Oligodeoxynucleotide DNA	MyD88	cell compartment
TLR 10	Unknown	unknown	Unknown

(Sources: Waltenbaugh, 2008)

2.1 Toll Like Receptors

There are ten different TLR genes present in human and there is strong correlation among the different TLRs. These ten TLRs are located in different chromosomes. TLR 1, 2, 3, 6 and 10 are present in the chromosome number 4, TLR4 in chromosome 9, TLR7 and 8 in X chromosome and TLR9 on chromosome 3. These receptor genes are not like that of HLA and KIR because they are locus specific, presents in the single chromosome but TLRs are present in the different chromosome set. So, linkage study is much more complicated for the TLRs.

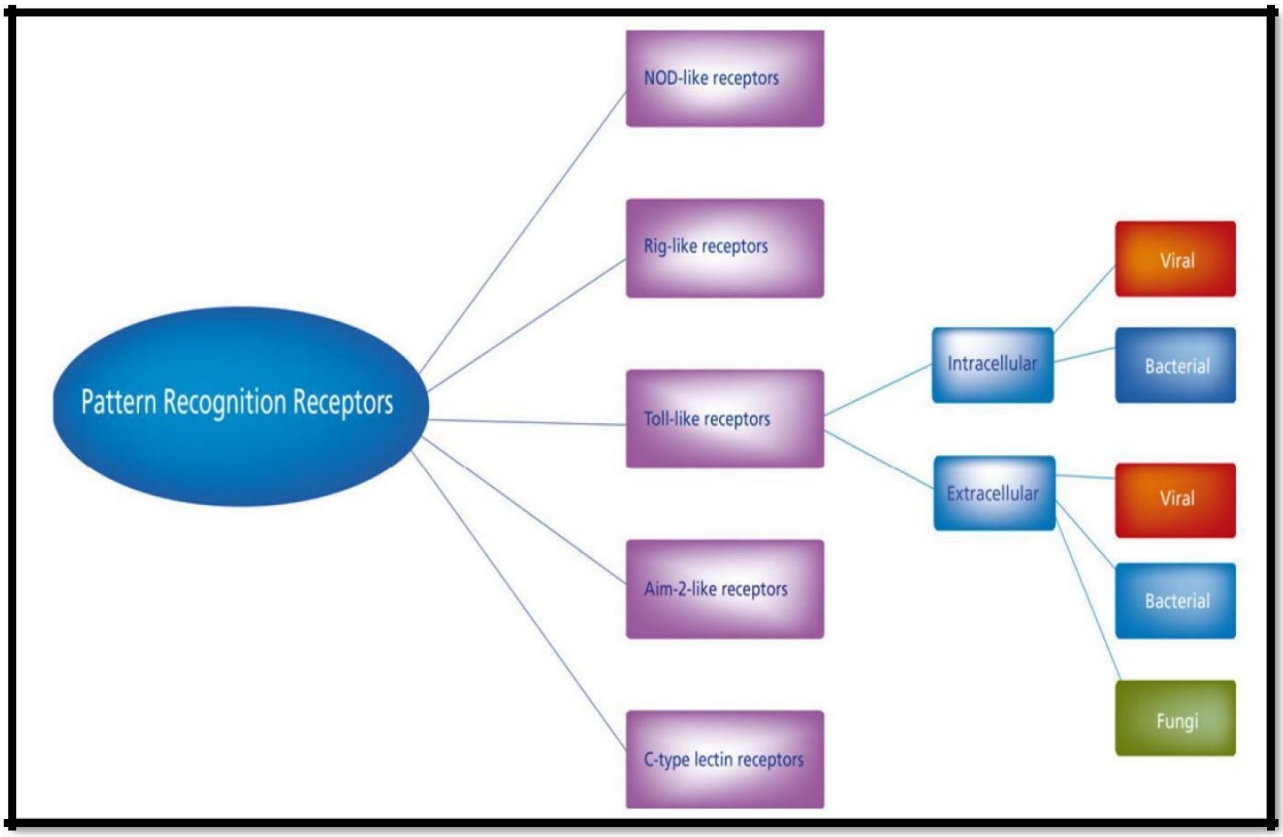

Fig. 1: Pattern recognition receptors and their function against antigens (Rietdijk *et. al.*, 2016)

2.2 Structure of Toll like receptors

Structural studies of TLR-ligand complexes have become an attractive area of research as the structural information is critical in understanding the innate immunity as well as designing novel drugs **(Mi Sun Jin, 2008)**. TLRs are type I transmembrane glycoproteins composed of extracellular, transmembrane and intracellular signaling domains **(Gay and Gangloff, 2007)**. The extracellular domain contain leucine-rich repeat (LRR) and are responsible for binding so-called pathogen associated molecular patterns (PAMPs) **(Janeway, 1989., Medzhitov, 2001)**. The extracellular domains of all TLR family proteins contain 16–28 LRRs **(Matsushima *et. al.*, 2007)**. On the basis of their sequences and structural patterns, LRR family proteins can be classified into seven subfamilies such as RI-like (ribonuclease inhibitor-like),CC (cysteine containing), PS (plant specific), SDS22-like, bacterial, and TpLRR (Treponema pallidum LRR) **(Kobe and Kajava, 2001., Matsushima *et. al.*., 2007)**. TLRs, typical subfamily proteins, have LRR modules of 24 amino acids with the conserved motif of xLxxLxxLxLxxNxLxxLPxxxFx.

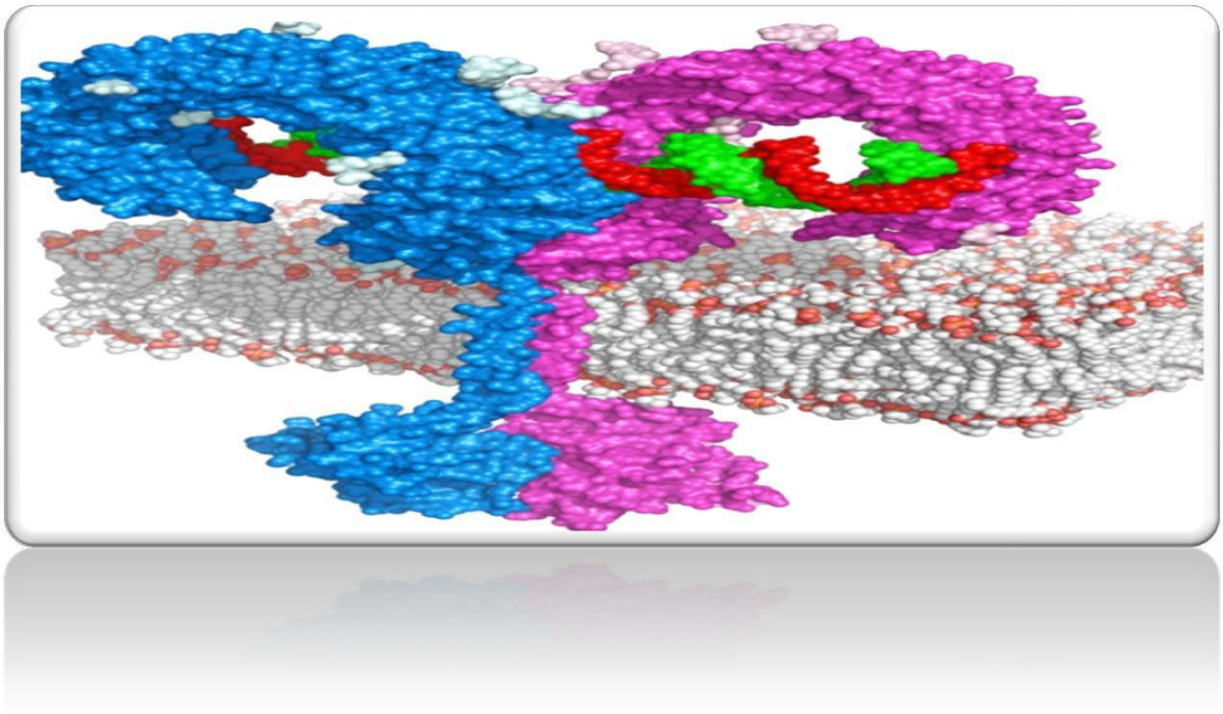

Fig 2: Structural model of the full-length TLR3/dsRNA (Sources: Botos, 2011)

2.3 Signalling pathways of TLR

The activation pathway of TLR signalling originates from the cytoplasmic TIR domains. The downstream signaling pathway via TIR domain, a TIR domain-containing adaptor, and MyD88 was first characterized to play a crucial role. In addition, recent accumulating evidence indicates that TLR signaling pathway consists of a MyD88-dependent pathway that is common to all TLRs, and a MyD88-independent pathway that is restricted to the TLR3- and TLR4 **(Akira *et. al.*, 2001., Takeda and Akira, 2004).** There are other similar pathways present that help in TLR signaling.

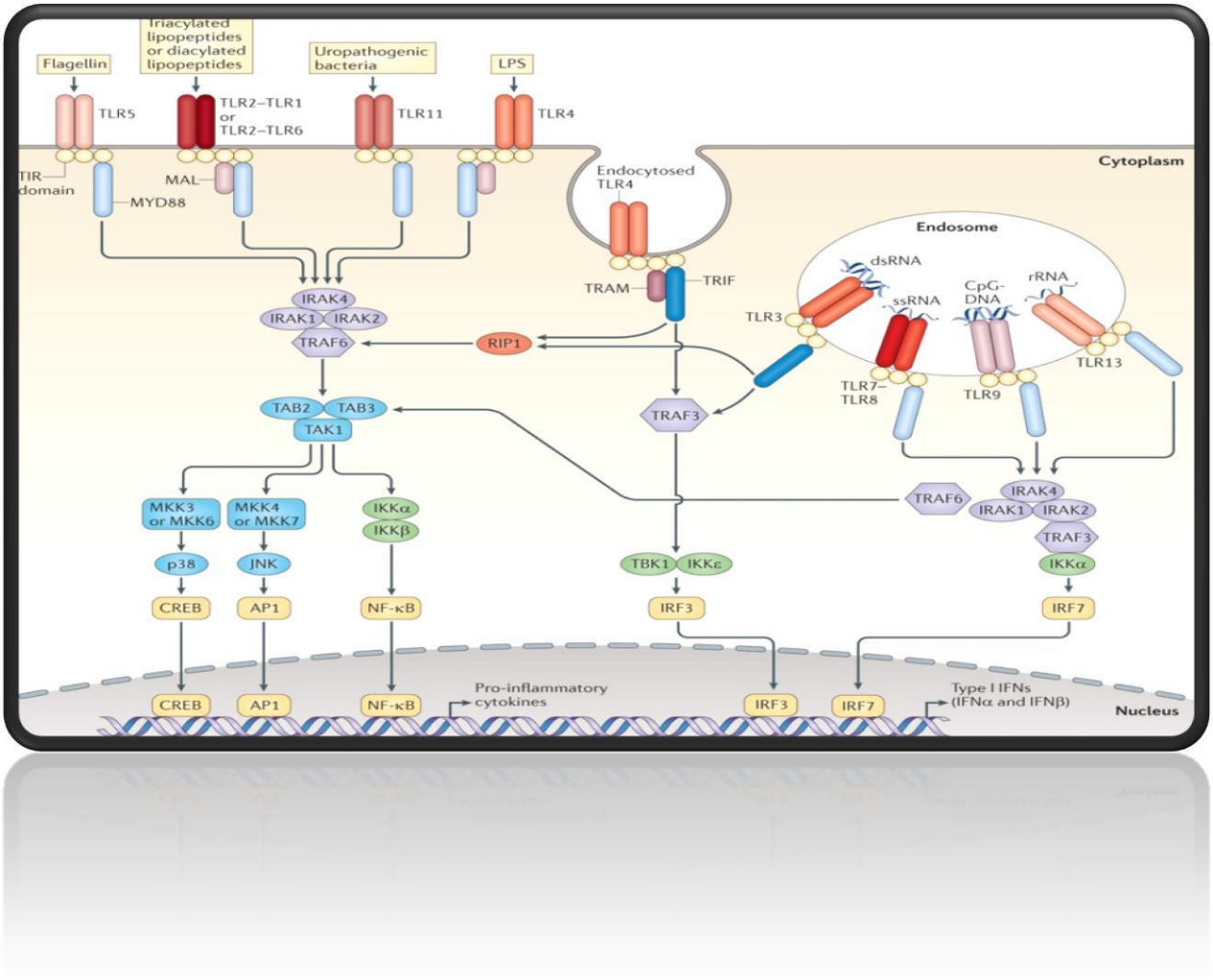

Fig 3: Mammalian TLR signalling pathways (Sources: O'Neill, 2013)

2.4 MyD88-dependent pathway

MyD88 pathway contains the TIR domain in the C-terminal position, and a death domain in the N-terminal portion. After TLR engagement with the pathogen, MyD88 forms a complex with IRAK kinase family members, known as the Myddosome **(Kawasaki, 2014)**. During the complex formation IRAK4 activates IRAK1, which is then autophosphorylated. IRAK1 associates with the RING-domain E3 ubiquitin ligase TRAF6. TRAF6 is a member of the tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family that initiates cytokine signaling pathways **(Arch, 1998)**. TRAF6, along with ubiquitin-conjugating enzyme UBC13 and UEV1A, promotes K63-linked polyubiquitination of both TRAF6 and TAK1 protein kinase complex **(Kawasaki, 2014)**. Then TAK1 then activates two different pathways that lead to the activation of IKK complex-NF-κB pathway and -MAPK pathway. TAK1 then binds to the IKK complex through ubiquitin molecule after that allows it to phosphorylate and activate IKKβ. The IKK complex phosphorylates the NF-κB inhibitory protein IκBα, which undergoes proteasome degradation, allowing NF-κB to translocate into the nucleus to induce proinflammatory gene expression **(Akira, 2006 ., Kawai, 2010)**.

2.5 Other molecules

Tollip (Toll-interacting protein) is another protein that complex with IRAK- 1. Tollip-IRAK-1 complex is then recruited to the IL-1 receptor complex. IRAK-1 then phosphorylated and leads to the rapid dissociation of IRAK-1 from Tollip, thereby inducing activation of TRAF6. Tollip also negatively regulates the TLR- mediated signaling pathways (**Zhang, 2002., Bulut, 2006)**.

2.5.1 MyD88-independent pathway

Another pathway for TLR signalling is MyD88-independent pathway. This pathway activates through two different molecules, one is TIRAP/Mal and another is TRIF. TIRAP/Mal specifically interacts with TLR4, and then involved in the TLR4-mediated MyD88-independent signaling pathway **(Horng, 2002)**.

TRIF interacts with TRAF6 and TRAF3. TRAF6 recruits another molecule that is RIP- 1 kinase which again interacts and activates the TAK-1 complex leading to the activation of NF-κB and MAPKs and production of inflammatory cytokines **(Akira, 2006., Kawai, 2010)**. On the other

hand, TRAF3 activates IKK related kinase along with NEMO for IRF3 phosphorylation. Subsequently, IRF3 forms a dimer and translocate into the nucleus from the cytoplasm and induces the expression of type- I IFN genes for the production of cytokines.

2.6 BALANCED ACTIVATION BETWEEN MyD88- AND TRIF-DEPENDENT PATHWAYS

TLR4 activates both the MyD88-dependent and TRIF-dependent pathways. Activation of these pathways is controlled by several other molecules to induce appropriate signaling. Balanced production of inflammatory cytokines and type- I IFN is very important for controlling the tumor cell growth and autoimmune diseases. In case of TLR4 signalling TRAF3 has been shown to be incorporated into the MyD88 complex as well as with the TRIF complex. TRAF3 within the MyD88 complex has been degraded and causes activation of TAK1. Thus it plays a vital role for inhibiting the MyD88- dependent pathway along with its role in promoting TRIF-dependent pathway activation. NRDP-1, an E3 ubiquitin ligase is consisting of two molecules that bind and ubiquitinates MyD88 and TBK1. It is inducing the degradation of MyD88 and augmenting the activation of TBK1. This attenuates inflammatory cytokine production and induces preferential type I IFN production **(Wang, 2009)**.

2.7 Negative regulation of TLRs

The excessive activation of TLR signaling which associated with autoimmunity and inflammatory diseases is negatively regulated by a number of other molecules through various mechanisms to prevent or terminate the excessive immune responses that lead to the detrimental results. Negative regulatory proteins target each of the key molecules present in TLR signaling pathway. Excessive activation of MyD88-dependent pathway is suppressed by ST2825, SOCS1, and Cbl-b, and activation of TRIF-dependent pathway is suppressed by SARM and TAG molecules **(Palsson, 2009., Han, 2010)**. These molecules associate with MyD88 or TRIF to prevent them from binding to TLRs or downstream molecules. TRAF3 activation is negatively regulated by SOCS3 and DUBA **(Kayagaki, 2007)**. A20, USP4, CYLD molecules target the TRAF6 and inhibit the molecules for cytokine production **(Skaug, 2011., Yuk, 2011., Kondo, 2012)**. Now TAK1 activation is inhibited by other molecules TRIM30α and A20 **(Shi, 2008)**. Not only these signaling molecules but the transcription factor NF- κB is also suppressed by Bcl-

3, IκBNS, Nurr1, ATF3, and PDLIM2. While IRF3 activation is inhibited by Pin1 and RAUL **(Saitoh, 2006)**. The stability of mRNAs encoding signaling molecules is regulated by miRNAs such as miR-146a, miR-199a, miR-155, miR-126, miR-21, miR-29, miR-148/152, and miR-466l **(Kondo, 2012)**. In addition to the mRNAs stability for signaling molecules, cytokine production is regulated by Regnase-1 and TTP **(Kondo, 2012)**.

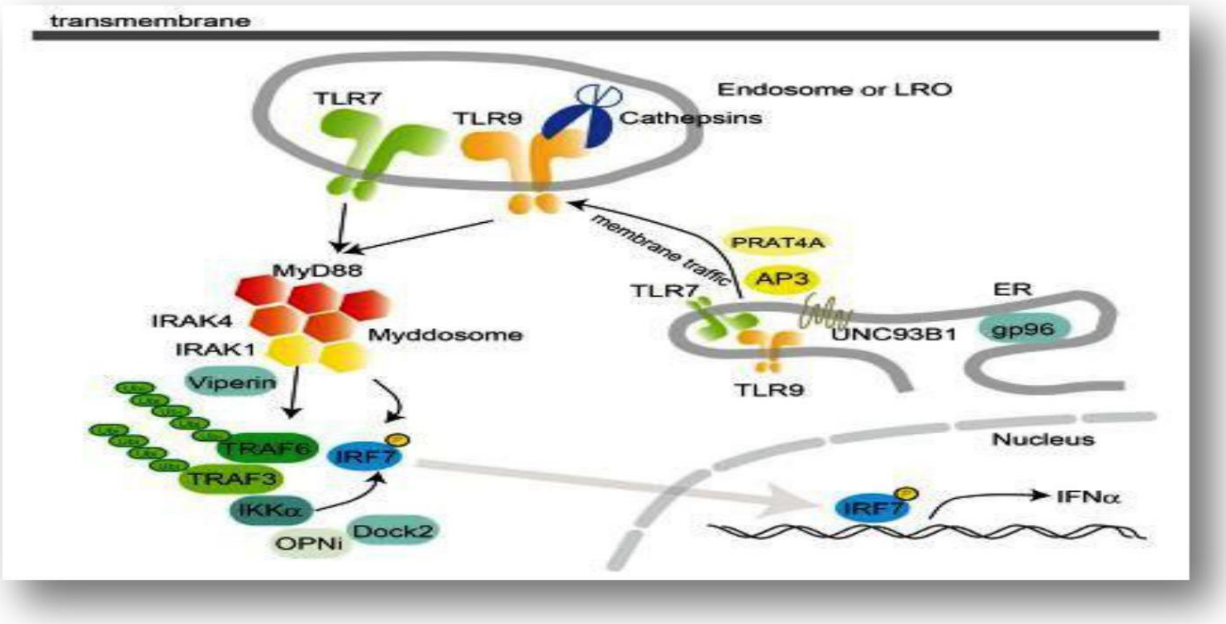

Fig 4: TLR signaling and membrane processing in pDCs Polymorphic variation of the TLRs in Human populations (sources: Kawasaki T, 2014)

Toll like receptors recognize very conserve molecular patterns. Ten different TLR genes are associated with different diseases in human populations. Different polymorphic varieties of the ten different TLR genes have been identified. Some polymorphic TLR genes may undergo mutation or single nucleotide polymorphisms in the TLR genes and causes diverse susceptibility for particular diseases. Synonymous substitution in the TLR genes produces no change in the production of amino acid whereas nonsynonymous substitution produces substitution in the amino acid **(Cheung, 2002)**. Different SNPs have been identified in human for the different

TLR genes and correlation study done on the disease association among different populations. Different diseases are caused by particular pathogens and the TLRs identified through the receptors and send the signals via IRAK or MyD88 pathway. So, the mutation in the coding or some conserve sequence region in the TLR genes causes the modification of the amino acid of particular region of the receptors and modified it. Thus the substitute pattern may induce the susceptibility for a particular disease and make it epidemic in the population **(Yi-Tzu Lin, 2012).**

Work on different SNPs study revealed that different polymorphic variety of TLR genes associate with the different diseases. Out of ten TLR polymorphic varieties, TLR4 has been extensively studied in different populations. The present investigation has elaborately studied the TLR polymorphic regions and how these relate to different diseases and correlate the association among the gene and disease.

2.8 TLR1 and disease association (cluster of differentiation 281-CD281)

TLR1 receptor mainly recognizes peptidoglycans and lipoproteins in association with the TLR2. It is mainly found on the surface of macrophage and neutrophil **(Farhat, 2008**) and designated as CD281. Structure of TLR1 consists of 786 amino acid residue of which extracellular domain contains 581-amino acid (leucine-rich), a 23-amino acid transmembrane domain and a 181-amino acid cytoplasmic domain (**Hawn, 2005**). It is situated on the chromosome number 4p14. There are different types of nonsynonymous polymorphic mutations found in gene sequence of TLR1, susceptible to different diseases like sepsis, leprosy, and candidemia. It has been found that TLR1 variants associated with leprosy. *Mycobacterium tuberculosis* is also inducing TLR1 for the susceptibility of the tuberculosis (**Hawn, 2003**). In case of TB lack of surface expression was found for TLR1 negative cell (**Uciechowski, 2011**). Some of the SNPs found in case of TLR1 gene causes susceptibility for that disease. Three common SNPs are found in the TLR1 of which -7202G/A, Asn248Ser, Ile602Ser are susceptible to sepsis and leprosy.

2.9 TLR2 and disease association (cluster of differentiation 282-CD282)

TLR2 is expressed on the cell surface of different cell types except T cells. This receptor recognizes lipoproteins from different bacteria like *Borrelia, Treponema* and *Mycobacterium.* Common SNPs found on the TLR2 are Arg677Trp, Arg753Gln. Mutation on the Arg753Gln make less responsive to the bacterial infection. Arg677Trp mutation on TLR2 suppresses the Nf-

kB signalling and makes susceptible to *Mycobacterium leprae* (**Kang, 2002**) and Tuberculosis (**Bochud, 2003**) and also increase the susceptibility for leprosy (**Kang, 2002**) and tuberculosis (**Ben-Ali, 2004**). TLR2 is also associated with other diseases like Lyme disease, urinary tract infection and staphylococcal infection.

2.10 TLR3 and disease association (cluster of differentiation 283-CD283)

There are 100 of SNPs are found in the TLR3 but none of the mutations are associated or susceptible with any diseases. Out of which four mutational regions are detected for the disease susceptibility for TLR3 gene. Asn284Ile, Tyr307Asp, Leu412Phe, and Ser737Thr are the four SNPs commonly found for TLR3 gene. But neither of these is related with each other and nor common with any diseases. Association has been found with Leu412Phe that correlates with the colorectal cancer and it has been found that 50% of the patients have the mutation for that disease (**Gorbea, 2010**). Pro554Ser, a rare mutation in the TLR3 gene was found to be associated with the herpes simplex virus that is not found in the healthy individuals by impairing the function of the signalling molecules (**Zhang, 2007**).

2.11 TLR4 and disease association (cluster of differentiation 284-CD284)

TLR4 have been extensively studied for association of various diseases in the different population. TLR4 is the cell surface receptor and recognize bacterial polysaccharide, LPS, taxol (**Akashi, 2001**) and other pathogens. The first identified TLR gene polymorphism encodes an Asp299Gly mutation in TLR4. This polymorphism is associated with a decreased signalling response to LPS *in vitro* and decreased airway response to inhaled bacterial LPS (**Arbour, 2000**). Thr399Ile Asp299Gly are two most common identified TLR4 gene SNPs found in the populations .This two are the co- segregating missense mutation (**Arbour, 2000**). Asp299Gly are related with different diseases like atherosclerosis, crohn‘s disease and asthma. Studied revealed that the patients with atherosclerosis has the higher level frequency of TLR4 (**Kiechl, 2002**). It has been found that for sepsis TLR4 is present in higher frequency when compared to the control. These common SNPs are found in higher frequency in some of the populations where they have increased the disease probability for that population. Indeed, it is not only related to atherosclerosis or sepsis but also with the other diseases common in different regions.

2.12 TLR5 and disease association (cluster of differentiation 285-CD285)

TLR5 recognizes the bacterial flagellin as a ligand for the receptor (**Akira, 2006**). TLR5 is expressed on the basolateral, but not the apical side of intestinal epithelial cells (**Takeda and Akira, 2003**). Only one common SNP found in the TLR5 gene which is a stop codon mutation Arg392Stop. This mutation in the TLR5 gene causes less responsive to bacteria like *Legionella pneumophila* which is responsible for pneumonia. This allelic variation also transmitted in the case of SLE where it protects the population from infection.

2.13 TLR6 and disease association (cluster of differentiation 286-CD286)

No extensive data till date have been found regarding TLR6 gene polymorphism or any disease relationship. TLR6 has expressed on the cell surface and recognize the lipopeptides and lipotechoic acid (**Irvine, 2013**). One common SNP found on TLR6 is Ser249Pro where serine changed to proline at 249 positions. It is also related with decreased risk of asthma (**Tantisira, 2004**). TLR6 Ser249Pro SNP has been associated with susceptibility to infection with aspergillosis (**Kesh, 2005**). The allelic variation in the TLR6 also caused the ventricular wall thinning (**Sales, 2010**).

2.14 TLR7 polymorphism and disease association (cluster of differentiation 287-CD287)

TLR7 was found on the endosomal part in the human. They mainly recognize single stranded RNA (ssRNA) as their ligands. There are three common variants found in TLR7. The common SNPs are Gln11Leu, 1-120T / G found in intron 1 and a synonymous SNP in TLR7 (**Schott, 2007**). The common polymorphism Gln11Leu is associated with the Hepatitis C infection and enhances the viral infection. It reduces the expression of the IFN- γ (**Askar, 2010**). It has also been provided evidences that TLR7 also related with the HIV infection (**Oh, D, 2009**).

2.15 TLR8 and disease association (cluster of differentiation 288-CD288)

TLR8 gene mainly encoded by the X chromosome and also known as CD288, it senses the bacterial nucleic acid as their ligands. TLR8 is highly expressed on monocytes, macrophages and dendritic cells and in the lung tissue (**Alexopoulou, 2012)**. TLR8 gene variation or the single

nucleotide polymorphism made susceptible for the disease. Met1Val and 129G/C are two common polymorphic variety found in the TLR8 gene. Study on the Met1Val SNP analysis on pulmonary tuberculosis patients provides evidence that this variation increase the disease susceptibility of the male patients (**Dalgic, 2011)** Association with TLR7 suggested that the frequency is highest in case of HCV (Hepatitis C virus patients) **(Chiou-Huey Wang, 2011).** As it senses the nucleic acid in the endosome compartments it senses the HIV-1 nucleic acid (**Akira, 2006**).

2.16 TLR9 and disease association (cluster of differentiation 289-CD289)

TLR9 gene encodes the receptor that resides in the endosome. It recognizes the CpG DNA as their ligands. As the bacterial DNA contain high amount of this DNA motifs so its automatically stimulate TLR9 and induce production of inflammatory cytokines such as IL-12 and TNF-a (**Akira, 2006**). The mutational regions which are associated with various diseases like cerebral malaria, lupus nephritis, cervical cancer. There are evidences that mutations like -1237T/C +1174G/A +2848G/A increased the frequency for the symptomatic malaria in the Ghanaian children (**Omar, 2012**).SNPs like -1237T/C +1174G/A increased the IFN-γ level in case of cerebral malaria in Ugandan children (**Sam-Agudu, 2010**) (**Nadia, 2010**). Common mutation likes 2848G/A in the TLR9 causes increased risk of cervical cancer and also induce the HPV infection (**Zeng-Zhen Lai, 2013**). A rare TLR9 SNP Pro99Leu prevents the receptor from being activated by ligands as it binds to the ligand normally (**Kubarenko, 2010**).

2.17 TLR10 and disease association (cluster of differentiation 290-CD290)

The lack of sufficient data for the TLR 10 reveals close examination of this receptor and lots of works have to be done on this. TLR 10 is one of the members of the TLR family that resides in the endosomal membrane and recognizes profilin as their ligand. The two common SNPs found in the TLR10 are Pro344Pro and Iso775Val which those are related with the asthma (**Lazarus, 2004**).

2.18 Correlation among the TLR polymorphic varieties and diseases

Till to date it was found that TLRs are the main components of innate immunity. But somehow it is also strongly correlated with the adaptive immune system. Vertebrate immune system is very specialized system, thus it comprised multiple defensive system to protect our body. So, in course of the vertebrate evolution various modifications occurred as the pathogens also modified itself in the evolutionary adaptive processes. As the TLRs are much conserved molecular pattern from teleost to human, there was very less change of the structural identity of this molecular marker. Some in course of the evolution has been deleted from the organisms and some are modified. Mutations are also played a very vital role for the adaptation of these receptors. Also, environmental factors are responsible for the modifications. Therefore, mainly ten TLRs still in humans are recognized (now TLR11 and TLR12) and various mutational regions or single nucleotide polymorphisms are found in the populations.

TLRs are very common in case of inflammation. As we know that mutations almost created a negative pressure on the receptors and if this negative selection (**Smirnova, 2001**) acts on it during infection it may be deleted from the population (**Smirnova, 2003**). But it does not occur in the population as well as in the case of immune system. Sometimes it has also been seen that the disease that related with the Single nucleotide polymorphism for certain TLRs may not be related in the other population.

2.19 TLRs and rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of synovial membrane of the joints caused by the infiltration of activated immune cells including CD4+ T cells, B cells, and antigen-presenting cells. It typically results in warm, swollen, and painful joints, thereby causing disability, deformities, premature deaths, and economic loss. Estimates of heritability suggested that genetic factors contribute more or less 50% to the risk of developing RA. Indeed, the development of autoimmune diseases like rheumatoid arthritis (RA) depends on the interaction between the genetic background and our surrounding environment.

2.19.1 Markers of RA

During the onset of the disease, various serological factors like CRP, anti-CCP, rheumatic factor (RF) are produced and identified as markers of the disease. C- Reactive proteins are produced in the hepatocytes of the RA patients due to the influences of certain cytokines like IL-6, TNF-α etc (**Kim, 2015**).The high risk concentration of this protein is above 3.0 mg/L. Rheumatoid factors are the autoantibodies, produce against IgG and present in lymphoid follicles of synovial area (**Dissick, 2010**). This marker is found to be positive in over 70% of RA patients. CD14-positive cells (monocytes) from the bone marrow stimulate RF-producing B cells (**Hirohata, 1996**). IgM-RF is also one of the major markers and can be detected in 60-80% of RA patients. It was measured up to 50IU/ml (**Nell-Duxneuner, 2010**). The physiological role of RFs is to enhance the clearance of immune complex by increasing its avidity and size, also to help the B cells to uptake the immune complex, and efficiently present to T cells.

Anti-CCP is another well known marker of RA pathogenesis. It is sensitive and specific than RFs. Citrullinated proteins, a non standard amino acid, are produced by post translation modification of arginine by peptidylarginine deaminase (PADI) enzymes. The apoptotic cells also activate the enzyme. So, when apoptotic cells are not cleared properly, the level of this protein and enzyme are raised in the inflamed area (**Vossenaar, 2004**). These proteins are mainly found in the form of filaggrin and cyclic citrullinated proteins. Autoantibodies are produced by the immune system against this altered peptide in case of RA in the synovial tissue and increase the severity of the disease. Connection to this, antibodies has been produced against this altered circular protein in the body and it has been the prime marker for the disease. Anti- CCP is locally produced in RA joints and very high sensitivity and specificity for the diagnosis of the disease (**Mimori, 2005**). The antibody titer has a prognostic value in destruction of the joints in this disease with 88% sensitivity and 98% specificity.

Now-a-days, it has been observed that in addition to the above mentioned serological markers, various different genetic markers are detected for the pathogenesis of the RA. Polymorphism of the markers in different populations around the world predicted the susceptibility or resistant to the disease. Certain allelic variation or mutation may influence the prognosis of the disease in

various populations. Recently two different markers have been identified which may influence susceptibility or prognosis of rheumatoid arthritis.

Genetics of TLR with RA

2.19.2 Epidemiology of RA

RA is the most common inflammatory joint disease with prevalence between 0.5% and 1% worldwide (**Dejaco *et. al*., 2006**).Epidemiological data showed that Native American populations such as Pima Indians have a high prevalence of RA and it is low in countries like China, Japan and Africa compared to Caucasians (**Wolfe, 1968**). RA can occur at any age, but its incidence increases with age and may vary depending upon the type of classification criteria used and demographics of the population studied (**Symmons, 1994**). The peak age of onset has risen to 50 years or more and is more common in women than men with a ratio of 3:1 (**Young, 2000., Symmons, 2002., James, 2004**). Several prevalence and incidence studies of RA have been reported during the last decades, suggesting a considerable variation of the disease occurrence among different populations.

2.19.3 Disease etiology

RA is an autoimmune disease of unknown cause (**Rindfleisch and Muller, 2005**) and interaction between genetic and environmental factors play an important role in the development of disease in susceptible individuals (**Jawaheer *et. al*., 2004**).

2.19.4 Genetic factors

Family and twin studies indicate that first degree relatives of patients with RA have an increased frequency of developing this disease, particularly if the patients had severe disease or were seropositive for rheumatoid factor (**Lawrence, 1970**). Identical twins have higher concordance rates of the disease compared to non-identical twins, supporting genetic susceptibility (**Lawrence, 1970; Silman, 1993**). However, RA is a polygenic and genetically heterogeneous disease and non-inherited factors are also of great importance. In RA, the causative role of different genes may vary between individual patients and various combinations of polymorphisms in a selection of different genes (genotype) may predispose to the clinical picture

(phenotype). Some genes are responsible for severity of the disease rather than occurrence. Only few genes have been consistently associated with RA.

The major Histocompatibility complex (MHC) is a large genetic region on the short arm of chromosome 6, which has been consistently linked to RA. A large part of the MHC comprises human leukocyte antigen (HLA) genes, which encode individual‘s tissue type and are divided into class I (HLA-A, HLA-B, HLA-C) and class II (HLA-DR, HLA-DQ, HLA-DP) genes. The encoded proteins are crucial in determining the individual‘s immune response to antigenic stimuli. HLA class II genes, in particular HLA-DR4 and HLA-DRβ1, have been strongly linked to RA. Particular HLA-DRβ1 molecules in RA share a sequence that influences the peptides that are bound and viewed by the immune system. This core amino acid sequence is named the ―shared epitope‖ and these epitopes have been linked with both predisposition to, and severity of RA **(Gregersen, 1987., Del Rincon, 2003.,Gorman, 2004)**.

2.19.5 Role of Environment in case of Rheumatoid Arthritis

The main environmental factors that affect the patients are the non-inherited factors like smoking and infections play a major role in the etiology of RA. Epstein-Barr virus (EBV), Parvovirus B19, *Mycobacterium tuberculosis*, *Escherichia coli* and *Proteus mirabilis* have all been implicated as possible trigger factors for RA, but the results have been inconsistent **(Holoshitz,1986., van Eden, 1988.,Venables, 1988., Ebringer, 1989., Albani,1995., Rashid, 2007)**. Environmental agents are considered as triggers rather than as being directly involved in the disease process and complex interplay between genetic and environmental factors are probably important for the initiation of the disease process in susceptible hosts. Certain viruses and bacterial agents contain identical peptide sequence to auto antigen and infection with these microbial agents can induce an immune response that cross-reacts with the auto antigen, termed ―antigen mimicry‖. Antigen mimicry is one hypothesis to explain induction of autoimmunity by environmental triggers. Another concept proposes that a local immune response to any environmental agents may release pro-inflammatory cytokines to up regulate antigen-presenting capacity resulting in an immune mediated inflammatory cascade (**Feldman, 1989)**. Hormonal factors may also play a possible role in the etiology of the disease as suggested by increased female preponderance, high incidence during the premenopausal or post-partum period and protective effect of oral contraceptive pills presumably due to its progesterone content (**Lahita,**

1990). Diet and stress have also been considered to play a possible role in the disease expression (**Buchanan, 1991)**. Vitamin D and its metabolites may have an inverse relationship with disease activity in inflammatory polyarthritis or RA, due to their immunomodulatory effects (**Patel, 2007)**. The higher consumption of olive oil, oil-rich fish, fruit, vegetables and beta-cryptoxanthin may have a protective effect on the development of RA, whereas lower consumption of foods rich in antioxidants, could be associated with an increased risk of RA, but the results were inconclusive. Also, high intake of red meat and low intake of vitamin C might play a role in the development of inflammatory polyarthritis (**Pattison, 2004)**.

2.19.6 Cellular destruction in case of Rheumatoid Arthritis

The most pronounced and fundamental pathology in RA is destruction of articular cartilage and subchondral bone by ectopic and hyperplastic synovium. T lymphocytes and macrophages are also seen in large numbers along with dendritic cells, plasma cells and B-lymphocytes in the synovial fluid and membrane. The lining layer of the synovial membrane, which is normally two cells thick, becomes much thickened with increased numbers of both macrophage like and fibroblast-like cells (**Isaacs, 2011)**. In case of RA, the synovium becomes highly vascular with increased number of new blood vessel formation termed ―angiogenesis‖. The junction between synovial tissue, cartilage, and the bare area of bone within the joint capsule is prone to develop erosions early in RA. The synoviocytes proliferate as the disease progresses and invade the adjoining articular cartilage, where the secretion of cytokines, and cartilage and bone-degrading enzymes, results in characteristic destructive changes of RA. The invading, hyperplasic synovium is called pannus and the zone of invasion is called cartilage-pannus junction. The proliferative ―pannus‖ behaves as a locally invasive malignancy, burrowing into and destroying articular cartilage and subchondral bone. Synovial membrane, lines the tendons and bursae, also develops similar proliferative changes leading to destruction and deformity **(Szekanecz, 2001., Yamanishi, 2001., Isaacs, 2011)**. Although the pathogenesis of RA is still unclear (**Hayer *et. al*., 2005**), genetic studies have pointed to the association of RA with the HLA–DRB1 alleles. The HLA–DRB1 alleles are encoding for the SE. The SE hypothesis postulates that the SE motif is directly involved in the pathogenesis of RA by allowing the presentation of an antigenic peptide to T cells. The Ag could be either an exogenous Ag, such as a viral protein, or an endogenous protein. Recently, a number of possible endogenous Ags including citrullinated

protein, human cartilage glycoprotein, and heavy chain binding protein, have been identified (**Blass *et. al*., 1999**). Ag-activated CD4+ T cells stimulate monocytes, macrophages, and synovial fibroblasts to produce the cytokines IL -1, IL-6, and TNF α through cell-surface signaling by means of CD69 and CD11, as well as through the release of soluble mediators such as interferon-γ and IL-17. IL -1, IL-6 and TNF α that drive inflammation in RA. TNF α and IL-1 stimulate synovial fibroblasts, osteoclasts and chondrocytes that release matrix metalloproteinases, in particular stromelysin and collagenases. These enzymes degrade connective-tissue matrix and are thought to be the main mediators of joint damage in RA. Furthermore, TNF α and IL-1 inhibit the production of tissue metalloproteinase inhibitors by synovial fibroblasts. These activated macrophages, lymphocytes, and fibroblasts, as well as their products can also stimulate angiogenesis, which may explain the increased vascularity found in the synovium of patients with RA. Endothelial cells in the synovium are activated and express adhesion molecules that promote the recruitment of inflammatory cells such as neutrophils into joints. Neutrophils release elastase and protease, which degrade proteoglycan in the superficial layer of cartilage.

Rheumatoid synovium contains a number of pro and anti-inflammatory cytokines, which are mainly of T-cell and macrophage origin. Prominent pro-inflammatory cytokines are TNF-α, IL-1, IL-6, IL-8, IL-12, IL-15, IL-18 and interferon-gamma (IFN-γ), whereas the main anti-inflammatory cytokines are IL-4, IL-10, IL-11, IL-13, TGF-β, and cytokine neutralizing factors such as soluble TNF-α receptors and IL-1 receptor antagonist (IL-1ra). An imbalance between pro and anti-inflammatory cytokines may be the main pathogenic mechanism in RA as pro-inflammatory mediators, in particular TNF-α and IL-1, appears to play a major role in the immune mediated inflammatory cascade leading to the various articular and systemic manifestations **(Feldmann,1996., Fox, 1997., Zhang, 2001., Isaacs, 2011)**. Nitric oxide, prostaglandins, leukotrienes, and free oxygen radicals are the other pro-inflammatory factors present within the RA synovium. Rheumatoid synovium is characteristically highly vascular with angiogenesis and this is stimulated by various factors including hypoxia, soluble factors such as vascular endothelial growth factor (VEGF) and soluble vascular cell adhesion molecule-1 (VCAM-1), which stimulate endothelial cell growth. There are other adhesion molecules that are abundantly present on the vascular endothelium such as E-selectin and intercellular adhesion

molecules (ICAMs). Their expressions are stimulated by proinflammatory cytokines, particularly TNF-α and IL-1, resulting in the recruitment of inflammatory cells via specific receptors. Chemokines such as monocyte chemotactic protein-1 (MCP-1), IL-8 and MCP-2 are highly expressed in RA synovium and they stimulate progression of inflammatory cells into the joint **(Liao, 1995., Panayi, 2001., Isaacs, 2011)**.

Tissue hyperplasia and lymphocyte proliferation as a result of immune response are normally counteracted by programmed cell death or apoptosis to prevent over accumulation of cells. In rheumatoid joints, apoptosis is actively inhibited despite the presence of pro-apoptotic stimulants such as hypoxia and TNF-α in rheumatoid synovium. Impaired synoviocytes apoptosis may contribute to the pathogenesis of RA (**Isaacs, 2011)**.

The exact mechanism of cartilage and bone destruction in RA are not understood, but may be related to a variety of destructive enzymes secreted by pannus. The important ones are MMPs, which include collagenases, stromelysin and gelatinases, and serine and cysteine proteases such as cathepsins. These enzymes destroy the articular cartilage by acting upon collagen and proteoglycan matrix but are normally controlled by physiological inhibitors such as TIMPs. An impaired regulatory mechanism between these destructive enzymes and their inhibitors may partly be responsible for the destructive nature of the disease (**Goldring, 2000., Gravallese, 2000.,Tak, 2000., Isaacs, 2011)**. Other destructive factors include the cytokines TNF-α and IL-1, which activate osteoclasts leading to bone resorption. Bone destruction may also be mediated by factors such as osteoclast differentiation factor (ODF) or TNF-related activation induced cytokine (TRANCE) and receptor activator of nuclear factor κB ligand (RANKL). ODF interacts with membrane RANK that is present on osteoclast precursors, resulting in their differentiation and activation and subsequent bone destruction. The combination of TNF-α, IL-1 and ODF probably contributes to periarticular as well as systemic osteoporosis in RA. Activated CD4+ T cells express osteoprotegerin ligands that stimulate osteoclastogenesis, which then leads to bone degradation.

Activated CD4+ T cells also stimulate B cells through cell-surface contact and through the binding of αLβ2 integrin, CD154 (CD40 ligand), and CD28, to produce immunoglobulins,

including ACCP2A and RF. The precise pathogenic role of RF is unknown, but it may involve the activation of complement through the formation of immune complexes (**Anderson, 2004., Choy and Panayi, 2001**).

2.19.7 Role of different cells in case of RA.

2.19.7.1 Monocytes/Macrophages

Macrophages (MΦ) have phagocytic capacity and are central effectors of synovitis (**Haringman *et. al.*, 2005**). They are found both in the synovial tissue and SF. There are two types of macrophages in the RA synovial tissue. The macrophage-like type A synoviocytes in the lining and the sublining macrophages migrated as monocytes from the circulation and are diffusely distributed in the synovium. Both types have multiple functions such as clearance of immune complexes, antigen presentation (MHC class II are over expressed on MΦ), mediation and regulation of local and systemic inflammation, tissue remodeling through release of different cytokines and growth factors (TNFα, IL-1, IL-6, IL-10, IL-13, IL-15, IL-18 and GM-CSF), mediation and regulation of monocyte migration, stimulation of angiogenesis by chemokines and chemo attractants, tissue degradation and post-injury tissue remodelling by matrix metalloproteinases (MMPs) (**Kinne *et. al.*, 2007**). They express several markers of the resident macrophage population including CD68, CD163 and CD14 (**Bartok and Firestein, 2010**). In addition to the monocytes/MΦs central role in inflammation, they are also involved in bone erosions due to their ability to differentiate into osteoclasts. Upon stimulation with TNF-α, IL-1, IL-6 and IL-17 synovial fibroblasts and activated T cells can up regulate RANKL expression on their surface which can engage its receptor RANK on the surface of monocytes and drive them into osteoclastogenesis (**Davignon *et. al.*, 2013**).

2.19.7.2 Fibroblast-like synoviocytes

FLS are non-phagocytic mesenchymal-derived cells. The FLSs found in the lining layer are highly activated and exhibit features with aggressive invasive properties. They are important in both initiation and perpetuation of RA and can contribute to the maintenance of chronic inflammation through cell–cell contact and through elaboration of soluble products. In response to environmental stimuli and interactions with various cell types in the inflamed synovium, FLS

can secrete several mediators like cytokines, chemokines, growth factors and several other proinflammatory molecules like prostaglandins and leukotrienes. It has recently been shown in a SCID mouse model of arthritis that FLSs can migrate to a distant unaffected joint and invade and degrade the cartilage and thereby promote articular involvement (**Lefevre *et. al*., 2009**). In a very recent study, citrullinated fibronectin (cFn) was shown to inhibit apoptosis and increase proinflammatory cytokine secretion of RA FLSs (**Fan *et. al*., 2012**). This could be one possible explanation for the increased number of FLSs that contribute to the hyperplasia in RA synovial membrane.

2.19.7.3 T and B cells

The T cells constitute around 30-50% of all cell types in the sublining and the majorities are CD4+ with T helper (Th) 1 phenotype (**Bartok and Firestein, 2010**). T cells are identified as CD3+ cells in the synovial tissue and are either CD4+ Th cells, CD8+ cytotoxic T cells or CD4+ regulatory T cells (**Wagner *et. al*., 1998**). The Th1 subset mediates cellular immunity and is defined by IFNγ secretion. The Th2 is involved in humoral immunity and forms mainly IL-13 and IL-4, while Th17, the newest member of the T cell family is identified through its signature cytokine, IL-17. Th17 cells are important promoters of autoimmunity in RA (**Gaffen, 2009**). Synovial-derived T cells have a phenotype that indicate chronic immune activation but express low levels of cytokines and show signs of anergy (**Cope, 2002**).

B cells and plasma cells are mainly found in the sublining layer of synovial membrane. Around 5% of sublining synovial cells is B cell. The pathogenic roles of B cells in autoimmune disorders have historically been attributed to autoantibody production that would drive the inflammation locally either in soluble form or as immune complexes (**Marston *et. al*., 2010**). B cells contribute to RA through both antibody-dependent and antibody independent mechanisms. Examples of antibody-independent functions are antigen presentation, T cell activation and polarization, organisation of other inflammatory cells and dendritic cell modulation. B cells display considerable phenotypic diversity (**Anolik *et. al*., 2009**).

2.19.7.4 Neutrophils

The phagocytic neutrophils are the most numerous and most important cells in innate immune responses. In the RA joint neutrophils are the first cells to be recruited at the sites of

inflammation and accumulate mainly in the inflamed SF and to a lesser extent in synovial membrane at the site of active destruction where they phagocyte immune complexes and release degrading proteases (**Cascao *et al*., 2010**). Resting peripheral blood neutrophils are relatively short lived while primed and activated neutrophils within tissues undergo molecular changes that extend their life span and alter their molecular properties, thereby allowing them to carry out many functions. Delayed apoptosis, together with synthesis of inflammatory mediators like IL-8, TNF-α, IL-1, IL-6, IL-12, TGF-β and BLyS, and ability to present antigen to T cells via MHC II, makes tissue neutrophils capable of driving inflammatory processes. Several recent reports have suggested a possible direct contribution of neutrophils in early RA pathophysiology and bone remodelling (**Poubelle *et. al*., 2007**) by mediating Th17-responses (**Cua and Tato, 2010**), expressing PRRs (**Hayashi *et. al*., 2003; Kerrigan *et. al*., 2009., Greenblatt *et. al*., 2010**) and mediating bone resorption via activating osteoclastogenesis (**Chakravarti *et. al*., 2009**). Neutrophil adheres to the endothelial wall using selectins, integrins and adhesion molecules to pass from the peripheral blood to the site of inflammation. Rolling arrest precedes transmigration through the endothelial lining of the blood vessel, and chemo taxis to sites of inflammation, for example the joint.

2.19.7.5 Dendritic cells

DCs play an essential role in the initiation and perpetuation of inflammatory arthritis by presentation of arthritogenic antigens to auto-reactive T cells. Through their potent antigen-presentation ability they stimulate naïve T cells, direct effecter cell‘s function and polarize the T cell repertoire towards the Th1, Th2, or Th17 phenotypes. Myeloid DCs (mDCs) are considered especially important in promoting synovial inflammation. Plasmacytoid DCs (pDCs) are recruited in RA ST and comprise an antigen presenting cell (APC) population. That might contribute to the local inflammatory environment, particularly as a result of their capacity to produce cytokines *in situ* such as IFN-α, IFN-β, Il-15, Il-18 and Il-23p19. The number of synovial pDCs is specially increased in RA patients that are ACPA positive (**Lebre and Tak, 2009**).

2.19.7.6 NK cells

Several reports have indicated that NK cells may have direct or indirect role in RA (**Ahern and Brennan, 2011**). Dalbeth and Callan reported that a subset of NK cell (CD56bright) is greatly expanded within inflamed (synovium) joints (**Dalbeth and Callan, 2002**), in which they produce more IFN-γ compared to the blood NK cells from the same patients (**Aramaki *et. al*., 2009**). Moreover, these NK cells could induce the differentiation of monocytes into DCs. The communication between NK cells and other cell types through cytokines and chemokines make a potential risk for autoimmune diseases. Other example of this phenomenon is the crosstalk between NK cells and myeloid DCs, referred to as ―DC editing,‖ which may lead to NK cell activation and DC maturation. In this way, activated NK cells may in turn kill immature DCs that fail to undergo proper maturation (**Moretta *et. al*., 2006**). Furthermore, it has been reported that NK cells can function as APCs in some instances, which complicate the involvement of these cells in the immune responses (**Hanna *et.al*., 2004**).

Yen *et al*. have found that patients with RA complications have an expansion of unique population of CD4+CD28− T cells which is uncommon in healthy individuals (**Yen *et. al*., 2001**). Interestingly, CD4+CD28− T cells are functionally distinct from classical CD4+ TH cells and share some features with NK cells. For instance, they do not express CD40 ligand, but express CD57 (an NK cell marker), and produce large amounts of IFN-γ, and produce granzyme B and perforin (**Yen *et. al*., 2001**). One of the most potent osteoclastogenic cytokines which is pivotal in the pathogenesis of RA is TNF-α (**Di Santo, 2006**). TNF-α induces receptor acquisition by NK cells and the combination of TNF-α and IL-15 can enhance this effect (**Lee *et. al*., 2010**). It has been demonstrated that NF-κB is an important factor in regulation of NK cell growth and differentiation. NF-κB is activated in presence of TNF-α plus IL-15(**Lee *et .al*., 2010**).

2.19.8 Diagnosis

2.19.8.1 Laboratory diagnosis

Bony erosions and deformities seen in case of RA are largely irreversible. Initiation of therapy within three months after the diagnosis of RA is crucial since a delay of as little as three months in the introduction of these medications result in substantially more radiographic damage at five

years. Therefore, early diagnosis, although challenging, is critical. Laboratory quantifications of antibodies and inflammatory markers provide a way for early diagnosis of RA.

2.19.8.2 RF antibodies

The serum of RA patients contain a variety of Antibodies (Abs) directed against self-antigens. The most widely known of these Abs are the RF and anti CCP antibodies. Rheumatoid factors (RF) are autoantibodies directed against the Fc portion of IgG and found in every immunoglobulin subclass (IgM, IgA and IgG). However, the IgM class is being the most common (**Halldorsdottir *et. al*., 2000**). Rheumatoid factor is a well-established diagnostic and prognostic test in Rheumatoid Arthritis. Normal human lymphoid tissue commonly possesses B lymphocytes with RF expression on the cell surface. However, RF is not routinely detectable in the circulation in absence of an antigenic stimulus. Modified IgG could be a stimulus to RF production and may become an important component of RA pathogenesis (**Newkrik *et. al*., 2003, Das *et. al*., 2004**). This concept is supported by studies that observed an association of RF and more severe RA with autoantibodies to advanced glycated end product-damaged IgG or agalactosyl IgG. Co-stimulation of B cells, perhaps mediated by toll-like receptors (TLRs), may allow B cells with low affinity receptors for IgG to become activated. TLRs are components of the innate immune system, and they provide signals after engaging various bacterial and viral products (**Shlomchik *et. al*., 1993, Rifkin *et. al*., 2005**). CD14-positive cells (monocytes) from the bone marrow stimulate RF-producing B cells (**Hirohata *et. al*., 1995)**. Synovial fluid RF may be produce by synovium-derived CD20-negative and CD38-positive plasma cells (**Van Esch *et. al*., 2003).** Circulating B cells require interleukin-10 (IL-10) for RF production (**Perez *et. al*., 1995**). Cigarette smoking, a risk factor for more severe RA, is associated with an increased prevalence of RF (**Padyukov *et. al*., 2004**). RFs possess significant heterogeneity related to mutations within heavy and light chain genes (**Youngblood *et. al*., 1994**). Thus, IgM RFs from patients with RA react with a variety of antigenic sites on autologous IgG (**Carson, 1993**). The potential physiological role of IgM RF includes the following:

1) Binding and processing of antigens embedded in immune complexes.
2) Presentation of antigens to T lymphocytes in presence of HLA molecules.
3) Immune tolerance.
4) Amplification of the humoral response to bacterial or parasitic infection.

5) Immune complex clearance.

2.19.8.3 Anti-cyclic citrullinated peptide (CCP) antibodies

Anti-citrullinated protein antibodies are highly specific for RA (**Masson-Bessiere *et. al*., 2001**). Citrulline, the antigenic determinant for this Abs, is a nonstandard amino acid. It does not incorporate into proteins during translation. It can, however, be generated post-translationally by enzymatic citrullination (deimination) of arginine residues. The citrullination is catalyzed by peptidyl arginine deiminase; arginine residues on fibrin and fibrinogen may be favoured sites for deimination within rheumatoid joints (**Van Boekel *et. al*., 2002; Vossenaar and van Venrooij, 2004, Vossenaar *et. al*., 2004, Vossenaar and Robinson, 2005**).

An enzyme linked immunosorbent assay (ELISA) was developed to detect antibodies directed against filaggrin derived from human skin and has high specificity and sensitivity for the diagnosis of RA (**Palosuo *et. al*., 1998**). The target amino acid in filaggrin is citrulline, a post-translationally modified arginine residue (**Schellekens *et. al*., 1998**). Subsequently, an ELISA assay for the detection of antibodies to a cyclic peptide containing citrulline was made commercially available, easier to standardize, and also has high sensitivity and specificity for the diagnosis of RA. This has become the assay for the detection of anti-cyclic citrullinated peptide (anti-CCP) antibodies.

2.19.9 Different Inflammatory markers in case of RA (ESR and C-reactive protein)

While multiple blood markers of inflammation have been identified and shown to be useful in the evaluation and treatment of RA, to date, ESR and CRP have been most commonly studied and used in clinical practice. Elevation of ESR and CRP are the strongest predictors of persistent, progressive disease in RA. If they are elevated in early disease and do not show any improvement with therapy, this may lead to joint damage and other worse outcomes (**James *et. al*., 2004; Lindqvist *et. al*., 2005**). ESR and CRP are very sensitive to change in disease activity. The ESR, an indirect assessment of inflammation, measures the distance that RBCs fall in a capillary tube over the course of an hour. The presence of inflammation causes the cells to fall more quickly due to the action of inflammatory proteins, such as fibrinogen or immunoglobulins, blocking the normal charge inhibition on RBCs. In many RA studies, an ESR level greater than

20 to 30 mm/h has been considered abnormal; however, considerable individual variability between normal and abnormal tests exists.

CRP is a pentameric protein released in response to inflammatory stimuli. CRP levels are a more accurate measure of inflammation than the ESR. Measuring CRP in inflammatory conditions is preferred over the ESR as CRP responds much more quickly to inflammatory stimuli and can, therefore, be used as a timely marker of active inflammation. The CRP level that has been determined to be abnormal in RA studies is generally greater than 1.0 mg/dL. But caution must be taken when interpreting this value as many clinical labs report CRP in mg/L, resulting in a 10-fold higher value that may still be a normal result.

2.19.9.1 Assessment of Disease Activity

In case of RA, measurement of disease activity at specific point of time or at a regular intervals helps to evaluate the disease progression and it is vital to assess treatment response, outcomes and prognostic factors. Various methods have been introduced and validated to measure disease activity in RA over the last few decades. These methods have been designed and modified to evaluate three different but interrelated aspects of the disease progression: clinical, radiological and functional.

2.19.10 Measurement of clinical disease activity

In the early 1990s, core sets of disease activity measures have been proposed by the American College of Rheumatology (ACR, formerly ARA), European League Against Rheumatism (EULAR) and World Health Organization (WHO) / International League of Associations for Rheumatology (ILR), to standardize the disease activity assessments in the clinical trials involving RA patients **(Tugwell, 1982., Felson, 1993., Boers, 1994)**. These measures included swollen joint count (SJC), tender joint count (TJC), and patient assessment of pain, global assessment of disease activity by the patients (PGA) and by the evaluators (EGA) and acute phase reactants such as erythrocyte sedimentation rate (ESR) and C - reactive protein (CRP). The core set also included structural damage on radiographs and functional status (**Aletaha and Smolen, 2006., Tugwell and Bombardier, 1982**). These measures are also very useful and crucial to assess disease activity and treatment response in day-to-day clinical practice.

2.19.10.1 Pain, Swollen and tender joint counts

Pain is the serious symptom for patients with RA and it is measured on a 100-mm visual analogue scale (VAS), evaluating symptom for one week before the study point. Horizontal VAS is more commonly used than vertical scales and there are also other reliable methods of pain assessment such as, arthritis impact measurement scale (AIMS) and McGill pain questionnaire (**Aletaha and Smolen, 2006**).

A number of different joint indices and counts have been developed over the years and they vary by the number of joints assessed or by the way several joints are aggregated to represent joint regions (**Aletaha and Smolen, 2006**). Ritchie *et al.,* introduced a graded tender joint count, assessing 26 joint areas with grades ranging between 0 to 3 depending upon the severity of joint tenderness (**Ritchie *et. al*., 1968**). Further modifications of the joint indices and simplifications of the extensive joint counts were carried out by other groups over the years, reducing the number of joints assessed (**Egger *et. al*., 1985; Fuchs *et. al*., 1989; van der Heijde *et. al*., 1992**). These simplified joint counts have been validated and are reliable and easy to use in clinical practice (**Prevoo *et al*., 1993; Smolen *et. al*., 1995**).

2.19.11 Disease activity scores and indices

Composite disease activity scores have been developed over the years to give reliable identification of disease activity and to overcome methodological problems. These scores use special formulas integrating SJC, TJC, ESR or CRP and GH to measure overall disease activity (**Aletaha and Smolen, 2006**). Van der Heijde *et al*, introduced disease activity score (DAS) in 1990 based on 44-swollen joint count (**van der Heijde *et. al*., 1990., van der Heijde *et. al*., 1993;**). This was later modified to include the reduced 28-joint count, DAS28, which shows similar validity and reliability compared to DAS and has been widely used (**Prevoo *et. al*., 1993; Prevoo *et. al*., 1995; Smolen *et. al*., 1995**). Both DAS and DAS28 have been modified in several ways to exclude the assessment of GH (DAS-3 and DAS28-3) and to include CRP instead of ESR (DAS-CRP and DAS28-CRP) (**Aletaha and Smolen, 2006**). Formulae to calculate DAS28 with 4 or 3 variables and with ESR or CRP

2.19.12 Criteria to assess disease activity

After the introduction of the composite disease activity indices, a number of criteria have been validated, based on DAS, DAS28, SDAI and CDAI, to assess different levels of disease activity including remission (**van Gaestel *et. al*., 1996., Smolen *et. al*., 2003., Balsa *et. al*., 2004., Fransen *et. al*., 2004., Paulus, 2004., Fransen and van Riel, 2005., Aletha *et. al*., 2005**).

EULAR has adapted disease activity criteria based on DAS and DAS28, which have been widely used in several studies.

EULAR criteria based on DAS

DAS < 1.60 - remission

DAS ≥1.60 and ≤ 2.40 - low disease activity

DAS >2.40 and ≤ 3.70 - moderate disease activity

DAS> 3.70 - high disease activity

The United States (US) Food and Drug Administration (FDA) has also proposed remission criteria, which is based on ACR remission criteria, but also takes into account the structural damage on x-rays and treatment status at the time of assessment. According to this, 5 out of 6 ACR remission criteria have to be fulfilled plus radiographic arrest for ≥ 6 months with no drug therapy (**Paulus, 2004**).

2.19.13 Radiological progression

Conventional radiography has been traditionally used to assess structural damage in RA. X-rays of hands and feet and/or large joints have been used to define radiological damage at a given point of time as well as progression of structural damage over a period of time. The advantage of radiographic assessment of disease progression over other methods is that the damage seen on x-rays largely irreversible and it represents the cumulative measure of disease activity and destructive process over time. Another major advantage is that apart from providing permanent records, radiographs can also be randomized and blinded for clinical investigations of new therapeutic agents in clinical trials (**Wollheim *et. al*., 1988., van der Heijde, 2000**). It has been widely recognized that radiological damage on x-rays has to be quantified to define the disease status of the patients and more importantly to assess disease progression, treatment response and outcome (**Weisman, 1987., van der Heijde, 2000., Rau and Wessenberg, 2005**). Semi-

quantitative methods have been developed to translate the amount of structural damage on x-rays into a score value as no truly quantitative methods are available(**Rau and Wessenberg, 2005**). There are lot of abnormalities that can be seen on radiographs in patients with RA among which erosions and, to a lesser extent, JSN are widely accepted to be included in the scoring methods as they give reliable and additive information on radiological progression (**Sharp *et. al.*, 1985., Fries *et .al.*, 1986**).

2.19.14 Included Joints

It is known that synovial joints can be affected in RA but not feasible to include all joints in scoring radiological damage. Hands (including wrists) and feet have been chosen to represent the overall radiological status of the disease as they are the most commonly involved joints in a majority of patients with RA, wherein erosions and JSN can be seen very early (**Scott *et. al.*, 1986., Drossaers- Bakker *et. al.*, 2000**).The joints that are usually evaluated in the scoring methods include PIP joints, MCP joints, IP joints of thumbs, wrist joint as a whole or as individual joints, MTP joints and IP joints of the 1st toe(**van der Heijde *et. al.*, 1999**). RA is typically a symmetrical polyarthritis, radiological changes can appear asymmetrically. So both hands and feet should be included in the radiographic evaluation (**van der Heijde *et. al.*, 1999**). Postero-anterior (PA) views of the hands and feet x-rays are the most commonly used technique for radiographic assessment (**Mewa *et. al.*, 1983**).

2.19.15 Different Scoring methods for RA

Several scoring methods have been developed and subsequently modified over the last few decades to quantify the radiographic damage in RA (**Steinbrocker *et. al.*, 1949., Kellgren, 1956**). The global method of scoring have been designed to score erosions and JSN together with one overall score, while the composite method scores erosions and JSN individually with a separate score for each that are added together at the end to give a overall score. Although there are several scoring methods available to measure radiographic damage, the modifications made by Larsen and Sharp mainly SvdH have been the most commonly used practice. Each of these scoring methods has their own advantages and disadvantages. The advantage of Larsen‘s score is that an experienced reader can perform it quickly, whereas SvdH method is more time consuming (**Sharp *et. al.*, 2004., Rau and Wessenberg, 2005**). However, inclusion of soft tissue

swelling in the Larsen's score may lead to a relatively high baseline score, decreasing with response to treatment. This may reduce the total possible increased score due to progressive damage, contributing to low sensitivity to change (**Rau and Wessenberg, 2005**). It has been shown that SvdH method is better than others in respect to its sensitivity to detect a real change in x-ray progression over time (sensitivity to change) and in detecting changes that are clinically meaningful, termed minimal clinically important difference (MCID) i.e. smallest radiographic change that necessitates the physicians to alter their treatment (**Pincus *et. al*., 1997., Paimela *et. al*., 1998., Lassere *et. al*., 1999., Drossaers- Bakker *et. al*., 2000., Bruynesteyn *et. al*., 2002., Bruynesteyn *et .al*., 2004., Sharp *et. al*., 2004., Guillemin *et. al*., 2005**).

2.19.16 Assessment of function

Functional assessment in patients with RA is a vital component in the evaluation of disease progression as it significantly correlates with disease activity, structural damage and long-term outcomes (**Wolfe and Hawley, 1998., Barrett *et. al*., 2000., Scott *et. al*., 2000., Young *et. al*., 2000**). The Health Assessment Questionnaire (HAQ) or HAQ-disability index (HAQ-DI) is a 20-question instrument, which assess the degree of difficulty a patient has in accomplishing his or her tasks in eight functional categories such as dressing, rising, eating, walking, hygiene, reaching, gripping and usual day to day activities. For each question there is a four-level difficulty scale ranging from 0 to 3. The final score is the mean of the highest scores across eight categories and it ranges from 0 to 3, with higher levels indicating more disability (**Fries *et al*., 1980., Ramey *et. al*., 1992**). The HAQ has been modified several times subsequently to simplify it and to make it user friendly and also to include other domains such as depression and anxiety (**Pincus *et. al*., 1983., Pincus *et. al*., 1999., Wolfe *et. al*.,2004**).It has been shown that during the early stages of the disease (<5 years duration), the HAQ score is mainly influenced by joint pain and swelling due to inflammation, which can improve with treatment (reversible); whereas in the late stages, the HAQ scores strongly correlate with structural damage (irreversible) and so the reversibility of HAQ in patients with established RA may not be as significant as in early RA (**Aletha *et. al*., 2006**).

The Arthritis Impact Measurement Scale (AIMS) is another form of patient self reported functional questionnaire, which include assessment of depression and anxiety (**Meenan *et. al*.,**

1980). There are longer and shorter versions of the AIMS, which have been used to evaluate function in patients with arthritis including RA (**Aletha and Smolen, 2006**).

Objective quantitative instruments have also been used to assess function and these include measures of grip strength and locomotion (Pincus and Callahan, 1992) using a vigorimeter or a dynamometer, indicating the pressure attained by squeezing a compressible rubber bulb (**Jones *et. al*., 1991; Pincus *et. al*., 1991**).

2.20 Relation of TLRs with RA

TLRs constitute one of the major markers of innate immunity which recognize various conserved antigens like LPS, bacterial flagellin, double stranded DNA, RNA. These markers are expressed on a variety of cell types such as NK cells, monocytes, epithelial cells etc. TLR1, 2, 4, 5 and 6 are expressed on the cell surface and they interact with ligands found on the surface of pathogens. In contrast, TLR3 and 7, 8 and 9 are located intracellularly on endosomal membranes and their ligands must be taken up into the endosome in order to activate the downstream signaling pathways (**Huang, 2009**). Binding of the TLRs with its corresponding ligands results in the activation of either the MyD88-dependent or the MyD88-independent pathways. The MyD88-dependent pathway leads to the activation of NF-κB and promotes in cytokine gene expression (**Huang, 2009)**.

TLR2 and TLR4 are primarily expressed in the blood monocytes of RA patients. Expressions of TLRs can also be found in the synovial tissue. (**Radstake, 2004; Huang, 2009)**. Endogenous TLR ligands in synovial tissue of RA patients include fibrinogen, heat shock protein 60 and 70, and fibronectin. These ligands activate certain TLRs in the synovial tissue and induce the production of various cytokines.

A Plethora of researches are being conducted throughout the World to explore the association of TLR with RA. Non-missense single nucleotide polymorphism in TLR4 was identified to be risk factors for the development of RA in Chinese Han population (**Yang, 2013**) yet several other studies have reported the lack of association with RA (**Xu, 2012**).

Cytokines such as TNF, IFN- y, IL-6 also regulates the expression of TLRs in the synovial fluid in case of RA pathogenesis (**Steiner, 1999., Radstake, 2004**). Chamberlain *et. al*., in 2012 reported strong correlation of TLR5 and TNF-α with RA progression. TLR5 has been postulated to be a TNF responsive gene and it is possibly linked to RA progression through induction of angiogenesis (**Chamberlain, 2012**). Study with transmission disequilibrium test on the French families revealed no association with the major RA related TLRs (TLR1, 2, 6 and 9) with RA (**Orozco, 2005; Jaen, 2009**).

It can be inferred from the different reports that TLR plays significant roles in RA pathogenesis. Genetic variations in the TLRs may influence the susceptibility or resistant to the disease in various populations. Auto reactivity of the synovial cells and production of the cytokines in RA pathogenesis significantly related with the markers activation. Autoimmune cells in the synovial tissue trigger TLR for the signalling and influence the cytokine production for the disease progression. So, signalling pathways can modulate the expression and cytokine production for the improvement of the disease. Initial screening of TLRs is needed in various populations, as that may reveal the association of RA with the populations and may help for controlling of the disease.

2.21 TLRs and typhoid fever

Free living organisms have the ability to cope up with the new environment by modifying their gene expression patterns (**Groisman and Mouslim, 2006**). Enteric fever is an important infection now-a-days among the populations in endemic countries like India (**Meltzer and Schwartz, 2010**). *Salmonella enterica* serotype *typhi* (*S. typhi*) is a gram negative bacteria that is restricted to human and causes a wide range of food and water-borne diseases ranging from self-limiting gastroenteritis to systemic typhoid fever (**Manon, 2012)** . The occurrence of typhoid fever is less in developing and industrialized countries, but it is high prevalent in countries like India and South- East Asia (**Meltzer and Schwartz, 2010)**. According to Crump *et. al*., typhoid fever caused 21,650,974 illnesses and 216,510 deaths during the year 2000 (**Crump, 2004)**. The Poor sanitation, lack of a safe drinking water supply, unhygienic condition and low socio economic conditions have amplified the disease progression in India which increased the morbidity and mortality among population (**Kanungo, 2008)**.

The virulence factors of different Salmonella serotypes can perform a powerful model for studying the host adaptation mechanisms, because these pathogens are physiologically well characterized for genetic analysis (**Baumler, 1998)**. The Salmonella genus is divided into two distinct species, *Salmonella bongori* and *Salmonella enterica*. The serotype typhi and paratyphoid A, B and C is present in human and in other higher primates (**De Jong, 2012**). Salmonella produces multiple Pathogen associated molecular patterns (PAMPs) like flagella, fimbrae, LPS (Vi antigen), and bacterial DNA and develop survival mechanism from the host cells by producing superoxide dismutase, salmonella containing vacuole, type I secretion system etc.(**Ibarra and Steele-Mortimer, 2009)**. These virulence factors have been recognized by the pattern recognition receptors like Toll like receptors (TLRs) (**De Jong, 2012)** which initiate an immune response and form a link between the innate and adaptive immunity (**Kawai and Akira, 2010)**. Primarily TLR4 and TLR5 play a major role in activation of the immune responses against LPS and flagellin. TLR4 polymorphisms among Asian Malay population express a higher risk for typhoid infection in case of *S. typhi* (**Bhuvanendran, 2011).** Genetic association study among the Vietnam population could not prove any association of TLR5392STOP stop codon with typhoid fever patients (**Dunstan, 2005)**. The modulation or variation of binding site of TLR gene receptors against the LPS, flagellin or other antigens of *Salmonella typhi* evokes host immune response during typhoid fever (**Sivaji, 2016)**. Several association studies have been conducted on TLR with typhoid fever on a different population of the worldwide, especially on TLR4 and TLR5. So, a complete screening of all ten TLR genes on typhoid patients is needed to interpret if there is any association present with other TLRs.

2.21.1 Toll-Like Receptor 4 (TLR4) and Typhoid Fever

The detection of genotype and mutation study within the TLR4 gene in typhoid fever patients and controls in the Vietnamese population postulate that genetic variations present within TLR4 may affect the recognition of *S. typhi* and altering activation of innate immunity and hence severely affecting the first line of defense against this pathogen. Out of the ten mutations identified seven are novel mutations found in the Vietnamese population. Besides the two common polymorphisms that has been reported (Asp299Gly and Thr399Ile), most polymorphisms within TLR4 occur at low frequencies in different populations in the world. Therefore it is difficult to establish their role in genetic susceptibility to infectious disease. The

TLR family has been described as type I transmembrane pattern recognition receptors (PRR) that contain varying numbers of extracellular N-terminal leucine-rich repeat (LRR) motifs, followed by a cysteine-rich region, a transmembrane domain, and an intracellular Toll/IL-1 R (TIR) motif. Several lines of evidence argue that TLRs play an important role in innate immunity, and changes in TLR structure could potentially lead to functional changes of those receptors. The extracellular TLR4 region which contains Glu24-Lys631 is the functional domain for LPS and MD-2 binding. It was identified five low frequency missense mutations (Ser73Arg, Ala97Val, Tyr98Cys, Thr175Ala, Thr399Ile) in the ectoplasmic LRR domain. The amino acid substitutions may alter protein structure and function as the structure and side chains of some of the substituted amino acids differ from wild-type TLR4. One of these, Ser73Arg, showed a slightly higher frequency in cases of typhoid fever than controls. These LRR region mutations may potentially alter phosphorylation of TLR4 altering downstream signaling of inflammatory mediator activation, ultimately contributing to disease susceptibility. The mutations Thr399Ile and Asp299Gly, which also lie in the ectoplasmic domain, are significantly associated with a blunted response to inhaled LPS and a variety of diseases. A mutation in the hydrophobic region adjacent to the transmembrane domain of TLR4 did not respond to LPS. A low frequency missense mutation (Val651Phe) in the transmembrane domain of TLR4 was identified and the possibility exists that it may alter the function of TLR4 in response to LPS produced by the *S typhi* (**Hue *et. al*., 2009**).

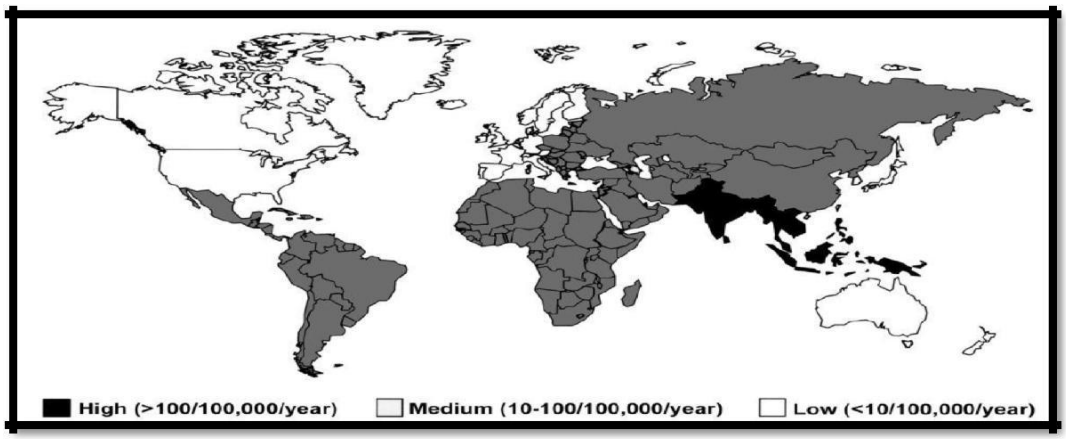

Fig 5: Geographical distribution of Typhoid fever (Source: Crump et al. 2004).

2.21.2 Toll-Like Receptor5 (TLR5) and Typhoid fever

TLR5 is a good candidate gene to use in the study of genetic association of typhoid fever. In one case of *in vitro study*, Flagellin from *S. typhimurium* binds to TLR5 and activates proinflammatory cytokines in the intestinal epithelia. In another *in vivo* murine studies, including experiments that involved Salmonella infections indicate that flagellin is an important stimulant of both innate and adaptive immune responses. In the third case, the murine TLR5 gene lies within a locus that is associated with the susceptibility to Salmonella infection.

In addition, TLR5 is associated also with legionnaire‘s disease caused by infection with *Legionella pneumophila*. Legionella and Salmonella are similar in that they both are gram-negative, flagellated pathogens, but, most importantly they occupy the same intramacrophage niche within the host (**Dunstan *et. al*., 2005**).

The presence of antiflagellin antibody responses in patients with typhoid fever clearly indicates that flagellin is expressed *in vivo* in humans and therefore is available for interaction with TLR5. TLRs also regulate innate immune responses and also play a crucial role in the initiation of adaptive immunity. TLRs influence the activation of adaptive immune responses by two mechanisms. Primarily, TLRs initiate signalling pathways by up-regulating co- stimulatory molecules, and this leads to the maturation of dendritic cells. In addition, TLR-induced cytokines, mostly interleukin 6 (IL-6) are essential if T helper cells want to overcome the suppressive effect of CD4+ and CD25+ regulatory T cells and to generate pathogen specific adaptive immune responses. Individuals who have one or two copies of the stop codons in TLR5, have significantly decreased IL-6 production after stimulation with flagellin, and this decreased production affect the mechanism of TLR-activated adaptive immunity. There may be sufficient redundancy in the TLR pathway to obviate the requirement of TLR5 for a protective immune response to *S. typhi*. The frequency of stop codon in TLR5, which functions as a dominant negative and severely impairs signalling, was not significantly associated with typhoid fever. Despite *in vitro* and murine studies describe the recognition of Salmonella flagellin by TLR5; this pattern recognition molecule may not play an important role in TLR- stimulated innate immune responses to human infection with *S. typhi*. Initiation of these responses may rely on other TLRs recognizing different bacterial ligands (**Dunstan *et. al*., 2005**).

2.21.3 Association of Toll like receptors with HIV

Toll like receptors regulates both the innate and adaptive immune response and polymorphism in the TLR genes has been investigated in case of various diseases (**Schwartz, 2005**). Susceptibility to the human immunodeficiency virus (HIV) infection and disease progression are variable among different populations and also it has been genetically determined (**Willie, 2014**). A small percentage (0.2%) of HIV-1 sero-positive patients is able to control the HIV-1 infection over several years. The adult HIV prevalence at national level has 0.26% in 2015 (**India HIV Estimations, 2015**). It defines that they can maintain a viral load which is fewer than 50 copies of HIV-1 RNA per ml **(Nunez, 2011)**. Different TLRs expressed on different cell types in the human immune system and up regulated by the effect of cytokines. IFN- γ has also induced the expression of TLR4 in peripheral blood monocytes (**Mita, 2001**).

Several association studies have been reported in case of TLRs with HIV disease progression. It has been reported from the previous study that depletion of CD4+ cells in HIV positive individuals release some viral proteins that directly activates the TLR4 (**Brenchley, 2006**). According to **Baenziger *et. al.***, in murine model the chronic activation of TLR7 leads to immune dysregulation that is almost similar found among humans (**Chang, 2009**). Several other TLRs are also associated with HIV disease progression. It was also reported that some polymorphism in TLR3 gene (Leu412Phe) plays a protective role against the disease (**Huik, 2013**). Asp299Gly, Thr399Ile the two variants which recognize lipopolysaccharide (LPS) is associated with increased infection risk in HIV patients (**Papadopoulos, 2010**). According to **Martinelli *et. al*.,** pDCs which normally secretes the IFN- γ and activates the natural killer cells also gets suppressed due to gp120 viral envelope produced by the HIV virus. It also inhibits the TLR9 mediated induction of proinflammatory cytokines in pDCs (**Martinelli, 2007**). Some of the polymorphic variation in the TLRs which related to disease progression or as a set point for the disease depends on the ethnicity among different populations of the world (**Mackelprang, 2014**).