2. Review of literature

2.1 Epidemiology of Tuberculosis

Worldwide, tuberculosis remains one of the leading infectious disease causing morbidity and death. *M. tb*, the etiological agent of tuberculosis has evolved together with human since the beginning of our civilization. Discovered by Robert Koch in 1882, *M. tb* is particularly a successful pathogen as it has evolved multiple mechanisms effectively in counteracting both robust host defense mechanisms and antibacterial activities of series of antibiotics. However even after 130 years of its discovery and more than five decades of anti TB chemotherapy, this epidemic is still worsening. Even more alarming is the fact that incidences of tuberculosis is augmented in recent years, largely owing to co-infection with HIV, emergence of MDR and XDR strains of *M. tb*, immigration, increased trade, and globalization.

According to a study by Kochi in 1991, one third of the global population is infected with tuberculosis (Kochi et al. 1991). Developing countries are most affected by this infectious bacilli which encounters 95% of 8 million new cases and 98% of 1.9 million deaths annually (World Health Organization. Global Tuberculosis Control : Surveillance, Planning, Financing.WHO Report 2002. WHO/CDS/TB/2002.295). Taking into consideration, the rate of aggravation of this disease especially in resource poor developing countries, in 1993 WHO declared tuberculosis a global emergency, which was an unprecedented step in public health. In 1994 a framework was launched to effectively control tuberculosis, which comprises the five essential elements of TB control policy. These elements were: (i) Commitment of government to control tuberculosis in a sustainable fashion since it is claiming serious magnitude of human lives globally; (ii) diagnosis through quality assured sputum-smear microscopy to confirm tuberculosis especially among the symptomatic patients who are cautious enough to refer themselves to the health centers; (iii) treatment through standardized short-course chemotherapy under proper case management conditions, including direct observation of treatment (Medot-Pirenne, Heilman et al. 1999, Huang, Pan et al. 2006) functioning drug supply system that can procure and supply drugs regularly; and (Huang, Pan et al. 2006) a standardized recording and reporting system allowing assessment of treatment results and aid in assessing the effectiveness of the programme performance in totality. This program was named DOTS and it was rapidly implemented by many countries.

Since 2006, WHO has re-assessed the progress towards achieving the impact targets related to incidence, prevalence, mortality and morbidity due to tuberculosis. The ultimate goal set by Stop TB partnership is
to reduce the frequency of occurrence of disease by 1 tuberculosis patient per million annually by 2050. The Stop TB Strategy, launched by WHO in 2006 sets out the major interventions that should be implemented to achieve the Millennium Development Goals (MDGs) and Stop TB Partnership. These are divided into six broad components: (i) pursuing high-quality DOTS expansion and enhancement which may include drug susceptibility tests, MDR-TB treatment, local analysis and routine collection of data; (ii) addressing TB/HIV, MDR -TB; since co-infection with HIV has aggravated the cases of tuberculosis; TB control should be closely linked to HIV/AIDS control. For treatment of MDR-TB there should be high detection and cure rates of all new TB cases so as to avoid drug resistance in patients. However MDR-TB can be treated effectively by continuous and sustained procurement and supply of second line drug by health centers. (iii) improvising primary health care by strengthening health care centers. (Huang, Pan et al. 2006) Engaging all health care providers to make TB control programme more effective; (Huang, Pan et al. 2006) empowering people with tuberculosis, and communities through partnership; and (Huang, Pan et al. 2006) enabling and promoting research to improvise the tuberculosis treatment.

2. 2 TB is a leading killer of people with HIV

The HIV/AIDS epidemic has been the major cause of the surge in TB cases over the past few decades. Probability to develop active TB during HIV-TB coinfection is increased 50 times due to impaired immune response of HIV patient than people who are HIV-negative. Hence, enhanced susceptibility to TB infection and progression to active TB in HIV-infected populations is another serious health problem throughout the world.

HIV infection is also associated with rifampin resistance (Lutfey, Della-Latta et al. 1996; Munsiff, Joseph et al. 1997; Ridzon, Whitney et al. 1998) and malabsorption of anti-TB medications (Peloquin, Nitta et al. 1996; Gurumurthy, Ramachandran et al. 2004; Tappero, Bradford et al. 2005). In Tugela Ferry cluster, all the XDR TB patients were tested positive to HIV (Gandhi, Moll et al. 2006). In 2004 there 13% of adult tuberculosis patients were co-infected with HIV (Dye et al. 2006).
2.3 Problems faced in TB Control

Tuberculosis (Humbert, Deng et al. 2002) is a growing international health concern, especially with the increasing prevalence of multidrug-resistant MDR-TB and high rate of a co-infection with HIV. Today it is the deadliest disease in the world. Development of potent anti-TB drugs without cross-resistance with known antimycobacterial agents is the need of the hour.

The major problem that prevents more effective TB control is the tenacious ability of \textit{M. tb} to persist in the host and to develop drug resistance, often due to poor compliance to lengthy therapy leading to emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains. There is an urgent need to understand the molecular mechanisms of host-pathogen interaction, which would hopefully provide significant insight into the identification of druggable targets and therefore may be helpful in treatment of XDR and MDR tuberculosis.

2.4 Plan to Stop TB (2006-2015)

Proper treatment of tuberculosis has been deterred due to multiple reasons, the major reason being emergence of Multi Drug Resistant strains (MDR-TB). Poor adherence to the therapeutic regimen, improper prescription, suboptimal dosing, insufficient monitoring of therapy by clinicians and mal-absorption can result in partial suppression of bacterial growth and the emergence of resistant organism. Poorly functioning TB control programs, medication stock-outs, lack of staff, and inadequate access for patients because of distance or lack of transportation to medical facilities further complicate the problem. Most astonishing is the fact that global and national policies rely only on sputum smear to diagnose TB disease. This policy has led to improper diagnosis of MDR/XDR TB who do not receive second line of drug hence are improperly treated and continue to be active carrier of disease. Radiology studies, laboratory studies, or smear status can be useful in predicting drug resistance. The prolonged treatment (typically two years) with a combination of drugs including second line drugs is the only way to treat MDR-TB, but non-compliance further accentuates drug resistance giving rise to Extremely Drug Resistant (XDR-TB) tuberculosis strains.
XDR-TB is defined by WHO global task force as resistance to at least rifampin and isoniazid plus resistance to fluoroquinolones and to at least one of the injectable drugs capreomycin, kanamycin and amikacin. There are an estimated 40,000 new XDR-TB cases annually. XDR-TB is extremely difficult to cure because the remaining classes of drugs are inefficient, highly toxic and expensive. MDR-TB thus remains a serious threat and continues to jeopardize TB control programme (Dorman, Chaisson et al. 2007).

2.5 The pathogen: \textit{M. \textit{tb}}

\textit{Mycobacterium} is a non-sporeforming, aerobic, slow-growing, facultative intracellular pathogen, with a doubling time of about 20 hours. It has a unique lipid rich cell wall due to the dominant presence of mycolic acids that make up more than 50\% of its dry weight enabling it to retain basic dyes carbol fuschin even in the presence of acidic alcohol, also called as acid fast staining. \textit{M. \textit{tb}} is a single species by DNA-DNA hybridization studies with more than 99.9\% identity at the nucleotide level and an identical 16S rRNA sequence (Ernst, Trevejo-Nunez et al. 2007). A large number of different strains of \textit{M. \textit{tb}} exist that can be distinguished from one another on the basis of restriction fragment length polymorphism (RFLP). Other members of this group are \textit{Mycobacterium africanum}, \textit{Mycobacterium microti}, \textit{Mycobacterium bovis}, and \textit{Mycobacterium canettii} which differ remarkably with respect to their host range and pathogenicity. \textit{M. \textit{tb}}\textit{H37Rv} is the most pathogenic strain. The genome was cracked 93 years after this strain was first isolated in 1905. The genome of \textit{M. \textit{tb}} has been sequenced and shown to be 4.41 Mb in size and to contain about 4000 protein-coding genes of which 52\% have been assigned a function. It has a high G+C content of 65.6\%, a parameter associated with an aerobic metabolism. The annotation has identified 4,043 genes thought to encode 3,993 proteins and 50 stable RNAs (Cole et al. 2002). Close to 250 genes are annotated as encoding enzymes for the metabolism of fatty acids, comprising 6\% of the total coding capacity. Only 376 putative proteins share no homology with known proteins and presumably are unique to \textit{M. \textit{tb}} (Camus, Pryor et al. 2002). The availability of this type of information is important for identifying genes that code for virulence factors and antigens against which host immunity is directed. The genome sequence is also important for identifying targets for chemotherapy.
2.6 Granuloma Formation

Granuloma is a medical term for an inflammatory reaction that can occur in both infectious and non-infectious diseases where the foreign substance be it bacteria, fungi, keratin or suture fragments are surrounded by the immune cells that are unable to eliminate them but only restrict them in a defined domain.

Granuloma are chronic inflammation critical for the containment of infection. Once the lung resident macrophages are infected by mycobacteria, a series of chemokines are produced which attract naive monocytes, lymphocytes, and neutrophils (Van Crevel, Ottenhoff et al. 2002). Macrophages respond to infection by initiating the inflammatory cascade needed for the extravasation of leukocytes at sites of infection. After this initial encounter between \( M. \text{tb} \) and the lung macrophages, focal lesions composed of macrophage derived giant cells and lymphocytes begin to form, capable of just containing the bacteria but not eliminating it. It also reduces the rate of bacterial multiplication and prevents tissue damage or inflammation elsewhere in the host body. However, whether the infection develops into full blown disease or not depends upon the strength of host immune response. In hosts which have strong immune system, latent infection results in asymptomatic and non-transmissible state and finally granulomas will heal, leaving small fibrous and calcified lesions. On the contrary, in immuno-suppressed hosts, liquefied granuloma centers serve as the medium for the revival and replication of the \( M. \text{tb} \), which then escape to other tissues, resulting in the exaceberation of the infection.

2.7 Virulence in \( M. \text{tb} \)

(Mahairas, Sabo et al. 1996)Mahairas et.al.in 1996 elucidated the functional basis for attenuation of virulence in \( M. \text{bovis} \) by comparing BCG and \( M. \text{bovis} \) genomic sequences using subtractive hybridization which revealed that three regions of difference designated as RD1, RD2, and RD3 that are responsible for observed attenuation. (Behr, Wilson et al. 1999). Others (Gordon, Eigelmeier et al. 2001), later identified 16 large deletions, including RD1-RD3, present in BCG/ Bovis genome. Eleven out of 16 deletions were exclusive to \( M. \text{bovis} \). Other 5 deletions were found to be unique to BCG. RD1 is one of the most vulnerable locus which was approximately absent from all substrains of BCG that clearly indicated its significance in attenuation leading to advent of BCG which is the only vaccine against tuberculosis used
worldwide. Later, it was documented that RD1 was deleted quite early in passaging during the development of BCG. (Behr and Small 1999). When RD1-containing vector was cloned in BCG, its virulence was partially restored (Pym, Brodin et al. 2002). Of greater significance was the observation that attenuation in M. tb was restored after deletion of RD1 (9,454 bp) (Lewis, Liao et al. 2003).

Importantly, deletion of RD1 from M. tb attenuates the organism and conversely, incorporation of the RD1 region from M. tb into Bacillus Calmette–Gue’rin restores ESAT-6 and CFP-10 expression and increases virulence and immunogenicity (Pym, Brodin et al. 2002; Lewis, Liao et al. 2003; Pym, Brodin et al. 2003). ESAT-6 and CFP-10 are antigens expressed from genes in RD1 region secreted from M. tb and forms a 1:1 heterodimeric complex with each other and play a key role in the virulence of the infection. Analyses of the M. tb genome have suggested that genes neighboring the esat-6-cfp-10 operon may be important for ESAT-6 and CFP-10 secretion (Tekaia, Gordon et al. 1999; Pallen et al. 2002).

2.8 Pathology

M. tb has evolved to avoid its clearance by innate and adaptive mechanisms of immunity in immunocompetent humans and to induce lung pathology which ensures transmission by aerosol route. However infection need not necessarily be active disease, infection may lie latently in the patient asymptomically. In most cases of M. tb infection, the individual is asymptomatic and noninfectious. The immune response mounted to the infection is generally successful in containing, although not eliminating, the pathogen. Macrophages accumulate at the site of bacterial implantation and phagocytose them. These bacilli harboring macrophages are then surrounded by lymphocytes and fibroblasts to form granuloma. Granuloma are the hallmark of tuberculosis that are meant to contain the infectious bacilli, wall them off from the robust host immune response and thereafter persist in the host lifelong. (Dannenberg et al. 1989). Latent infection may become active disease on abrogation of host immunity which may be due to multiple reasons like co-infection with HIV, poor health, aging, drug abuse and poor diet.
2.9 Role of T cell Subsets in protection against Mycobacterial Infection

The protective response to *M. tb* requires cell-mediated immunity (Flynn and Chan 2001; Kaufmann 2001; North and Jung 2004; Lazarevic, Nolt et al. 2005). This intracellular pathogen, usually reside within macrophages, and hence T-cell effector mechanisms, rather than antibody response, are required to control the infection. Within 1 week of infection with virulent *M. tb*, the number of activated CD4⁺ and CD8⁺ T cells in the lung-draining lymph nodes increases (Feng, Bean et al. 1999; Serbina, Liu et al. 2000). About 3 weeks post-infection, both CD4⁺ and CD8⁺ T cells migrate to the lungs, and demonstrate an effector/memory phenotype (Serbina, Flynn et al. 1999). This indicates that activated T cells migrate to the site of infection and interact with antigen-presenting cells. The granuloma contains both CD4⁺ and CD8⁺ T cells (Randhawa et al. 1990; Flynn, Goldstein et al. 1992) that are likely to participate in containing the infection within the granuloma.

CD4⁺ T cells play a critical role in protection against tuberculosis through production of a vital cytokine like IFN-γ. This cytokine induces apoptosis in infected macrophages, one of the mechanism suggested to be important in controlling *M. tb* infection (Keane, Balewicz-Sablinska et al. 1997; Balewicz-Sablinska, Keane et al. 1998). The importance of CD4⁺ T cell was further determined from the observation that in the absence of Class I-dependent immunity, lung infection progresses to a 1 log higher level than in wild type mice and can be controlled at a stationary level for a long period of time but when Class II-dependent immunity is absent, infection progresses rapidly and animal succumbed to infection. The infection process downregulates the surface expression of MHC II molecules on the macrophages minimizing the host immune activation and production of inhibitory cytokines, such as TGF-β, IL-10 or IL-6, which further reduce T-cell stimulation (Gong, Zhang et al. 1996; Hirsch, Ellner et al. 1997; Rojas, Olivier et al. 1999). Some antigens from the phagosome could enter the cytoplasm and be presented on MHC Class I molecules to CD8⁺ T cells. In humans and mice, antigens recognized by these cells have been identified (Stenger, Mazzacarro et al. 1997; Tan, Canaday et al. 1997; Zhu, Stauss et al. 1997; Lewinsohn, Alderson et al. 1998; Mohagheghpour, Gammon et al. 1998; Lewinsohn, Briden et al. 2000). CD8⁺ T cells are thus induced during the infection. They contribute to anti tuberculosis immunity by producing IFN-γ and cytolytic activity (Caruso, Serbina et al. 1999; Scanga, Mohan et al. 2000). In humans, CD8⁺ T cells also produce granulysin, which enters the macrophage via the perforin pore. Granulysin was shown to be directly toxic to *M. tb* and represents a mechanism by which CD8⁺ T cells can contribute to clearance of the bacilli within cells (Stenger, Mazzacarro et al. 1997; Stenger, Niazi et al. 1998).
2.10 Effectors molecules

Nitric oxide: Nitric oxide (Huang, Pan et al.) and its metabolites represent one of the major antimicrobial defense mechanisms of macrophages. NO is formed when the guanidine nitrogen of L-arginine is oxidized by a family of isoenzymes known as NO synthases (NOSs). Exposure to NO at low concentrations, e.g., 100 ppm, killed more than 99% of M. tb in culture (Bobe, Benihoud et al. 1999). There are several mechanisms by which NO may affect antimicrobial activity. One of the most common mechanisms employed by NO and other Reactive Nitrogen Intermediates (RNI) is to modify pathogen DNA, protein, and lipids which are present either intracellularly or on the microbial surface. NO modifies DNA by either deaminating the purine and pyrimidines in DNA or completely removing it creating abasic sites or creating breaks in the DNA strands. Further Peroxynitrite, ONOO· can nitrosylate tyrosine residues, which can disrupt the tyrosine phosphorylation-dependent signaling pathways (Bryk, Griffin et al. 2000). The induction of apoptosis and bactericidal activity of B10R macrophages harboring H37Rv M. tb, is attributed to NO production (Rojas, Barrera et al. 1997). B10R macrophages is a cell line derived from bone marrow of mice strain B10A. Bcgr that is resistant to BCG. Previously it has been studied that intra-cellular NO production by macrophages is more likely to be bacteriostatic than bactericidal (Rhoades and Orme 1997). Interestingly, whereas BCG was susceptible to ONOO·, the Erdman strain of M. tb and a clinical isolate M160 were resistant to it. A possible mechanism for the NO₃⁻ resistance is by the ability of M. tb peroxiredoxin alkylhydroperoxide reductase subunit C (AhpC) to catalytically detoxify NO₃⁻ (Bryk, Griffin et al. 2000). It has been observed that even after drug treatment, iNOS deficient mice are more vulnerable to tuberculosis as compared to wild type (MacMicking, North et al. 1997), clearly intimating that tuberculocidal activity of drugs is enhanced with the aid of iNOS-derived NO. On the other hand, M. tb has evolved clever ways to evade the toxic effects of RNI. Ehrt and coworkers showed that a novel M. tb genes, noxR1, noxR3 conferred resistance to RNI and ROI (Reactive Oxygen Intermedeates) (Ehrt, Shiloh et al. 1997; Ruan, St John et al. 1999). Similarly, the M. tb peroxiredoxin gene Ahpc (alkyl hydroperoxide reductase subunit C) prevented RNI-induced necrosis and apoptosis in human cells (Chen, Xie et al. 1998). Ahpc has also been shown to detoxify NO₃⁻ to NO₂⁻ (Bryk, Griffin et al. 2000).

In hypoxic conditions, nitrate (NO₃⁻) a degradation product of NO, is reduced by the tubercle bacilli to nitrite (NO₂⁻) with the help of nitrate reductase at a rate that is significantly greater than in aerobic conditions attributing the shift of the M. tb from aerobic active phase to a state of dormancy (Wayne, Hayes et al. 1998).
2.11 Cytokines and Chemokines:

**Interferon-γ:** In humans, the importance of IFN-γ (Jouanguy, Altare et al. 1996) and IL-12 (Altare, Durandy et al. 1998; de Jong, Altare et al. 1998) in host defense against mycobacterial infection has been shown previously. The initial wave of protective immunity involves the generation of Th1 CD4+ T cells, which secrete interleukin 2 (IL-2) and gamma interferon (IFN-γ) (Barnes, Abrams et al. 1993; Orme, Andersen et al. 1993). NK cells are also potent producers of IFN-γ in the absence of fully developed adaptive T-cell immunity in response to either IL-12 and IL-18 (Iho, Yamamoto et al. 1999) or as a result of direct exposure to mycobacterial oligodeoxynucleotides (Garcia, Uyemura et al. 1999). IFN-γ is also produced by lung macrophages in *M. tb*-infected mice (Wang, Lafuse et al. 2000). γδ T cells, CD1-restricted T cells, γδ T cells may directly recognize small mycobacterial proteins (Janis, Kaufmann et al. 1999) and non protein ligands (Constant, Davodeau et al. 1994; Tanaka, Morita et al. 1995; Kaufmann et al. 1996) in the absence of antigen-presenting molecules. In mycobacterial infections, CD4-CD8- double negative, CD4+ or CD8+ single positive and γδ T cells interact with CD1 (Rosat, Grant et al. 1999). However, CD1-restricted T cells produce IFN-γ and have cytotoxic ability (Stenger, Mazzaccaro et al. 1997).

**Transforming Growth Factor-β (TGF-β):** TGF-β seems to counteract protective immunity in tuberculosis. TGF-β suppresses cell-mediated immunity in T cells. TGF-β inhibits proliferation and IFN-γ production, antagonizes antigen presentation in macrophages, production of proinflammatory cytokine, and cellular activation (Toossi, Ellner et al. 1998). Mycobacterial products induce production of TGF-β by monocytes and dendritic cells (Toossi, Young et al. 1995). Interestingly, LAM (Lipoarabinomannan) from virulent mycobacteria selectively induces TGF-β production (Dahl, Shiratsuchi et al. 1996). TGF-β is produced in excess during tuberculosis and is expressed at the site of infection (Toossi, Young et al. 1995; Condos, Rom et al. 1998). In addition, TGF-β may be involved in tissue damage and fibrosis during tuberculosis, as it promotes the production and deposition of macrophage collagenases (Toossi, Ellner et al. 1998) and collagen matrix (Roberts, Sporn et al. 1986). Naturally occurring inhibitors of TGF-β eliminate the suppressive effects of TGF-β on mononuclear cells from tuberculosis patients and in macrophages infected with *M. tb* (Hirsch, Ellner et al. 1997). Within the anti-inflammatory response, TGF-β selectively induces IL-10 production, and synergistically suppresses IFN-γ production (Othieno, Hirsch et al. 1999). TGF-β may also interact with IL-4. Paradoxically, in the presence of both cytokines, T cells may be directed towards a protective Th1-type profile (Erard, Garcia-Sanz et al. 1999).
**Interleukin-23:** Interleukin-23 (IL-23) is a heterodimeric cytokine consisting of two subunits, one called p40, which is shared with another cytokine, IL-12, and another called p19 (the IL-23 alpha subunit). Upon exposure of dendritic cells (DCs) to *M. tb*, IL-12p70 and IL-23 are induced (Khader, Pearl et al. 2005; Lockhart, Green et al. 2006; Wozniak, Ryan et al. 2006). When CD4⁺ T cells are primed with cognate antigen from infected DCs and then get restimulated, the efficient generation of Th17 cells is dependent upon the presence of IL-23 during the initial priming (Khader, Pearl et al. 2005; Lockhart, Green et al. 2006). Similar are the observations, when *M. bovis* BCG is used (Cruz, Khader et al. 2006). IL-23 along with IL-6 and TGF-β1, stimulate and differentiate naive CD4⁺ T cells into Th17 cells. Th17 secret proinflammatory cytokine IL-17. It also stimulates the production of other proinflammatory molecules like IL-1, IL-6, TNF-α, and NOS-2, resulting in inflammation. Upon aerosol infection, the absence of IL-23 leads to ablation of the Th17 response and significant loss of IL-17mRNA expression in the lung which clearly demonstrates that IL-23 is essential for the activation of Th17 and induction of IL-17 (Khader, Pearl et al. 2005). This fits with other observations wherein the continuous function of Th17 cells is associated with IL-23 (Stockinger, Veldhoen et al. 2007). In humans the critical role for IL-23 is in the initiation of Th17 cells has been reported (Chen, Tato et al. 2007; Wilson, Boniface et al. 2007). It is likely that IL-23 is required for optimal Th17 responses to mycobacterial infection in humans. IL-23 is also able to induce IFN-γ-producing Th1 cells, a key player in host response against *M. tb* as in the absence of IL-23, residual Th1 response to *M. tb* is lost. (Khader, Pearl et al. 2005).

Since IL-23 is responsible for the persistence and function of Th17 cells (Stockinger, Veldhoen et al. 2007), it is also likely a key player in inflammation. There is no difference in extent of lung consolidation in either the wild type or mutant strain which is IL-23p19 gene-deficient in murine model of tuberculosis but as and when disease progresses, the severity of the inflammation is aggravated and the extent of fibrin deposition is ablated due to absence of IL-23 (Khader, Pearl et al. 2005). Therefore it is clear that IL-23 acts in a complex manner in the control of mycobacteria-induced inflammation.

**Interleukin-17** (IL-17): IL-17 is recognized as an inflammatory cytokine capable of inducing chemokine gradients and initiating inflammation, particularly in the lung (Miyamoto, Prause et al. 2003; Kolls and Linden 2004; Sergejeva, Ivanov et al. 2005). In particular, the γδ T cell population is the primary source of *M. tb*-induced IL-17 in the mycobacterial infection model (Lockhart, Green et al. 2006; Umemura, Yahagi et al. 2007). Following intra-tracheal delivery of BCG, IL-17 mRNA can be detected 1 day post-infection and in the absence of this cytokine, the induction of chemokines and early neutrophil accumulation is reduced (Umemura, Yahagi et al. 2007). DC harboring the *M. tb* bacilli induces IL-17 in T cells from uninfected mice which is largely due to IL-23-dependent induction of IL-17 especially in the γδ T cell population (Lockhart, Green et al. 2006). The invariant natural killer T (iNKT) cells in the lung...
recognize lipopolysaccharide and pathogen glycolipids. These cells produce IL-17 upon stimulation and are required for the airway neutrophilia induced by these products (Michel, Keller et al. 2007). The balance between Th1 and Th17 is critically controlled by the relative levels of IL-12 and IL-23 induced by mycobacterial-infected cells. In primary *M. tb* infection, Th17 and Th1 cells are induced with the same kinetics but there are 5-10 fold more Th1 than Th17 cells (Khader, Pearl et al. 2005; Khader, Bell et al. 2007). In BCG infection the Th17 is rapidly suppressed by the Th1 response (Cruz, Khader et al. 2006) and this may be related to differential induction of IL-12 and IL-23 by BCG compared to *M. tb* (Wozniak, Ryan et al. 2006). It has been well documented that while IFN-γ dramatically increases IL-12p70 production by BCG-infected DCs, it also reduces IL-23; conversely IL-17 limits IL-12 production while augmenting IL-23 (Cruz, Khader et al. 2006). This clearly suggests that the cross-regulation of IL-12 and IL-23 by each other and by IFN-γ and further suggesting that, IL-17 is critical for the inflammatory outcome of any mycobacterial infection. However, complete absence of IL-17 results in reduced mononuclear and polymorphonuclear infiltration early in the BCG model (Umemura, Yahagi et al. 2007).

2.12 Discovery of Mesenchymal stem cells

130 years ago a German pathologist Cohnheim suggested the presence of nonhematopoietic stem cells in bone marrow which may be the source of fibroblasts that deposit collagen fibers as part of the normal process of wound repair (Phinney, Prockop et al. 2007). The work of Friedenstein and colleagues later gave the evidence that bone marrow contains cells that can differentiate into other mesenchymal cells, as well as fibroblasts (Friedenstein, Gorskaja et al. 1976). It was established that the cells isolated by Friedenstein’s method were multipotential in nature and had the property to differentiate into osteoblasts, chondrocytes, adipocytes, and even myoblasts, currently referred as mesenchymal stem cells (MSCs). (Phinney, Prockop et al. 2007).

2.13 *In vitro* characteristics of MSCs

In adult, stem cells of mesenchymal lineage (MSCs) are mainly confined to the bone marrow (BM), where they constitute a small proportion (0.1%-0.01%) of total BM cells. Bone marrow aspiration is
expanded through serial passaging in plastic culture in appropriate culture media, where they multiply and
grow as adherent cells. Non adherent cells are washed away, leaving adherent, fibroblast-like cells. The
cultures are fed by replacement of complete alpha-medium on a weekly basis. Confluent stromal layers so
obtained are trypsinized for at least 3 consecutive weeks to ensure the substantial depletion of
contaminating haematopoietic and other cell populations leading to morphologically heterogenous
population of cells (Javazon, Beggs et al. 2004). Other ways to isolate MSCs from different organs is to
use the method of negative selection to enrich MSCs, whereby cells from the hematopoietic lineage are
removed (Baddoo, Hill et al. 2003); and using antibodies to positively select MSCs (Jones, Kinsey et al.
2002; Gindraux, Selmani et al. 2007).

MSCs and MSC-like cells can be isolated from various sites other than the bone marrow, including
adipose tissue, amniotic fluid, peritoneum, fetal tissues, and show phenotypic heterogeneity. Phenotypically,
there is no surface marker that is unique to MSCs hence there characterization is done by
assessing the presence and absence of group of specific markers. There is now agreement for human
MSCs that they lack the hematopoietic markers CD14, CD34 and CD45, while being positive for CD44,
CD71, CD73, CD90, and CD105 (Dominici, Le Blanc et al. 2006). However, in mice MSC do not
express the hematopoietic markers CD11b, CD34, and CD45, but are positive for CD9, Sca-1, and CD44
((Eliopoulos, Stagg et al. 2005; Uccelli, Moretta et al. 2008). Hence, it can be concluded that
mesenchymal precursor cells are phenotypically heterogeneous. Beside, there morphologic
characterization, they can be identified by their intrinsic property to differentiate into bone, fat, and
cartilage tissue in vitro.

2.14 Immunological phenotype and functions of MSCs

Human and murine MSCs are generally considered to be poorly immunogenic cells because the immune
phenotype of MSCs.MHC class I which has low surface density on MSCs may activate T cells, but, in the
absence of costimulatory molecules, a secondary signal would not be engaged, leaving the T cells anergic
(Javazon, Beggs et al. 2004). On the other hand, suppression of T cell proliferation did not require MHC
restriction but could also be mediated by allogeneic MSCs (Krampera, Glennie et al. 2003; Le Blanc,
Tammik et al. 2003). Not much is known about the molecular mechanism(s) responsible for MSCs
suppressive effect on T cell proliferation. Many previous reports have documented that inhibition of T
cell proliferation by MSCs depends on cell-to-cell interaction and release of soluble factors (Di Nicola,
Transforming growth factor–β (TGF-β), hepatocyte growth factor, indoleamine 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2) have been reported to mediate T-cell suppression by MSCs (Young, Wright et al. 1996; Di Nicola, Carlo-Stella et al. 2002; Meisel, Zibert et al. 2004; Aggarwal and Pittenger 2005). Another potent candidate, soluble factor responsible for T-cell suppression is nitric oxide (Huang, Pan et al.) because it is known to inhibit T-cell proliferation (Mazzoni, Bronte et al. 2002); (Albina, Abate et al. 1991); (Lejeune, Lagadec et al. 1994; Young, Wright et al. 1996; Bobe, Benihoud et al. 1999; Medot-Pirenne, Heilman et al. 1999; Angulo, de las Heras et al. 2000). MSCs can modulate many T-cell functions including cell activation (Bartholomew, Sturgeon et al. 2002; Di Nicola, Carlo-Stella et al. 2002) which appears to be independent of MHC matching between the MSCs and the T cells. The immunomodulatory or migratory function of MSCs is attributed to the soluble factors like growth factors, cytokines, chemokines and proteases produced by it. (Kim, Yoo et al. 2005; Lee, Seo et al. 2006). MSCs have been shown to express a restricted pattern of chemokine receptors, including CXCR4, allowing them to migrate to tissues upon specific chemotactic triggers (Tan, Canaday et al. 1997; Sordi, Malosio et al. 2005; Honczarenko, Le et al. 2006; Lee, Hsu et al. 2006). These features are likely to represent the basis for MSCs homing to multiple organs where they undergo a program of tissue-specific differentiation (Liechty, MacKenzie et al. 2000).

2.15 Effect of MSCs on different cell types

Previous study have documented that the allogeneic MSCs inhibited B cell activation, proliferation and IgG secretion and enhances CD40 expression and CD40 ligand ectopic hyperexpression on BXSB-derived B cells (Deng, Han et al. 2005). Mice of the BXSB strain develop spontaneous autoimmune disease which is strikingly accelerated in males due to the presence of the Yaa gene on the Y chromosome. There is striking enhancement in peripheral monocytes initially at the age of 2 months and later they become deficient in pre-B-cells after about 4 weeks for whole life. Stimulated B lymphocytes proliferation, soluble CD40 ligand and cytokines (IL-2 and IL-4) is reported to be inhibited by human MSCs (Deng, Han et al. 2005). MSC stifles B cell differentiation to plasma cells which secrete antibody. Chemokines like CXCL12 and CXCL13 play a crucial role in B cell placement in secondary lymphoid organs (Corcione, Benvenuto et al. 2006). MSCs have been reported to suppress B cell chemotaxis to the destined organ in response to chemokines, due to release of multiple MSCs-derived soluble factors. Previously, it has been documented that both murine and human B lymphocytes whether T cell-dependent
or T cell independent are responsive to MSCs inhibition. (Corcione, Benvenuto et al. 2006). Also, it has been reported that MSCs suppress IL-2 or IL-15 mediated NK proliferation. (Krampera, Cosmi et al. 2006; Sotiropoulou, Perez et al. 2006; Spaggiari, Capobianco et al. 2006). MSCs either autogenic and allogenic are highly amenable to lysis NK cells activated by IL-2 This is due to abjected surface expression of HLA class I molecules (Poggi, Prevosto et al. 2005; Sotiropoulou, Perez et al. 2006; Spaggiari, Capobianco et al. 2006). Incubation of MSCs with IFN-γ partly protect MSCs from NK cell-mediated lysis. This is due to up regulation of HLA class I on MSCs (Spaggiari, Capobianco et al. 2006). Similarly as observed for T cells, soluble factors like TGF-β1 and PGE₂ play a key role in the suppression of NK cell proliferation by MSCs (Sotiropoulou, Perez et al. 2006). Immunomodulatory properties exhibited by MSCs impair maturation and function of dendritic cells. They also inhibit hMSCs and human B-cell proliferation, their differentiation, and chemotaxis (Aggarwal, Pittenger et al. 2005; Beyth, Borovsky et al. 2005; Jiang, Ma et al. 2005; Corcione, Benvenuto et al. 2006).

Dazzi and colleagues documented that the cell cycle in DC was arrested in the G0/G1 phase, upon interaction with MSCs (Ramasamy, Lam et al. 2007). MSCs effect the maturation of monocyte-derived myeloid DC. Surface markers like CD11c, CD83, MHC class II alongwith costimulatory molecules are downregulated on cross-talk with MSC. This is accompanied with suppression ofIL-12 production after TLR-mediated DC activation (Zhang, Ge et al. 2004; Beyth, Borovsky et al. 2005; Jiang, Ma et al. 2005; Maccario, Podesta et al. 2005). Upon interaction with MSCs, DC either abrogate or aggrevate soluble factor production like myeloid DC produce less amount of TNF-α while plasmacytoid DC produce increased amount of IL-10 (Aggarwal, Pittenger et al. 2005). This effect, in turn, led to decreased IFN-γ production by Th1 cells, increased IL-4 secretion by Th2 cells, and an increased number of regulatory T cells (Aggarwal and Pittenger 2005; Maccario, Podesta et al. 2005). Release of soluble factor like PGE-2 and cell-to-cell contact is necessary feature of inhibition of DC function and differentiation (Aggarwal, Pittenger et al. 2005; Jiang, Ma et al. 2005).

### 2.16 Therapeutic value of MSCs

Till date clinical aspect of MSCs was focused on their ability to promote structural (Pereira, Halford et al. 1995) and functional repair of damaged tissues owing to their ‘stem-cell-like’ properties (Orlic, Kajstura et al. 2001). However, recently discovered immunomodulatory properties of MSCs support their possible use as a therapy for immune-mediated diseases. Their poor immunogenicity *in vitro* makes them useful
for preclinical (Bartholomew, Sturgeon et al. 2002), clinical studies (Tse, Pendleton et al. 2003) in human (Le Blanc, Rasmusson et al. 2004). However autologous MSCs are preferable over allogeneic MSCs since some studies have challenged the tenet that allogeneic MSCs are poorly immunogenic (Eliopoulos, Stagg et al. 2005; Nauta, Westerhuis et al. 2006). Recent studies have shown that MSCs from patients with autoimmune disease have a normal ability to support haematopoiesis and immunomodulatory activity (Bocelli-Tyndall, Barbero et al. 2006), and have a normal cell-surface and molecular phenotype (Kastrinaki, Sidiropoulos et al. 2008).

For an ideal treatment, MSCs should be able to provide both systemic and local therapeutic effects. MSCs migrate in response to several chemokines that bind to cognate receptors expressed on their cell surface (Sordi, Malosio et al. 2005) and lead to the activation of matrix metalloproteinases that degrade the basement membrane and allow subsequent extravasation from blood vessels. The ground breaking knack of MSCs to modulate immune response in vivo was first investigated by Bartholomew and colleagues. Their foremost observation is the therapeutic effect of MSCs, in stroke experienced rats, depended on the release of anti-apoptotic, anti-inflammatory and trophic molecules (Li, Chen et al. 2002) and in Experimental Autoimmune Encephalomyelitis (EAE) model of multiple sclerosis, (Zappia, Casazza et al. 2005) where MSCs induced T cell anergy and inhibited them from proliferation in response to antigen.

The therapeutic effect endowed by MSCs not only depends on its multipotentiality but also on its anti-apoptotic and anti-inflammatory properties. Like in acute renal failure model, administration of MSCs increased the recovery of renal function through the inhibition of production of proinflammatory cytokines, such as IL-1β, TNF-α and IFN-γ, and through an anti-apoptotic effect on target cells (Togel, Hu et al. 2005).

Clinical studies. Osteogenesis imperfecta has been successfully treated in children by administration of MSCs. This treatment was based on the capability of MSCs to engraft in bone of the recipient following its systemic administration. (Horwitz, Prockop et al. 1999). Systemic infusion of allogeneic MSCs has also led to encouraging results in patients with cancer who underwent high-dose chemo therapy, through the acceleration of bone-marrow recovery (Koc, Gerson et al. 2000). However, the in vivo immunosuppressive effect of infused MSCs has only been successfully shown so far in acute, severe graft versus host disease (GVHD) (Le Blanc, Tammik et al. 2003), for which the effect was probably due to the inhibition of donor T-cell reactivity to histocompatibility antigens of the normal tissues of the recipient. The immunomodulatory potential of MSCs is currently being tested for the treatment of Crohn’s disease, where these cells could also contribute to the regeneration of gastrointestinal epithelial cells (Okamoto, Yajima et al. 2002). These results indicate that the clinical use of MSCs has therapeutic potential.
2.17 Conclusions and future perspectives

Overall, the current data indicate that although application of bone marrow-derived MSCs is majorly due to their stem-cell-like qualities, their therapeutic effect can result from other characteristics, such as their anti-proliferative and anti-inflammatory properties. MSCs seem to nonspecifically target cells of the immune system. Ultimately, the immune-suppressive activity of MSCs provides a tool for inducing peripheral tolerance following systemic injection and seems to depend on the capacity to ‘freeze’ immune-competent cells through the inhibition of cell division, thereby preventing their responsiveness to antigenic triggers while maintaining them in a quiescent state. In addition, the evidence of clinical efficacy of MSCs in different experimental models almost only during the acute phase of disease and the limited evidence of trans-differentiation indicate that the therapeutic effectiveness of MSCs relies heavily on their ability to modify the microenvironment of injured tissues. These events occur through the release of anti-inflammatory cytokines, and anti-apoptotic and trophic molecules that promote the repair and protection of damaged tissues. So, the therapeutic plasticity of MSCs might be seen as a recapitulation of the physiological activity of stromal cells in the Hematopoietic Stem Cell (HSC) niche. Here, stromal cells contribute to the generation of the niche by regulating the size of the pool of HSCs and by providing signals necessary to maintain HSCs in a non-proliferating state and refractory to differentiation stimuli, but also to support HSC survival through trophic and anti-apoptotic molecules (Stenger and Modlin 1998; Stier, Ko et al. 2005; Wilson and Trumpp 2006; Kiel and Morrison 2008). The final outcome of the immunomodulatory activity of MSCs is likely to be significantly influenced by the microenvironmental cues encountered following In vivo administration. To elucidate these microenvironmental cues further, studies should address the impact of MSCs on physiological immune response in vivo — for example, on Th1- and Th2-cell responses or responses against viral and bacterial pathogens — and examine the molecular pathways that are activated in MSCs by environmental triggers. Also, it will be relevant to understand the mechanisms that regulate the behavior of MSCs under conditions of stress, such as during inflammation and tissue injury. Another fundamental question involves whether, after in vivo administration, MSCs can engraft into tissues where they might exert their bystander effects inside ectopic niches, as has been shown for neural stem cells. A key related issue that needs to be addressed for clinical purposes concerns the immunogenicity of MSCs, which might restrict engraftment in allogeneic environments. MSCs have been effectively used to evade host immune surveillance. This behavior could also be explained by the possibility that MSCs exert their therapeutic effects through a ‘touch and go’ mechanism — that is, through their rapid migration to the damaged organ and subsequent clearance following the release of stress-induced therapeutic molecules. Despite the limitations in our existing
knowledge of this matter, the capacity of MSCs to exert their therapeutic plasticity through bystander mechanisms also might indicate that persistent engraftment at the site of damage is not a mandatory prerequisite for having an effect on injured cells and possibly local progenitors during acute stress conditions. A final issue of crucial importance concerns the safety profile of injected MSCs. Although their use in most haemato-oncological conditions has been considered safe so far, their long-term effects on immune function and tumorigenic risk are still unknown. An understanding of these issues will help in better and effective application of MSCs as a therapeutic drug and in other clinical therapies.