Review of Literature

2.1 Introduction

Tuberculosis (TB) remains a major global health problem. It is spread from one person to another by breathing-in droplets from an infected person's cough or sneezes. The disease is caused by slow growing, acid-fast bacillus *Mycobacterium tuberculosis*, first identified as a pathogen by Robert Koch in 1882. It mainly affects lungs and causes Pulmonary TB, which is 70% of all TB cases. The symptoms of TB are coughing for three or more weeks, chest pain, weight loss, fatigue, fever, night sweats, chills and loss of appetite. It can spread to other body organs as well and cause extra pulmonary TB, which may include infection in lymph nodes, pleura, bone, meninges, kidneys and spine etc. The risk of TB increases with the following condition, like HIV/AIDS, diabetes, end stage kidney disease, certain cancers, cancer treatment (chemotherapy), drugs to prevent rejection of transplanted organs, some drugs used to treat Rheumatoid arthritis, Crohn’s disease and Psoriasis, malnutrition and very young age or advanced age.

Most of the infected population neither develops disease nor becomes infectious, because the bacteria generally stay latent (inactive) after they invade the body and only 10% of people infected with TB will ever develop the active disease. The remaining 90% have, what's called latent TB infection; they show no signs of infection and would not be able to spread the disease to others. This clinical latency often extends to lifetime of the individual. However, reactivation of the latent infection can occur in immuno-compromised state, and turns into active tuberculosis, which can be fatal if left untreated. Medication can help in getting rid of the inactive bacteria before they become active. Anti-tuberculous treatment for active disease goes for 6-9 months and the drugs taken are Rifampicin, Streptomycin, Pyrazinamide and Ethambutol. TB was virtually wiped out with the help of antibiotics developed in the 1950s, but the disease has re-emerged in potent new forms, multidrug-resistant TB (MDR, resistant to Rifampicin + Streptomycin) and extensively drug-resistant TB (XDR, resistant to Rifampicin + Streptomycin + any one drug of Quinolone family)
+any one of second-line anti-TB injectable drugs, like Kanamycin, Capreomycin or Amikacin). Today, these new and dangerous forms of the disease have created a public health crisis in many large cities worldwide.

2.1.1 Epidemiology of Tuberculosis
Tuberculosis (TB) ranks as the second leading cause of death from a single infectious agent, after the human immunodeficiency virus (HIV). One third of the world’s population is infected with TB. About 8.6 million developed the disease in 2012, including 1.1 million cases of HIV. In 2012, 1.3 million people died from TB, including approximately three lakhs people who were HIV-positive (CDC, 2013, www.who.int/tb). The TB mortality rate has decreased 45% since 1990, and the 2015 global target of a 50% reduction in mortality is now within reach (WHO, Global Tuberculosis Report 2012; WHO, Global Tuberculosis Report 2013). Globally, in 2012, an estimated 4, 50,000 people developed multidrug-resistant TB (MDR-TB) and there were an estimated 1, 70,000 deaths from MDR-TB. About 3.6% of newly diagnosed TB cases and 20% of those previously treated for TB had MDR-TB. The highest levels of MDR-TB are found in Eastern Europe and Central Asia. On average, an estimated 9.6% of MDR-TB cases are XDR-TB. In 2011, out of the estimated global annual incidence of 9 million, TB cases, 2.3 millions were estimated to have occurred in India only. Indian TB prevalence rate is 3.1 million cases and the mortality rate is 0.32 million per year (TB India Annual report, 2013 RNTCP Programme).

2.1.2 Pathology of Tuberculosis
2.1.2.1 Infection and disease progression
The *M.tb* bacilli are inhaled from air droplets and reaches to the alveolar/air sacs, where infection begins. It multiplies within unactivated alveolar macrophages until the macrophages burst. Other macrophages also begin to extra vasate from peripheral blood and phagocytose bacilli, but being unactivated, cannot destroy the bacteria. The next stage of infection is when lymphocytes begin to infiltrate and specifically T-cells, recognize processed and presented *M.tb* antigen in context of MHC molecules, which leads to T-cell activation and release of cytokines, especially IFN-γ, which further leads to activation of macrophages. Activated macrophages may release lytic enzymes and reactive intermediates (ROI, RNI) that facilitate the development of
immune pathology. Activated macrophages and T-cells also secrete cytokines that may also play a role in the development of immune pathology, including Interleukin-1 (IL-1), tumor necrosis factor (TNF), and IFN-γ. By six to eight weeks post-infection, antigen presenting Dendritic cells (DCs) reach lymph nodes where T lymphocytes are activated and recruited. Resident dendritic cells are also infected by *M. tuberculosis* and migrate to the lymph node to prime naive T cells. Also, at this stage, tubercle formation begins, the center of the tubercle is characterized by "caseation necrosis", meaning, it takes on a semi-solid or "cheesy" consistency. *M.tb* cannot multiply within these tubercles because of the low pH and anoxic environment. *M.tb*, however, can persist within these tubercles for extended periods.

The *M.tb* still continues to replicate in the unactivated macrophages surrounding the tubercles. When the tubercle grows in size, it invades a bronchus and infection spread to other parts of the lung. Slowly tubercle may invade an artery or other blood supply line. The hematogenous spread of *M.tb* may result in extra pulmonary tuberculosis otherwise known as milliary tuberculosis. The secondary lesions caused by milliary TB can occur at almost any anatomical location, but usually involve the genitourinary system, bones, joints, lymph nodes and peritoneum. The lesions are of two types: 1. Exudative lesions result from the accumulation of Perimorphonuclear neutrophils around *M.tb* and the bacteria replicate with virtually no resistance and become "soft tubercle; 2. Productive or granulomatous lesions occur when host becomes hypersensitive to tuberculoproteins and there forms a "hard tubercle" (Ulrichs and Kaufmann, 2006). On later stages, the caseous centers of the tubercles liquefy. This liquid is very conducive to *M.tb* growth, and the organisms begin to rapidly multiply extracellularly. After some time, the large antigen load causes the walls of nearby bronchi to become necrotic and rupture and form cavity. This also allows *M.tb* to spill into other airways and rapidly spread to other parts of the lung. When the primary lesion heals, it becomes fibrous and calcifies. When this happens the lesion is referred to as the Ghon complex. Depending on the size and severity, the Ghon complex may never subside. Typically, the Ghon complex is readily visible upon chest X-ray. Small metastatic foci containing low numbers of *M.tb* may also calcify. However, in many cases, these foci will contain viable organisms. These foci are referred as Simon foci. These Simon foci are also visible upon chest X-ray and are often the site of disease reactivation.
Chapter 2

Review of Literature - Tuberculosis

Figure 2.1 Dynamics of tuberculosis infection based on clinical, epidemiological studies in humans. Upon infection with *M*. *tuberculosis*, immune system is able enough to contain the infection through granuloma formation, which results in the establishment of latent TB infection (LTBI), but in cases of immuno-compromised state (HIV/AIDS), no granuloma forms and TB transmission & dissemination is accelerated. People with LTBI may or may not develop TB disease. In some people, the tubercle bacilli overcome the immune system and multiply, resulting in progression from LTBI to active TB disease (self-drawn).

2.1.2.2 Role of Granuloma

Granuloma protects the host or promotes the infection or the tissue pathology, depending on the stage of disease and whether *M*. *tuberculosis* bacilli are being controlled by innate and/or adaptive immune responses or whether the disease has progressed to active TB (Barry et al 2009). It is a fine balance between host containment of infection and protection of *M*. *tuberculosis* from IFN-γ producing lymphocytes. Human TB granulomas are composed of a central mass of infected macrophages, stimulated macrophages that have differentiated into multinucleated giant cells, epithelioid cells and foamy macrophages loaded with lipid droplets, and neutrophils (Russell et al 2009), again surrounded by lymphocytes, largely CD4⁺ T cells, in addition to CD8⁺ T cells and B cells, and by fibroblasts, which create a peripheral fibrotic capsule. Although, T cells appear to have limited antigen-presenting cell function in the granuloma (Egen et al 2011). The granuloma is maintained by
delayed type hypersensitivity (DTH) response to the persistent presence of the antigens and immunostimulatory lipids, as *M.tb* can survive within macrophages and extracellularly within the granulomas. The granuloma environment is considered hypoxic in guinea pigs, rabbits, non-human primates, and human beings (Via et al 2008; Aly et al 2006), which is an important consideration for mycobacterial metabolism and antibiotic therapy efficacy. *M.tb* adapt to this environment by preferentially using fatty acids in their metabolism, slowing down active replication, and increasing cell wall thickness, entering a so-called dormant state (Boshoff et al 2005). The lesion at the primary site of implantation is termed the *Ghon focus*. Bacilli may spread before the formation of these organized granulomas via the lymphatics to regional lymph nodes, resulting in granulomatous lymphangitis and lymphadenitis, which along with the Ghon focus, is called the primary Ranke’s complex. In chronic lesions, *M.tb* growth is predominantly in the foamy macrophages at the periphery of lesions or in chronic granulomas that have cavitated and accessed an airway. Histologically, there are different types of granulomas. Initially, epithelioid cells may be surrounded by an acellular necrotic region with a ring of B and T lymphocytes. The granulomas can displace parenchymatous tissue and may necrotize, caesate or calcify. Caseous granulomas might turn calcified during chronic or latent infection. Other types of granulomas may not have a necrotic area and are composed primarily of macrophages and a few lymphocytes.

Immuno-compromised state, such as HIV or other infection, old age, malnutrition, malignant disease, immunosuppressive medication, or new infection can lead to reactivation or secondary disease (Verver et al 2005). The most common form of secondary tuberculosis is usually limited to the lung, and lesions begin as an exudative bronchopneumonia and progress to classical caseous granuloma formation, followed by massive necrosis and cavity formation. TB reactivation occurs in the lungs in 80% of the cases and 20% at other tissue sites (e.g., pleural space, lymph nodes, bone, kidney, etc) (Frieden et al 2003). There is eventual breakdown of the fibrous capsule and communication occurs with airways. These events allow for rapid growth of extracellular bacilli and spread into airways leading to transmission or intracellular spread.
Chapter 2

2.2 Immune response in tuberculosis infection

The immune responses to tuberculosis can be grouped into two parts – Innate and Adaptive.

2.2.1 Innate Immunity

Innate immunity plays an important role in the host defense against M. tuberculosis, and the first step in this process is recognition of M.tb by cells of the innate immune system. The initial interaction with surface receptors influences the subsequent fate of M. tuberculosis within the macrophage. Interactions with the constant regions of immunoglobulin receptors (FcRs) and Toll-like receptors stimulate host-defense mechanisms, whereas, those with complement receptors, promote mycobacterial survival (Kleinnijenhuis et al 2011; Kaufmann et al 2004).

2.2.1.1 The Toll-Like Receptors (TLRs)

TLRs are evolutionarily conserved receptors, homologues of the Drosophila Toll protein, discovered to be important for defense against microbial infection. TLRs represent the family of pattern recognition receptors (PRRs) expressed on the cell membrane or on the membrane of endocytic vesicles of mainly immune cells, including macrophages and dendritic cells (DCs). TLRs recognize highly conserved structural motifs, known as pathogen- associated microbial patterns (PAMPs), which are exclusively expressed by microbial pathogens. PAMPs include various bacterial cell wall components, such as lipopolysaccharide (LPS), peptidoglycan (PGN) and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA (Kleinnijenhuis et al 2011; Takeda and Akira, 2004).

TLR signaling consists of at least two distinct pathways: a MyD88-dependent pathway that leads to the production of inflammatory cytokines, and a MyD88-independent pathway associated with the stimulation of IFN-β and the maturation of dendritic cells. Upon activation by PAMPs, the TLRs hetero- or homodimerize and promote recruitment of adaptor proteins via cytoplasmic TIR domain. TLR4 and TLR2 signaling requires the adaptor TIRAP/ Mal, which is involved in the MyD88-dependent pathway. Subsequently, IL-1 receptor associated kinases (IRAK), TNF receptor associated factor (TRAF) 6, TGF β- activated protein kinase 1 (TAK-1), and mitogen activated protein (MAP) kinase are recruited in a signaling cascade leading to activation and nuclear translocation of transcription factors, such as the nuclear
transcription factor NF-κB. This leads to the transcription of genes involved in the activation of the innate host defense, mainly the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-12 and nitric oxide. In addition to MyD88, TLR4 can induce intracellular signals through a second pathway, which is mediated by the adaptor molecule Toll/IL-1R (TIR) domain-containing adaptor inducing interferon (IFN) -β (TRIF). This signaling cascade is shown to be involved in the LPS-induced autophagy (Xu et al 2007; Takeda and Akira, 2004; Reinout et al 2002).

Figure 2.2 Schematic model representing Toll Like Receptors (TLRs) and integrated signaling pathways induced by diverse mycobacterial antigens. M.tb and its antigens are recognized by pattern recognition receptors (PRRs) present on the surface of macrophages and dendritic cells that activate the immune response. TLRs, NOD2, Dectin-1, and Mincle are pro-inflammatory, while DC-SIGN and MR (mannose receptor) repress inflammatory signals. Complement receptors (CRs) are primarily responsible for uptake of opsonized M. tuberculosis and Mannose receptors (MRs) and scavenger receptors (SRs) are for the uptake of non-opsonized M. tuberculosis. TLRs play a central role in immune recognition of M. tuberculosis. In the context of CD14, TLR2 binds lipoarabinomannan and a 19-kDa M. tuberculosis lipoprotein, TLR4 binds to a heat shock protein (HSP) 60/65 and 38-kDa
antigen and TLR9 binds to M. tuberculosis DNA. TLR activation by mycobacterial antigens leads to an intracellular signaling pathway that culminates in the production of pro-inflammatory cytokines in macrophages and dendritic cells through MAPK and NF-κB pathways. The integrated signaling affects both, the initial host response and the onset and character of adaptive immunity (self-drawn).

Table 2.1: Different Pattern Recognition Receptors involved in the innate immune recognition of *Mycobacterium tuberculosis* (Kleinnijenhuis et al 2011)

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Cells on which they are expressed</th>
<th>PAMP/ligand</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-2</td>
<td>Macrophages and DCs</td>
<td>LAM, LM, 38KDa and 19KDa mycobacterial glycoprotein</td>
<td>TNF-α production, IL-1β production, IL-12 release in macrophages</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Placenta and myelomonocytic leukocytes</td>
<td>Hsp 60/and65</td>
<td>TNF-α production</td>
</tr>
<tr>
<td>TLR-9</td>
<td>DCs, B cells, Natural killer cells, monocytes</td>
<td>Unmethylated CpG motifs in bacterial DNA</td>
<td>IL-12 secretion</td>
</tr>
<tr>
<td>NOD (Nucleotide Oligomerization domain) like receptors (NLRs)</td>
<td>Intracellular receptor</td>
<td>Bacterial peptidoglycans</td>
<td>Involved in recognition of mycobacteria, form different variants of the inflammasome containing NLRP1, NLRP3, NLRC4 and adaptor protein ASC, involved in the production of IL-1β</td>
</tr>
<tr>
<td>C-type lectins (eg., mannose receptor)</td>
<td>Alveolar macrophages</td>
<td>Polysachharides, such as ManLAM, PIMs</td>
<td>Cell invasion and induces phagocytosis, limited phagosome-lysosome fusion, anti-inflammatory cytokine response</td>
</tr>
<tr>
<td>DC-SIGN (Dendritic cell-specific adhesion molecule-3), CD209</td>
<td>Dendritic cells</td>
<td>Non-integrin ManLAM, Lipomannans, α-glucan</td>
<td>Dendritic cell migration, DC-T cell interaction, anti-inflammatory immune response by induction of IL-10, immunosuppressive by acetylation of NF-κB subunit p65 via Raf-1</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>Macrophages, DCs, neutrophils and a subset of T cells</td>
<td>α-glucan</td>
<td>Innate immune recognition, induces Th1 and Th17 responses</td>
</tr>
</tbody>
</table>
2.2.1.2 Consequences of host-pathogen interactions

(i) Macrophage activation and Phagocytosis: Macrophages are the main effector cells involved in killing of *M. tuberculosis*. Lymphocyte products, mainly IFN-γ, and proinflammatory cytokines, like TNF-α, are important for macrophage activation. In addition, vitamin D also seems to be involved in macrophage activation. The active metabolite of vitamin D, 1, 25-dihydroxyvitamin D3 is shown to enhance macrophage phagocytosis of *M. tuberculosis* and increases the production of antimicrobial peptide cathelicidin and killing of *M. tuberculosis* (Selvaraj et al 2011). Phagocytes such as macrophages and dendritic cells, upon activation, generate ROI, NO and related RNI via NOS2 using L-arginine as the substrate, which are involved in the host defense against *M. tuberculosis*, both *in-vitro* and *in-vivo*, particularly in the murine system (Raja et al 2004) in both acute and chronic persistent infection and also in host defense in human tuberculosis (Nicholson et al 1996). When phagocytosed by macrophages, bacteria typically enter specialized phagosomes that undergo progressive acidification, followed by fusion with lysosomes. However, mycobacteria arrest phagosomes at an early stage of maturation, and thereby block the digestive activity of acidic hydrolases (Sabine and Schnappinger, 2009).

*Nramp1*, which codes for natural-resistance-associated macrophage protein (*Nramp*) also known as mycobacterial susceptibility gene, *Bcg*, is an interesting gene involved in macrophage activation and mycobacterial killing (Blackwell et al 2000). The protein is an integral membrane protein, which belongs to a family of metal ion transporters. These metal ions, particularly Fe$^{2+}$, are involved in macrophage activation and generation of toxic antimicrobial radicals (Zwilling et al 1999). Following phagocytosis, *Nramp1* becomes part of the phagosome. *Nramp1* mutant mice display reduced phagosomal maturation and acidification (Hackam et al 1998). Genetic variation in *Nramp1* may affect the outcome of infection with *M. tuberculosis* (Cervino et al 2000).

(ii) Apoptosis due to *M.tb* infection: Apoptosis of phagocytic cells may constitute another effector mechanism for the infected host to limit outgrowth of *M. tuberculosis*, as it prevents dissemination of infection (Lee et al 2009). In addition, apoptosis of infected cells reduces viability of intracellular mycobacteria, while necrosis of infected cells does not (Behar et al 2011).
TNF-α is required for induction of apoptosis in response to infection with *M. tuberculosis* (Stenger, 2005). Pathogenic *M. tuberculosis* strains induced significantly less host cell apoptosis than related attenuated strains, because pathogenic strains lead to selective induction and release of neutralizing soluble TNF-α receptors. Release of TNF-α receptors, in turn, is regulated by IL-10 production. Thus, pathogenic strains of *M. tuberculosis* selectively induce IL-10, leading to decreased TNF-α activity and reduced apoptosis of infected cell (Balcewicz-Sablinska et al 1998; Keane et al 1997). Independent of cytokine production, LAM (lipoarabinomannan) may prevent in-vitro apoptosis of *M. tuberculosis*-infected cells in a Ca\(^{2+}\) dependent mechanism (Rojas et al 2000). In addition, increased expression of Fas ligand in infected macrophages may also contribute to decreased macrophage apoptosis (Mustafa et al 1999). However, in cases where necrosis is induced, the *M. tuberculosis* infection is regulated by lipid mediators, eicosanoids, prostaglandin E2 (PGE2) (pro-apoptotic), and lipoxin A4 (LXA4) (pro-necrotic). This regulation plays a major role in determining the outcome of infection (Divangahi et al 2010). The *Alox5*−/− (arachidonate lipoxygenase) mice is more resistant to *M.tbc* infection, while *Ptges*−/− (prostaglandin E synthase) mice is more susceptible, because, by activation of 5-lipoxygenase pathway, *M. tuberculosis* infection not only inhibits macrophage apoptosis, but also prevents cross presentation of *M. tuberculosis* antigens by DCs, thus, impeding the initiation of T cell immunity (Divangahi et al 2010; Bafica et al 2005).

Autophagy is another cellular process by which a cell degrades its own intracellular components and is an immune defense mechanism. It may also be induced by a variety of methods in macrophages, most immunologically relevant of which are IFN-γ and activation of TLRs (Gutierrez et al 2004; Xu et al 2007).

(iii) **Persistence and survival of Mycobacterium tuberculosis in host cell:** *M. tuberculosis* has acquired different mechanisms for its intracellular survival in the host cell. Lysosomal degradation of *M. tuberculosis* is also impeded by a molecule, named tryptophan-aspartate containing coat protein (TACO), which is recruited to the phagosome in a cholesterol dependent manner (Ferrari et al 1999). The pathogen possesses specialized iron-scavenging molecules to compete for iron with its host (Collins et al 2001). Iron is also required in various host-defense mechanisms (Schaible et al 1999). Persistent microbes proceed to a stage of Dormancy with a
reduced metabolic activity that facilitates their survival under conditions of nutrient and oxygen deprivation. These bacteria can persist without producing disease and, therefore, create a state of Latency. In-vitro experiments indicated that mycobacteria switch to lipid catabolism and nitrate respiration to ensure their survival (McKinney et al 2000; Weber et al 2000). Lipids are abundant in the caseous detritus of granulomas, providing a rich source of nutrients during persistence.

2.2.2 Adaptive Immunity
The adaptive immune or specific immune response consists of antibody responses and cell-mediated responses, which are carried out by different lymphocyte cells, B cells and T cells, respectively. B cells are the major cells involved in the creation of antibodies that circulate in blood plasma and lymph, where they bind specifically to the foreign antigens. Binding of antibody inactivates viruses and microbial toxins by blocking their ability to bind to receptors on host cells. These antibody responses are also called humoral immunity. Cell-mediated immunity does not involve antibodies but rather involves the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T lymphocytes, and the release of various cytokines in response to an antigen. A key feature of the adaptive immune system is the development of immunological memory, in which each pathogen is “remembered” by a signature antibody. Memory cells remain ready to respond rapidly and efficiently to a subsequent encounter with a pathogen. This so-called secondary response is often stronger than the primary response to infection. In principle, three processes contribute to the initiation of adaptive immunity: antigen presentation, co-stimulation and cytokine production.

(i) Antigen Presentation
The basis of adaptive immunity lies in the capacity of immune cells to distinguish between the body's own cells and infectious pathogens. Unlike B cells, T cells fail to recognize antigens in the absence of antigen presentation, with the important exception of the superantigens. The T cell receptor is restricted to recognizing antigenic peptides only when bound to appropriate molecules of the major histocompatibility complex (MHC), also known in humans as Human leukocyte antigen (HLA). During M. tuberculosis infection, first, MHC class II molecules present mycobacterial proteins to antigen specific CD4+T cells. These antigens are
processed in phagolysosomal compartments in professional antigen-presenting cells. Second, MHC class I molecules, expressed on all nucleated cells, are able to present mycobacterial proteins to antigen-specific CD8+ T cells. This mechanism allows presentation of cytosolic antigens, which may be important as certain mycobacterial antigens may somehow escape the phagosome (Mazzaccaro et al 1996). Third, non-polymorphic MHC class I molecules such as type I CD1 (-a, -b, and -c) molecules, which are expressed on macrophages and dendritic cells, are able to present mycobacterial lipoproteins to CD1-restricted T cells. This mechanism of antigen presentation activates a larger fraction of T cells at an earlier point in the infection, before even antigen specificity has developed. A fourth pathway may involve non-polymorphic MHC class Ib molecules (Lewinsohn et al 1998). The expression of particular class I and class II MHC alleles in an individual determines the ability of that individual to respond to particular (mycobacterial) antigen or epitope. Certain allelic human leukocyte antigen (HLA) variants have been shown to be associated with tuberculosis (Ravikumar et al 1999; Goldfeld et al 1998). Pro-inflammatory cytokines, primarily IFN-γ, stimulate expression of MHC while anti-inflammatory cytokines inhibit its expression. *M. tuberculosis* blocks the presentation of its antigens to T cells by reducing the expression of MHC Class II molecules on the surface of infected macrophage (Gercken et al 1994; Pancholi et al 1993).

(ii) Costimulation:
Antigen presentation only leads to T-cell stimulation in the presence of particular costimulatory signals. The most well-known costimulatory signals for T-cell stimulation are B-7.1 (CD80) and B-7.2 (CD86). These molecules are expressed on macrophages and dendritic cells and bind to CD28 and to CTLA-4 on T cells. Interestingly, *in-vitro* infection of monocytes with *M. tuberculosis* leads to diminished expression of B-7.1b (Saha et al 1994). On the other hand, *M. tuberculosis* infection of dendritic cells induces expression of B7.1, CD40, and ICAM-1. In the absence of proper costimulatory signals, antigen presentation may lead to increased apoptosis of T cells (Hirsch et al 2001; Hirsch et al 1999).

(iii) Cytokines production:
Cytokines are small-secreted proteins produced by the cells of the immune system and act both, in the early stages of the infection to initiate the immune response and
at later stages to sustain and regulate it. When the immune system is fighting pathogens, cytokines signal immune cells such as T cells and macrophages to travel to the site of infection. In addition, cytokines activate those cells, stimulating them to produce more cytokines.

2.2.3 Cytokines are further categorized as pro-inflammatory and anti-inflammatory based on their function.

2.2.3.1 Pro-inflammatory cytokines

(i) Tumor Necrosis Factor -α

*M. tuberculosis* induces TNF-α secretion by macrophages, dendritic cells, and CD4+ T cells (Henderson et al 1997). TNF-α, a prototype pro-inflammatory cytokine is required for control of acute *M. tuberculosis* infection. TNF-α acts as a mediator of macrophage activation, apoptosis induction and also involved in the granuloma formation (Stenger, 2005). TNF-α synergistically act with IFN-γ to induce NOS2 expression (Chan et al 1992). In the absence of TNF-α or the 55-kDa TNF receptor, the NOS2 expression is delayed in response to *M.tb* infection, granulomas that do form are disorganized, with fewer activated or epithelioid macrophages (Flynn et al 1995). This shows that TNF-α affects cell migration and localization within tissues in *M. tuberculosis* infection. TNF-α influences the expressions of adhesion molecules, chemokines as well as chemokine receptors. TNF-α knockout mice (Bean et al 1999; Kaneko et al 1999) or the TNF-α receptor p55 display an increased susceptibility for mycobacteria (Senaldi et al 1996).

(ii) Interleukin-1β

Interleukin-1 beta (IL-1β) is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis. It is primarily secreted by monocytes, and dendritic cells (Dahl et al 1996). In tuberculosis patients, IL-1β is expressed aberrantly and at the site of disease (Bergeron et al 1997). IL-1α and β double knockout mice and IL-1βR1 deficient mice (which do not respond to IL-1) display an increased mycobacterial growth and also defective granuloma formation after infection with *M.tb* (Yamada et al 2000; Juffermans et al 2000).
(iii) Interleukin-6
IL-6 has multiple roles in the immune response, including inflammation, hematopoiesis, and differentiation of T cells and exhibit both pro- and anti-inflammatory properties generally produced at early stage of mycobacterial infection and at the site of infection (VanHeyningen et al 1997; Law et al 1996). Some reports have indicated that IL-6 may be harmful in mycobacterial infections, as it inhibits the production of TNF-α and IL-1β, whereas other reports support a protective role for IL-6. The IL-6 gene disrupted mice display increased susceptibility to infection with M. tuberculosis (Ladel et al 1997), which seems relate to a deficient production of IFN-γ early in the infection, before adaptive T-cell immunity has fully developed (Saunders et al 2000). Following low dose aerosol infection, early increases in lung burden, as well as decreased IFN-γ production, were observed in the IL-6-/mice, compared to control mice, suggesting that IL-6 is important in the initial innate response to the pathogen. Once acquired immunity is developed, the survival of IL-6-/mice is increased with intact memory response (Saunders et al 2000).

(iv) Interleukin-12
IL-12 is a key player in host defense against M. tuberculosis and has a crucial role in the induction of IFN-γ production. In tuberculosis, IL-12 has been detected in lung infiltrates, in pleurisy, in granulomas, and in lymphadenitis (Lin et al 1996). The expression of IL-12 receptors is also increased at the site of disease (Zhang et al 1995). IL-12 knockout mice are highly susceptible to mycobacterial infections (Cooper et al 1997). An IL-12R defect has also been identified in a patient with abdominal tuberculosis (Altare et al 2001). Apparently, IL-12 is a regulatory cytokine, which connects the innate and adaptive host response to mycobacteria and exerts its protective effects, mainly through the induction of IFN-γ (Cooper et al 1997). Early administration of IL-12 to M.tb infected BALB/c mice resulted in significantly decreased bacterial numbers and increased mean survival time, although the mice still succumbed to the infection (Flynn et al 1995). In contrast, IL-12 had only marginal effects on the bacterial numbers in C57BL/6 mice (Cooper et al 1995), perhaps reflecting the more naturally resistant phenotype of this strain compared to BALB/c.
(v) **Interleukin-18**

IL-18, a novel pro-inflammatory cytokine that shares many features with IL-1 (Dinarello et al 1998) was initially discovered as an IFN-γ inducing factor, synergistic with IL-12. It has been found that IL-18 also stimulates the production of other pro-inflammatory cytokines, chemokines, and transcription factors (Netea et al 2000). IL-18 knockout mice are highly susceptible to BCG and *M. tuberculosis* (Sugawara et al 1999), *M. tuberculosis*-mediated production of IL-18 by peripheral blood mononuclear cells is reduced in tuberculosis patients, and this reduction may be responsible for reduced IFN-γ production (Vankayalapati et al 2000).

(vi) **Interferon-γ**

IFN-γ is a key cytokine in control of *M. tuberculosis* infection. This cytokine is produced by both CD4+ and CD8+ T cells upon infection with tubercle bacilli (Orme et al 1998; Serbina and Flynn 1999) as well as by NK cells. T cells expressing γδ T-cell receptors (γδ T cells) and CD1- restricted T cells, may produce IFN-γ during early infection. IFN-γ is required for the macrophage activation and NOS2 expression. Although IFN-γ production alone is insufficient to control *M. tuberculosis* infection, it is required for the protective response to this pathogen. IFN-γ is the cytokine most invariably detected as protein or mRNA at the sites of human *M. tuberculosis* infection (including in the lung, bronchoalveolar lavage (BAL) fluid, TB pleuritis fluid, and lymph nodes) and in the responses of PBMCs to mycobacterial antigens. IFN-γ is also important for endosome maturation (Russell et al 2010) and the induction of antimicrobial peptides 1 (Fabri et al 2011). To date, IFN-γ gene knockout (GKO) mice are the most susceptible to virulent *M. tuberculosis* (Cooper et al 1993; Flynn et al 1993). Individuals defective in genes for IFN-γ or the IFN-γ receptor are prone to serious mycobacterial infections, including *M. tuberculosis* (Ottenhoff et al 1998). *M. tuberculosis* bacilli grew essentially unchecked in the organs of gene knockout mice, and although granulomas formed, they quickly became necrotic. However in case of *M. tuberculosis*-infected NOS2-/mice, the mean survival time is at least twice that of GKO mice (MacMicking et al 1997), suggesting that there are IFN-γ dependent, NOS2-independent mechanisms of protection against tuberculosis.
2.2.3.2 Anti-Inflammatory Cytokines

The pro-inflammatory response, which is initiated by \textit{M. tuberculosis}, is antagonized by the anti-inflammatory cytokines. Soluble cytokine receptors (e.g., soluble TNF-\(\alpha\) receptors I and II) prevent binding of cytokines to cellular receptors, thereby blocking further signaling e.g., IL-1\(\beta\) is counteracted by a specific antagonist, IL-1Ra. Apart from that, three anti-inflammatory cytokines, IL-4, IL-10, and transforming growth factor beta (TGF \(\beta\)), may inhibit the production or the effects of pro-inflammatory cytokines in tuberculosis.

(i) \textit{Interleukin-10}

IL-10 is secreted by many immune cells including macrophages, neutrophils, DCs, B cells, and T cells (Saraiva and Garra, 2010). IL-10 directly inhibits CD4\(^{+}\) T cell responses, as well as APC function of macrophages and dendritic cells and the production of cytokines such as IL-12, thus, inhibiting the development of Th1 responses infected with mycobacteria (Rojas et al 1999). IL-10 antagonizes the pro-inflammatory cytokine response by down regulation of production of IFN-\(\gamma\), TNF-\(\alpha\), and IL-12 (Fulton et al 1998). In human tuberculosis, IL-10 production is higher in anergic patients, both before and after successful treatment, suggesting that \textit{M. tuberculosis}\- induced IL-10 production suppresses an effective immune response (Boussiotis et al 2000). IL-10 is an immunosuppressive cytokine essential for dampening the immune response and limiting the host immune pathology to numerous intracellular pathogens and gut flora, but, if overproduced, IL-10 can contribute to chronic infection. In addition, IL-10 can inhibit the killing of intracellular pathogens by macrophages, induction of nitric oxide, and production of TNF (Moore et al 2001).

(ii) \textit{Transforming Growth Factor \(\beta\)}

TGF \(\beta\) is present in the granulomatous lesions of tuberculosis patients and is produced by human monocytes and dendritic cells after stimulation with \textit{M. tuberculosis} or lipoarabinomannan (Toossi et al 1995; Dahl et al 1996). It inhibits T cell responses to \textit{M. tuberculosis} as well as participates in macrophage deactivation by inhibiting IFN-\(\gamma\) induced NOS2 production (Rojas et al 1999). Regulation of this cytokine is very complex and occurs at various levels. The \textit{in-vivo} role of TGF \(\beta\) in
protection or pathology in tuberculosis has not been directly tested. Lipoarabinomannan (LAM) from virulent mycobacteria selectively induces TGF β production (Dahl et al 1996). TGF β suppresses cell-mediated immunity in T cells and IFN-γ production; in macrophages it antagonizes antigen presentation, pro-inflammatory cytokine production, and cellular activation. In addition, it is also involved in tissue damage and fibrosis loss, as it promotes the production and deposition of macrophage collagenases and collagen matrix (Tossi et al 1998). TGF β and IL-10 seem to synergize: TGF β selectively induces IL-10 production, and both cytokines show synergism in the suppression of IFN-γ production (Othieno et al 1999). TGF β may also interact with IL-4 and paradoxically in the presence of both cytokines, T cells may be directed towards a protective Th1-type profile (Erard et al 1999).

(iii) Interleukin-4
The deleterious effects of IL-4 in intracellular infections (including tuberculosis) are suppressed IFN-γ production and macrophage activation. In mice infected with M. tuberculosis, and reactivation of latent infection are both associated with increased production of IL-4. Similarly, overexpression of IL-4 aggravates tissue damage in experimental infection (Howard et al 1999). Conversely, IL-4/- mice displayed normal instead of increased susceptibility. IL-4 production did not seem to promote cellular immunity to mycobacteria in two studies, suggesting that IL-4 may be a consequence rather than the cause of tuberculosis development (North et al 1998). In contrast, a recent study on IL-4 knockout mice showed increased granuloma size and mycobacterial outgrowth after airborne infection (Sugawara et al 2000). Compared with control mice, production of pro-inflammatory cytokines was increased in these animals and accompanied by excessive tissue damage. In human studies, a depressed Th1 response but not an enhanced Th2 response was observed in PBMC from tuberculosis patients (Bhattacharya et al 1999; Zhang et al 1995). Elevated IFN-γ expression was detected in granulomas within lymph nodes of patients with tuberculous lymphadenitis, but, little IL-4 mRNA was detected (Lin et al 1996). These results indicated that in humans a strong Th2 response is not associated with tuberculosis.
Figure 2.3 Cellular immune response against M. tuberculosis and its regulation. Inhalation of M. tuberculosis aerosols leads to infection of resident lung alveolar macrophages (1a), neutrophils (1b) and lung DCs (1c), that results in production and secretion of antimicrobial peptides, cytokines, and chemokines. The balance between lipid mediators, prostaglandin E2 (proapoptotic) or lipoxin (LX) A4 (pronecrotic) within infected macrophages decides the downstream pathways either to the induction of apoptosis or necrosis. Infected apoptotic cells can be taken up by resident lung DCs or efferocytosed by uninfected lung macrophages (1c). Within 8-12 days, infected DCs migrate to the local lung-draining lymph nodes under the influence of IL-12(p40) and IL-12(p70) and chemokines CCL19 and CCL21 (2) in order to drive naive T cell differentiation toward a Th1 phenotype (3). Within 14-17 days of initial exposure, these protective antigen-specific Th1 cells migrate back to the lungs in a chemokine-dependent manner (4) and secrete IFN-γ, which further leads to macrophage activation, cytokine production, the induction of microbicidal factors including iNOS (5) and, thus, control bacterial growth. The two specific regulatory pathways (IL-10 and regulatory T cells) normally serve to limit host-induced immune pathology and promote M.tb persistence. The IL-10 induction during infection leads to inhibition of macrophage effector functions, with reduced bacterial killing and impaired secretion of cytokines/chemokines. IL-10 can also block chemotactic factors that control DC trafficking to the draining lymph nodes. In the lymph nodes, both IL-10 and regulatory T cells can block the differentiation of naive T cells to IFN-γ producing Th1 cells, predominantly through direct effects on the DC (self-drawn).
2.2.4 Chemokines

Chemokines are tiny protein molecules (8 to 10 kilodaltons) secreted by cells to induce chemotaxis in nearby cells. The role of certain chemokines is considered pro-inflammatory, with the proteins being recruited to an infection site during an immune response, while other chemokines are thought to have a homeostatic role, controlling cell migration as part of normal tissue growth and maintenance. The action of a chemokine is mediated when it interacts with a chemokine receptor, which is a member of the G protein-coupled receptor (GPCR) family. These are transmembrane receptors that are coupled to intracellular G-protein, which stimulates signal transduction pathways inside the cell when it is activated.

Upon phagocytosis of *M. tuberculosis*, or stimulation with LAM, macrophages produce IL-8 (Juffermans et al 1999). This production is substantially blocked by neutralization of TNF-α and IL-1β, indicating that IL-8 production is largely under the control of these cytokines (Zhang et al 1995). Pulmonary epithelial cells also produce IL-8 in response to *M. tuberculosis*. In tuberculosis patients, IL-8 has been shown to be present in bronchoalveolar lavage fluid, lymph node and plasma (Sadek et al 1998). A second major chemokine is monocyte chemoattractant protein (MCP-1), which is produced by and acts on monocytes and macrophages. *M. tuberculosis* preferentially induces production of MCP-1 by monocytes (Kasahara et al 1994). In murine models, deficiency of MCP-1 inhibited granuloma formation (Lu et al 1998). Also, C-C chemokine receptor 2-deficient mice, which fail to respond to MCP-1, display reduced granuloma formation and suppressed Th1-type cytokine production and die early after infection with *M. tuberculosis* (Peters et al 2001; Boring et al 1997). In TB patients, concentrations of MCP-1 were found to be elevated in broncho-alveolar lavage fluid, serum, and pleural fluid (Kurashima et al 1997; Juffermans et al 1999; Mohammed et al 1998). A third chemokine is RANTES, which is produced by a wide variety of cells and which shows promiscuous binding to multiple chemokine receptors. In human patients, RANTES has been detected in alveolar lavage fluid. Apart from IL-8, MCP-1, and RANTES, other chemokines (such as CCL-2, CCL-3, CCL-4, CCL-5 and MIP-1α, MIP-1β) may be involved in cell trafficking in tuberculosis (Ragno et al 2001).
2.2.5 Cells involved in immune response against *M. tuberculosis*

Both CD4\(^+\) and CD8\(^+\) T cells have a role in fighting *M. tuberculosis* infection. Mycobacteria specific CD4\(^+\) T cells are typically of the helper T cell (Th1) type, and they are potent producers of IFN-\(\gamma\). CD8\(^+\) cells just like CD4\(^+\) T cells can produce IFN-\(\gamma\), but their main function is target cell killing (Flynn and Chan, 2001; Kaufmann et al 1999). By lysing host cells, CD8\(^+\) cells could facilitate the translocation of *M. tuberculosis* from incapacitated cells to more proficient effector cells (Kaufmann et al 1999). Recently Human CD8\(^+\) cells that express granulysin and perforin have been shown to kill *M.tb* directly (Kaufmann et al 1999; Stenger and Modlin, 1998). Granulysin is responsible for bacterial killing and it gains access to *M. tuberculosis* that resides within macrophages through pores formed by perforin. Neutrophils and cytotoxic T cells also contribute to killing of *M. tuberculosis*. Neutrophils show more phagocytic activity and also transport the live bacteria from peripheral tissue to lymphoid organs (Eum et al 2010), whereas, cytotoxic CD8\(^+\) T cell - mediated killing of infected host cells. B cells play a greater role in the host defense against *M. tuberculosis* infection. Follicle-like B cell aggregates in the lungs of TB patients and in the granulomas of mice infected with *M. tuberculosis* have been observed. B cells and their antibodies are likely to orchestrate local host defense and/or immunomodulation in the lung of *M. tuberculosis*– infected hosts by the induction of cytokines, such as IL-10, possibly by the engagement of distinct Fc-gamma receptors (Fc\(\gamma\)R) by antibodies produced by B cells during *M. tuberculosis* infection (Ulrichs et al 2004; Maglione and Chan, 2009; Maglione et al 2008).T cells that express \(\gamma\delta\) TCR, also participate in the immune response against *M. tuberculosis* (Kaufmann et al 1996). \(\gamma\delta\) T cells are stimulated by a unique group of non-proteinaceous antigens that contains phosphate. These phospholigands include different prenylpyrophosphates and nucleotide conjugates, all of which are abundant in mycobacteria. The phospholigands stimulate \(\gamma\delta\) T cells that express the \(V\gamma2\delta2\) chain combination independently of their fine antigen specificity. These cells readily produce IFN-\(\gamma\) after stimulation with phospholigands and express granule dependent mycobactericidal activity (Behr-Perst et al 1999). The \(\gamma\delta\) T cells could, therefore, be responsible for the first line of defense against tuberculosis.
Figure 2.4 Cellular outcomes of *M. tuberculosis* infection and the role of the adaptive immune system. The combination of innate and adaptive responses influences the macrophage response to TB infection. Mycobacterial antigens residing in the early phagosome are processed by MHC class II leading to potent CD4+ T cell stimulation. Phospholipids of mycobacteria stimulate γδ T cells in the absence of known antigen presentation molecules. Presentations of proteins by MHC class I and glycolipid by CD1 is more complex and requires cross priming. Mycobacteria infected macrophages undergo apoptosis. Resulting extracellular vesicles carry antigens to bystander dendritic cells. Uptake of these vesicles results in glycolipid presentation through CD1 and protein presentation through MHC class I. This two-cell mechanism can explain stimulation of MHC class I restricted CD8+ T cells and of CD1 restricted T cells (self-drawn).

T cells with specificity for mycobacterial glycolipids presented by CD1 molecules seem to have a unique role in human tuberculosis (Schaible et al 2000; Ulrichs et al 2000). Group1 CD1 molecules, comprises (CD1a, CD1b and CD1c, are found in primates and guinea-pigs and not in mice (Park et al 2000; Ulrichs et al 2000). Generally CD1 glycolipid- specific T cells produce IFN-γ and express cytolytic activity. In mycobacterial infections, several different T-cell subsets have been found to interact with CD1, including CD4^+^CD8^−^ (double-negative) T cells, CD4 or CD8 single-positive T cells, and γδ T cells (Rosat et al 1999). CD1-restricted
T cells display cytotoxic activity and are able to produce IFN-γ (Stenger et al 1997). Group1 CD1 molecules typically present glycolipids that are abundant in the mycobacterial cell wall, such as phosphatidylinositol mannosides, lipoarabinomannan, mycolic acids and hexosyl-1-phosphoisoprenoids (Ulrichs et al 2000). The different CD1 molecules display distinct intracellular locations, with CD1a being almost exclusively expressed on the cell surface and in the early recycling endosome, CD1b residing primarily in late endosomes/lysosomes and CD1c being localized on the cell surface and in endosomes at different stages of maturation (Schaible et al 2000; Sugita et al 2000). CD1a and CD1c have ready access to mycobacterial glycolipids as M.tb arrests phagosomal maturation at early stages. CD1 molecules are abundantly present on DCs, virtually absent in macrophages and CD1b surface expression is down regulated in cells infected with M. tuberculosis. The transfer of glycolipids from infected macrophages to bystander DCs, therefore, constitutes an important mechanism for promoting CD1 presentation. Group 2 CD1 molecules seem to have a minor role in tuberculosis (Rolph et al 2001).

2.3 Global gene expression studies in tuberculosis

Genome wide approaches enable us to extend our understanding of the cross talk between the intracellular pathogens and their host cells and identify novel mechanisms of bacterial evasion and immunological elimination. The initial interaction between the macrophage and the mycobacterium is thought to play a key role in determining the outcome of infection. Ragno et al (2001) have studied the change in the gene expression profiles in THP-1 cells after 1hr, 6hr and 12hr post-infection with M. tuberculosis, at the levels of transcriptome and proteomic both and found that atleast 375 human genes are involved in immunoregulation, among which, IL-1β showed the highest induction ratio with a 100 fold at 6hr and 400 fold by 12hr. There were no other genes found in the microarray studies, which were consistently down regulated, suggesting that at the early time points there is wide scale switching on of genes encoding proteins involved in the cell migration and homing.
Table 2.2: Gene expression profile of early response genes in THP-1 monocytic cells after *M. tuberculosis* infection (Ragno et al 2001)

<table>
<thead>
<tr>
<th>Class</th>
<th>Induced genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokines</td>
<td><em>IL</em>-8, Osteopontin, <em>MCP</em>-1, <em>MIP</em>-1α, RANTES, <em>MIP</em>-1β, <em>MIP</em>-3α, <em>MPIP</em>-1, PARC, GRO-β, GRO-α, <em>MCP</em>-2, I-309, eosinophil attracting chemokine, eotaxin</td>
</tr>
<tr>
<td>Cell migration</td>
<td>MMP-9 (14 fold), VEGF &amp; its receptor Flk-1, CCR3 chemokine receptor, VCAM-1 cell adhesion molecules, integrin α3</td>
</tr>
<tr>
<td>Cytokines</td>
<td><em>IL</em>-1β (433 fold), <em>IL</em>-2, TNFα (18 fold), MgSOD (macrophage enzyme)</td>
</tr>
</tbody>
</table>

Volpe et al (2006) studied the transcription patterns of human monocyte derived macrophages upto 7 days after infection (7dpi) with *M.tb* and found 858 human genes, which were involved in immunoregulation. Pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 were consistently up-modulated in human macrophages as well as in THP-1 cells during *H₃₇Rᵥ* infection and they also investigated expression of some *M.tb* genes involved in the bacterial response, i.e., *sig A, sig E, sig G, acr, sod C* (3 regulatory genes) and 16S rRNA as an internal control.

Thuong et al (2008) studied macrophage transcriptional profiles in individuals with 3 different clinical forms of TB: latent, pulmonary, meningeal TB, and found more than 38,500 genes from *ex-vivo M.tb* stimulated macrophage in 12 subjects. Gene expression profiles in *M.tb* stimulated and unstimulated macrophages were
compared and 1,608 and 199 genes were found that were differentially expressed by more than 2- and 5- folds, respectively. Both Microarray and RT-PCR results showed that expression of CCL1 (CC chemokine family) distinguished the above three clinical groups and 6 Single Nucleotide Polymorphisms (SNPs) were found to be associated with CCL1 TB. Mistry et al (2007) performed whole blood transcriptional profiling from individuals with 4 clinical TB groups (active, latent, cured and recurrent TB) and found 9 genes, which could distinguish the above groups. Jacobsen et al (2011) identified up regulation of particular discriminating immunoregulatory genes, including JAK3, SOCS3, and IL2RA in T cells of active TB patients compared with latent TB patients.

2.4 Antigens of *Mycobacterium tuberculosis*

Till today, a number of mycobacterial proteins or antigens have been identified, which have immunological relevance. Cell wall (but perhaps not cytoplasmic) polysaccharides, proteins, and peptides all have been shown to be antigenic proteins. Some of them are PPD, Ag85, MPT64, PE/PPE, ESX-family proteins etc (Cole et al 1998). Some antigens responsible for the latency of the bacteria are: hspX, Ag85B, Ag85A, TB10.4etc. Basically PE/PPE proteins, ESAT-6 and CFP-10 coded by ESX systems in mycobacteria etc. show antibody mediated as well as T cell mediated immune response, which are found only in pathogenic mycobacteria.

2.4.1 Early Secreted Antigen Target-6 (ESAT-6)

ESAT-6 (Early Secreted Antigenic Protein-6) is a small secreted protein of *Mycobacterium tuberculosis* crucial for virulence and immunity (Pallen, 2002). ESAT-6 can induce specific T cell response and N terminal of ESAT-6 is associated with it (Wang et al 2009). Multiple T cell epitopes can be found within the ESAT-6 protein, and can be recognized by IFN-γ- secreting T cell lines from donors with various HLA-DR phenotypes.
Figure 2.5 Genomic localization of M. tuberculosis antigens in the genome. Gene encoding ESAT-6, together with its partner CFP-10 (Culture Filtrate Protein-10), locates within the RD1 (region of deletion) of M. tuberculosis genome encoded by Rv3875 and Rv3874, respectively; belonging to a 9455 bp fragment covering genes for Rv3871–Rv3879c. A specialized transport system ESX-1, dedicated to the secretion of CFP-10/ESAT-6 complex, consists of genes Rv3866–Rv3883c. Genes within ESX-1 indispensable for the secretion of ESAT-6/CFP-10 complex are Rv3866, Rv3868, Rv3869, Rv3870, Rv3871, PE35 (Rv3872), Rv3872 and MycPI (Rv3883c). PPE-68 (Rv3873), nonessential for the ESX-1 secretion system, might function as a gating protein to forestall the secretion of premature proteins. The secretion of CFP-10 and ESAT-6 relies intimately on the proteins encoded by the distant Rv3613c–Rv3616c operon (self-drawn).

RD1 is closely related to the virulence of M. tuberculosis. RD1 can be found only in pathogenic Mycobacterium bovis, M. tuberculosis, and absent in attenuated BCG. The molecular mass of ESAT-6 is 9903.87Da. The amino acids sequence of ESAT-6 is highly conserved among mycobacterium species, identity of M. bovis, M. kansasii, M. marinum, M. smegmatis, and M. leprae with M. tuberculosis being 100%, 98%, 92%, 72% and 38%, respectively. ESAT-6 and its putative chaperone CFP-10 form a tight 1:1 heterodimers. ESAT-6 and CFP-10 are co-transcribed and secreted and can form protein complex. Both ESAT-6 and CFP-10 belong to ESAT-6/WXG-100 superfamily and controlled
by the same promoter. WXG-100 superfamily proteins are characterized by a conserved WXG motif, about 100 amino acids long, and co-transcribing pairs with its partners, and locating downstream of a PE, PPE gene couplet. Therefore, ESAT-6 and CFP-10 are co-transcribed and secreted and can form protein complex, even in low pH circumstance, such as within mature phagosome. C-terminal 11 residues of ESAT-6 are dispensable for the interaction with CFP-10 (Lightbody et al 2008; Brodin et al 2005). ESAT-6 based novel vaccines are under intensive exploration, including combining the DNA vaccine encoded by ESAT-6 (DNA-E6) with BCG immunization(Fan et al 2007), recombinant BCG expressing ESAT-6, fusing ESAT-6 with another antigen of M.tuberculosis, such as MPT64, Ag85, and ubiquitin-fused ESAT-6 DNA vaccine. Ruchi et al (2008) have shown that recombinant BCG- rBCG85C overexpressing Ag85C shows more efficacy in controlling the infection in guinea pigs against M.tuberculosis infection compared to the parental BCG vaccine, which was associated with the reduced secretion of pro-inflammatory cytokines,-IL-12, IFN-γ, TNF-α, moderate levels of anti-inflammatory cytokines-TGF β and up regulation of inducible nitric oxide synthase (iNOS).

2.4.1.1 Role of ESAT-6 in M.tuberculosis invasion

(i) ESAT-6 modulates macrophage signaling

Recombinant CFP-10 and ESAT-6 (r-CFP-10-ESAT-6 or rCE) can enhance the TNF-α release from human monocytes and THP-1 cells alone in a dose- and time dependent fashion. However, prolonged rCE treatment during monocyte to macrophage differentiation can suppress the TNF-α production stimulated by LPS (Feng et al 2008). ESAT-6 can affect the signaling pathway, especially the ERK1/2 MAPK pathway, in macrophages to modulate the activation of macrophages but, whether ESAT-6/ CFP-10 can be a signaling molecule capable of activating macrophages via boosted IFN-γR1 expression, is controversial (Ganguly et al 2007; Guo et al 2010).

ESAT-6 plays a key role in IL-1β secretion and inflammatory response. ESAT-6 activates the NLRP3/ASC inflammasome and caspase-1, and promotes the IL-1β maturation. ESAT-6 can also facilitate the delivery of mycobacterial PAMPs, such as Ag85 into the macrophage cytosol, which further promotes caspase-1 activation and IL-1β secretion. Ectopic expression of the ESAT-6/CFP-10 fusion proteins in macrophages reduced the expression levels of autophagy- related genes.
(ATGs), $atg5$, $atg8$ and $atg12$, and so regulates the autophagosomes formation (Zhang et al. 2012). ESAT-6 can induce the apoptosis of THP-1 human macrophages by upregulating the expression of caspase 1, 3, 5, 7 and 8 genes. Among them, the activation of caspase-8 indicates that ESAT-6 might induce apoptosis via the receptor-mediated extrinsic pathway, but not the intrinsic mitochondrial pathway (Derrick and Morris, 2007).

**(ii) ESAT-6 modulates TLR- NF-κB cross talk**

ESAT-6 can promote both Th17 and Th1 responses via TLR-2/MyD88 signaling crucial against *M. tb* infection. In BCG-vaccinated or LPS-treated DCs, ESAT-6 can significantly suppress the up regulation of miR146a, a negative feedback regulator in TLR signaling by inhibiting IL-1R associated kinase (IRAK)-1 and TRAF6, thereby impairing the activity of NF-κB and ultimately increasing the expression of NF-κB-dependent genes IL-6 and TGF β. IL-6 and TGF β can create an environment favorable for the differentiation of Th17 cells (Akira et al. 2001). After TLR signaling, ESAT-6 can inhibit the activation of transcription factor NF-κB and interferon regulatory factors (IRFs), thereby, lowering the expression of the NF-κB-dependent genes IL-12, IL-6 and TNF. ESAT-6 directs TLR signaling pathway by direct binding to TLR2, activating PI3K-AKT-dependent kinases, instead of MyD88-IRAK-IKK signaling pathway, that precludes the interaction between the adaptor MyD88 and downstream kinase IRAK4, thereby abolishing otherwise NF-κB activation. The C-terminal amino acid residues 90–95 in ESAT-6 are essential for the attenuation effect on TLR-2 signaling pathway (Pathak et al. 2007). Furthermore, the ESAT-6, CFP-10 and ESAT-6/CFP-10 complex inhibit the production of lipopolysaccharide (LPS)-induced reactive oxidative species (ROS) in RAW 264.7 macrophages, which downregulated the NF-κB p65 binding activity, thus, inhibiting the expression of NF-κB-dependent genes.

**(iii) ESAT-6 modulates TCR signaling pathway**

ESAT-6 can inhibit IFN-γ production by T cells at transcriptional level by inhibiting the expression of ATF-2 and c-Jun, two positive transcription factors of IFN-γ gene expression. The ESAT-6 can also bind to CD3+ T cells, inhibit the expression and proliferation of T cell activation markers, CD25 and CD69, without affecting TCR-
induced ZAP70 phosphorylation, suggesting that ESAT-6 might directly inhibit human T cell responses by affecting TCR signaling pathways downstream of ZAP70 (Wang et al. 2009). Recombinant ESAT-6 can inhibit the secretion of IL-12 induced by multiple TLR ligands (Pathak et al. 2007), suggesting the indirect inhibition of T cell IFN-γ secretion by reducing IL-12 production by APCs (Wang et al. 2009).

(iv) *ESAT-6 modulates MAPK signaling pathway*

ESAT-6 can induce the phosphorylation of extracellular signal regulated kinases 1/2 (ERK1/2) in the cytoplasm and antagonize the phosphorylation of ERK1/2 induced by LPS in the nucleus via certain phosphatase(s). Moreover, ESAT-6 down-regulated the expression of LPS induced gene c-Myc in an ERK1/2-dependent fashion. The c-Myc gene is a MAP kinase inducible transcription factor that promotes cell proliferation and apoptosis (Tsai et al. 2009). ESAT-6 regulates gene expression within macrophages by limiting the ERK1/2 activation (Ganguly et al. 2007). ESAT-6 induce IL-8 expression by increasing IL-8 gene transcription and mRNA stability. ESAT-6 induction of IL-8 promoter activity is dependent on nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) binding and sensitive to pharmacological inhibition of PKC and ERK and p38 MAPK pathways. ESAT-6 activate ERK- and p38- MAPK phosphorylation, and rapidly induced reactive oxygen species (ROS) production.

2.4.2 CFP-10

In the mycobacterial cell, CFP-10 is also responsible for the virulence of the pathogen. Both ESAT-6 and CFP-10 are interdependent on each other for stability. *M. tb* secretory protein, CFP-10/MTSA-10 leads to modulation of host cell signalling through down regulation of innate ROS levels in the cultured host cell. The reduction in ROS levels due to CFP-10 treatment of cells is the likely cause for increased activity of the phosphatases and consequent decrease in both tyrosine and serine/threonine phosphorylation of the cellular proteins (Basu et al. 2006). This ROS suppression, caused by CFP-10, attenuate both, the magnitude and kinetics of substrate phosphorylation, by ERK. Because ERK, along with other effector molecules, carries the signal to the nucleus, CFP-10 could lead to further down regulation of the activation markers and early response genes. CFP-10 when present alone on the cell surface, exert an inhibitory effect on the macrophage ROS
generation that is similar to the one obtained with the CFP-10–ESAT-6 complex. It is CFP-10 in the complex that plays the role of modulator of immune function. In addition, CFP-10 is responsible for preventing digestion of the CFP-10–ESAT-6 complex with lysosomal enzymes (cathepsins L and S), suggesting inhibitory/regulatory role of CFP-10 in the antigen processing (Basu et al 2006).

Table 2.4: Virulence factors of *Mycobacterium tuberculosis* (Sakamoto et al 2012)

<table>
<thead>
<tr>
<th>Factor (Gene)/ Proteins</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ESAT-6 (esxA) and CFP-10 (esxB)</td>
<td>T cell stimulation, elicitation of DTH, Downregulate ROS production in macrophages, Block TLR2-mediated signaling, Pore formation, apoptosis, cytolysis</td>
</tr>
<tr>
<td>2 α-crystallin (acr)</td>
<td>Antigenic; potential role in triggering latency</td>
</tr>
<tr>
<td>3 Antigen 85 complex (fbpA, fbpB, fbpC)</td>
<td>Antigenic; mediates attachment to macrophages,</td>
</tr>
<tr>
<td>4 Erp (erp)</td>
<td>Required for intracellular growth</td>
</tr>
<tr>
<td>5 Cholesterol transporter (Mce4)</td>
<td>Major cholesterol uptake system, important for survival during chronic phase</td>
</tr>
<tr>
<td>6 Enzymes and lipid carriers (lgr locus)</td>
<td>Cholesterol metabolism; important for growth</td>
</tr>
<tr>
<td>7 Isocitratetlyase (ICI I)</td>
<td>Allows shift to use of fatty acids as major carbon source; important for chronicity, persistence,</td>
</tr>
<tr>
<td>8 Catalse-peroxidase-peroxynitritase (KatG)</td>
<td>Protection against ROI and RNI</td>
</tr>
<tr>
<td>9 Alkyl-hydroperoxideructase (ahp C)</td>
<td>Protection against ROI and RNI</td>
</tr>
<tr>
<td>10 Superoxide dismutases (sod A, sod C)</td>
<td>Protection against ROI and RNI</td>
</tr>
<tr>
<td>11 Nitric oxide reductase (noxR3)</td>
<td>Protection against ROI and RNI</td>
</tr>
<tr>
<td>12 Lipoarabimannan, such as Mannose-capped (ManLAM)</td>
<td>Inhibition of DC maturation; induction of IL-10, Inhibition of phagolysosomal fusion, protection against ROI; inhibition of protein kinase C activity; block transcription of IFN-γ-inducible genes.</td>
</tr>
<tr>
<td>13 Mycolic acids</td>
<td>Role in granuloma formation, macrophage activation required for mycobacterial survival, Biofilm formation</td>
</tr>
<tr>
<td>14 Trehalosedimycolate</td>
<td>Granuloma formation, proinflammatory, cachexia, Decrease in NAD, Damage to host cell membranes, damage to mitochondria, induction of apoptosis, inhibition of phagosomal- lysosomal fusion</td>
</tr>
<tr>
<td>15 Phenolic glycolipids</td>
<td>Immunosuppression</td>
</tr>
<tr>
<td>16 Sulfolipids</td>
<td>Increase macrophage infectivity, impair macrophage activation by inhibiting phagosomal maturation, blocks priming by IFN-γ</td>
</tr>
</tbody>
</table>
2.5 Animal models of TB

A number of animal models have been tested for their ability to study the pathogenesis and immune responses to *Mycobacterium tuberculosis* infection. Different animal models that have been used in recent years are mice, guinea pig, rabbit and Non-human primates (NHP). Among them, inbred mice are most popular model because of cost effectiveness. In terms of understanding susceptibility of the disease, guinea pig and rabbit are considered to be better animal models for TB research. On the other hand, the genetic manipulations in mice are easier, which makes them model of choice for infection studies (Gupta et al 2005).

2.5.1 Comparison between different animal models of tuberculosis

(i) Mouse:

Advantages/similarities to human TB: Like most humans, mice are able to generate a strong immune response to *M. tuberculosis*, resistant to low dose inocula and can contain the infection without developing active, disseminated disease. Infection is likely to revive/reappear, as the animal grows old. A huge database of immunological reagents is available for all the parameters of mice model. Role of various immunological mechanisms including that of Th1/Th2 paradigm and the role of CD4+ and CD8+ T cells, Toll-like receptor system have been well established. Mice are cost effective, both in terms of purchase and housing.

Disadvantages: Resistance of mice to tuberculosis represents one of the significant disadvantages of the mouse model.

(ii) Guinea pig:

Advantages/similarities to human TB: GPs are hormonally and immunologically closer to humans than rodents. They require an exogenous supply of ascorbic acid (vitamin C) in the diet, like human beings. They are corticosteroid - resistant like humans and non-human primates and show striking similarities in response of lung to inflammatory stimuli. They respond quite well to anti-tuberculosis antibiotics and are of prime importance for the search of drugs against MDR resistant strains of *M. tuberculosis*.

Disadvantages: There is paucity of readily available immunological reagents that are required for the qualitative and quantitative evaluation of the immune responses. GPs have high cost compared with rodents and they need excellent husbandry practices.
(iii) Rabbit:

**Advantages/similarities to human TB:** Rabbit is an excellent laboratory model for TB because it displays a spectrum of disease that represents many specific stages of the human disease. They show relative pathogenicity, including disease severity with strain genotype, which may help in identifying stage-specific \( M. \text{tuberculosis} \) genes important in human disease (Bishai et al 1999; Converse et al 1998).

**Disadvantages:** Lack of immunological reagents and high cost of purchasing are the most prime disadvantages. Extensive BSL-3 space and caring is required for rabbits compared with an equal number of mice or guinea pigs.

(iv) Monkey (Non-human Primate):

**Advantages/similarities to human TB:** The non-human primate, monkey, is similar to humans in terms of pathology and immunology. There is availability of immunologic and other reagents, ease of procedural manipulations, the ability to perform experimental infections and schedule endpoints, tissue availability, and potential to conduct studies with simian immunodeficiency virus (SIV) co-infection, that make it a suitable model for tuberculosis in AIDS patients.

**Disadvantages:** High cost of purchasing and containment of biohazards are the main disadvantages of this animal model. In addition, concerns about animal right activists, and the general perception that monkeys are exquisitely susceptible to tuberculosis have limited the use of this model.

2.5.2 Mouse tuberculosis as a model of the human TB disease:

The growth of \( M. \text{tuberculosis} \) in mice has been well characterized. The intravenous and aerogenic routes of infection are most commonly used. Given below are the number of factors which influences the course and rate of mycobacterial infections:

(i) **Virulence of the M.tb strain:** The virulence of a given strain of \( M. \text{tuberculosis} \) further depends extensively on experimental conditions, such as the route of infection, the manner of preparation of the suspension, and the dispersion and size of the suspension. Some host-associated factors such as severe malnutrition (affects cell mediated immunity), stress, terminal cancer, diabetes, kidney failure, degenerative lung disease and chronic vitamin D deficiency can also influence virulence.

(ii) **Organ in which bacilli are lodged:** Bacilli in the liver do not multiply as fast as in the spleen. Bacteria may also infect phagocytic cells in the lungs and kidney
tissues. These bacteria only grow in immuno-competent mice but may eventually cause abscesses.

(iii) **Size of the inoculum:** There is an inverse relationship between the inoculum size and the time duration in which the inoculum grows in the mouse to a size that triggers acquired immunity.

(iv) **Intensity of the immune response:** The genetic differences among various inbred strains of mice influence the course of disease.

*M. tuberculosis* infection in mice is dependent on the genetic background of the animal. Inbred mouse strains can be ranked by their median survival time following infection, although susceptibility of inbred mice can vary depending on the route of infection, the dose, and the strain of *M. tuberculosis* used (Table 1) (Chackerian et al 2003). Medina and North (1998) identified susceptibility variations in several inbred mouse strains based on differences to *bcg* gene (resistance is controlled by a single genetic locus, *Nramp1*) and histocompatibility complex haplotype effects.

**Table 2.5:** List showing the susceptibility status of inbred mice and their genetic background

<table>
<thead>
<tr>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Sn</td>
<td>BALB/c*</td>
<td>A/J</td>
</tr>
<tr>
<td>BALB/c</td>
<td></td>
<td>C3H*</td>
</tr>
<tr>
<td>C57BL/10</td>
<td></td>
<td>CBA</td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td>DBA/2J</td>
</tr>
</tbody>
</table>

*Most laboratories have found BALB/c mice to be susceptible following intravenous infection, although they are generally more resistant than C3H or CBA mice (Flynn et al 1995). After aerosol infection, the survival of BALB/c mice is similar to C57BL/6 mice. * Includes the C3H substrains: C3H/HeJ, C3H/SnJ, C3H/HeOuJ, C3HeB/FeJ and C3H.SW-H2b.

**2.5.2.1 Different routes of infection and disease progression**

The infection in mice initiated via the respiratory route does not stay confined to the lungs but disseminates via lymph and blood to infect other organs. Infection in the liver and spleen, for example, is evident by about day 15 (Chackerian et al 2002), and progresses until about day 20 when further *M.tb* growth ceases and infection is held at a stationary level, almost certainly because of the expression of systemic immunity (Mogues et al 2001). Because of the short period of *M.tb* growth in the liver and spleen, the level of stationary infection reached in these organs is much lower than that reached in the lungs, whereas, in the case of infection through intravenous
(i/v) route (Medina et al 1996; Medina et al 1999), the bulk of an i/v inoculum is implanted in the liver (95%) and spleen (about 4%); however, only 0.1% is implanted in the lungs that is responsible for the disease that follows. To ensure that the lung is infected with $10^2$ colony forming units of \textit{M.tb}, it is necessary to give about $10^5$ viable bacilli via i/v route. It is in the lungs that the pathogen grows progressively for the longest period of time (20 days) before further \textit{M.tb} growth ceases and stationary infection ensues. This compares with only about 10 days of progressive growth in the liver and spleen before infection is controlled and held at a stationary level in case of infection via i/v route (Medina et al 1999).

\textbf{2.5.2.2 Pattern of infection in resistant mice vs susceptible mice}

In resistant C57BL/6 and BALB/c strains, initiation of infection via the respiratory route with a small number ($10^2$ or fewer) of \textit{M.tb} is followed by about a twenty-day period of log linear bacterial growth in the lungs. The average doubling time of the pathogen during this progressive phase of infection is about twenty-eight hours. The progressive phase ends with the inhibition of further \textit{M.tb} growth, which is followed by a stationary level of infection that persists from day twenty until the mice succumbs in about ten-twelve weeks’ time. On the other hand, \textit{M.tb} growth in the lungs of susceptible mice (DBA/2J mice) is not inhibited, but progresses at a slower rate until death. Growth is fast enough after three weeks of infection to result in a 2 log higher level of lung infection than in resistant strains (Medina et al 1996; Medina et al 1999).

\textbf{2.5.2.3 Pattern of infection in the mouse versus rabbit and guinea pig}

In all the three host species, lung infection progresses for about 20 days before further \textit{M.tb} growth is inhibited and infection is held at a stationary level. Thus, in all three host species, it is apparent that immunity is not expressed in the lungs until 3 weeks of infection and that it is incapable of causing infection to resolve. Therefore, according to the ability to control lung infection, guinea pigs are not more susceptible to tuberculosis than mice of resistant strains, and are even less susceptible than mice of susceptible strains that are incapable of stabilizing lung infection. On the other hand, guinea pigs show progressive disease in the spleen, a characteristic that has limited relevance to the disease in most susceptible humans (Schell et al 1974; Alsaadi et al 1973; Lurie et al 1955).
2.5.2.4 Lung Pathology in the mouse versus guinea pig and rabbit

As in mice, stationary lung infection in the rabbit and guinea pig causes progressive pathology, which is different from that which develops in the mouse. The macrophages that accumulate at sites of infection are not distributed among intact air sacs, but form true granulomas composed of compact aggregates of \textit{M.\textit{tb}} infected macrophages. The granulomas appear to be anatomically distinct structures, resembling those that form early in the lungs of \textit{M.\textit{tb}} infected humans. The centers of granulomas can undergo necrosis and liquification, and this can lead to the formation of cavities, particularly in the lungs of rabbits. There is no doubt that the histopathology of tuberculosis in the rabbit and guinea pig is more human like than in the mouse during early stages of infection. The reason for this is not known, but it is worth considering two key differences between the lungs of mice and of guinea pig, rabbits, and other larger mammals. First, although the lungs of mice are smaller, they are not structurally equivalent to a small piece of guinea pig or rabbit lung. Instead, the mouse lung has a miniaturized architecture, with alveoli that are a fraction of the volume of those in the lungs of rabbits and guinea pigs, which are smaller, in turn, than those in the human lung. The second difference is that the lungs of rabbits and guinea pigs, but not of mice, contain intrapulmonary lymphoid tissue in the form of bronchus-associated lymphoid tissue (BALT). In the mouse lung, in contrast, BALT is inducible and is formed in response to infection. It seems possible that some \textit{M.\textit{tb}}-induced granulomas in guinea pigs, rabbits, and humans form within BALT. Infection-induced pathology in all these host species is of a chronic type that leads to lung consolidation.

2.6 Tuberculosis infection and gene disrupted mice:

In the last decade, large amount of thrust was given in understanding the molecular mechanism of immune response during \textit{M.\textit{tb}} infection. In post-genomic era, the use of gene-disrupted mice has led to new insights into how several immunological components protect the mammalian host against \textit{M.\textit{tb}} infection. Several studies using gene knockout mice provided a lot of information about the specificity and importance to different components of immune response (Gallegos et al 2011; reviewed in Tani and Schurr, 2005; Flynn et al 1993; Green et al 2012).
Table 2.6: List demonstrating the response of different gene knockout mice to *M. tb* infection

<table>
<thead>
<tr>
<th>Gene knock out mice/Category</th>
<th>Function</th>
<th>Response to Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lymphocyte Immunity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHC Class II</td>
<td>Presentation of antigens to CD4(^+) T cells</td>
<td>+++</td>
</tr>
<tr>
<td>MHC Class I (beta 2m)</td>
<td>Presentation of antigens to CD8(^+) T cells</td>
<td>++</td>
</tr>
<tr>
<td>TAPI1</td>
<td>Transport of peptide antigens</td>
<td>+</td>
</tr>
<tr>
<td><strong>Cytokines and associated response elements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>Macrophage activation</td>
<td>++++</td>
</tr>
<tr>
<td>IFN-(\gamma)R1</td>
<td>Receptor for IFN-(\gamma)</td>
<td>+++</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal transducer and activator (downstream of IFN-(\gamma))</td>
<td>+++</td>
</tr>
<tr>
<td>LRG-47</td>
<td>IFN-inducible GTP-binding protein</td>
<td>+++</td>
</tr>
<tr>
<td>IRF-1</td>
<td>Interferon regulatory factors, signaling molecule for IFN-(\gamma)</td>
<td>+++</td>
</tr>
<tr>
<td>IL12-p70</td>
<td>Differentiation of CD4(^+) T cells to Th1 cells</td>
<td>+++</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>Pro-inflammatory cytokine, macrophage activation</td>
<td>++++</td>
</tr>
<tr>
<td>TNFr</td>
<td>Receptor for TNF-(\alpha)</td>
<td>++++</td>
</tr>
<tr>
<td>LT1(\alpha)</td>
<td>Cytotoxicity</td>
<td>+++</td>
</tr>
<tr>
<td>iNos</td>
<td>Synthesis of reactive nitrogen intermediates</td>
<td>++</td>
</tr>
<tr>
<td>IL12-p40</td>
<td>Subunit of IL-12p70 and IL-23</td>
<td>++</td>
</tr>
<tr>
<td>STAT4</td>
<td>IL-12R signaling molecule</td>
<td>++</td>
</tr>
<tr>
<td>IL-18</td>
<td>IFN-(\gamma) inducing factor</td>
<td>+</td>
</tr>
<tr>
<td>IL-6</td>
<td>Pro-inflammatory cytokine</td>
<td>+</td>
</tr>
<tr>
<td>IL-1(\beta/\beta)</td>
<td>Pro-inflammatory cytokine</td>
<td>+</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Receptor for IL-1</td>
<td>+</td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2 cell type cytokine</td>
<td>+</td>
</tr>
<tr>
<td><strong>Cellular migration and granuloma formation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein</td>
<td>++</td>
</tr>
<tr>
<td>CCR2</td>
<td>Receptor for MCP-1</td>
<td>+++</td>
</tr>
<tr>
<td>CCR-5</td>
<td>Coreceptor for HIV-1</td>
<td>+</td>
</tr>
<tr>
<td>CXCR-3</td>
<td>T cell migration</td>
<td>+</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Adhesion molecule</td>
<td>++</td>
</tr>
<tr>
<td>CD44</td>
<td>Adhesion of leukocytes</td>
<td>++</td>
</tr>
</tbody>
</table>

* Effect on susceptibility: ++++ extreme increase; +++ large increase; ++ moderate increase; mild increase
Chapter 2  
Review of Literature- Nuclear matrix protein and Gene regulation  

2.7 Nuclear matrix proteins and Gene regulation

2.7.1 Nuclear Matrix

Nuclear matrix is a proteinaceous RNA- rich network of thick polymorphic fibres, underlying which are thinner core filaments. The network of fibers and filaments that constitutes the nuclear matrix supports the structural properties of the nucleus as a cellular organelle and accommodates modifications in gene expression, associated with proliferation and differentiation and changes necessary to sustain phenotypic requirements in specialized cells (Philimonenko et al 2001). The nuclear matrix also contains molecules involved in mRNA processing including splicing factors and the heterogeneous nuclear ribonucleoproteins (hnRNPs) important in RNA biogenesis (Mattern et al 1996; Blencowe et al 1994). DNA polymerase, nascent DNA fragments and other factors involved in replication are also the essential component of nuclear matrix. Nuclear matrix also participates in spatially organizing DNA sequences in the nucleus.

2.7.2 Matrix attachment regions (MARs)

Chromatin is organized in loop domains that are formed by the periodic attachment of chromatin, at DNA sequences called matrix (or scaffold) attachment regions (MARs), to nuclear-matrix sites. Specific MAR binding proteins have been reported to bind to these MARs (Bode et al 1992). All the cellular processes are highly co-ordinated and programmed and this demands the genome to be organized as a set of genes and gene clusters. Such an orderly arrangement of nuclear domains is brought about by anchorage of specific sequences to the matrix at interphase (Berezney and Coffey, 1974) and chromosomal scaffolds during mitosis (Mirkovitch et al 1984). These signature sequences, known as S/MARs (Scaffold/ Matrix Attachment Regions), serve as boundary elements that punctuate chromosomal DNA into topologically restricted functional units, defining borders between chromatin domains (Breyne et al 1992). MARs have been assigned by characteristic MAR recognition signature (MARS) that consists of two degenerate sequences ‘AATAAYAA’ and ‘AWWRTAANNWWGNNNC’. These are the cis elements mostly flanking transcribing regions and occur in introns, centromeres and telomeres (Bode et al 2000; Van Drunen et al 1999; Bode et al 1995). They represent 200–300 bp, AT-rich DNA sequences and occur at an average of 1 for every 30 kb of eukaryotic DNA. Several studies also indicate the insulator nature of these elements that control
Chapter 2  Review of Literature- Nuclear matrix protein and Gene regulation

multiple gene expression, by serving as Locus Control Regions (LCRs) (Fields et al 2004; Li et al 2002; Grosveld et al 1987). MARs are also known to aid cell specific expression by their co-habitation with enhancers and reduce the position effect variegation from local chromatin structures (Forrester et al 1994; Blasquez et al 1989). Thus, they orchestrate topological organization of functional chromatin domains. Since MARs organize and govern the accessibility to local chromatin structures, they are also targets for viral integration and replication. Most of these integration sites (95%) have been shown to be flanked by S/MARs, around 1 kb region of integration that could serve as promoters (Johnson and Levy, 2005; Kulkarni et al 2004).

Figure 2.6 Model demonstrating nuclear matrix and MAR Binding proteins. MAR-matrix attachment regions, MARBPs- matrix attachment binding proteins (self drawn).

2.7.2.1 Role of MARs in transcriptional regulation

The base unpairing property is a common feature of MARs; they unwind as a consequence of stress that might be imposed by supercoiling or by association with protein factors. The base unpairing regions (BURs) in MARs are the regions that become base unpaired under negative superhelical strain (Bode et al 1996). MARs are bound to the nuclear matrix, either constitutively or transiently. The higher-order chromatin structure of interphase and metaphase chromosomes is likely to be
maintained by constitutive MARs. The dynamic associations of transient MARs are more likely to be implicated in genomic function, as they correlate with transcription or replication of the genetic loci with which they are associated (Heng et al 2004). MARs can either activate or repress transcription depending upon the assembly of factors recruited at the site. Few examples of transcriptional regulation by MARs are human β-globin gene cluster, regulation of Th2 cytokine gene locus etc (Ostermeier et al 2003). The molecular mechanisms by which MARs reorganize higher-order chromatin structure have been investigated in detail at the mouse Th2 cytokine locus, which contains a cluster of co-coordinately regulated genes, *Il4, Il13* and *Il5*, in a region of about 120 kb (Ottaviani et al 2008). These genes are silent in naive T cells but expressed in Th2 cells. Following activation of Th2 cells, expression of the nuclear matrix protein SATB1 is rapidly induced, and MARs within the locus mediate the formation of small loops by anchoring the loops onto a common protein core associated with SATB1 (Cai et al 2006). Studies from our lab have shown that MARs can stimulate HIV-1 LTR transcription in the absence of functional Tat (Kulkarni et al 2004). Thus, MARs itself can act as promoter and can, therefore, influence the transcription of genes, which harbor them.

### 2.7.3 Nuclear Matrix-associated MAR Binding Proteins (MARBPs)

The specific set of proteins that tether matrix attachment regions MARs to the nuclear matrix and form topologically independent loop domains of chromatin are known as MAR binding proteins (MARBPs). Associations of MARs and MARBPs have been shown to play an important role in gene regulation by altering chromatin in stage and tissue specific manner. Several studies report the ability of these MARBPs to control gene expression by binding to MAR sequences within the regulator regions of the gene and activate or repress the gene expression (Sinha et al 2010; Singh et al 2009). They are often found in a complex with co-activators or co-repressors, modulating gene function by remodeling or covalently modifying the chromatin structure. Most of these MARBPs are drastically affected upon malignant transformations. Aberrant gene expression gives rise to malignancies and abnormal cell cycle progression. Therefore, understanding the nature and order of these proteins assumes greater importance in the current scenario of chromatin biology and disease manifestation. The MARBPs have also been identified to play major role in normal tissue differentiation and organogenesis. Certain novel isoforms of MARBPs have been
suggested to play key role in disease manifestation. MARBPs with their newly discovered role as linkers between chromatin remodeling, signal transduction and cell cycle regulation form an important part of chromatin biology. Till today, a number of MARBPs have been identified, namely, Bright, SATB1, Cux/CDP, SMAR1, topoisomerase II, histone H1, lamin B1, SAF-A, HMG-I(Y), hnRNP-A1/B1, AP1, nucleolin. PARP-1, Ku autoantigen, p53, SAF-A, Nucleolin, CTCF, YY1, PCNA, etc.

2.8 SMAR1 – a nuclear matrix associated MARBP

SMAR1 was identified as novel DNA binding protein from murine thymocyte expression library screening (Chattopadhyay et al 2000). The human homolog of SMAR1 named BANP was isolated as a BTG3 associated nuclear protein using yeast two-hybrid approach (Birot et al 2000). SMAR1 gene encodes a 2.1 kb long mRNA that corresponds to 60 kDa full length protein (548 amino acids). SMAR1 transcript is differentially expressed in all organs, like heart, lung, thymus, spleen, liver, brain and kidneys. However, the expression is high in thymus as compared to other organs (Chattopadhyay et al 2000). SMAR1 shares significant homology with other MARBPs, such as Cux/CDP, Bright and SATB1, in the MAR binding domains. The MAR binding domain of SMAR1 resides within 352-394 amino acids and is identical to that of SATB1. An extended stretch of same region (328-420 aa) shares significant homology with Cut repeat box I (330-371 aa) and box II (365-381 aa) of Cux/CDP. In addition to this, 398- 456 aa region of SMAR1 is homologous to the tetramer domain of Bright (Kaul-Ghanekar et al 2005; Chattopadhyay et al 2000). The small domain of SMAR1 within 160-350 aa contains nuclear localization signal (NLS). The 160-350 aa region of SMAR1 also contains arginine-serine rich motifs and serves as a protein interaction domain, while the C-terminal region 350-548 aa is characterized as a DNA binding domain (Jalota et al 2005; Rampalli et al 2005). Additionally, SMAR1 also contains the BEN domain residing between 218-291 amino acid residues (Abhiman et al 2008). The BEN domain is predicted to mediate protein-DNA and protein-protein interactions during chromatin organization. The C-terminal region of the BEN domain overlaps with the MAR-binding region in SMAR1. The BEN domain is highly conserved and is predicted to be present in proteins that functions as transcription factors. It also functions as an adaptor for the higher-order chromatin structure and recruitment of chromatin modifying factors in transcriptional regulation.
2.8.1 Biological functions of SMAR1

Owing to its DNA binding ability, our lab has deciphered several important biological role of SMAR1. Our lab has showed in several model systems that how this protein transcriptionally regulates different genes in context dependent manner. Discussed below are few examples of gene regulation by SMAR1.

2.8.1.1 Role of SMAR1 in regulation of immune responsive genes

Previous studies from our lab has shown that SMAR1 is directly associated with the recombination of antigen receptor genes in T cells. The phenotype of SMAR1 transgenic mice demonstrated evident organomegaly of lymph nodes and spleen accompanied by follicular hyperplasia, suggestive of a hyper-responsive immune system. The over expression of SMAR1 perturbs T cell development during transition from DN (double-negative) to DP (double-positive) stage. SMAR1 transgenic mice exhibit a selective increase in the population of T cells expressing early markers, such as CD4/CD62-L, CD4/CD45RB, and CD4/CD44. A decreased frequency of T cells expressing commonly used Vs, particularly, V5.1, 5.2, 8.1, 8.2, and 8.3 in thymus and lymph nodes of SMAR1 transgenic mice, has been observed (Kaul-Ghanekar et al 2005). In T cell, transcriptional repressor role of SMAR1 has been documented through the interaction with MARβ region where SMAR1 physically associates with negative regulator Cux, especially through its arginine-serine rich (RS) domain, and both the proteins synergistically function to repress Eβ mediated transcription (Kaul-Ghanekar et al 2005). Both the proteins form a ternary complex with MARβ and...
negatively regulate the transcription mediated by Eβ enhancer. Previously, it was shown that at the DP stage of T cell development, V(D)J recombination is halted and MARβ is induced (Kaul-Ghanekar et al 2004).

2.8.1.2 Role of SMAR1 in tumorigenesis: Although SMAR1 was identified in T cells, subsequent studies in human cancer cell line demonstrated its ability to regulate cellular proliferation and growth. Ectopic expression of SMAR1 leads to retarded proliferation of melanoma and breast cancer cells. Studies have shown that SMAR1 work in concert with another tumor suppressor, p53, wherein its ectopic expression in cancer cell lines stabilizes p53 to induce growth arrest and minimal domain responsible for this function is arginine-serine domain (Jalota et al 2005). Later studies have shown the interaction of SMAR1 with p53 and MDM2 to regulate cell cycle (Pavithra et al 2009). SMAR1 expression is down regulated in many transformed cancer cell lines, suggesting its important role in cellular functioning. A study by Singh et al (2007) characterized SMAR1 promoter and demonstrated the presence of p53 binding site on SMAR1 promoter, and there is feedback loop by which each of these proteins regulate each other. In addition, our lab has shown that SMAR1 is stress responsive protein, and maintain cellular homeostasis under the conditions of DNA damage (Singh et al 2009).

2.8.1.3 SMAR1 regulates NF-κB signaling: SMAR1 binds to the MAR present in IκB- promoter, recruits HDAC1, forms a repressor complex together with p65/p50 at the locus and, thus, inhibits transcription. In contrast, SMAR1 inhibited TNF-α mediated activation of NF-κB. Real time PCR array revealed that SMAR1 represses a set of NF-κB target genes that promote tumorigenesis. Inversely, knockdown of SMAR1 by specific siRNAs resulted in direct induction of these genes, which were further induced in the presence of TNF-α. Thus, NF-κB activity and SMAR1 expression are directly and functionally co-related (Singh et al 2009).
Figure 2.8 Multifunctional role of SMAR1 in diverse cellular pathways. SMAR1 interacts with co-repressor molecules like Sin3A and HDAC-1 to regulate the transcription of genes. Given above is the model showing the association of SMAR1 with DNA and its interaction with other components of transcriptional machinery (self-drawn).

2.8.1.4 SMAR1 regulates cell cycle arrest and apoptosis: SMAR1, upon interaction with another tumour suppressor protein p53, activates it, which in turn, activates p21. The activation of p21 might inhibit the positive regulators of the cell cycle, which delays cells from proceeding from G2 to M phase and, thus, delay tumour growth in mouse melanoma model (Kaul et al 2003). On mild damage, SMAR1 modulates the cellular response to genotoxic stress by a dual mechanism. First, SMAR1 interacts with p53 and facilitates p53 deacetylation through recruitment of HDAC1 and generates an anti-apoptotic response by specifically repressing Bax and Puma expression by binding on their promoters. Reduction in the expression of SMAR1 by shRNA leads to significant increase in p53-dependent apoptosis. However, on severe DNA damage, SMAR1 is sequestered into the Promyelocytic Leukemia nuclear bodies (PML-NBs). This facilitates p53 acetylation and transactivation of BAX and PUMA leading to apoptosis in cancer cells. Silencing of PML by specific siRNA abrogates DNA damage induced apoptosis through trans-repression of BAX and PUMA by SMAR1. Thus, sequestration of SMAR1 into the
Promyelocytic leukemia - nuclear bodies (PML-NBs) acts as a molecular switch to dictate p53-dependent cell arrest and apoptosis on DNA damage (Sinha et al. 2010). Ectopic expression of SMAR1 targets proteosomal degradation of p300, suppresses p53 acetylation by p300 and, thereby, inhibits genotoxic stress induced apoptosis. Conversely, reducing the expression of SMAR1 by specific shRNA induces apoptosis through increased expression of p300, p53 acetylation and transactivation of p53 apoptotic targets like Bax, Puma and miR-34a, even in the absence of genotoxic insult (Sinha et al. 2012).

2.8.1.5 SMAR1 as a repressor of HIV-1 LTR promoter: SMAR1 represses HIV-1 LTR promoter both in presence and absence of transactivator Tat. SMAR1 maintains the latent state of viral transcription by recruiting HDAC1-mSin3a corepressor complex, which is dislodged from the LTR-MAR upon activation by PMA and TNF-α (Kadreppa et al. 2010).
<table>
<thead>
<tr>
<th>Section</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK</td>
<td>4-6, 8-9, 11-20, 22, 23, 25-27, 29-39, 41-42, 44, 46</td>
</tr>
<tr>
<td>Col.</td>
<td>7, 10, 21, 24, 28, 40, 43, 45</td>
</tr>
</tbody>
</table>