Chapter 2 REVIEW OF LITERATURE

2.1 Tuberculosis

Tuberculosis is defined as a chronic communicable disease caused by *Mycobacterium tuberculosis*. Tuberculosis remains one of the main threats to mankind and is one of the world’s leading causes of death due to infection.

2.1.1 Historical Prospective

Tuberculosis has threatened the human survival even before the recorded history. An early progenitor of *M. tuberculosis* was probably contemporaneous and co-evolved with early hominids in East Africa, three million years ago. The modern members of *M. tuberculosis* complex seem to have originated from a common progenitor about 15,000 - 35,000 years ago (Gutierrez et al 2005). The disease was widespread in Egypt and Rome (Zink et al 2003, Donoghue et al 2004), it existed in America before Columbus (Salo et al 1994, Konomi et al 2002, Sotomayor et al 2004), and in Borneo before any European contact (Donoghue et al 2004). The TB epidemic in Europe, known as the Great White Plague, started at the beginning of the 17th century and continued for the next 200 years. Death from TB was considered inevitable and by 1650, TB was the leading cause of mortality. Tuberculosis was also known as phthisis in Greeks and tabes in Roman history.

TB was documented in Egypt, India and China as early as 5,000, 3,300, and 2,300 years ago, respectively (Daniel 2006). In India written proof about TB was found in Charak and Sushrut Sanhita (1000B.C.) and has been mentioned by the name “Rajyakshma”. Since then many references were written for TB in the ancient writings. Although there is evidence of the presence of the disease in pre-historic Asia, it was only towards the end of the 19th century that the incidences were observed in India and China.

2.1.2 Epidemiology of tuberculosis: World

Tuberculosis (TB) remains a major global health problem. The latest estimates indicates that there were 9.0 million new TB cases in 2013, globally, which shows a rate of 126 cases per 100,000 population. Out of 8.6 million incident cases, an estimated 0.5 million
were children and 2.9 million occurred among women. A total of 1.3 million TB deaths occurred in 2013, out of which, 1.0 million were HIV-negative people and 0.3 million were reportedly HIV-associated TB deaths. Most number of cases and deaths occur among men, but the burden of disease among women was also high. In 2013, there were an estimated 2.9 million cases and 410,000 TB deaths among women and an estimated 530,000 cases and 74,000 deaths among children. Approximately 75% of total TB deaths occurred in the African and South-East Asia regions. Most of the estimated number of cases are reported from Asia (56%) and the African Region (29%), while very low number of cases have so far been reported from Eastern Mediterranean, the Americas (3%). India and China alone accounted for 24% and 11% global cases, respectively. The five countries with the largest number of incident cases in 2013 were India (2.0 million–2.3 million), China (0.9 million–1.1 million), Nigeria (0.34 million–0.88 million), Pakistan (0.37 million–0.65 million) and Indonesia (0.41 million–0.52 million). The number of incident TB cases relative to population (the incidence rate) varies widely among countries, the lowest rates are found predominantly in high-income countries, including most countries in Western Europe, Canada, the United States of America, Japan, Australia and New Zealand. In these countries, the incidence rate per 100,000 population has been less than 10 cases in 2013 (WHO 2014).

The number of TB deaths per 100,000 population averaged 13 globally in 2013. There is considerable variation among countries ranging from under 1 TB death per 100,000 population (examples include most countries in Western Europe, Canada, the United States of America, Australia and New Zealand) to more than 40 deaths per 100,000 population in much of the African Region as well as three high burden countries in Asia (Bangladesh, Cambodia and Myanmar) (WHO 2014).
Figure 2.1- TB incidence rate globally in 2013. (Global Tuberculosis Report, WHO 2014)

Figure 2.2- TB mortality rate globally. (Global Tuberculosis Report, WHO 2014)
2.1.3 Epidemiology of tuberculosis: India

TB is one of the leading causes of mortality in India, killing 2 persons every three minute or nearly 1,000 every day (RNTCP 2013). India ranked first in reference to annual incidence rates of 2-2.4 million in the world. India and South Africa accounted for about one-third of global TB deaths (WHO 2013). In India, tuberculosis condition is alarming as 1.8 million tuberculosis cases occur annually, which is 1/5\textsuperscript{th} of the worlds new TB cases and 2/3\textsuperscript{rd} of the cases in the south-east Asia region. This makes India the highest TB burden country in the world. It is estimated that every 2 of 5 Indians are infected with the TB bacillus. There is a strong chance that out of them, at least, 10% will develop TB disease during their life time (RNTCP 2013).

2.1.4 Mycobacterium tuberculosis

*Mycobacterium tuberculosis* (*M.tb*) is a pathogenic bacterial species of the family Mycobacteriaceae and the causative agent of most cases of tuberculosis (TB). First discovered in 1882 by Robert Koch, *M.tb* emerged as a human pathogen in Africa around 70,000 years ago and then spread out of the continent following human migrations. It is now widely accepted that the ancients *M.tb* strains originated from environmental mycobacteria (smooth tubercle bacilli) (Gutierrez et al 2005, Hershberg et al 2008, Supply et al 2013).

With Ziehl-Neelsen stain *M.tuberculosis* looks rod shaped, straight or slightly curved with beaded or barred appearance. They are aerobic non-motile, non-sporic bacilli having a dimension of 2-3\textmu m X 0.2-0.4\textmu m, arranged singly or in groups. They are acid fast due to presence of mycolic acid in cell wall and are Gram-positive.
2.1.5 Transmission of tuberculosis

TB is an airborne disease and commonly affects the lungs, referred to as pulmonary TB disease. *M.tuberculosis* is carried in airborne particles, called droplet nuclei, of 1–5 microns in diameter. Infectious droplet nuclei are generated when persons who have pulmonary or laryngeal TB disease cough, sneeze, shout, or sing. Depending on the environment, these tiny particles can remain suspended in the air for several hours. *M. tuberculosis* is transmitted through the air, not by surface contact. Transmission occurs when a person inhales droplet nuclei containing *M.tuberculosis* and the droplet nuclei traverse the mouth or nasal passages, upper respiratory tract and bronchi to reach the alveoli of the lungs.

2.1.6 Pathogenesis of tuberculosis

*M.tb* infection occurs when few tubercle bacilli dispersed in the air from a patient with active pulmonary TB reach the alveoli of the host. Here, *M.tb* is quickly phagocytized by alveolar macrophages that most often can kill the entering bacteria (Urdahl et al 2011). If the bacilli can escape from this first line of defense, it starts actively replicating in macrophages, diffuse to nearby cells including epithelial and endothelial cells, reaching in few weeks of exponential growth a high bacterial burden (Wolf et al 2008). During these early steps of infection, *M.tb* can diffuse to other organs through the lymphatics and by haematogenous dissemination where it can infect other cells (Balasubramanian et al...
Thereafter, when the adaptive immune response come into action, migration of neutrophils, lymphocytes and other immune cells to the site of primary infection form a cellular infiltrate that later assumes the typical structure of a granuloma (Ottenhoff and Kaufmann 2013). Fibrotic components cover the granuloma that becomes calcified such that bacilli remain encapsulated inside and protected by the host immune response. This primary lesion, classically termed as the Ghon complex (Ghon 1923, Ober 1983), was thought to be the “sanctuary” of *M.tb* during latent infection, with bacilli persisting in a dormant, non-metabolically active state, for years, decades, or most often for lifetime. In this scenario, when, during latent infection, for unknown reasons, bacilli would start replicating inside this primary lesion, active disease would develop (Bishai 2000). During latent infection most bacilli persist in a dormant state with very less number of *M.tb* found in an active replicating state. These replicating bacilli, known as “scouts”, are processed and killed by the host immune defenses and as a result they are responsible for the induction of the large number of effector/memory T cells directed against *M.tb* antigens that are found in the peripheral blood (Chao and Rubin 2010).

As, during latent TB dormant bacteria constantly replenish the bulk of actively replicating bacilli readily killed by the host. When, for any reason, host immune responses fail to control these scouts, uncontrolled bacterial replication promotes diseases manifestations and active disease ensues (Gengenbacher and Kaufmann 2012).

![Figure 2.4](source.png)

Figure 2.4- Transmission of tuberculosis and model of latent and active tuberculosis.  
(Source: Gengenbacher and Kaufmann 2012)
2.2 Leprosy

Leprosy was the most feared infectious disease in the world. Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, an acid-fast, rod-shaped bacillus. The disease mainly affects the skin, the peripheral nerves, mucosa of the upper respiratory tract and also the eyes. Leprosy is also known as Hansen’s disease, after Gerhard Armauer Hansen, the Norwegian scientist who first discovered the bacillus in 1873 and identified it as the causative agent of leprosy.

2.2.1 Historical Prospective

Leprosy has effect on human since the known history. Term leprosy was originated from Greek word lepra. In Bible leprosy was described as the punishment of god for some transgression. At the time, many thought leprosy was a “curse of God”. Leprosy was recognized in the ancient civilizations of China, Egypt and India. The first known written mention of leprosy is dated 600 BC. Leprosy is mentioned as kushtha by the renowned Indian physician, Sushruta, in his book *Sushruta Samhita* as early as 600 B.C. As per hindu mythology lord Rama was also have kustha and take treatment of chaulmoogra oil, description about this also mentioned in Shustruta Samhita.

2.2.2 Epidemiology of leprosy: World

Although most of the countries of the world achieve the elimination goal of leprosy but some regions still show some increase in the new case detection rate. In 2012 both prevalence and new case detection rate observed to be highest as compared to the 2011. The prevalence of leprosy in 2012 was 1,81,941 (0.34), compared to 1,89,018 (0.33) at the end of the first quarter of 2013. Compared to the previous years, in 2012 an increase was observed in the number of new cases reported almost exclusively in the South-East Asia Region. Marginal increases in the number of new cases were also noted in the African and Western Pacific Regions (WHO 2013). While in 2013 decrease in new case detection was observed compared to 2012. The South-East Asia Region accounted for 72% of new cases detected worldwide, with 16% from Americas, 9% from the Africa Region and 2% each from the Eastern Mediterranean and Western Pacific Regions. Côte d’Ivoire, Democratic Republic of Congo, Ethiopia and Madagascar, reported more new cases in 2013 compared to previous year. The new cases reported from 14 countries
account for 96% of total new cases reported worldwide. 81% of all new leprosy cases in 2013 occurs in 3 countries, India, Brazil and Indonesia (Leprosy report WHO 2014).

### 2.2.3 Epidemiology of leprosy: India

As per WHO report 2014 India is one of the 16 countries who still reported more than 1000 cases during 2013. South East Asia region shows increase in the new case detection rate, India reported highest number of new leprosy cases followed by Brazil and Indonesia. India alone accounted for 58.85% of the global leprosy burden. As per NLEP 2014 report, 1.27 lakh new cases were detected during the year 2013-14, which gives Annual New Case Detection Rate (ANCDR) of 9.98 per 100,000 population. This shows decrease in ANCDR from 2011-12 which were 10.35 per 100,000. A total of 0.86 lakh cases are on record as on 1st April 2014, giving a Prevalence rate (PR) of 0.68 per 10,000 population. 33 States/ UTs had already achieved the level of elimination i.e. PR less than 1 case per 10,000 population. One State (Chhattisgarh) and One U.T. (Dadra & Nagar Haveli) has remained with PR between 2 and 4 per 10,000 population. Three States, Odisha, Chandigarh and Lakshadweep, which have achieved elimination goal earlier have shown slight increase in PR with 1-2 in the current year 2013-2014. Only 14 districts with ANCDR more than 50/100,000 population are in Chhattisgarh (2), Gujarat (4), Maharashtra (3) West Bengal (1), Dadra & Nagar Haveli (1) Orissa (2) and Delhi (1). The above data shows that we have controlled the situation but still a lot of work is to be done for complete eradication of leprosy from the world (NLEP 2014).

### 2.2.4 *M. leprae*

The etiologic agent of leprosy, *Mycobacterium leprae* bacillus, also known as Hansen’s bacillus, is an intracellular microorganism, ranges from 2 to 7 micrometers in length and 0.3 to 0.4 micrometers in width. The most natural and congenial environment for *M. leprae* is eukaryotic cell, which, in most cases, is found in humans, but occasionally may be found in the nine-banded armadillo, the mouse footpad and, to a lesser degree, the monkey. The leprosy bacillus is an obligate intracellular parasite. It is a slow growing bacterium that multiplies with a generation time of 12.5 days with its long incubation period, which is approximately three to five years. It grows best at 27–30°Celsius (80–
86°F Fahrenheit), hence, it has predilection for cooler areas of the human body, such as skin, peripheral nerves and the airway mucosa and top areas, like finger tips (Britton and Lockwood 2004, Britton 2010).

Figure 2.5- *M.leprae* in skin lesion and Structure of *M.leprae*
(Source- Leprosy,book by Alfica Sehgal, CH publication,USA)

### 2.2.5 Transmission of *M.leprae*

Exact mechanism of transmission of the leprosy is still not clear. Theories suggest that the possible way of transmission of leprosy is direct person to person contact or contact with the product of respiratory secretion from the infected person due to the presence of live *M.leprae* bacilli. There are evidences that shows that contact with the soil containing bacilli to be also responsible for transmission. It is observed that the bacillus may be kept viable up to 24 hours outside of the body, in nasal secretion or droplets, which supports this hypothesis.

### 2.2.6 Pathogenesis of leprosy

Pathogenesis of infection with *M.leprae* is not understood very well, but on the basis of studies, it is believed that clinical outcome of infection depends on the host immune response to the leprosy bacillus. *M.leprae* probably enters the body by way of the nose and cuts of the body and then spreads to the skin and nerves via circulation (Scollard
The onset of leprosy is insidious, it affects nerves, skin and eyes. It may also affect mucosa (mouth, nose, pharynx), testes, kidney, voluntary/smooth muscles, reticulo-endothelial system and vascular endothelium. After entering into host, three stages of pathogenecity could follow, which depends on the cell mediated immunity of the host. If CMI is present, then the bacteria are engulfed by the macrophages and other immune cells. In more than 90% cases of infection, this phenomenon happens and host never develops active disease. In the event of compromised CMI of the host, *M.leprea* start dividing inside the host macrophages and transported to various parts of the body and may result in severe form of the disease, known as lepromatous form. In other cases, where some patients have low CMI, not enough to completely destroy the pathogen, but can resist the infection and allows a limited, but variable, multiplication of the bacilli, develope the tuberculoid or paucibacilary (PB) form of the disease. This response keep the infection localized to a small area and the disease may be some what generalized, with leprosy lesions scattered in different places. This form of the disease falls in the “borderline” category. Borderline leprosy is immunologically unstable. Besides these, there are two groups based on immunological spectrum, ranging from those with a strong CMI at the tuberculoid end, to the more serious types, with a CMI deficit at the lepromatous or infectious end. There is another group, which is not considered as part of the spectrum. In its early stage, it is not known whether it will progress into the spectrum to become tuberculoid or lepromatous, so it is known as the indeterminate form of the disease (Scollard et al 2006).

Pathogens do not cause so much damage directly, rather than immunology of the host itself. Reactions developed due to host immune response against pathogen during treatment, can cause very serious damage to the host. Type 1 reaction or reversal reaction are generally suffered by borderline patients as a result of delayed type hypersensitivity. More than 30% of the patients of borderline leprosy are at the risk of developing reversal reactions during their treatment. Type 1 reaction leads to inflammation of skin and nerve. The skin lesions become acutely inflamed and oedematous and may ulcerate. These types of reactions finally leads to permanent nerve damage (Manandhar et al 1999). Type 2 reactions, also known as erythema nodosum leprosum (ENL), affect 50% of lepromatous and 10% of borderline lepromatous cases (Pocaterra et al 2006). Patients with ENL
reaction shows high infiltration in the skin and very high BI. These reactions are systematic disorder and affect many organs. These reactions produce fever, skin pain and tender red papules or nodules. Due to involvement of subcutaneous tissues, reaction causes tethering and fixation of joints which finally results in loss of function. ENL reactions may also produce uveitis, neuritis, arthritis, dactylitis, lymphadenitis and orchitis. The recurrent inflammation of organs leads to blindness and sterility (Scolard et al 2006).

![Diagram]

Figure 2.6- Classification of leprosy disease, based on CMI of host and AFB (Source-Davey 2012 from pharmacotherapy)

2.3 Immune system

Immunity is defined as defence system of the host evolved to protect the host from invading pathogenic microorganisms. All multicellular organisms including plants posses their own intrinsic mechanisms of resistance against microbial infection. The immune system consists of cells, tissues and molecules, capable of specifically recognizing and eliminating limitless variety of foreign invaders. Immune response has two related activities, at first step it recognizes the forigen molecule and second, it respond against the molecule by recruiting variety of cells and molecules to mount appropriate action, known as effector response. Second and changed exposer to the same molecule induce memory response, which is rapid and hightened immune reaction that serves to eliminate the pathogen and prevent disease.
2.3.1 Innate Immunity

Innate immunity is known as the first line of defense against infection. It is also known as natural or native immune response. The components of the system are present before the exposure to the infection. It consists of a specific pattern of disease resistance mechanism that are common to all foreign invaders. Innate immunity of the host consists of anatomic, physiological, phagocytic and inflammatory barriers. The components of innate immunity recognize structures shared by a variety of microbes. Innate immune system consist of pattern recognition receptors (PRRs), phagocytic cells, like neutrophils, macrophages, NK cells and complement system. Several circulating proteins, like mannose binding lectin (MBL) and C reactive proteins (CRP) plays a very important role as innate immunity component against the infection.

2.3.2 Adaptive Immunity

Adaptive immunity, also called specific or acquired immunity, is capable of recognizing and selectively eliminate specific foreign microorganisms and molecules. Adaptive immune response is not similar to all the members of a species but are reactions to specific antigenic challenges. Adaptive immune system have four main characteristic, the antigenic specificity, diversity, immunological memory and self nonself recognization. Adaptive immune system works through the cooperation of two types of cells, one is lymphocytes and the other is antigen presenting cell. Two major groups of cells are B lymphocytes and T lymphocytes. T cells are the central component of cell mediated immunity, they are also divided in two broad categories, T helper and T cytotoxic cells. They are distinguished by the presence of CD4 or CD8 membrane glycoproteins on their surfaces. B Cells are the major cells involved in the creation of antibodies that circulate in blood plasma and lymph, known as humoral immunity.

2.3.3 Host immune response and tuberculosis

Epidemiological data indicates that protective immunity against *M.tb* exists in most exposed humans. The proportion of non immuno compromised individuals who undergo primary infection or reactivation disease, following *M.tb* infection, is low. Only 5% to 10% of *M.tb* infected individuals develop tuberculosis (TB) disease during their lifetime (Bates 1980, Comstok et al 1974). Development of *M.tb* infection to primary, latent or
active tuberculosis form depends on the complex mutual interplay of host and pathogen and efficiency of protective human immunity against M.tb.

2.3.4 Innate immunity against M.tuberculosis

Upon entry into the host lungs by aerosol inhalation, M.tuberculosis interacts with various receptors, like pattern recognition receptors (PRRs), such as toll-like receptors (TLR), (Trinchieri and Sher 2007), complement receptor-3 (Schlesinger et al 1990), mannose receptor, scavenger receptor (Ernst 1998), DC-specific intercellular-adhesion-molecule-3-grabbing nonintegrin on the surface of macrophages and DC. Lung surfactant protein D binds to M.tuberculosis surface lipoarabinomannan and limits the intracellular growth of M.tuberculosis by increasing phagosome lysosome fusion (Ferguson et al 2006). In addition, cytosolic nucleotide binding and oligomerization domain-like receptors, such as NOD2 that recognizes muramyl dipeptide and also C-type lectin dectin-1 that interacts with M.tuberculosis, cooperate with TLR-2 to activate NF-kB and mediate pro-inflammatory cytokine and antimicrobial responses against tuberculosis (Divangahi et al 2008). TLRs and NF-kB pathway activation promotes nuclear translocation of NF-kB, which leads to (i) production and secretion of many proinflammatory mediators, including cytokines TNF-α, IL-1, IL-12, IL-18 and chemokines, which attract neutrophils, natural killer (NK) cells, T cells, and more DC and macrophages to the infection site (Korbel et al 2008, Liu and Modlin 2008) and nitric oxide. It is of interest to note that M.tuberculosis secreted protein ESAT-6 inhibits the activation of NF-kB by preventing interaction between MyD88 and downstream kinase IRAK4 (Pathak et al 2007) and (ii) Upregulation of the expression of vitamin D receptor (VDR) and the vitamin D-1-hydroxylase genes, which converts pro-vitamin D into the active form 1,25(OH)2D3 and leads to induction of the antimicrobial peptides cathelicidin and β-defensin to kill intracellular mycobacteria (Liu et al 2006, Liu et al 2007, Chocano-Bedoya and Ronnenberg 2009).
Figure 2.7- Innate immune response against M.tb (Source- Dheda et al 2010)

NADPH oxidase 2, which mediates phagocytic killing of ingested pathogens, like M. tuberculosis, via reactive oxygen species, interacts with TLR2 and affects VDR mediated antimicrobial peptide production (Yang et al 2009). The DC specific intercellular-adhesion molecule-3 grabbing non-integrin signal pathway activation leads to production of IL-10 and transforming growth factor-β (TGF-β), which suppress the immune response (Korbel et al 2008). Beside these receptors, several cells have direct role against pathogen. Neutrophils are thought to contribute to the control of infection through production of chemokines (Riedel and Kaufmann 1997), the induction of granuloma formation (Ehlers et al 2003) and transfer of their own microbicidal molecules to infected macrophages (Tan et al 2006). The secretory proteins, M. tuberculosis secreted antigen (MTSA-10) and 6 kDa early secretory antigenic target (ESAT-6), activate macrophages, dendritic cells and mast cells for the liberation of pro-inflammatory mediators (Munoz et al 2003, Trajkovic 2004).

Macrophages are the paradigmatic cell with regard to M. tuberculosis infection. Alveolar resident macrophages are the primary cell type involved in the initial uptake of M.tb. After that, dendritic cells and monocytes derived macrophages take part in the phagocytic process. Nitric oxide is the primary product of inducible nitric oxide synthase (iNOS, NOS2 isoform) expressed by monocytes and macrophages, which can kill M.tb at low
concentrations. The relevance of nitric oxide in human protective immunity against *M.tb* has accumulated slowly. Nitric oxide is released from *M.tb* infected blood monocytes (Sharma et al 2004, Bose et al 1999), Alveolar macrophages (Nicholson et al 1996, Rich et al 1997, Carranza et al 2005) and respiratory epithelial cells (Roy et al 2004, Kwon et al 1999) and is increased in exhaled air droplets of TB patients (Wang et al 1998). Macrophages are heterogeneous and have different roles during *M.tb* infection. IL-23 producing type1 macrophages promote Th1 immunity against *M.tb* and IL-10 producing type2 macrophages suppress immunity to *M.tb*. The type2 macrophages have been shown to induce CD4+ T cells to adopt a Treg CD25+FoxP3+TGF-β1+ suppressor phenotype (Savage et al 2008).

Among others, the 19kDa lipoprotein and lipoarabinomanann (LAM) activate macrophages through TLR2, promoting the production of IL-12 and inducible nitric oxide synthase (iNOS) (Brightbill et al 1999). The cellular cholesterol present in the macrophage cell membrane is an essential molecule for the internalization of the bacteria (Gatfield and Pieters 2000).

The internalization of *M.tb* into human and murine dendritic cells has been observed in several *in vitro* (Fortsch et al 2000, Giacomini et al 2001, Hanekom et al 2003,) and *in vivo* (Jiao et al 2002, Pedroza- González et al 2004, García-Romoet al 2004) studies. In a protective immune response, dendritic cells induce maturation of T cells towards a Th1 profile by secreting cytokines, such as IL-12, IL-18, IL-23, and probably IFN-α and β, but not IFN-γ (Wozniak et al 2006, Kadowaki et al 2001, Kalinski et al 1999). Th1 cells expand in response to the BCG antigens presented by the dendritic cells in the lymphoid nodules and migrate towards infection sites, such as lung tissue, where they liberate IFN-γ and activate local macrophages that control bacilli replication (Humphreys et al 2006). Natural killer cells play a very important role in the development of innate immune response. Human natural killer cells have been shown to have an enhanced cytotoxicity for macrophages infected with *M.tuberculosis*. They also optimize the ability of CD8+ T lymphocytes to produce IFN-γ and to lyse *M. tuberculosis* infected cells, thus, connects innate to adaptive immune responses (Vankayalapati et al 2002, 2004). Epithelial cells are able to establish an initial pro-inflammatory environment by secreting IL-8 (Wickremashinge et al 1999) and inducing the production of nitric oxide (NO) (Roy et al
Neutrophils have been reported to have four known human neutrophil defensins peptides (Ganz et al 1990), of which three (HNP-1, HNP-2 and HNP-3) were found to be active against *M. avium* and *M. tuberculosis* (Ogata et al 1992, Miyakawa et al 1996). Human alveolar epithelial cells infected with *M. tb* were also found to express human beta-defensin, HBD-2 (Rivas-Santiago et al 2005). It is clear that several pathways and cell types interact together to mediate innate immunity against *M. tuberculosis*. Any defect in these pathways and non-functioning of any cell type may be a possible mechanism, due to which many individuals selectively develop active TB disease.

### 2.3.5 Acquired immune response against *M. tuberculosis*

There is general consensus that cell-mediated immunity (CMI) is the primary host defence mechanism against intracellular pathogens such as *M. tb*. CD4+, CD8+, and γδT lymphocytes play an important role in the protection from *M. tb* (Kaufmann 2001). *M. tb* infected macrophages and dendritic cells of the innate immunity present antigens to T cells and B cells to MHC class CD1 restricted CD4 T cells and MHC class CD2 restricted CD8 T cells that recognize peptide antigens and the γδ T lymphocytes as well as the CD1-restricted specific T lymphocytes produce IFN-γ and constitute the protective immunity. The CD4+ Th1 cells mount a much stronger IFN-γ response than CD8+ T cells after mycobacterial infection (Nagai et al 2007) and are thought to play a prominent role in protection (Kaufmann and Michael 2005). The CD8+ cytolytic T lymphocytes (CTL) secrete granulysin, granzymes and perforins to kill mycobacteria-infected cells and are capable of immune protection against secondary mycobacterial challenge in the absence of CD4+ T cells (Nagai 2007). The CD4+ T helper cells can be differentiated into Th1, Th2, Th17 and T-regulatory cells. The Th1 cells produce cytokines, notably IFN-γ, TNF-α, IL-2, lymphotoxin and granulocyte-macrophage colony-stimulating factor (GM-CSF), which promote the stimulation of Th1 cells, CTL, maturation and activation of macrophages as well as granulocytes. The Th2 cells produce B cell-stimulation factors, such as IL-4, IL-5, IL-10 and IL-13, which promote antibody production, but suppress the Th1 type immune response. The Th17 cells, a distinct subset of helper T cells, produce unique cytokines of IL-17, IL-21 and IL-22, which stimulate defensin production and recruit neutrophils and monocytes to the site of inflammation and are
involved in the early phase of host defence. The CD4+, CD25+, FoxP3+, natural Treg cells are characterized by TGF-β and IL-10 production (Kaufmann and Parida 2008, Dorhoi and Kaufmann 2009), while Treg cells also co-produce IFN-γ (Trinchieri 2001). In addition to CD4+ Treg cell subsets, CD8+ Treg cells are also described, which could inhibit T cell proliferation and cytokine production (Joosten and Ottenhoff 2008). The FoxP3 expressing Treg cells are expanded during TB infection (Scott-Browne et al 2007) and inhibit human memory T cells to produce IFN-γ in response to M.tb antigens (Li and Wu 2008). CD4+, CD25+ FoxP3+ Treg cells also produce TGF-β to down regulate CD4+ T cell response and suppress the effector-immune response and induce bacillary dissemination and disease manifestation (Sharma et al 2009, Rahman et al 2009). High percentage of Treg cells characterized as CD4+CD25highCD39+ was also identified in active TB patients (Chiacchio et al 2009). Clearly, T cells play a central role in host defence and mucosal immunity against *M.tuberculosis*.

Figure 2.8- Immune response against *M.tuberculosis* (Source-Tuberculosis, by Reichman Hershfield, Informa Healthcare, USA).
2.3.6 Immune response against leprosy

The immunological response mounted by the host dictates the clinical phenotype of the host that develops. People with leprosy show a spectrum of clinical types. In case of *M. leprae*, this appears to take place mainly through TLR2/TLR1 heterodimer and leads to monocyte differentiation into macrophages and dendritic cells (Krutzik et al 2003, Krutzik et al 2005). The differentiated dendritic cells present antigen and leads to the activation of naive T cells by IL-12 secretion. The IL-12βR2 portion of the IL-12 receptor is expressed more on Th1 lymphocytes, preferentially shifting the immune response further towards a Th1 response. CD4+ cells are found mainly within the granuloma and CD8+ cells in the mantle area surrounding it (Modlin et al 1988). T cells in tuberculoid granulomas produce the antimicrobial protein granulysin (Ochoa et al 2001). Lepromatous disease is characterized by poor granuloma formation. In lepromatous disease mRNA production is predominantly for cytokines IL-4, IL-5 and IL-10 (Yamamura et al 1991). IL-4 has been shown to downregulate TLR2 on monocytes
(Brightbill et al 1999) and IL-10 suppress the production of IL-12. This is associated with a preponderance of CD8+ lymphocytes in lepromatous lesions (Libraty et al 1997). Johnson et al. reasoned that the TLR response may be critical during acute infection, but a moderation of the innate response may be beneficial in chronic infectious diseases, such as leprosy (Johnson et al 2007). Consistent with this hypothesis is the finding that TLR2/1 activation can lead to tissue injury, including nerve damage in leprosy (Oliveira et al 2003). TLR2 and TLR1 were more strongly expressed in lesions from the localized tuberculoid leprosy form as compared to the disseminated lepromatous leprosy form of the disease. The ability of *M. leprae* to upregulate tryptophan aspartate containing coat protein (TACO), known to be expressed in macrophages containing *M. leprae* in vitro and in disease lesions (Suzuki et al 2006), was shown to down regulate TLR2-mediated signalling (Tanigawa et al 2009). Investigations showed that plasmacytoid dendritic cells are not involved in the immune response against *M. leprae*, whereas, FoxP3 positive cells were present in 95% of the cases in a retrospective immunohistochemical study with an average density of 2.9% of the infiltrate. Their distribution was not related to granulomatous structures or special locations (Massone et al 2010).

![Figure 2.10- Metabolic pathways of immune response against leprosy.](Source-Goulart and Goulart 2008).
2.3.7 Immunology of leprosy reactions

Type 1 reactions are delayed hypersensitivity reactions that occur in borderline disease. *M. leprae* antigens have been demonstrated in the nerves and skin of patients experiencing type 1 reactions. The antigens were localized to schwann cells and macrophages (Lockwood et al 2002). Schwann cells have been shown to express TLR2 receptors (Oliveira et al 2003). *M. leprae* infection may lead to the expression of major histocompatibility complex (MHC) II on the surface of the cells and this may give rise to antigen presentation, which triggers CD4 lymphocyte killing of the cell (Ochoa et al 2001). Immunohistochemistry studies show higher TNF-α staining in the skin and nerves during type 1 reaction compared with non-reactional controls (Khanolkar-Young et al 1995). There is a shift towards increased Th1 immunity and lesions in reaction, expressing pro-inflammatory cytokines IFN-γ, IL-12 and the oxygen free radical producer inducible nitric oxide synthase (Little et al 2001). The expression of mRNA of various chemokines including IL-8, monocyte chemoattractant protein-1 and regulated upon activation normal T-cell expressed and secreted (RANTES) is higher in the skin during reaction (Kirkaldy et al 2003). Treatment of the reaction causes clinical improvement, but changes in the inflammatory cytokines lags behind by some considerable time and in some, may remain unchanged (Anderson et al 2005). Type 2 or erythema nodosum leprosum (ENL) reactions occur in borderline lepromatous and lepromatous disease. High levels of circulating TNF have been demonstrated in the plasma of some individuals with type 2 reactions (Sarno et al 1991). *In vitro* peripheral blood mononuclear cells from individuals with ENL reactions secrete increased amounts of TNF following stimulation with *M. leprae* (Barnes et al 1992). The use of thalidomide and pentoxifylline have been shown to reduce the levels of TNF *in vivo* in subjects whose ENL has shown clinical improvement (Sampaio et al 1998, Moreira et al 1998). Haslett et al. 2005 has demonstrated low TNF levels in individuals with milder ENL reactions and found that the levels increased during therapy with thalidomide (Haslett et al 2005). This effect has been noted in toxic epidermal necrolysis as well as other diseases (Wolkenstein et al 1998). Type 2 reactions with systemic involvement may produce the high circulating TNF levels previously seen and that this may not be the case in milder
forms of the condition. The balance and complex interaction of cytokines, chemokines, adhesion molecules, their receptors and the cells of the innate and adaptive immune system, all play a role in ultimately determining the particular immune response of the individual to the organism.

2.4 Cytokines

Cytokines are small, secreted polypeptides that regulate essentially all functions of the immune system. They are known as messengers of the immune system that contribute to inflammatory responses through activation of the host’s immune cells. Cytokines are potent, low-molecular-weight protein, cell regulators, produced transiently and locally by numerous cell types. Now cytokines are recognized as multifunctional proteins whose biological properties suggest a key role in hematopoiesis, immunity, infectious disease, tumorigenesis, homeostasis, tissue repair, cellular development, and growth (Thomson and Lotze 2003, Oppenheim et al 2001). Cytokines participate in determining the nature of the immune response by regulating or controlling cell growth, differentiation, activation, immune cell trafficking and location of immune cells within the lymphoid organs (Gouwy et al 2005). Cytokines are host-derived products that enhance the recruitment of circulating leukocytes as a response to the presence of pathogens. Chemokines and cytokines provide a complex network of signals that can either activate or suppress inflammatory responses (Borish and Steinke 2003).

2.4.1 Cytokines against tuberculosis

Recognition of *M. tuberculosis* by phagocytic cells leads to cell activation and production of cytokines, which in itself induces further activation and cytokine production in a complex process of regulation and cross-regulation. This cytokine network plays a crucial role in the inflammatory response and the outcome of mycobacterial infections (van Crevel et al 2002). Upon infection, macrophages are activated to produce proinflammatory cytokines, including TNF, IL-1, IL-6, IL-12, and IL-18 and the regulatory IL-10. Chemokines relevant to *M. tuberculosis* infection include IL-8 (CXCL8), monocyte chemoattractant protein 1 (MCP-1, CCL2), RANTES (CCL5) and CXCL10 (IP-10) (Azad et al 2012). The efficient induction of Th1 immunity is decisive for the defence against *M.tb*. The classical cytokines produced in response to *M.tb* infection are IL-2,
IFN-γ, IL-6, IL-1α/β, IL-12, and TNF-α (Fenhalls et al. 2002, Herrera et al. 2009, Kellar et al. 2011, Unsal et al. 2005, Guler et al. 2011, Zhang and Rom 1993). The function of IFN-γ in response to pathogens has been extensively studied and is critical in the regulation of T cell responses in mycobacterial disease (Cooper 2009). IFN-γ is produced by activated T cells, NK cells and macrophages and it is essential for the activation of phagocytes and antigen presentation. It also promotes cellular proliferation, cell adhesion and apoptosis. In macrophages, IFN-γ induces respiratory burst contributing to the production of RNIs and ROIs (Cooper et al. 2002). Activated macrophages produce immunomodulatory and chemotactic molecules that promote upregulation of TNF-α receptor and NRAMP-1. The production of large amounts of ROIs and NO by innate immune cells is considered one of the most important effects of IFN-γ. The IFN-γ also augments antigen presentation, leading to recruitment of CD4+ T cells and/or cytotoxic CD8+ T cells, which participate in mycobacterial killing and also prevents exhaustion of memory T cells (Chan and Flynn 2004, Cooper 2009). IFN-γ induces the transcription of more than 200 genes in macrophages including upregulation of MHC class II expression and the production of antimicrobial effectors, such as oxygen radicals and nitric oxide. A major effector mechanism responsible for the antimicrobial activity of IFN-γ, in association with TNF-α is the induction of the production of nitric oxide and other reactive nitrogen intermediates (RNI) by macrophages via iNOS (Russel et al. 2009, Scanga et al. 2001). TNF-α, produced by macrophages, dendritic cells and T-cells is another cytokine that has a major protective role against M.tuberculosis infection, both in mice and humans (Bean et al. 1999, Keane 2005). TNF-α also contributes significantly to the development of immunopathology associated with TB (Flynn and Chan 2005). The TNF-α produced by the infected macrophages induces the expression of chemokines, such as IL-8, MCP-1 and RANTES, which provide signals for migration of immune cells to the sites of M.tuberculosis infection (Algood et al. 2003). TNF-α also initiates cell migration and formation of microbicidal granulomas, while disruption of TNF-α responses leads to overgrowth of the mycobacterial pathogens (Chan and Flynn 2004, Cooper 2009). Both T cell and macrophage derived TNF-α are required for sufficient and long term protection against M.tuberculosis infection (Saunders et al. 2005). IL-12p70 is the critical factor that drives the generation of IFNγ producing T cells that are thought to
be essential for bacterial control (Cooper et al 2007). Indeed, deficiencies in the IL-12 or IFNγ signaling pathways have been associated with human susceptibility to tuberculosis (Filipe-Santos et al 2006). The importance of IL-12 is also evident from increased susceptibility of mice and humans deficient in IL-12 responses to mycobacterial infections (Jouanguy et al 1999). Individuals with defects in the production of IL-12 or its receptor are highly susceptible to active TB disease (Lichtenauer et al 2003). IL-23 and IL-27 appear to play mostly a regulatory role during mycobacterial infections. During \textit{M. tuberculosis} infection in the absence of IL-27 signaling, T cells express less IFNγ on a per cell basis (Pearl et al 2004). IL-6 is produced in response to \textit{M.tb} in early phases of infection. The absence of this cytokine, in the low dose of \textit{M.tb} mice model infection, promotes a delayed IFN-γ response in the lung and a slight increase in the \textit{M.tb} burden (Saunders et al 2000, Appelberg et al 1994). The evidence supports the idea that IL-6 is critical in the modulation and maintenance of the IL-17-producing cells in response to \textit{M.tb} infection in mice (Jones et al 2010).

\textit{M.tuberculosis} is a strong inducer of both IL-1α and IL-1β at the site of infection (Mayer-Barber et al 2010, Mayer-Barber et al 2011). Inflammatory monocytes/macrophages and DCs are the major sources of both IL-1α and IL-1β in the lungs of \textit{M.tb} infected mice (Mayer-Barber et al 2011). Mice deficient in IL-18 had lower IFNγ responses when compared to wild-type mice, despite this, the control of \textit{M.tuberculosis} bacterial burden was only modestly impaired in the absence of this cytokine (Sugawara et al 1999, Kinjo et al 2002). Mice deficient in IL-18 were extremely and acutely susceptible to aerosol infection with \textit{M.tuberculosis} to a similar extent as MyD88 and IL-1β deficient mice (Schneider et al 2010). IL-10 has been shown to modulate the activity of phagocytes in the lung by negatively impacting their ability to secrete TNF and IL-12p40 (Turner et al 2002, Beamer et al 2008) and by blocking the maturation of the phagosome (O’Leary et al 2010). IL-10 can have a negative impact in the recruitment of T cells into the lung by inhibiting the expression of T cell recruiting chemokines. IL-10 may also act to prevent strong activation of T cells thereby ensuring their survival and possibly limiting immunopathological consequences (Redford et al 2010). IL-2, IL-6 and IL-9 were only significantly increased in plasma of active TB patients and the two factors were consistently highly secreted after \textit{M.tb} antigen stimulation (Yu et al 2012).
2.4.2 Cytokines against leprosy

Predominance of IL-2, TNF and IFN transcripts in tuberculoid lesions and IL-4 and IFN in lepromatous ones, gene expression profiles consistent with Th-1 and Th-2 patterns, respectively (Arnoldi et al 1990, Flad et al 1990, Mutis et al 1993, Sieling and Modlin 1994, Yamamura et al 1991). CD4 clones isolated from lesions of TT patients secreted primarily IFN, whereas, a CD4 clone from a lesion of LL patients produced predominantly IL-4 (Sieling et al 1994) and CD8 clones, isolated from LL patients, likewise, generate large amounts of IL-4 (Salgame et al 1991). Further studies have also indicated that IL-12 and IL-18 promote resistance to *M. leprae* and are highly expressed in tuberculoid lesions (Garcia et al 1999, Sieling and Modlin 1994). Studies have also indicated that IL-12 and IL-18 promote resistance to *M. leprae* and are highly expressed in tuberculoid lesions (Garcia et al 1999, Sieling et al 1994). Circulating leukocytes and T cell lines from tuberculoid patients stimulated by *M. leprae* in vitro have generally been found to produce a Th1 cytokine pattern, while, leukocytes and T cell lines from lepromatous patients generally produce a Th2 cytokine pattern (Misra et al 1995, Nath et al 2000). However, leukocytes from approximately 40% of all patients produced a mixed Th0 cytokine profile of IFN, IL2 and IL4 (Misra et al 1995). Fractionated *M. leprae* antigens have also been found to stimulate IFN in vitro with leukocytes from tuberculoid patients (Dockrell et al 1996). Short and long-term intradermal administration of IFN resulted in an influx of mononuclear cells and an increase in CD4/CD8 ratio in the lesions. In lepromatous patients, intradermal injections of IL2, generated apparent increases in cell-mediated immunity within the skin lesions (Kaplan et al 1989) and resulted in increased levels of antibodies to *M. leprae* antigens (Kaplan et al 1991). TNF levels were highly variable with a tendency to be lower in LL patients than BT and BL patients. TNF-α were significantly low in controls compared to patients (Jadhav et al 2011). IFN-γ production induced by recombinant proteins was not significantly different between the three different groups in Bangladeshi population (TT/BT, HHC and EC). IFN-γ cannot be used as a single biomarker to discriminate between leprosy patients (TT/BT) and those merely exposed to *M. leprae* (EC). In striking contrast to IFN-γ, the concentrations of IL-1β, macrophage inflammatory protein-1β (MIP-1β or CCL4) and monocyte chemotactic protein-1 (MCP-1 or CCL2) were significantly enhanced in
TT/BT patients after stimulation with *M. leprae* WCS compared to Bangladeshi exposed contacts. AUC (areas under the curve) ranging from 0.89 (IL-1β) to 0.94 (MIP-1β) indicating good to excellent discrimination between the TT/BT and EC groups in Bangladesh. Combination of the three biomarkers enhanced this diagnostic ability even more as evident from the AUC value (Geluk et al 2012). The healthy contacts showed a significant increase in the expression of IL-17 isoforms as compared to the patient groups. Detection of clinical disease was associated with lowered IL-17 expression, IL-17 release was also influenced by the leprosy spectrum was indicated by BT patients who showed significant increase in expression of IL-17 isoforms, IL-17A, IL-17C, IL-17D and IL-17F, as compared to the multibacillary LL patients. Healthy contacts showed higher expression as compared to BT and LL. Moreover, within the leprosy groups, IL-21 and IL-22 were significantly higher in BT patients as compared to LL patients. Skin biopsies did not reveal differences in the leprosy types and only IL-21 showed significantly higher expression in dermal lesions of BT cases as compared to normal skin samples. It is of interest that BT skin lesions showed statistically significant increase in the expression of IL-1β as compared to normal skin. IL-23A showed significant increase in healthy contacts and BT groups, as compared to LL patients. IL-27 and IL-2 have been reported to negatively regulate Th17 cells paradoxically, the expression of these cytokines was also higher in healthy contacts as compared to leprosy types, but did not show differences within the leprosy groups even though expression was lower in LL (Laurence et al 2007, Fitzgerald et al 2013, Saini et al 2013). Human monocytes stimulated with PGL elicited very low levels of the proinflammatory cytokines TNF-α, IL-1β and IL-10. The levels of IFN-γ in these cell culture supernatants were below the limit of detection in both, test and the control wells. PGL-1 elicited negative regulatory molecules MCP-1 and IL-1 at levels comparable to those induced by LPS (Manca et al 2012). TNF-α was detected in 78% of skin biopsies, iNOS in 78% and TGF-β in 94%. All three molecules were detected at higher levels in patients with BT leprosy. TNF-α was localised within macrophages and epithelioid cells in the granuloma, in the epidermis and in dermal nerves in a few cases. TNF-α, iNOS and TGF-β were all significantly associated with type 1 reaction. The three cytokines TNF-α, iNOS and TGF-β detected by immunohistochemistry showed significant association with the presence of
skin reaction (Lockwood et al 2011). IL-10 and IL-15 induced monocytes to express CD209 receptor, IL-10 also induced coexpression of CD163, the hemoglobin scavenger receptor in leprosy patients (Kristiansen et al. 2001). IL-15 induced higher expression of the costimulatory molecule CD40, while both CD209 cell populations expressed monocyte specific markers CD14, CD16 (FcγRIII) and CD64 (Montoya et al 2009). Interleukin-4 is highly expressed in skin lesions of multibacillary patients, induce the expression of CD209 on human schwann cells and subsequently helped in schwann cell binding to *M.leprae*, whereas Th1 cytokines did not induce CD209 expression on these cells (Teles et al 2010). *M.leprae* stimulated cells produced almost 20 times more IgM in the presence of IL-5, whereas, there was no significant difference in IgA or IgG production. A role for IL-5 has been demonstrated to stimulate IgM production from B cells against *M.leprae* (Ochoa et al 2010). Expression of CCL2, CCL3, CCL7, IL1β, IL6 and IL8 mRNA were significantly down-regulated in *M.leprae* infected THP-1 cells when compared to control. A borderline decrease was observed for CCL7, TNF and IL6. Expression of CCL2, CCL3 and CCL4 estimated in nerve biopsies of the patients, showed down-regulation, while IL1β and SOD2 were borderline significant, confirming a repression of these genes by *M.leprae* ex vivo, as was observed in vitro in THP-1 cell line. Expression of other cytokine genes IL10, IL12 were also less expressed in leprosy patients when compared to healthy contacts (Guerreiro et al 2013). The difference of CXCL10 median plasma concentration among type 1 reaction patients and their matched controls was highly significant. IL6 also showed statistically significant differences between type1 reaction patients and matched controls. The higher levels of CXCL10 detected in type1 reaction patients suggests its involvement in the type1 reaction immunopathology probably by attracting Th1 type cells to the reactional inflammatory sites in the skin (Stefani et al 2009). Higher levels of TNF-α and IFN-γ were found in PB patients, whereas, higher levels of IL-1β and IL-10 were found in MB cases. No significant difference was noted among the TNF-α, IFN-γ, IL-1β and IL-10 cytokine levels between cases of TT & BT leprosy and between BL & LL cases. The bacillary index (BI) showed a positive correlation with the levels of TNF-α and IFN-γ and a negative correlation with the levels of IL-1β and IL-10. The studied cytokines TNF-α,
IFN-γ, IL-1β and IL-1 were raised in reactional cases as compared to non-reactional cases (Madan et al 2011).

2.5 Chemokines

Chemokines are a large family of small cytokines. These are small molecular mass chemotactic cytokines of molecular weight 8 to 14 kDa that are rich in basic amino acids and contain conserved cysteine motifs forming essential disulfide bonds between the first and third and the second and fourth cysteine residue. They are single polypeptides ranging from 70 to 100 amino acids in length and share varying degrees (20%-95%) of amino acid sequence identity that mediate constitutive recruitment of leukocytes from the blood into tissues. Since the identification of the chemokines CXCL8 (IL-8) and CCL2 (MCP-1) in the late 1980s, the chemokine superfamily has expanded rapidly (Rollins 1997, Yoshie et al 2001, Zlotnik and Yoshie 2000, Zlotnik et al. 2006). An initial wave of chemokine discovery occurred in the early 1990s, when some chemokines that attracted neutrophils and monocytes were discovered. Their identification was facilitated by the abundance of their transcripts in activated cells that participate in inflammatory responses. Their receptors were soon identified and were found to be a subgroup of a G protein-coupled receptors (GPCRs) (Vassilatis et al. 2003, Yoshie et al 2001). The chemokines were originally described as proinflammatory cytokines, however, recent studies indicate that their biological activities reach beyond that category. Chemokines also play critical roles in development (Raz and Mahabaleshwar 2009) and homeostasis where they guide cells during immune surveillance for pathogens by interacting with antigen presenting cells residing in these tissues. Some chemokines have roles in promoting angiogenesis or guide cells to tissues that provide specific signals critical for cellular maturation. Other chemokines are inflammatory and they function mainly as chemo attractants for leukocytes from the blood to sites of infection or tissue damage (Mantovani 1999, Zlotnik and Yoshie 2000, Moser et al. 2004, Zlotnik et al. 2011, Zlotnik & Yoshie 2012). Five subfamilies of chemokines, CXC, CC, XC, CX3C, and CX, have been recognized on the basis of the arrangement of the two N-terminal residues of four conserved cysteines. One and three amino acids separate the first and second cysteines in the CXC and CX3C chemokines, respectively, whereas the two cysteines are
adjacent to each other in the CC subfamily. The XC (or C) subfamily lacks the first and paired third cysteine residues. The fifth subfamily, CX, which has so far been identified only in zebrafish, lacks one of the two N-terminal cysteine residues but retains the third and fourth (Nomiyama et al. 2008), however, there is no evidence that this latter kind of chemokine exists in mammals. Chemokines can also be functionally classified into several groups, based on their mode of expression and function (Zlotnik and Yoshie 2000, Moser et al. 2004, Mantovani et al. 2006).

![Diagram of chemokine receptors and ligands]

Figure 2.11- Types of chemokines and their receptors
(Source- Hisayuki Nomiyama et al 2013)

In the last several years, there has been substantial progress in the field of chemokine research, and there is growing evidence that chemokines play an important role in the organization of the immune system (Baggiolini et al 1997). Chemokines play essential roles in both innate and adaptive immunity (Yoshie et al 2001, Luster 2002, Coelho et al 2005). Chemokines of the CC family attract and activate lymphocytes, monocytes/macrophages, basophils, eosinophils, dendritic cells and NK cells, whereas CXC chemokines mainly attract and activate neutrophils, but some also activate NK cells and T cells (Zlotnik and Yoshie 2000). The CC chemokine subfamily, has general chemotactic activity for mononuclear cells (Roth et al 1995, Taub et al 1993), but also induce activation and proliferation of T cells (Taub et al 1996) and of macrophages (Lima et al 1997, Fahey et al 1992). MIP-1α promotes Th1 cell differentiation (Karpus
These CC chemokines, play significant roles in granuloma formation (Chensue et al 1999). The ability of CC chemokines to attract and activate T cells and monocytes suggests that chemokines may have a role in modulating immune responses to bacterial infection (Mendez-Samperio 2008).

### 2.5.1 Monocyte chemoattractant protein-1/CCL2

The monocyte chemoattractant protein-1 (MCP-1/CCL2) is a member of the C-C chemokine family and a potent chemotactic factor for monocytes (Cochran et al 1983). CCL2 is the first discovered human CC chemokine. The encoding gene of CCL2 is located on chromosome 17 (Chr.17, q11.2), human MCP-1 is composed of 76 amino acids and is 13 kDa in size. The sequence homology between CCL2 and other family members is high and varies between 61% for CCL8 and CCL4 and 71% for CCL7 (Van Coillie et al 1999). CCL2 is produced by a variety of cell types, either constitutively or after induction by oxidative stress, cytokines, or growth factors. Analysis of CCL2 has resulted in the identification of two regions of the primary structure that are critical for biological activity (Beall et al 1996). The first region consists of the sequence from Thr-10 to Tyr-13, whereas the second region that also appears to be functionally important, consists of residues 34 and 35 (Ebisawa et al 1994). NMR experiments, the solution structure of CCL2 dimer, has been determined (Handel and Domaille 1996). These studies indicated that the secondary structure of CCL2 consists of four regions of β-sheet. These include residues 9-11 (β0), 27-31 (β1), 40-45 (β2), and 51-54 (β3). In addition to the four strands of sheet, there are two helical regions. A long helix extends from, approximately, residue 58 to residue 69. Moreover, it was also found that residues 6-16 are involved in the dimerization interface of CCL2 (Zhang and Rollins 1995). The residues involved in the interface include Asn6, Ala7, Val9, Cys11, Tyr13, Asn 14, Phe15, and Thr16 near the N-terminus, and Glu 50, Ile51, and Cys 52. The overall secondary and quaternary structures of CCL2 monomers and dimers resemble RANTES and MIP-1β (Meunier et al 1997). The protein complex appears elongated with the two monomers oriented to give a fairly large pocket. Structures of monomeric and dimeric CCL2 in two crystal forms, namely, I and P forms, respectively, have also been determined (Lubkowski et al 1997). CCL2 is produced by many cell types, including fibroblasts, endothelial, epithelial, mesangial, smooth muscle, astrocytic, microglial and
monocytic cells (Cushing et al 1990, Standiford et al 1991, Brown et al 1992, Barna 1994). However, monocyte/macrophages are found to be the major source of CCL2 (Yoshimura et al 1989 A,B).

2.5.2 Monocyte chemoattractant protein-1 in Immunology

CCL2 regulates the infiltration and migration of monocytes, natural killer (NK) cells and memory T lymphocytes. All the functions of CCL2 were first identified on the basis of in vitro assay using purified protein, which were reproduced and confirmed later in vivo (Fuentes et al 1995, Gunn et al 1997). CCL2 mediates its effects through its receptor CCR2, and unlike CCL2, CCR2 expression is relatively restricted to certain types of cells. There are two alternatively spliced forms of CCR2, namely, CCR2A and CCR2B, which differ only in their C-terminal tails (Charo et al 1994). CCL2 has been demonstrated to recruit monocytes into foci of active inflammation (Ajuebor et al 1998). CCL2 secreted in or injected into skin arrives in the draining lymph nodes, where it can be presented on the surface of high endothelial venules (HEVs) for recruitment of lymphocytes. It was found that CCL2 was the main chemokine responsible for recruiting monocytes (Palframan et al 2001).

Apart from recruiting and directing leukocyte movement, several lines of evidence indicate that CCL2 might influence T-cell immunity. CCL2 expression is associated with the development of polarized Th2 responses (Chensue et al 1995, Handel and Domaille 1996) and it also enhances the secretion of IL-4 by T cells (Karpus et al 1997). In Th2 immune-mediated diseases, such as asthma, CCL2 is expressed at high levels and its neutralization in animal models ameliorates disease (Gonzalo et al 1998). Other chemokines and their receptors are linked to specific responses of Thelper cells (Sallusto et al 1998). In contrast to other chemokines of the CC family, which trigger the Th1 phenotype upon their interaction with CCR5 on Thelper cells (Van Coillie et al 1999), CCL2 acts as a potent factor in the polarization of Th0 cells toward a Th2 phenotype (Gu et al 2000). The T-lymphocyte differentiation process is initiated by the ligation of the T-cell receptor (TCR). Cytokines present during the initiation of a T cell response determine the development of the particular T-helper subset (Rogge et al 1997). Polarization of T-cell subsets occurs in the secondary lymphoid organs to which Th0 cells preferentially migrate. Memory lymphocytes and effector precursor cells, in contrast, migrate to
peripheral tissues (Picker and Butcher 1992). It is likely that, given their different effectors function, Th1 and Th2 cells are differentially recruited to peripheral sites of infection (Lichtman and Abbas 1997). It has been shown that Th1 cells, but not Th2 cells, express a functional ligand for P and E selectin and therefore, are selectively recruited to sites where Th1 immune responses occur (Astrup et al 1997). There may be a direct role for CCL2 in the development of Th2 response. It appears that CCL2 can directly activate the IL-4 promoter, as IL-4 production is increased in cells that are given a primary TCR stimulus in the presence of CCL2. A higher level of CCL2 augments the Th2 response (Karpus and Kennedy 1997). These findings provide an important clue as to why there is a switch from Th1 to Th2 cytokine response in HIV-1 disease. The reciprocal inhibition between Th1 and Th2 cytokines, such as IL-4, is a major factor that governs Th2 differentiation and inhibits the development of IFN-γ-secreting cells (Brown and Hural 1997). This may be important for the effective regulation of the immune response to viruses. Moreover, Th1 and Th2 cells, because of their different chemokine receptor expression pattern induced at least in part by CCL2, are likely to have different susceptibility to HIV strains that use different fusion coreceptors.

2.5.3 MCP-1 and other chemokines in tuberculosis

The contribution of chemokines in the control of \( M. tb \) infection has been supported by several \textit{in vitro} and \textit{in vivo} studies (Sadek et al 1998, Mayanja-Kizza et al 2001, Algood et al 2003). Once the macrophage engulfs \( M. tb \), it produces several cytokines and chemokines which induce the development of proinflammatory responses. \( M. tb \) infection of macrophages induces the production of various chemokines, including CCL2, CCL3, CCL5, CCL7, CCL12, CXCL2, CXCL8, and CXCL10 (Algood et al 2003). Chemokines relevant to \( M. tb \) infection include IL-8 (CXCL8), monocyte chemoattractant protein 1 (MCP-1/CCL2), RANTES/CCL5, and CXCL10 (IP-10) (Azad et al 2012). These chemokines are closely related with activation of microbicidal responses promoting the migration of different cell subpopulations to the \( M. tb \) infected tissues to form granulomas (Serbina et al 2008). Several studies have investigated the effects of chemokines in the function and recruitment of monocytes following infection with \( M. tb \). They promote activated macrophages, monocytes, dendritic cells, polymorphonuclear cells (particularly
neutrophils) and T lymphocytes migration to bronchoalveolar spaces during pulmonary TB (Gonzalez-Juarrero et al 2003). Inflammatory monocytes infiltrates are significantly reduced in CCR2 deficient mice infected with \textit{M.tb}. During TB infection in normal mice, recruited monocytes express CCR2, which has different agonists, CCL2, among them (Peters et al 2004). In line with observations, it is accepted that CCL2 is a central activator of macrophages. Secreted chemokines play a significant role in the recruitment of effector T cells to the site of \textit{M.tb} infection (Deshmane et al 2009). \textit{In vitro} analysis has demonstrated that TLRs (TLR1, TLR2, TLR3, TLR4, and TLR-9) are relevant in the signaling for CCL2 induction through different transcription factors, including NF-kB and MAP kinases (Tsuboi et al 2002, Fietta et al 2002). A direct correlation between elevated CCL2 levels in TB and severity of the disease has been reported (Hasan et al 2009). It is of importance that CCL2 exerts functional activity in the recruitment of both Th1 and Th2 cells and facilitates the polarization of naive T cells to Th2 cells as a result of IL-4 upregulation. In this perspective, increased CCL2 levels may promote an excessive polarization to Th2 responses, resulting in a defective control of \textit{M.tb} infection (Siveke and Hamann 1998, Mendez et al 2011, Hussain et al 2011). CCL5 is a chemokine produced by a variety of cells including macrophages, fibroblasts, endothelial cells, platelets and eosinophils. CCL5 exerts chemotactic activity on dendritic cells, T lymphocytes, NK cells, mast cells and polymorphonuclear cells to inflamed or infected tissues. The expression and functional activities of CCL5 have been studied in experimental models of infection with mycobacteria. CCL5 blockade affects the recruitment of cells and the formation of granulomas induced by \textit{M.bovis} antigens (Chensue et al 1999, Vesosky et al 2010). CCL5 is important in early responses to \textit{M.tb} due to its role in the recruitment of IFN-\(\gamma\) producing T cells to form lymphocyte enriched granulomas (Vesosky et al 2010). In contrast, there is one study that suggested that CCR5 and their ligands (including CCL5) are not essential to the development of protective responses to \textit{M.tb}. In this study, significant differences in the response to the pathogenic H37RV strain of \textit{M.tb} between CCR5 deficient mice and wild-type mice were not detected (Badewa et al 2005). CXCL8, CXCL9 and CXCL10 were higher in patients with active TB compared to both the healthy controls and patients who take treatment of 6 months (Almeida et al 2009). Significantly elevated plasma levels of IL-2, IP-10,
CXCL11 and CXCL12 were present in both patients with tuberculosis and in a sub-group participant with latent tuberculosis infection, who showed a higher level of IFN-γ producing cells by ELISPOT assay compared with other latently infected individuals (Yu et al 2012). CCL19 (MIP-3β) and CCL21 have been shown to be essential in the trafficking of IFN-γ+ T cells from mediastinal lymph node to the lungs of M.tb infected mice (Khader et al 2009). In tuberculosis patients, IL8 has been found in bronchoalveolar lavage fluid (Kurashima et al 1997, Sadek et al 1998), lymph nodes (Bergeron et al 1997) and plasma (Friedland et al 1995, Juffermans et al 1999). Patients who died from tuberculosis showed higher concentrations of IL8 (Friedland et al 1995). Interestingly, following antituberculous treatment, concentrations of IL8 remain elevated in alveolar lavage fluid (Kurashima et al 1997) and serum (Friedland et al 1995) for months. In this context, it has been demonstrated that CCL19 and CCL21, CC-chemokines are induced in the lungs and secreted within granulomatous lesions after infection with M.tb (Schreiber et al 2006). Recently, production of CCL20 in both peripheral blood mononuclear cells and monocyte derived macrophages from active pulmonary tuberculosis patients after in vitro stimulation with the 30 kDa antigen of M.tb has been demonstrated (Lee et al 2008). At present, it has been reported that CCL3, CCL4 and CCL5 are released by human alveolar macrophages upon infection with M.tb and that these proteins are the major CC chemokines produced in response to mycobacterial infection (Mendez-Samperio 2008). During infection, mycobacteria induce increased expression of not only the CC chemokine subfamily members, but also of the CXC chemokine subfamily members, such as CXCL10 and CXCL8 (Zhang et al 1995). A significantly increased resistance to M.tb, H37Rv has been observed after the stimulation of macrophages with CXCL7 (Khajoee et al 2006). Monocytes secrete CXCL8 in response to M.tb (Zhang et al 1995). CXCL8 is known to be involved in inflammatory conditions following infection by M.tb (Friedland et al 1992), also facilitates the elimination of microorganisms by increasing the efficiency of bactericidal activity by enhancing non-oxidative mechanisms (Nibbering et al 1993). Neutrophils themselves secrete CXCL8 and CXCL9 upon stimulation with M.tb (Zhu and Friedland 2006). A number of laboratory animal studies have been conducted to investigate CXCL10 production in response to mycobacterial infection. These studies showed that CXCL10
secretion is induced in *M.tb* stimulated macrophages (Orme and Cooper 1999). CXCL10, in addition to its chemotactic properties, is also involved in the stimulation of natural killer and T cell migration in *M.tb* infection (Zhu and Friedland 2006). It has been demonstrated that following interaction with *M.bovis*, the human monocytes produce significant amount of CXCL10 and that the type1 IFN may play an important role to regulate the expression of CXCL10 in *M.bovis* BCG infection (Mendez-Samperio et al 2004).

### 2.5.4 MCP-1 and other chemokines in leprosy

Macrophage inflammatory protein-1β (MIP-1β/CCL4) and monocyte chemotactic protein-1 (MCP-1/CCL2) were significantly enhanced in TT/BT patients after stimulation with *M.leprae* WCS compared to exposed healthy contacts (Geluk et al. 2012). Expression of MMP3, MMP13, CCL22 were seen to be highest in PBMC cultures of healthy contacts as compared to leprosy patients. The MMP3 and MMP13 showed statistically significant decrease in BT patients. CCL22 showed decrease in LL patients as compared to healthy subjects but did not discriminate the leprosy types. In contrast, skin lesions showed distinct differences in chemokine expression. MMP3, CCL20 and CCL22 showed increase in BT as compared to LL lesions. The higher expression of MMP3 and CCL20 in BT patients as compared to normal skin is suggestive of a possible role in the trafficking of relevant cells to the sites of tuberculoid leprosy granulomas (Saini et al 2013). Expression of CXCL10 (IP10) was observed to be increased in tuberculoid skin lesions compared to lepromatous lesions (Kaplan et al 1987). Significantly elevated plasma levels of CXCL10 were observed in association with type1 reactions (Stefani et al 2009). CXCL10 showed significantly greater median levels in patients with type1 reactions than in any other group. CXCL10 levels in LL patients with type2 reactions were not significantly different compared to those of LL patients without type2 reactions. Median CXCL10 levels were higher in all leprosy patient groups as compared to healthy controls. Serum CXCL10 was significantly higher during type1 reactions in BL and in BT patients. In general, BT patients showed a more consistent association of CXCL10 elevation with histopathological and clinical evidence of type1 reactions. mRNA expression studies done on biopsy specimens showed that CXCL10 levels were 16 fold higher in biopsy specimens from patients with type1 reactions than in
biopsy from patients without type 1 reactions. CXCL10 mRNA levels ranged from 2.5 to 5.2 fold higher in biopsy specimens taken during type 1 reactions than in biopsy specimens taken from the same individual prior to the reaction. The strong association between elevated CXCL10 and clinical type 1 reactions suggests that this chemokine may be useful as a laboratory marker to aid in the diagnosis of type 1 reactions (Scollard et al 2011). mRNA expression of CCL2, CCL3, CCL7, IL1β, IL6 and IL8 were significantly down regulated in *M. leprae* infected THP-1 cells, when compared to control. A borderline decrease was observed for CCL7, TNF and IL6. Gene expressions in nerve biopsies of the patients were also estimated, which indicates down regulation of CCL2, CCL3 and CCL4, while IL1β and SOD2 were of borderline significant, confirming repression of these genes by *M. leprae* ex vivo, as was observed invivo in THP-1 (Guerreiro et al 2013). The difference of CXCL10 median plasma concentration among type 1 reactions and their matched controls was highly significant. IL6 also showed statistically significant differences between type 1 reactions patients and matched controls. The higher levels of CXCL10 detected in type 1 reactions suggest its involvement in the type 1 reactions immunopathology, probably by attracting Th1 type cells to the reactional inflammatory sites in the skin (Stefani et al 2009).

### 2.6 MCP-1 gene polymorphism -2518 A/G and -362G/C in Tuberculosis

Robin and Saxena in 1999 identified two novel polymorphisms in the distal regulatory region of the *MCP-1* gene by direct sequencing PCR at positions -2518 (A/G) and -2076 (A/T) relative to the major transcriptional start site of the gene. Polymorphism at position -2518 (A/G), which is present in the distal regulatory region, was found to affect the transcriptional activity of monocyte MCP-1 production. The -2518G allele is associated with increased MCP-1 production in response to different stimulating agents (Robin and Saxena 1999). Flores-Villanueva et al 2005 first reported the association of -2518A/G genotype with tuberculosis, they said that allele G of the *MCP-1* promoter enhancing region is strongly associated with increased odds of developing active pulmonary tuberculosis after infection in Mexicans and Koreans. Persons with the *MCP-1* genotypes
AG and GG were 2.3 and 5.4 fold and 2.8 and 6.9 fold more likely to develop tuberculosis than those with the AA genotype in Mexicans and Koreans, respectively. They also reported that carriers of the GG genotype had the highest MCP-1 levels, followed by those with AG and AA genotypes. There was a significant negative correlation between MCP-1 and IL-12p40 levels in persons with the GG genotype, but not in those with the AA or AG genotypes (Flores-Villanueva et al 2005). In contrast to the earlier report of Flores-Villanueva that suggested an association of $MCP-1 -2518G$ with susceptibility to TB in Mexicans and Koreans, an opposite association of $MCP-1 -2581G$ was found with resistance to TB in the Ghanaian population and no effect at all on TB in Russian population. Another polymorphism, $MCP-1 -362G/C$, is found associated with protection from TB, both in the Ghanaian case-control sample and in nuclear families. The meta-analysis of the association between $MCP-1 -2518A/G$ variant and susceptibility to TB in five case-control studies of five ethnicities, including Ghanians, Russians, Chinese, Mexicans and Koreans, showed differences in associations between studies as indicated by a significant test for heterogeneity. Haplotype analysis showed that $MCP-1 -2581G/-362C$ alleles were strongly associated with resistance to TB (Thye et al 2008). Haplotype combination $-2518G/-362C/int1del554-567$ mediates stronger protection than does the $MCP-1 -362C$ allele alone (Intemann et al 2011). No association of $-2518A/G$ polymorphism was reported in hong cong Chinese patients (Chu et al 2007). Larcombe et al 2008 in their study in canadian population showed that, the Dené and Cree communities of Canada, which were reported to have highest rates of tuberculosis, were found to maintain the G allele, which is associated with high protein expression at a frequency of 91% and 78%, respectively, compared with 23% in the Caucasian cohort. The Dené and Cree cohorts had significantly more homozygotes (G/G) for MCP-1 -2518 than the Caucasian cohort, which may impair optimal macrophage function and facilitate containment of $M.tuberculosis$ (Larcombe et al 2008). While, in population of Zambia, it was reported that the odds of having TB was 2.8 fold higher in carriers of the -2518 AG single nucleotide polymorphisms in the promoter region of the CC chemokine ligand 2, than in those carrying the homozygous genotype AA (Buijistel et al 2008).
The -2518 MCP-1 GG genotype might be associated with susceptibility to pulmonary tuberculosis in Chinese Han population (Yang et al 2009). Findings suggest that persons bearing the MCP-1 genotype GG produce high concentrations of MCP-1, which increases the risk of active TB infection in Chongqing Han people. These findings are more significant in child patients than in adult patients with TB (Xu et al 2009). Joint effect between the -2518 MCP-1 genotype GG and the –1607 MMP-1 genotype 2G/2G consistently increases the odds of developing TB, 3.59 fold in Mexicans and 3.9 fold in Peruvians. Carriers of these susceptibility genotypes might be at increased risk of developing TB because they produce high levels of MCP-1, which enhances the induction of MMP-1 production by M.tb sonicated antigens to higher levels than in carriers of the other two-locus MCP-1/MMP-1 genotypes (Ganachari et al 2010). There were no significant differences found between PTB patients and control subjects regarding -2581 A/G single nucleotide polymorphism of CCL2 in population of Zahedan, Southeast Iran (Naderi et al 2011). In Tunisian population, it was found that the -2518 GG genotype was significantly over-represented in active pulmonary TB patients as compared to controls, and was associated with risk of development of TB (Selam et al 2011). Allele -2518G was associated with increased TB susceptibility. The odds of developing TB in genotypes GG were higher than those in homozygous AA, and the risk was higher in children than in adult. Cases of homozygous GG had the highest plasma levels of MCP-1, which increased the likelihood of developing TB (Xu et al 2009). Edwards et al 2012, in their study on the populations of Guinea-Bissau, Gambia, U.S. (African-Americans), and European ancestry from the U.S. and Argentina, tested the association of -2518A/G and -362G/C with tuberculosis, but they did not find any association in either of the populations studied (Edwards et al 2012).

The MCP-1 -2518GG genotype is associated with protection against pulmonary tuberculosis in Moroccan patients (Arji et al 2012). Three studies have been done so far to show the association of TB and -2518 A/G MCP-1 gene polymorphisms from India, in the first report of Alagarasu and Selvaraj, concluding that this polymorphism did not differ significantly between their choosen study groups, HIV infected persons with and without tuberculosis and healthy contacts (Alagarasu et al 2009). The genotypic and allelic frequencies of -2518A/G, -362G/C of CCL2 and -28C/G of CCL5 did not differ
significantly between cases and controls in the study carried out by Mishra and Tiwari in Sahariya tribe, a primitive tribe of North Central India, having a high prevalence of TB (Mishra et al 2012). In a study on south Indian population carried out by Singh et al also did not find any difference in genotype and allele frequency between patients and contacts for -2518 A/G polymorphism (Singh et al 2013). Presence of association between MCP-1, -2518G allele and the G carrier genotypes with susceptibility to developing TB is reported. In the stratified meta analysis of G allele significant associations was observed with disease in Asians and Latin-Americans (Hispanics) (Feng et al 2011). Another meta analysis showed that GG homozygote carriers had a 67% increased risk of TB, compared with the A allele carriers in the subgroup analysis by ethnicity. Significant elevated risks were found in Asians and Latinos, but not in Africans (Zhang 2012). Similar results were also reproduced in a recent meta analysis done by Gong et al where they reported that G allele of -2518A/G polymorphism is a risk factor for tuberculosis in Asians and Americans, but not in Africans and indicates that C allele of -362G/C polymorphism is a protective factor for tuberculosis (Gong et al 2013). All the above studies show varing relationships hence further studies in different populations are required.

2.7 MCP-1 gene polymorphism -2518 A/G and -362G/C in leprosy

No study has been done so far to find out the role of these polymorphisms in leprosy cases.