Chapter 2

Review of Literature
Tuberculosis: An Epidemic

Despite hopes of its eradication, tuberculosis (TB) remains a leading cause of death due to infectious diseases worldwide. The World Health Organization (WHO) declared TB an emerging public health problem in 1993, and TB has now reached alarming proportions with an estimated 8-10 million new cases and 2-3 million deaths annually. A long time of common life with men has endowed Mycobacterium tuberculosis (M. tuberculosis), the causal agent, with the best adaptation among all known human pathogens. Therefore, it has remained in a quiescent state within a large number of individuals, generating neither symptoms nor disease, but surviving and awaiting more suitable conditions to attack. Drug sensitive M. tuberculosis infection in an immunocompetent individual must be treated for at least 6 months with multiple antibiotics such as isoniazid, rifampin, pyrazinamide and ethambutol or streptomycin (Directly Observed Therapy Shortcourse- DOTS therapy) to achieve reliable clinical cure rates (Cohn et al., 1990; Iseman et al., 1993). The financial and logistical barriers to administering these drug regimens in the areas where TB is most prevalent are enormous. Although the disease is currently only endemic in developing countries, epidemics in the developed world are only prevented by ceaseless monitoring for infection and treatment of potentially exposed individuals. Thus, in addition to the mortality and morbidity it causes, M. tuberculosis inflicts a significant economic cost on the developed and developing world alike. Furthermore, microbial resistance to commonly used antituberculous drugs is increasingly becoming common, both in primary isolates and as a result of incomplete treatment (Surveillance, 1997). In addition, 50 million people may already be infected with multidrug-resistant (MDR) strains of M. tuberculosis. Therefore, development of new drugs active against M. tuberculosis, particularly drugs that would allow shorter courses of therapy, is a major priority of tuberculosis investigation and also the primary objective of WHO. A high prevalence of TB is also associated with human immunodeficiency virus (HIV) infection and AIDS and is now becoming the leading...
cause of death among HIV-positive individuals, with a fatality rate of 80% (World Health Organization, 1999).

The outcome of the battle between *M. tuberculosis* and the human host is determined by complex host-, environmental- and pathogen-factors, and clearly is a multifactorial disease. Much of the considerable variation in outcome of exposure and infection with *M. tuberculosis*, and also in TB disease severity can probably be attributed to variations in the interplay between pathogen, host and environment as shown in the figure below. When the interactions shift the balance in favor of *M. tuberculosis*, TB disease will develop. On the other hand, when the balance is in favor of the host, *M. tuberculosis* can be killed or contained by the host immune system. The coordinated response of the innate and adaptive immune systems is required for an effective host defense.

*Figure*: The interaction of host factors, pathogen factors and environmental factors play a key role in outcome of *M. tuberculosis* infection. Whether infection becomes symptomatic depends on innate immunity, with or without involvement of adaptive immunity.
*M. tuberculosis* is transmitted solely by direct human-to-human spread through the air via inhalation. The bacilli can remain in the air for prolonged periods of time. Dried bacteria may even survive for days to months if protected from sunlight. The bacteria can be inhaled by any bystander individuals (young and old) when active TB patients cough, sneeze or speak, especially in the close or intimate environment of the patients; e.g. family members and close contact at home, work or school. Intensity of exposure (the load/concentration of the mycobacteria inhaled) is related to the infectivity of the case. A study in The Gambia, West Africa showed that TB infection in children was directly related to the intensity of exposure of the child to the individual with infected TB, mostly in the form of parent-to child or vertical transmission. Infection in infants and young children up to 5 years may thus indicate recent transmission. Infected children represent a large proportion of the pool from which TB cases will arise. The World Health Organization (WHO) reported that 75% of TB cases are adults in their most productive life years. Recent studies in three countries in West Africa showed that the environmental risk factors for TB are associated with single marital status, crowding, and living in a rented house. As TB transmission occurs in a closed environment, reduction of crowding and improvements in housing with better ventilation are likely to have an essential impact. Furthermore, virulence of the *M. tuberculosis* is also associated with the development of TB. Recent epidemiological data suggest that differences in transmission and virulence among *M. tuberculosis* strains are related to the genetic background of the organisms. For instance, *M. tuberculosis* of the Beijing genotype which is highly prevalent in Asia and the former USSR is considered to be more virulent and elicits a non-protective immune response. In contrast, *M. canettii* is associated with a more favorable course while other genotypes caused intermediate clinical and pathological effects. A better understanding of differences in virulence between *M. tuberculosis* genotypes could have implications for TB control once established more precisely. Transmission of *M. tuberculosis* can be limited by proper TB control strategies, including active TB case detection (the so-called contact tracing), supervised treatment of sputum smear-positive cases and *M. bovis* Bacillus Calmette-Guérin (BCG) vaccination for neonates. BCG, discovered in 1921 by Albert Calmette and Camille Guérin, is the only vaccine currently available against TB. It is, however, difficult to control TB due to difficulties in TB diagnosis, (extreme) multi-drug resistance, lack of treatment compliance, resource-poverty and HIV co-infection.
Knowledge of the factors that influence progression of TB infection to disease will obviously be important to identify susceptible individuals and understand transmission patterns in the community.

Clinical appearance
Individuals with *M. tuberculosis* infection are in most cases asymptomatic and noninfectious. In active pulmonary TB, patients may present in the clinic with a wide clinical appearance spectrum. The pathogenic symptoms such as coughing and night sweating are the most common complaints reported by TB patients; coughing with blood (hemoptysis), and shortness of breath (dyspnea) are also reported. Systemic manifestations include wasting or weight loss (phtisis), fever, loss of appetite, and fatigue. Nonspecific hematological changes e.g. anemia, increase in the peripheral blood leukocyte count (leukocytosis), and low albumin are also common (Iseman *et al.*, 2000). The presence of an inflammatory process can be monitored by the increase of erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) level as these are markers for an acute phase response of inflammation and tissue injury (Okamura *et al.*, 1990). In lymphocytic pleural effusions, CRP can be used as a tool for diagnosis of TB pleuritis (Garcia-Pachon *et al.*, 2005). TB with other concomitant diseases such as HIV-infection, diabetes mellitus (DM) or malignancy may present with different clinical presentations. For example, HIV-positive TB patients develop a negative TST and cutaneous anergy indicating a strong association with immune dysfunction (Mukadi *et al.*, 1993). Patients with AIDS and TB are more likely to develop multifocal extrapulmonary disease and atypical chest X-ray radiography (CXR) (Bonadio *et al.*, 2005). TB patients with concomitant DM develop more cavitation nodules (Wang *et al.*, 2005), and tend to have higher body mass index (BMI) compared with TB patients without concomitant DM. Of clinical importance, the prominent weight loss (wasting) in TB disease can be masked by the fact that concomitant DM leads to higher BMI compared with healthy individuals (Alisjahbana *et al.*, 2007).

Chest X-ray examination
The airway is the usual port d'entrée of *M. tuberculosis* in humans. Lung tissue destruction such as lung infiltrates with or without cavities can be observed by CXR and is indicative of active TB. The severity of pulmonary TB can be classified based
on the CXR using the criteria from The National (USA) Tuberculosis and Respiratory Disease Association. Briefly, minimal lung disease or mild-TB is defined when CXR shows infiltrates of slight to moderate density, present in a small portion of both lungs with a total volume of infiltrate(s) of one lung, and no cavitations present. Moderate-TB is defined as scattered lesions of slight to moderate density present in a total volume of infiltrate(s) of one lung and /or dense, confluent infiltrates present in one third of the volume of one lung, with or without cavities not greater than 4 cm. Advanced –TB is defined as lesions more extensive than moderate TB.

Granuloma formation is the pathological hallmark of the host response to M. tuberculosis, characterized by an accumulation of infected phagocytes surrounded by activated monocytes, macrophages and lymphocytes (Gonzalez-Juarrero et al., 2001). When the infection is successfully contained, the granuloma shrinks and may eventually calcify. This calcification can be observed in CXR of individuals who did not develop active TB in the past indicating a successful containment of M. tuberculosis. On the other hand, destroyed macrophages and lymphocytes release large amounts of proteolytic enzymes (liquefaction process) which form a place where M. tuberculosis can grow extracellularly. Cavitation is a process in which liquefied granulomas breach the mucosal surface by tissue destruction, resulting in M. tuberculosis spread to other regions of the lung or to the environment by coughing. CXR is, thus, supportive for TB diagnosis, especially in a poor area where microscopy or culture of M. tuberculosis is lacking.

Detection of M. tuberculosis
The gold standard for diagnosis of course is the pathogen detection. It is, for diagnosis of TB, essential to obtain two consecutive sputa positive for acid-fast bacilli. The bacilli are easy to detect under the microscope, however, it is difficult to obtain sputum from TB patients. Better patient instruction may help to improve the volume and the quality of sputum samples which may increase the yield of sputum microscopy, and thus improve TB diagnosis (Alisjahbana et al., 2005). The culture of M. tuberculosis, which takes 4 to 6 weeks, gives a definite diagnosis. In poor areas where culture of M. tuberculosis is difficult or not routinely available, a patient with two sputum smears positive for the bacilli is also considered a definite case.
PCR and nucleic acid-based methods for detecting \textit{M. tuberculosis} DNA sequences require complex equipment and highly skilled staff. Also, they are expensive and unsuitable for routine diagnostic testing in developing countries (Beige \textit{et al.}, 1995). Rapid serological diagnostic tests such as the enzyme-linked immunosorbent assay (ELISA) and membrane chromatography tests, in contrast, are simple and inexpensive, and the latter can be point-of-care devices (Banica \textit{et al.}, 1994). A major problem encountered in serological techniques is the specificity and reactivity of antigens used. A majority of \textit{M. tuberculosis} antigens studied to date have homology with analogous proteins of environmental mycobacteria or other bacteria, resulting in unspecific reactivity to antibodies in patients with inactive TB or non-tuberculous infections (Bothamley \textit{et al.}, 1995; Lyashchenko \textit{et al.}, 1998). Hence, positive test results produced by these known antigens are generally unreliable.

Purified Protein Derivative (PPD), an ammonium sulphate precipitate of heat inactivated stationary phase cultures, a crude, poorly defined mixture of mycobacterial antigens containing both secreted and somatic proteins, has been used for TB diagnosis as a skin test regent for DTH responses since more than half a century. It contains many mycobacterial antigens, some of which are shared among pathogenic mycobacteria belonging to the \textit{M. tuberculosis} complex (\textit{M. tuberculosis}, \textit{M. bovis}, and \textit{M. africanum}), environmental nontuberculous mycobacteria (NTM), and the vaccine substrain \textit{M. bovis} bacilli Calmette-Guerin (Harboe \textit{et al.}, 1981). Thus although responsiveness to PPD is an important aid in the diagnosis of TB and can give an indication of exposure to mycobacteria, it is highly cross-reactive and it is often impossible to distinguish BCG vaccination and exposure to NTM from \textit{M. tuberculosis} infection (Fogan \textit{et al.}, 1969). It has been apparent, therefore, that a new diagnostic reagent with specificity for \textit{M. tuberculosis} and \textit{M. bovis} is needed to overcome the limitations of PPD. The recent identification of regions of the \textit{M. tuberculosis} genome that are missing from BCG and most NTM provides a new opportunity for the development of novel diagnostic tools (Brosch \textit{et al.}, 1998; Deuskar \textit{et al.}, 1991, Lyashchenko \textit{et al.}, 1998). One such region is the RD1 region, which is deleted from all BCG strains but present in the \textit{M. tuberculosis} complex (Harboe \textit{et al.}, 1996; Sorenson \textit{et al.}, 1995).
Diagnostic potential of culture filtrate proteins

The recent identification of regions of the *M. tuberculosis* genome that are missing from BCG and most NTM provides a new opportunity for the development of novel diagnostic tools (Brosch *et al.*, 1998; Deuskar *et al.*, 1991). In 1996, Stover and co-workers used subtractive hybridization to identify regions on the genome of *M. bovis* that were deleted in BCG (Mahairas *et al.*, 1996). These regions were called RD-1, RD-2, and RD-3, and the first two regions contained the genes for ESAT-6 and the 24-kDa antigen MPT64, which previously had attracted a great deal of interest as an antigen with diagnostic potential.

ESAT-6, is broadly recognized early during disease in different species infected with *M. tuberculosis* or *M. bovis* (Andersen *et al.*, 1995; Brandt *et al.*, 1996; Pollock *et al.*, 1997; van Pinxteren *et al.*, 2000), and this antigen is generally reported to trigger the release of high levels of IFN-γ by sensitized PBMC from TB patients (Mustafa *et al.*, 1998; Ravn *et al.*, 1999; Ulrichs *et al.*, 1998). This antigen discriminates TB patients from both BCG-vaccinated and *M. avium* patients and has therefore been suggested as a candidate for in vitro TB diagnosis (Ravn *et al.*, 1999; Ulrichs *et al.*, 1998).

Failure of BCG: need for new Vaccine(s)

TB remains a major cause of morbidity and mortality worldwide, in spite of the availability of excellent antimycobacterial therapies and a vaccine (Mycobacterium bovis bacillus Calmette-Guerin [BCG], since 1921), which is one of the most extensively utilized vaccines in the world. BCG was developed by attenuation of *M. bovis*, which is closely related to *M. tuberculosis* (>90% DNA homology) and is a part of the *M. tuberculosis* complex. French scientist Albert Calmette and Camille Guerin of Pasteur institute developed BCG at the start of 20th century by growing it on culture medium and monitoring its decrease in virulence in animals through this period (Calmette *et al.*, 1929). BCG is effective against severe forms of childhood tuberculosis but is of limited efficacy against adult pulmonary disease in endemic areas (Fine, 1995). Its protective efficacy ranges from 0 to 80% in different clinical trials (Fine, 1989; Orme, 1999). Several reasons for its variable efficacy have been proposed ranging from the influence of prior infection with environmental mycobacteria (Brandt *et al.*, 2002) to the absence of antigens that are protective
against *M. tuberculosis* (Fine, 1995; Harboe *et al.*, 1996). Reasons for the variation in protective efficacy includes: i) genetic variation in the population studied; ii) differences in BCG strains, doses and vaccination schedules; iii) administration of vaccine to individuals already infected and iv) interaction between the vaccine strain and environmental mycobacteria (Andersen, 2001; Kaufmann, 2001). More effective anti-tuberculosis vaccines are therefore urgently needed. The identification of mycobacterial antigens which induce protective immunity is crucial for developing such vaccines.

To meet the ever-growing need for an effective vaccine against tuberculosis, several antigen discovery programs were undertaken in the 1980s in an attempt to find new immunogens that could constitute subunit or recombinant vaccines to replace the live vaccine BCG. It was widely believed that protection against the disease required the induction of Th1-type immune responses against secreted or surface exposed polypeptides. The rationale for this was inspired by the long established finding that, in contrast to vaccination with live BCG, immunization with killed BCG confers little or no protection against infection with *M. tuberculosis*, thus giving rise to the notion that active secretion of antigenic proteins induces appropriate protective T-cell responses (Horwitz *et al.*, 1995). An obvious place to search for such antigens was in the culture supernatant of in vitro grown *M. tuberculosis*, particularly in the early phase of growth, because many apparently cytoplasmic proteins, such as certain chaperones and enzymes, accumulate there in the later stages (Orme *et al.*, 1993; Andersen *et al.*, 1991).

Consequently, short-term culture filtrate proteins were partially purified and their antigenicity assessed in proliferation and IFN-γ production assays with lymphocytes isolated from mice infected with *M. tuberculosis* (Andersen *et al.*, 1995; Andersen, 1994), and later from humans or cattle suffering from tuberculosis. In this way, some of the lower molecular mass CFP were found to induce impressive immune responses (Sorensen *et al.*, 1995).
Vaccines against TB

Three approaches have been used to produce new vaccines:

- nonliving vaccines, which include purified antigens (subunit vaccines) and DNA vaccines;
- live mycobacterial vaccines, which include rationally attenuated strains of *M. tuberculosis* produced by targeted deletion of virulence genes or BCG strains that have been genetically manipulated to express immuno-dominant antigens (recombinant BCG);
- the use of viral vectors, such as adenovirus or vaccinia virus.

Thus, BCG may continue to play an important role in immunization, either as a primer to be boosted by new components or as an integral component of new vaccines (Ly et al., 2008).

**Prime-boost vaccination strategies:** Repeated vaccination with the same vaccine results in higher levels of antibodies than following a single vaccination. Such homologous boosting is sufficient for organisms for which the protective immune response is dependent on humoral immunity. However, the induction of a strong, cellular immune response by homologous prime-boost vaccination is not as straightforward. Repeated homologous boosting with the same vaccine does not necessarily result in the magnitude of the cellular immune response induced, as pre-existing host immunity inhibits antigen presentation (McShane et al., 2005). The results from a number of studies are far from promising (Xing et al., 2007). With or without novel adjuvants, such as arabinomannan-tetanus toxoid conjugate, BCG boosters were unable to provide additional protection above the initial immunization in mice (Haile et al., 2005). In addition, a recent, large human trial clearly demonstrated that revaccination of school-aged children in Brazil did not confer additional protection over the placebo group (Rodrigues et al., 2005). Thus, the strategy of heterologous prime-boosting has been developed. Novel vaccine candidates with the potential to be used for boosting purposes are usually evaluated first in animal models as stand-alone vaccines for their immunogenicity and protective efficacy. Once they are proven as stand-alone vaccines, they are then tested using heterologous prime-boost protocols in which BCG is used as a priming vaccine (Dietrich et al., 2006; Andersen et al., 2007).
Modified BCG vaccines: The main rationale of recombinant BCG (rBCG) development is the hypothesis that the immunogenicity of BCG has been weakened by continuing attenuation and gene loss (Behr et al., 1997). Thus, adding deleted genes back into BCG or increasing the expression of selected protective antigens might improve the effect of BCG vaccination (Hernandez-Pando et al., 2007; Nasser et al., 2005).

Attenuated M. tuberculosis vaccines: Whole genome microarray analysis reveals the absence of 15-16 regions that have been deleted from the genome of M. bovis BCG, but are present in M. tuberculosis. These regions of difference (RD) are believed to encode at least 129 open reading frames, which include several regulatory genes (Mahairas et al., 1996; Behr et al., 1999). These missing regions may encode potential antigenic determinants that could increase the immunogenicity of a vaccine, perhaps explaining a portion of the variable efficacy of different BCG vaccines. Hence, the hypothesis that using a TB vaccine derived from the human tubercle bacillus would protect better than BCG has driven the testing of live-attenuated mutants of M. tuberculosis (Sambandamurthy et al., 2005; Hernandez et al., 2006).

DNA vaccines: DNA vaccination is one of the most published approaches to protect against virulent challenge in animal models. This may be due to relative ease of preparation of DNA vaccines, which avoids complicated protein purification and chemical adjuvant issues. DNA vaccines are also amenable to the insertion of biological adjuvants, such as CpG motifs or cytokine genes. The DNA vaccine approach is complicated by a great degree of variability in the outcome of animal experiments conducted in different laboratories, as well as by the real possibility that immunization of infected individuals may, in fact, induce unacceptable immunopathology (Lowrie et al., 2006).

Subunit (protein/peptide) vaccines: Subunit TB vaccines are based upon purified mycobacterial proteins and peptides

Vaccines in pipeline:
Recent years have seen a renewed effort to develop new vaccines against tuberculosis. As a result, several promising avenues of research have developed, including the
production of recombinant vaccines, auxotrophic vaccines, DNA vaccines and subunit vaccines. The availability of an effective, licensed vaccine by the year 2015 has been proposed as a realistic target within The Global Plan to Stop TB (Stop TB Partnership and WHO, 2006). To reach this goal, it is estimated that at least 20 vaccine candidates should enter phase I safety trials, with around half going forward for immunological evaluation in phase II trials and leading to four phase III efficacy trials. There are several vaccines which are currently under different stages of clinical trials, some of them are mentioned below.

**rBCG30**- The rBCG30 vaccine, has recently passed phase I clinical trials (Horwitz *et al.*, 2003; Horwitz *et al.*, 2005). This vaccine was derived from a vaccine strain of BCG, which has been genetically modified to overexpress the immunodominant antigen Ag85B (Horwitz *et al.*, 2003). The idea behind this vaccine was to improve BCG by expressing higher levels of an antigen that had already been shown to be protective. The improved efficacy of rBCG30 came as something of a surprise, since BCG possesses a functional gene for Ag85B. However, recent work has shown that overexpression of genes can alter the immune response to the antigens they encode (Rao *et al.*, 2003).

**rBCG::ΔureCllO+**- This BCG based vaccine takes a different approach. The rBCG::ΔureCllO+ vaccine is a urease-deficient mutant of BCG, which expresses the *Listeriolyis O* gene from *Listeria monocytogenes*. This bacteria is unable to arrest phagosome maturation (due to the urease deficiency), and is less virulent than wild-type BCG in immunodeficient *scid* mice. In addition, the decreased pH in the maturing phagosome provides optimal conditions for the listeriolyisin, which is thought to damage the phagosome membrane, allowing leakage and potentially increasing the amount of bacterial-derived antigen(s) available for presentation to CD8+ T cells via the cytosolic scavenger pathways (Conradt *et al.*, 1999, Grode *et al.*, 2002).

**MVA-85A**- In contrast to vaccines designed to replace BCG, late booster vaccines aim to take advantage of the widespread use of BCG by boosting immunity in young adults already primed by earlier vaccination in childhood. One of the first type of such vaccines to be tested in humans is MVA-85A (Goonetilleke *et al.*, 2003), a
recombinant, replication-deficient vaccinia virus, expressing the strongly immunogenic antigen 85A from *M. tuberculosis*. This vaccine has performed well in animal models and is now in phase I-b clinical trials.

*M. tuberculosis* 72f- Among new TB vaccines entering clinical trials *M. tuberculosis* 72f is a vaccine based on recombinant protein. In the past, recombinant protein vaccines have not been very successful at stimulating strong Th1 responses, due to the lack of adjuvant suitable for generating strong cell-mediated immune responses in humans without generating unacceptable side effects. However, new adjuvants are now entering the clinic and several of these generate strong Th1 responses, making them good candidates for vaccines against *M. tuberculosis*. Jointly developed by Corixa and GSK, this vaccine is a fusion molecule comprised of two proteins, with the PPE family member Rv1196 inserted into the middle of the putative serine protease Rv0125, which is thus present as two fragments. The adjuvant used contains the saponin derivative QS21 mixed with the TLR4 ligand monophosphoryl lipid A (Skeiky *et al.*, 2004). In addition to its activity for priming, this vaccine has also been demonstrated to have a BCG booster effect (Brandt *et al.*, 2004).

Ag85B-ESAT-6- Another recombinant protein, developed independently by the Statens Serum Institute, is a fusion molecule comprised of two immunodominant, secreted proteins from *M. tuberculosis* (ESAT-6 and Ag85B) and has proven highly efficacious in animal models ranging from mice to primates, more effective in fact, than the single antigens (Doherty *et al.*, 2004, Olsen *et al.*, 2004, Weinrich Olsen *et al.*, 2001). The vaccine has also proven effective as a booster for BCG and augments its efficacy even though the ESAT-6 component of the vaccine is not present in BCG.

**Vaccine potential of Culture filtrate proteins**
Growing mycobacteria release proteins into their surroundings (Abou-Zeid *et al.*, 1988; Collins *et al.*, 1988). These secreted proteins have been suggested as protective antigens responsible for rapid recognition of bacilli by host lymphocytes (Abou-Zeid *et al.*, 1988). This hypothesis has been supported by the finding that whereas immunization with live bacilli efficiently generated protective T lymphocytes, killed preparations did not (Orme, 1988).
Among many antigens present in the culture filtrate, members of Ag85 complex (Ag85A, B, and C) have probably been evaluated most extensively. Th1 type responses induced in experimental animals have been postulated to be responsible for the protective efficacy of Ag85A and Ag85B proteins (D’Souza et al., 2000). A level of protection superior to BCG has been demonstrated to be induced by vaccination with recombinant BCG over-expressing Ag85B (Horwitz et al., 2000). These findings argue strongly in favor of Ag85B as an important candidate for tuberculosis vaccine.

BCG vaccination of humans although induces T cell responses to Ag85 complex but protection remains incomplete. It is, therefore envisaged that in order to achieve more effective induction of cell-mediated immunity, including memory immunity, additional antigens may be required. These antigens could be the important T cell antigens missing in the genome of BCG. Several researchers have postulated that absence of many immuno-dominant proteins in BCG might be an important reason for its inability to prime a potent immune response that can protect against *M. tuberculosis* infection (Behr et al., 1999; Skjot et al., 2000; Andersen, 2001). The genes of these missing proteins are clustered in 16 regions of difference (RDs) and in total 129 open reading frames (ORFs) are missing in all BCG strains, indicating a more direct link with attenuation of BCG (Behr et al., 1999). Numerous studies have demonstrated the antituberculous vaccine potential of ESAT-6 and MPT-64, the proteins absent in BCG (Brandt et al., 2000; Morris et al., 2000; Weinrich Olsen et al., 2001). Recombinant BCG that secretes ESAT-6 in culture filtrate has been shown to exhibit increased protection against *M. tuberculosis* (Pym et al., 2003). Besides ESAT-6 and MPT-64, other proteins of RD regions such as CFP21 and CFP-10 are known to induce strong IFN-γ production, proliferation of T cells and moderate cytotoxic T cell activity in *M. tuberculosis* infected mice (Weldingh et al., 1998; Shams et al., 2004). These results indicate that it is worthwhile to evaluate the immunoprophylactic potential of these RD encoded proteins as DNA vaccines against experimental tuberculosis.

Till date, several mycobacterial secretory proteins e.g. MPT-64 (Kamath et al., 1999), Pst-S (Tanghe et al., 1999), Ag85A (Tanghe et al., 2000), Ag85B (Kamath et al., 1999), ESAT-6 (Mollenkopf et al., 2001), *M. tuberculosis* 8.4 (Coler et al., 2001) and
A number of studies document the interactions of \textit{M. tuberculosis} secretory antigens with various cells of the immune system (Harboe \textit{et al}., 1996; Sorenson \textit{et al}., 1995). For example, ESAT-6 has been designated as an important T cell antigen recognized by protective T cells in animal models of infection with \textit{M. tuberculosis} (Brandt \textit{et al}., 1996). Further, together with MPT64, ESAT-6 has been shown to have potential in the diagnosis of \textit{M. tuberculosis}, as they are recognized by T cells in animal models of \textit{M. tuberculosis} (Elhay \textit{et al}., 1998; Oettinger \textit{et al}., 1994). MPT64 has been evaluated as a skin test reagent in guinea pig model of tuberculosis (Haga \textit{et al}., 1995) and in humans (Roche \textit{et al}., 1996). ESAT-6 and MPT64 has consistently been shown to induce strong and TB-specific DTH responses in the guinea pig model of TB (Brandt \textit{et al}., 2000). In another study it was shown that the combination of ESAT-6 and CFP10 is highly specific for \textit{M. tuberculosis}, with a low reactivity in BCG-vaccinated individuals responsive to PPD (van Pinxteren \textit{et al}., 2000). The above volume of data indicated that secretory antigens are strong targets for B and T cell responses, and the potential of these antigens in regulating the immune responses needs to be explored further.

TB continues to present a formidable challenge to vaccinologists. Progress has been achieved over the last decade by combining fundamental research on the immune response to mycobacterial infection with empirical testing of novel candidates in animal models. Investigation of the immunogenicity of selected candidates in human trials represents the next important stage in vaccine development.
**M. tuberculosis** and HIV

Tuberculosis develops very rapidly and fatally in the context of HIV/AIDS. *M. tuberculosis* remains latent in humans and of more than 2 billion individuals infected with *M. tuberculosis* only approximately 10% will develop tuberculosis. In most of the cases, the pathogen is not eradicated, but is contained in distinct foci by the immune system. Infection with HIV results in the reemergence of latent TB and is contagious at all stages (Kaufmann *et al.*, 2005) Human immunodeficiency virus is a potent risk factor for tuberculosis. Not only does HIV increase the risk of reactivating latent *M. tuberculosis* infection it also increase the risk of rapid TB progression soon after infection or re-infection with *M. tuberculosis*. In individuals infected with only *M. tuberculosis*, the lifetime risk of developing TB ranges between 10% and 20%. In persons coinfected with *M. tuberculosis* and HIV, however the annual risk can exceed 10%. The TB burden in countries with a generalized HIV epidemic has therefore increased rapidly over the past decade, especially in the severely affected countries of eastern and southern Africa (Corbett *et al.*, 2003). Globally, an estimated 13% of adults with newly diagnosed tuberculosis were infected with HIV in 2004, but there was great variation among the regions, from 34% in the African region to 1.4% in the western pacific region. Rates of HIV infection in patients with tuberculosis have so far remained below 1% in Bangladesh, China, Indonesia, and Pakistan (Dye, 2006).

**MDR and XDR-TB**

The era of TB chemotherapy begun in the 1950s with the finding of Para-Aminosalicylate (PAS) by Lehmann (1944), followed by other findings including streptomycin by Waksman and Schatsz (1943), and isoniazid by Domagk (1952) (Iseman, 2000). Though an efficient TB treatment exists which makes TB a curable disease, TB nevertheless kills 5000 people every day (WHO report, 2006). The main therapeutic strategy, as defined and recommended by the WHO, is to use a combination of three or four different anti-TB drugs: isoniazid, rifampin, pyrazinamide and/or ethambutol. The course of TB treatment is lengthy (4-6 months), because there are several goals that need to be achieved e.g. to rapidly kill the massive numbers of bacilli, to prevent the emergence of clinically significant strains of drug-resistant mutants, and to effectively sterilize the disease sites (Iseman, 2000). In many parts of the world, access to TB drugs is limited, and compliance with the drug regimen is poor. The complicated regimen, the drugs' side effects, and the lengthy
treatment can trigger patients to fail to complete therapy (Mitchison et al., 2005). For the latter reason, a directly observed TB therapy short-course (DOTS) has been widely implemented. However, new drugs are urgently needed. Global surveillance has shown that multi-drug resistance (MDR) is becoming widespread and is a threat to TB control programs in many countries (Espinal et al., 2003; Johnson et al., 2006). MDR-TB is defined as being resistant to the first-line TB drugs e.g. isoniazid and rifampin. MDR-TB treatment therefore requires the use of second-line drugs which are less effective, more toxic and more expensive than the first-line. In a recent report summarized by the Centers for Disease Control (CDC) and the WHO, a survey of several international TB laboratories during 2000 - 2004 had determined that of 17,690 TB isolates, 20% were MDR and 2% were even extremely or extensively drug resistant (XDR) (Anonymous, 2006). XDR-TB has been identified in all regions of the world, prevalent mostly in Asia and in Eastern Europe, but so far only or mostly in HIV co-infected individuals. XDR-TB may emerge as a worldwide threat, raising concerns of future untreatable TB epidemics. No new anti-TB drugs have been licensed in the past decades. Ideally, new drugs should have a high activity to reduce the duration of treatment, the ability to kill persistent bacilli that might otherwise reactivate later in life and activity against multi-drug resistant TB strains (Mitchison et al., 2005). Recent findings reported the possibility of breakthroughs in the field of anti-TB drug discovery, thanks to whole genome sequencing and comparative genome analysis (Andries et al., 2005). R207910, a diarylquinoline, has been proposed as a novel drug that inhibits both drug-sensitive and drug-resistant M. tuberculosis in vitro by targeting the proton pump of adenosine triphosphate (ATP) synthase.

**Biology of TB**

*Mycobacterium* is a member of class Actinobacteria and is a facultative intracellular pathogen usually infecting mononuclear phagocytes (e.g. macrophages). It grows successfully in tissues with high oxygen content such as the lungs and is obligate aerobe having a generation time of 12 to 18 hours. It has a unique lipid rich cell wall that retains carbol fuchsin dye even in the presence of acidic alcohol, also called as acid fast staining. The mycobacterial cell envelope has been found to play a fundamental role in the physiology of these bacteria, both in pathogenicity and resistance to antibiotics. The cell envelope of *M. tuberculosis* is unique both in terms
of its molecular composition and the architectural arrangement of its constituents (Adam et. al., 1969). A key feature of the mycobacterial envelope is its high lipid content and the variety of lipid compounds with unusual structures (Lederer et. al., 1975).

*M. tuberculosis* is a member of *Mycobacterium tuberculosis* complex, defined as 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 et al., 2007). Other members of this group are *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium bovis*, and *Mycobacterium canetti*. The subspecies can only be distinguished by a limited number of phenotypic characteristics but differ remarkably with respect to their host range and pathogenicity. *M. microti* is an exclusively rodent pathogen, *M. canetti* and *M. africanum* are agents of human tuberculosis in sub-Saharan Africa while *M. bovis* infects a wide variety of mammalian species including humans.

**Main features of genome of *Mycobacterium tuberculosis***

The sequence of the complete genome of *M. tuberculosis* H37Rv comprises of 4,411,529 base pairs. It has a high G+C content of 65.6%, a parameter associated with an aerobic lifestyle. The complete genome sequence and annotation of H37Rv strain of *M. tuberculosis* was published in 1998 (Cole et al., 1998). The annotation has identified 4,043 genes thought to encode 3,993 proteins and 50 stable RNAs (Cole, 2002). The annotated genome shows some unique features. Close to 250 genes are annotated as encoding enzymes for the metabolism of fatty acids, comprising 6% of the total coding capacity. Among these are approximately 100 that are predicted to function in the β-oxidation of fatty acids, which is not strange given the fact that Mycobacteria contain examples of every known lipid and polyketide biosynthetic system, including enzymes usually found in mammals and plants as well as the common bacterial systems. With the completion of the genome sequence of the H37Rv strain of *M. tuberculosis* a vast body of information about the bacterium’s protein content, or proteome, became available. This provided us with insights into the biochemical and physiological processes that govern the life of the tubercle bacillus, highlighting the importance of lipid metabolism as well as indicating the
existence of two novel protein families, PE and PPE, with unusual amino acid sequences.

Another distinguishing feature of the *M. tuberculosis* genome is the presence of the unrelated PE and PPE families of acidic, glycine-rich proteins, named so because of the presence of Proline-Pro-Glutamine (PE) and Pro-Pro-Glu (PPE) sequences found in the two conserved N-terminal regions in each of these protein families that are approximately 110 and 180 amino acids long, respectively. Out of the 172 genes, 104 belong to the PE class and 68 to the PPE variety, and together they comprise over 4% of the genes in *M. tuberculosis*. The genes encoded by the 104 PE genes can be further subdivided into three classes, containing 29 proteins with the PE region alone, 8 proteins in which the PE region is followed by unrelated C-terminal sequences; and 67 proteins that form the PE-PGRS subfamily. This group of proteins has the conserved PE domain followed by C-terminal extensions with multiple repeats of Glycine-Gly-(-Alanine)Ala or Gly-Gly-Asparagine (Asn) that are in the PGRS (for “polymorphic GC-rich repetitive sequences”) domains (Smith, 2003). Nevertheless, functional information remains scarce for the PE-PGRS proteins. On the basis of sequence similarity to EBNA, the Gly- and Ala-rich Epstein–Barr nuclear antigen, it has been proposed that PE-PGRS proteins could act as proteasome inhibitors and block antigen presentation by the MHC I pathway. According to size variations observed in members of the PE-PGRS subfamily in clinical TB strains, and because of the fact that many of these proteins have been localized in the cell wall and cell membrane it can be hypothesized that at least some of these proteins may be involved in antigenic variation of *M. tuberculosis* during infection (Banu et al., 2002).

Among transcriptional regulatory proteins *M. tuberculosis* has thirteen putative sigma factors corresponding to 0.3% of the total genes and 22 other regulatory proteins, including 13 two component response regulators corresponding to 0.6% of the total coding capacity. The sequencing of the genome of *M. tuberculosis* has thrown up a lot of possibilities towards finding virulence determinants and answering questions relating to the pathogen’s success in establishing persistence. Among many putative virulence determinants of this bacterium, one is RD1 region.
RD1 Region in *M. tuberculosis*

The elucidation of complete genome sequence of H37Rv, the most widely used strain of *M. tuberculosis*, has broadened the horizons for the research and development of new prophylactic and therapeutic interventions against tuberculosis. In an attempt to identify factors leading to the attenuation of *M. bovis* BCG (BCG), Mahairas *et al.* carried out a relatively simple experiment where they compared the genomic sequences of BCG and *M. bovis*, by using subtractive hybridization, and found that there were three regions of difference (designated RD1, RD2, and RD3) present in the genome of *M. bovis*, but missing in BCG. It was later identified that there are actually 16 large deletions, including RD1-RD3, which were present in the *M. tuberculosis* genome but absent in BCG (Behr *et al.*, 1999, Gordon *et al.*, 2001). Eleven of these 16 deletions were unique to *M. bovis* whereas the remaining 5 deletions were unique to BCG. One of these 5 deletions, designated RD1 (9,454 bp), was absent from all BCG substrains currently used as tuberculosis vaccines worldwide, and it was concluded that the deletion of RD1 occurred very early during the attenuation of BCG, probably before 1921. RD1 region is a genetic locus encoding 9 proteins from Rv3871 through Rv3879. Two of the genes within RD1 esat-6 and cfp-10 (Early secretory antigenic target-6 kDa and culture filtrate protein of 10 kDa) encode extracellular proteins ESAT-6 and CFP-10. Both proteins have been investigated for their utility in diagnosing tuberculosis (Pollock *et al.*, 1997) and for differentiating latent infection from BCG vaccination (Lalvani *et al.*, 2001). The ESAT-6 and CFP-10 genes are cotranscribed in *M. tuberculosis* (Berthet *et al.*, 1998) and when coexpressed in *Escherichia coli*, they form a tight 1:1 complex (Renshaw *et al.*, 2002). Both these proteins are highly immunogenic and are found in the early culture filtrates of *M. tuberculosis* after short periods of growth and in the absence of obvious autolysis. Though none of the nine open reading frames (ORFs) that comprise RD1 have a biochemically assigned function, this region has been carefully scrutinized in *silico*. The predicted functions of several RD1 region genes suggest they may have roles in protein translocation. Further, as CFP-10 and ESAT-6 lack clear secretion signals, they may require a novel secretion machinery for export (Braunstein and Belisle, 2000), and components of RD1 may form that machinery (Cole *et al.*, 1998; Tekaia *et al.*, 1999; Gey Van Pittius *et al.*, 2001; Pallen, 2002; Lewis *et al.*, 2003). This notion was proved true when disruption of individual genes (Rv3870, Rv3871...
and *Rv3877* within this locus prevented secretion of ESAT-6 and CFP-10, providing the first genetic evidence that this region encodes for a secretion system.

Though a lot of work has been carried out to elucidate exact function(s) of CFP-10 and ESAT-6 in particular in *M. tuberculosis* virulence and RD1 region in general, but the picture remains unclear. However, some studies do indicate their active participation in the spread of intracellular bacilli, in one of the reports RD1 deletion mutants were able to infect alveolar lung epithelial cells but failed to lyse them resulting in reduced lung pathology (Hsu *et al.*, 2003). In another study RD1 mutant produced no discernible cytotoxicity in either peripheral blood mononuclear cells or human macrophage like THP-1 cells. Mutant bacilli were capable of intracellular growth within THP-1 cells, but were unable to spread to new cells (Guinn *et al.*, 2004). Complementation and gene disruption studies further established that RD1-encoded genes were required for the full virulence of *M. tuberculosis*. In one study, RD1 region was deleted from *M. tuberculosis* H37Rv and the resulting mutant had the same virulence phenotype as that of BCG strains (Lewis *et al.*, 2003). The other experiments used a knock-in approach, in which the RD1 region was inserted into the chromosome of BCG. These studies showed that the complemented strain was much more virulent than its attenuated parent (Pym *et al.*, 2002). Cox and others showed that disruption of individual RD1-region genes did not prevent production of ESAT-6 or CFP-10. However, an intact RD1 region was required to ensure that these proteins were secreted by the bacterium (Stanley *et al.*, 2003, Pym *et al.*, 2003).

RD1 region seems to be a major determinant of mycobacterial pathogenesis but the mechanism by which this system affects the biology of the host cell is unknown. Many groups have suggested that this system functions to modulate early events during *M. tuberculosis* infection. RD1 region therefore represents one of the most interesting genomic regions of *M. tuberculosis* since it seems to be simultaneously involved in enhanced virulence in immuno-compromised hosts and increased protection in immuno-competent hosts and presents a challenge to researchers to identify the tactics employed by *M. tuberculosis* to ensure its survival in the intracellular environment.
Mycobacterium tuberculosis infection

From the first exposure to M. tuberculosis in the airways, a series of immune responses are triggered. Briefly, M. tuberculosis infects and replicates within the phagosome of alveolar resident macrophages, macrophages that differentiate from blood monocytes that are recruited to the site of infection (Algood et al., 2003), as well as dendritic cells (DCs) (Gonzalez-Juarrero et al., 2001). These cells, responsible as the first barrier in the lungs of the host, develop natural defense mechanisms to eliminate the mycobacteria, and, in the case of DC, migrate to draining lymph nodes to prime or boost specific T cells. The intrinsic microbial capacity of phagocytes and the virulence factors of the ingested mycobacteria will determine intracellular mycobacterial survival. In most cases, mycobacteria can evade intracellular destruction, resulting in mycobacterial multiplication and disruption of the macrophages. Blood monocytes and other inflammatory cells that are attracted to the site of infection, however, could subsequently ingest the extracellular mycobacteria released from the disrupted macrophages. T cell immunity develops two to three weeks after infection and activate macrophages to eliminate the intracellular mycobacteria (Van Crevel et al., 2002). These activated infected-macrophages undergo apoptosis, which may prevent dissemination of infection and reduce viability of intracellular mycobacteria. Moreover, apoptosis may facilitate DC mediated cross priming of CD8 effector cells. Necrosis of infected cells in contrast, does not reduce viability of intracellular mycobacteria and allow extracellular release of M. tuberculosis (Molloy et al., 1994). The released bacteria are taken up by activated macrophages within the granuloma, and will be contained or destroyed (Saunders et al., 2000). The state of the mycobacteria within the granuloma (the so-called tubercle) in latent infection is not known. The latent stage of infection is associated with a few bacteria surviving and perhaps replicating inside the granuloma. This latent stage might also be in a dormant non-replicating state with a low metabolic activity for years and M. tuberculosis could be contained as long as the individuals remain immunocompetent. The establishment of latent infection coincides with nutrient limitation, low pH, hydrolytic enzymes, reactive nitrogen and oxygen species and reduced oxygen tension (Andersen et al., 2005).

Upon triggering of microbial pattern recognition receptors (PRR); e.g. Toll-like receptors (TLR) (Flynn et al., 2001), mannose receptors (Kang et al., 2005), C-type
lectins like DC-SIGN (Geijtenbeek et al., 2003), and NOD/NACHT receptors (Ferwerda et al., 2005), phagocytes including macrophages and dendritic cells (Ladel et al., 1997) are activated to produce an array of cytokines that may act in concert for optimal effector function of macrophages. These cytokines e.g. interleukin (IL)-12, IL-18, and IL-23 are recognized by complementary receptors (IL-12R, IL-18R, and IL-23R respectively) on type-1 helper T (Th1) cells and natural killer (NK) cells. The major pro-inflammatory cytokine, IL-12, links innate and adaptive immunity by driving the development of T cells and NK cells to produce Th1 pro-inflammatory cytokines, including IFN-γ and TNF, and regulates IL-17 production (Ottenhoff et al., 2005; Hoeve et al., 2006; Rogge et al., 1997). IFN-γ is the key activating cytokine and induces, in synergy with TNF, e.g. inducible isoform of nitric oxide synthetase (iNOS) expression in macrophages as the major antimycobacterial mechanism, at least in the mouse model. Thus, the elimination of \textit{M. tuberculosis} mainly depends on the success of the interaction between infected macrophages and T cells.

While studies in TB clearly showed that CMI plays an essential role in the control of infection, the humoral immune response in contrast is considered not to be associated with protection to TB. However, Th1 cells induces B cells to release antibodies of the immunoglobulin (Ig) G2 isotype, responsible for phagocyte activation and antibody-dependent cellular cytotoxicity (Jankovic et al., 2001). A strong antibody response is generated in the infected host, and B cells or antibodies may nevertheless assist in the control of infection although clearly playing only an accessory role. The humoral immune response facilitates immunodiagnosis of active TB (Senol et al., 2007). Several components of \textit{M. tuberculosis} are known targets for B cells, e.g. crude cell sonicates, culture filtrate, purified protein antigens, and cell wall lipids (Chan et al., 2000). The lipoprotein 38 kD, the most frequently studied serologically recognized antigen, is a component in different commercial TB serological tests. The practical use of serodiagnosis for active TB has, however, not been widely appreciated, partly because the reliability of current tests using protein antigens is not very satisfactory.

In a recent report by Azzurri et al., IgG antibody levels in plasma against ESAT-6, LAM and 38kDa Ag were higher in untreated patients than in community controls, representing serological correlates of active disease. These serological markers might be predictive in treatment outcome (Azzurri et al., 2006). However, these levels may
vary greatly depending on the stage of disease and depending on the structure of the antigens. Combinations of multiple selected antigens might give a higher sensitivity and specificity for screening strategy (Kanaujia et al., 2005).

**Granuloma Formation**

Infected macrophages in the lungs, through their production of chemokines, attract inactivated monocytes, lymphocytes, and neutrophils (Van Crevel et al., 2002), none of which kill the bacteria very efficiently (Fenton et al., 1996). Then, granulomatous focal lesions composed of macrophage derived giant cells and lymphocytes begin to form. This process is generally an effective means of containing the spread of the bacteria. As cellular immunity develops, macrophages loaded with bacilli are killed, and this results in the formation of the caseous center of the granuloma, surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes (Dannenberg et al., 1994). The strength of the host cellular immune response determines whether an infection is arrested here or progresses to the next stages. This enclosed infection is referred to as latent or persistent TB and can persist throughout a person’s life in an asymptomatic and non transmissible state. In individuals with efficient cell-mediated immunity, the infection may be arrested permanently at this point. The granulomas subsequently heal, leaving small fibrous and calcified lesions. However, if an infected person cannot control the initial infection in the lung or if a latently infected person’s immune system becomes weakened by immunosuppressive drugs, HIV infection, malnutrition, aging, or other factors, the granuloma center can become liquefied by an unknown process and then serves as a rich medium in which the now revived bacteria can replicate in an uncontrolled manner. At this point, viable *M. tuberculosis* can escape from the granuloma and spread within the lungs (active pulmonary TB) and even to other tissues via the lymphatic system and the blood (miliary or extrapulmonary TB). When this happens, the person becomes infectious and requires antibiotic therapy to survive (Dannenberg et al., 1994). The mycobacterial granuloma contributes to host protection through different mechanisms. Perhaps most importantly, granulomas prevent dissemination of bacteria. Often, the level of dissemination correlates better with clinical outcome than bacterial numbers. In addition to this barrier function, granulomas control bacterial numbers through a variety of mechanisms including the secretion of reactive nitrogen and oxygen species by T cell-activated macrophages. Granulomas also restrict tissue
damage by shielding the surrounding tissue from the chronic inflammation. The hallmark of human tuberculosis is the caseating granuloma with an acellular core of necrotic material that resembles cheese on gross examination (hence, the term caseation). Caseation necrosis contributes to creating infectious material that can be coughed up to spread mycobacteria to other individuals.

**Role of Cytokines during Mycobacterial Infection**

Recognition of *M. tuberculosis* by phagocytic cells leads to cell activation and production of cytokines, which in itself induces further activation and cytokine production in a complex process of regulation and cross-regulation. This cytokine network plays a crucial role in the inflammatory response and the outcome of mycobacterial infections. Some of the proinflammatory cytokines are discussed here:

**Tumour necrosis factor-α**- Stimulation of monocytes, macrophages (Valone *et al.*, 1988), and dendritic cells (Henderson *et al.*, 1997) with mycobacteria or mycobacterial products induces the production of TNF-α, a prototype proinflammatory cytokine. TNF-α plays a key role in granuloma formation (Kindler *et al.*, 1989; Senaldi *et al.*, 1996), induces macrophage activation, and has immunoregulatory properties (Orme *et al.*, 1999; Tsenova *et al.*, 1999). In mice, TNF-α is also important for containment of latent infection in granuloma (Mohan *et al.*, 2001). In tuberculosis patients, TNF-α production is present at the site of disease (Barnes *et al.*, 1993; Casarini *et al.*, 1999; Law *et al.*, 1996). Systemic spillover of TNF-α may account for unwanted inflammatory effects like fever and wasting. Clinical deterioration early in treatment is associated with a selective increase of TNF-α in plasma (Bekker *et al.*, 1998), and quick recovery is associated with a rapid decrease of TNF-α in plasma (Hsieh *et al.*, 1999). To limit the deleterious effects of TNF-α (Bekker *et al.*, 2000; Hernandez Pando *et al.*, 1994), systemic production of TNF-α is downregulated (Friedland *et al.*, 1995; Johnson *et al.*, 1994; Takashima *et al.*, 1990), and soluble TNF-α receptors which block TNF-α activity are increased (Juffermans *et al.*, 1998). KO mice which are unable to make TNF-α (Bean *et al.*, 1999; Kaneko *et al.*, 1999; Roach *et al.*, 2001) or the TNF-α receptor p55 (Flynn *et al.*, 1995; Senaldi *et al.*, 1996) display an increased susceptibility for mycobacteria. In line with this, the use of potent monoclonal anti-TNF-α antibodies in Crohn’s disease and rheumatoid arthritis has been associated with increased reactivation of
tuberculosis (including miliary and extrapulmonary disease) (Keane et al., 1997). In human tuberculosis, no TNF-α gene mutations have been found and no positive association has yet been established between gene polymorphism for TNF-α and disease susceptibility (Blackwell et al., 1997; Goldfeld et al., 1998).

**Interleukin-12**- IL-12 is a key player in host defense against *M. tuberculosis*. IL-12 is produced mainly by phagocytic cells, and phagocytosis of *M. tuberculosis* seems necessary for its production (Fulton et al., 1996; Ladell et al., 1997). IL-12 has a crucial role in the induction of IFN-γ production (O’Neill et al., 1998). In tuberculosis, IL-12 has been detected in lung infiltrates (Casarini et al., 1999; Taha et al., 1997), in pleurisy (Zhang et al., 1994), in granulomas (Bergeron et al., 1997), and in lymphadenitis (Lin et al., 1996). The expression of IL-12 receptors is also increased at the site of disease (Zhang et al., 1999). The protective role of IL-12 can be inferred from the observation that IL-12 KO mice are highly susceptible to mycobacterial infections (Cooper et al., 2000; Wakeham et al., 1998; Wang et al., 1999). In humans suffering from recurrent non-tuberculous mycobacterial infections, deleterious genetic mutations in the genes encoding IL-12p40 and IL-12R have been identified (Altare et al., 1998; Altare et al., 1998; De Jong et al., 1998; Frucht et al., 1996). These patients display a reduced capacity to produce IFN-γ (Ottenhoff et al., 1998). Recently, 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 (O’Neill et al., 1998; Sieling et al., 1994; Trinchieri et al., 1995) and which exerts its protective effects mainly through the induction of IFN-γ (Cooper et al., 2000).

**Interferon-γ** The protective role of IFN-γ in tuberculosis is well established (Flynn et al., 1993), primarily in the context of antigen-specific T-cell immunity (Anderson et al., 1997). Mycobacterial antigen-specific IFN-γ production in vitro can be used as a surrogate marker of infection with *M. tuberculosis* (van Crevel et al., 1999). In principal, naive (tuberculin skin test-negative) individuals do not show purified protein derivative (PPD)-stimulated IFN-γ production in vitro (van Crevel et al., 1999). However, in both PPD-positive and PPD-negative individuals, *M. tuberculosis* infected monocytes stimulate lymphocytes for the in vitro production of IFN-γ (Johnson et al., 1994). It has also been observed that PPD (consisting of
mycobacterial proteins) selectively induces IFN-γ production in PPD-positive individuals, while *M. tuberculosis* sonicate, which contains mycobacterial polyglycans and phospholipids, non-selectively induced IFN-γ production in PPD-positive and PPD-negative individuals alike. This *M. tuberculosis* sonicate stimulates production of monocyte-derived cytokines like TNF-α and IL-1β (van Crevel et al., 2000). These, as well as IL-12 and IL-18, may act as costimuli for antigen-independent IFN-γ production (Bancroft et al., 1991; Micaleff et al., 1996; O’Neill et al., 1998). In the absence of fully developed adaptive T-cell immunity, NK cells may be the main producers of IFN-γ either in response to IL-12 and IL-18 (Iho et al., 1999) or directly by exposure to mycobacterial oligodeoxynucleotides (Garcia et al., 1999). Second, lung macrophages were found to produce IFN-γ in *M. tuberculosis*-infected mice (Wang et al., 2000). This remarkable observation however needs confirmation. Third, T cells bearing limited T-cell receptor diversity, including T cells expressing γδ T-cell receptors (γδ T cells) and CD1-restricted T cells, may produce IFN-γ during early infection. γδ T cells may directly recognize small mycobacterial proteins (Janis et al., 1989) and non protein ligands (Constant et al., 1994; Kaufmann et al., 1996; Tanaka et al., 1995) in the absence of antigen-presenting molecules. In mice, a single priming with *M. tuberculosis* substantially increases the number of γδ T cells, but not the number of αβ T cells (CD4+ and CD8+ T cells) in draining lymph nodes (Janis et al., 1989). In mice infected with *M. tuberculosis*, γδ T cells accumulate at the site of disease (Griffin et al., 1991) and seem necessary for early containment of mycobacterial infections (D’Souza et al., 1997; Ladel et al., 1995). Like γδ T cells, CD1-restricted T cells do not react with mycobacterial protein antigens in the context of MHC class I or class II molecules. Instead, these T cells react with mycobacterial lipid and glycolipid antigens bound to CD1 on antigen-presenting cells (Moody et al., 2000; Moody et al., 2000; Porcelli et al., 1999; Sieling et al., 1995). CD1 molecules have close structural resemblances to MHC class I but are relatively nonpolymorphic. 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 (Porcelli et al., 1992; Rosat et al., 1999; Sieling et al., 1994). CD1-restricted T cells display cytotoxic activity and are able to produce IFN-γ (Stenger et al., 1997).
Interleukin-10- IL-10 is produced by macrophages after phagocytosis of *M. tuberculosis* (Shaw et al., 2000) and after binding of mycobacterial LAM (Dahl et al., 1996). T lymphocytes, including *M. tuberculosis*-reactive T cells, are also capable of producing IL-10 (Barnes et al., 1993; Boussiotis et al., 2000; Gerosa et al., 1999). In patients with tuberculosis, expression of IL-10 mRNA has been demonstrated in circulating mononuclear cells, at the site of disease in pleural fluid, and in alveolar lavage fluid (Barnes et al., 1993; Gerosa et al., 1999). Ex vivo production of IL-10 was shown to be upregulated in tuberculosis by some investigators (Hirsch et al., 1999; Torres et al., 1998), but this was not found by others (Lin et al., 1996). IL-10 antagonizes the proinflammatory cytokine response by downregulating the production of IFN-γ, TNF-α, and IL-12 (Fulton et al., 1998; Gong et al., 1996; Hirsch et al., 1999). Indeed, IL-10 transgenic mice with mycobacterial infection develop a larger bacterial burden (Murray et al., 1997). In line with this, IL-10-deficient mice showed a lower bacterial burden early after infection in one report (Murray et al., 1999), albeit normal resistance in two other reports (Erb et al., 1998; North 1998). In human tuberculosis, IL-10 production was higher in anergic patients, both before and after successful treatment, suggesting that *M. tuberculosis*-induced IL-10 production suppresses an effective immune response (Boussiotis et al., 2000).

Transforming Growth Factor-β- TGF-β also seems to counteract protective immunity in tuberculosis. Mycobacterial products induce production of TGF-β by monocytes and dendritic cells (Toossi et al., 1995). Interestingly, LAM from virulent mycobacteria selectively induces TGF-β production (Dahl et al., 1996). Like IL-10, TGF-β is produced in excess during tuberculosis and is expressed at the site of disease (Condos et al., 1998; Toossi et al., 1995). TGF-β suppresses cell-mediated immunity: in T cells, TGF-β inhibits proliferation and IFN-γ production; in macrophages it antagonizes antigen presentation, proinflammatory cytokine production, and cellular activation (Toossi 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 et al., 1998) and collagen matrix (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. tuberculosis* (Hirsch et al., 1997). Within the anti-inflammatory response, 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. Paradoxically, in the presence of both cytokines, T cells may be directed towards a protective Th1-type profile (Erard et al., 1999).

**Interleukin-4** - The deleterious effects of IL-4 in intracellular infections (including tuberculosis) have been ascribed to this cytokine’s suppression of IFN-γ production (Lucey et al., 1996; Powrie et al., 1993) and macrophage activation (Appelberg et al., 1992; de Waal Malefyt et al., 1993). In mice infected with *M. tuberculosis*, progressive disease (Hernandez pando et al., 1996) and reactivation of latent infection (Howard et al., 1999) are both associated with increased production of IL-4. Similarly, over-expression of IL-4 intensified tissue damage in experimental infection (Lukacs et al., 1997). Conversely, inhibition of IL-4 production did not seem to promote cellular immunity: IL-4−/− mice displayed normal instead of increased susceptibility to mycobacteria in two studies, suggesting that IL-4 may be a consequence rather than the cause of tuberculosis development (Erb et al., 1998; North 1998). In contrast, a recent study on IL-4 KO mice showed increased granuloma size and mycobacterial outgrowth after airborne infection (Sugawara et al., 2000). Compared with control mice, production of proinflammatory cytokines was increased in these animals and accompanied by excessive tissue damage. An increased production of IL-4 in human tuberculosis patients has been observed, especially those with cavitary disease (Sanchez et al., 1994; Schauf et al., 1993; Surcel et al., 1994; van Crevel et al., 2000). However, this is not a consistent finding (Barnes et al., 1993; Hernandez Pando et al., 1994; Lai et al., 1997; Lin et al., 1996), and it still remains to be determined whether IL-4 causes or merely reflects disease activity in human tuberculosis. Thus, the role of IL-4 in tuberculosis susceptibility is not yet entirely resolved.

**Role of T cell Subsets in Protection against Mycobacterial Infection**

The protective response to *M. tuberculosis* requires cell-mediated immunity. This pathogen is intracellular within the host, usually residing within macrophages, and thus T-cell effector mechanisms, rather than antibody, are required to control the infection. Within 1 week of infection with virulent *M. tuberculosis*, the number of activated CD4+ and CD8+ T cells in the lung-draining lymph nodes increases (Feng et
Between 2 and 4 weeks post-infection, both CD4+ and CD8+ T cells migrate to the lungs, and demonstrate an effector/memory phenotype (Serbina 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 et al., 1992) that likely participate in containing the infection within the granuloma and preventing spread or reactivation.

**CD4+ T cells**

*M. tuberculosis* resides primarily in a vacuole within the macrophage resulting in MHC Class II presentation of mycobacterial antigens to CD4+ T cells. This T-cell subset is of primary importance in the protection against *M. tuberculosis*. The primary effector function of CD4+ T cells is believed to be production of IFN-γ, and possibly other cytokines, sufficient to activate macrophages, which can then control or eliminate intracellular organisms. Although IFN-γ production by CD4+ T cells is a crucial effector function of this subset, these cells likely have other roles in controlling *M. tuberculosis* infection. The possible roles include apoptosis, which has been suggested to be important in controlling *M. tuberculosis* infection, (Keane et al., 1997, Balcewicz-Sablinska 1998) conditioning of antigen-presenting cells, help for B cells and CD8+ T cells, and production of other cytokines. Several reports have provided data that *M. tuberculosis* infection inhibits MHC Class II presentation of antigens to CD4+ T cells, which would contribute to the inability of the host to eliminate a persistent infection. The presentation of soluble ovalbumin to a T-cell hybridoma was reduced following *M. tuberculosis* infection of macrophages (Mazzaccaro et al., 1996). One mechanism by which *M. tuberculosis* infection might inhibit the recognition of macrophages by CD4+ T cells is by the downregulation of cell surface expression of MHC Class II molecules. However, the reports in the literature are somewhat contradictory on this point. Reiner et al (Hmama et al., 1998) used virulent *M. tuberculosis* and human macrophages to clearly demonstrate that the greatest reduction in MHC Class II expression occurred when the cells were also exposed to IFN-γ. Others have reported decreased gene expression using avirulent mycobacteria, such as *M. tuberculosis* H37Ra or BCG (Wojciechowski et al., 1999, Noss et al., 2000). Infection of DCs with *M. tuberculosis* did not result in diminished MHC Class II cell surface expression (Henderson et al., 1997, Stenger et al., 1998,
Bodnar et al., 2001). Thus, priming of CD4+ T cells, a major function of dendritic cells in vivo, is less likely to be affected by M. tuberculosis infection. This is supported by the fact that a strong CD4+ T-cell response is mounted to M. tuberculosis infection. Macrophages infected with M. tuberculosis also produce cytokines, such as TGF-β, IL-10 or IL-6, which can reduce T-cell stimulation (Gong et al., 1996, Hirsch et al., 1997, Rojas et al., 1999). It is clear that robust CD4+ T-cell responses are observed in patients infected with M. tuberculosis. The inability of the CD4 T cells to eliminate intracellular bacteria may be due to a lack of recognition or activation of infected macrophages.

**CD8+ T cells**

For many years, the focus of T-cell research in tuberculosis was the CD4+ T cell. In recent years, there has been increased attention paid to the CD8+ T-cell subset as well. CD8+ T cells recognize antigens presented on MHC Class I molecules, and these antigens are frequently derived from the cytoplasm of the cells. However, M. tuberculosis does not reside primarily in the cytoplasm, but in vacuoles inside the cell. Studies have suggested that the bacilli within the vacuoles may have access to the cytoplasm, perhaps via a pore in the membrane (Mazzaccaro et al., 1996; Teitelbaum et al., 1999). Presumably, antigens from the phagosome could enter the cytoplasm and be presented on MHC Class I molecules. Alternative Class I loading mechanisms for mycobacterial antigens have also been suggested (Canaday et al., 1999). It has been demonstrated that mycobacterial antigens derived from infected cells can be presented by MHC Class I to CD8+ T cells in humans and in mice, and antigens recognized by these cells have been identified (De Libero et al., 1988; Silva et al., 1994; Stenger et al., 1997; Tan et al., 1997; Zhu et al., 1997; Mohagheghpour et al., 1998; Lewinsohn et al., 1998; Lewinsohn et al., 2000). Studies in various mouse models have indicated that CD8+ T cells are required to control M. tuberculosis infection (Flynn et al., 1992; Behr et al., 1999; Sousa et al., 2000; van Pinxteren et al., 2000; Rolph et al., 2001). However, other researchers have reported that CD8+ T cells are not required for the control of M. tuberculosis infection (Mogues et al., 2001). Clearly, these cells are induced during the infection, and perhaps better induction, activation, or effector function by these cells could increase protection against tuberculosis. CD8 T cells also recognize various antigens from M. tuberculosis that are not presented by classical MHC Class I molecules, but by a closely related group of molecules, the
Class Ib molecules. These are non-polymorphic, and include the CD1 molecules (Porcelli et al., 1999) as well as H2-M3 (Chun et al., 2001) CD1 molecules primarily present lipid antigens from *M. tuberculosis* to CD8 T cells, thereby increasing the possible antigen source greatly. This is an exciting area of research, and may provide new vaccination possibilities. CD8 T cells can produce cytokines and act as cytotoxic cells. Cytokines produced by CD8 T cells in *M. tuberculosis* infection include IFN-γ and TNF-α, although there are likely more produced as well. IFN-γ production by CD8 T cells probably participates in activation of macrophages (Caruso et al., 1999; Scanga et al., 2000). Lysis of target cells by CD8+ T cells can occur via perforin and granzymes or the Fas/Fas L pathway. Lysis of infected cells may result in apoptotic cell death, which may be detrimental to *M. tuberculosis* survival or release of bacteria from an infected cell into an environment (the granuloma) rich in activated macrophages. However, 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. tuberculosis* and represents a mechanism by which CD8 T cells can contribute to killing of the bacilli within cells (Stenger et al., 1997; Stenger et al., 1998).

**Unconventional T cells**

T cells with specificity for mycobacterial glycolipids presented by CD1 molecules seem to have a unique role in human tuberculosis (Schaible et al., 2000; Park et al., 2000; Porcelli et al., 1999; Ulrichs et al., 2000). Group 1 CD1 molecules, comprising CD1a, CD1b and CD1c, are found in primates and guinea-pigs, but not in mice. Generally, CD1-glycolipid-specific T cells produce IFN-γ and express cytolytic activity. Group 1 CD1 molecules typically present glycolipids that are abundant in the mycobacterial cell wall, such as phosphatidylinositolmannosides, lipoarabinomannan, mycolic acids and hexosyl-1-phosphoisoprenoids. 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 (Sugita et al., 2000; Schaible et al., 2000). CD1a and CD1c have ready access to mycobacterial glycolipids as *M. tuberculosis* arrests phagosomal maturation at early stages. Mycobacteria shed glycolipids inside the phagosome, and these enter the late endosome/lysosome, where
they can also interact with CD1b. Vesicles containing shed glycolipids can be released, and therefore deliver their antigenic cargo to bystander cells. Group 1 CD1 molecules are abundantly expressed on DCs, but are virtually absent on macrophages, and CD1b surface expression is downregulated in cells infected with *M. tuberculosis* (Stenger *et al.*, 1998). The transfer of glycolipids from infected macrophages to bystander DCs therefore constitutes an important mechanism for promoting CD1 presentation. Group 2 CD1 molecules (CD1d) seem to have a minor role in tuberculosis (Rolph *et al.*, 2001). T cells that express a γδ TCR, also participate in the immune response against *M. tuberculosis* (Kaufmann 1996). In the mouse, these T cells partially protect against high, but not low, inocula of *M. tuberculosis*, and regulate granuloma formation (D’Souza *et al.*, 1997, Ladel *et al.*, 1995). Unlike mouse γδ T cells, human γδ T cells are stimulated by a unique group of non-proteinaceous antigens that contain phosphate, apparently independent of any restriction elements. These ‘phospholigands’ include different prenylpyrophosphates and nucleotide conjugates, all of which are abundant in mycobacteria. The phospholigands stimulate γδ T cells that express the Vγ2δ2 chain combination in dependently of their fine antigen specificity. Vγ2δ2 T cells comprise an important population of all γδ T cells, and constitute about 5% of all T cells in the peripheral blood in adults (Kaufmann 1996). This large γδ T-cell population readily produces IFN-γ after stimulation with phospholigands and expresses granule-dependent mycobactericidal activity (Behr-Perst *et al.*, 1999). γδ T cells could, therefore, be responsible for mobilizing the first line of defence against tuberculosis. In this respect, γδ T cells constitute an interesting target for a novel tuberculosis vaccine.

**Dendritic Cells**

The body is constantly exposed to a remarkable variety of infectious agents, such as viruses, bacteria, fungi and parasites. The individual pathogens differ with regard to the way of transmission, the mode of replication, the mechanism of causing disease and the type of host response they elicit. The first phase of host defense against invading microorganisms relies on the mechanisms of innate immunity, an evolutionary ancient and universal form of host protection. The signals resulting from detection of an invading pathogen by cells of the innate immune system must be conveyed to the lymphocytes of the adaptive immune system. This critical task is fulfilled by dendritic cells (DCs), specialized antigen-presenting cells, which provide
T cells not only with microbial antigen but also with information about the features of the pathogen, thus conducting the development of an appropriate cell-mediated immune response. Dendritic cells are derived from haematopoietic stem cells in the bone marrow and form a network of heterogeneous cell populations. In most tissues, DCs are present in a so-called 'immature' state, unable to stimulate T cells. Although these DCs lack the requisite accessory signals for T-cell activation, such as CD40, CD54 and CD86, they are extremely well equipped to capture antigens and a key event in the induction of immunity-antigens are able to induce full maturation and mobilization of DCs.

**Antigen capture and Processing**

Immature DCs have several features that allow them to capture antigen. First, they can take up particles and microbes by phagocytosis (Inaba et al., 1993; Moll et al., 1993; Reis e Sousa et al., 1993; Svensson et al., 1997) Second, they can form large pinocytic vesicles, in which extracellular fluid and solutes are sampled, a process called macropinocytosis (Sallusto et al., 1994). And third, they express receptors that mediate adsorptive endocytosis, including C-type lectin receptors like the macrophage mannose receptor (Sallusto et al., 1994) and DEC-205 (Jiang et al., 1995), as well as Fcg and Fce receptors (Sallusto et al., 1994). Macropinocytosis and receptor-mediated antigen uptake make antigen presentation so efficient that picomolar and nanomolar concentrations of antigen suffice, much less than the micromolar levels typically employed by other APCs. However, once the DC has captured an antigen, which also can provide a signal to mature, its skills to capture antigens rapidly declines, and now they assemble antigen–MHC class II complexes. The antigen enters the endocytic pathway of the cell. In macrophages most of the protein substrate is directed to the lysosomes, an organelle with only few MHC class II molecules, where the antigen is fully digested into amino acids. Not in DCs; the DC is able to produce large amounts of MHC class II–peptide complexes at a single brief stage of its life. Much of this success may be due to specialized, MHC class II-rich compartments (MIICs) that are abundant in immature DCs (Sallusto et al., 1994; Winzler et al., 1997; Nijman et al., 1995; Pierre et al., 1997). MIICs are late-endosomal structures that contain the HLA-DM or H–2M products, which enhance and edit peptide binding to MHC class II molecules. During maturation of DCs, MIICs convert to non-lysosomal vesicles that discharge their MHC–peptide complexes to the surface (Pierre et al., 1997; Cella et
Immature DCs have been compared to idling motors in neutral gear, constantly degrading MHC class II molecules in their MIICs. As soon as an antigen instructs the DCs to move into gear, fragments of antigen are loaded onto class II molecules and these complexes are sent to the cell surface, where they remain stable for days. To generate CD8 cytotoxic killer cells, DCs present antigenic peptides on MHC class I molecules, which can be loaded through both an endogenous and an exogenous pathway (Pamer et al., 1998; Rock et al., 1999). The endogenous MHC class I pathway operates through the degradation of cytosolic proteins and the loading of peptides onto newly synthesized MHC class I molecules within the endoplasmic reticulum. Antigen processing occurs first in the cytosol through an ATP-dependent proteolytic system, which starts by ubiquitin conjugation. DCs, similarly to B cells, constitutively express di-ubiquitin, which could permit more efficient antigen processing (Bates et al., 1997). This gene, also known as FAT10 (Liu et al., 1999), encodes a di-ubiquitin protein containing tandem head to tail ubiquitin-like domains, with the conservation of key functional residues. The ubiquitinylated proteins are directed to the proteasome, which cleaves the protein into peptides. The peptides are then translocated into the ER via ATP-dependent TAP1/2 transmembrane transporters and are trimmed into 8–10 mers, which accommodate the MHC class I-binding groove. However, before DC can complex processed peptide antigen to MHC molecules and display them at the cell surface, they must first undergo a process of functional maturation.

**DC Maturation and Migration**

In the course of maturation, DCs are subject to profound changes. The endocytic capacity is downregulated, while there is a marked up-regulation of MHC class II expression, from a relatively high constitutive level. The expression of inflammatory chemokine receptors, such as CCR1, CCR2, CCR5 and CCR6, and the responsiveness to their ligands decreases. At the same time, DCs acquire surface expression of CCR7 and responsiveness to its ligands CCL19 (Macrophage Inflammatory Protein-3b) and CCL21 (secondary lymphoid tissue chemokine, SLC) that are produced in the T cell areas of lymph nodes (Dieu et al., 1998; Saeki et al., 1999; Vecchi et al., 1999). As CCR7 also mediates the homing of T cells to secondary lymphoid organs (Willimann et al., 1998), it is a key receptor for the encounter of antigen-bearing mature DCs and responder T cells. In addition, mature DCs express enhanced surface levels of co-
stimulatory and adhesion molecules such as CD80, CD86 and CD40 (Banchereau et al., 2000). As a result of these multiple modifications, mature DCs acquire the distinct ability to trigger a primary T cell response. Moreover, DCs play a critical role in shaping the emerging immune response. Their activation by microbial antigens induces the production of cytokines such as IL-12, IL-18 or IL-10, which promote the development of either type 1 T helper (Th1) cells or type 2 T helper (Th2) cells (Reis e Sousa et al., 1997; Moser and Murphy, 2000). Th1 cells secrete interferon (IFN)-γ, which is effective against intracellular pathogens because it stimulates microbicidal activities in macrophages, whereas Th2 cells produce IL-4 and IL-5, which drive B cells to release IgG1 and IgE antibodies. IgE is involved in effector mechanisms protecting against worm infections.

NF-κB family of transcription factors in dendritic cells

Many inducers of DC maturation are also strong activators of NF-κB transcription factors (Baldwin et al., 1996; Ghosh, et al., 1998), suggesting that these factors may play a key role in DC maturation. The NF-κB family of transcription factors exist as homodimers or heterodimers of 5 distinct proteins (p50, p52, RelA, RelB and cRel), and play an important role in regulating inflammatory and immune-response genes (Ghosh, et al., 1998). Latent NF-κB transcription factors are typically present in the cytoplasm in a complex with inhibitory IkB proteins (Ghosh, et al., 1998). Stimulation of cells with NF-κB activators, including LPS, TNF and CD40L, results in phosphorylation of IkB proteins by the IKKβ (IkB kinase) complex (Karin and Ben-Neriah, 2000). Phosphorylated IkB proteins are rapidly degraded, allowing translocation of NF-κB complexes to the nucleus. All five members of the NF-κB family have been knocked-out in mice (Beg et al., 1995; Burkly et al., 1995; Caamano et al., 1998; Doi et al., 1997; Franzoso et al., 1998; Kontgen et al., 1995; Sha et al., 1995; Weih et al., 1995). These studies have identified key roles for NF-κB proteins in regulation of innate immunity (Alcamo et al., 2001) and control of lymphocyte function (Kontgen et al., 1995; Sha et al., 1995; Zheng et al., 2001). In addition, RelA deficiency resulted in embryonic lethality (Beg et al., 1995) as a consequence of TNF-induced hepatocyte apoptosis (Alcamo et al., 2001; Doi et al., 1999), and cRel+/− B cells were found to have a high rate of cell death during activation (Grumont et al., 1999). These findings therefore indicate an important role for NF-κB in inhibition of
apoptosis. However, studies of RelA− fibroblasts have shown an essential role for NF-κB in regulation of Fas expression, suggesting that NF-κB proteins may also have a pro-apoptotic function (Ouaaz et al., 1999). Studies of RelB−/− mice have indicated a specific requirement for this protein in development of CD11c+CD8α− but not CD11c+CD8α+ DCs (Burkly et al., 1995; Weih et al., 1995; Wu et al., 1998). However, other than RelB, the function of other NF-κB subunits in DCs is not known.

**T cell Priming**

DCs that migrate from tissues to lymph nodes have a life expectancy of 2–3 days and can therefore be viewed as disposable packets, each carrying a given amount of peptide-MHC complexes, costimulatory molecules, and cytokines. These packets are assembled during DC maturation, a process which is initiated by pathogens and/or inflammatory stimuli. Maturation coordinately regulates DCs' antigen capturing, processing and presentation, expression of costimulatory molecules, cytokine production, and life-span.

The ability to prime naive CD4+ T cells constitutes a unique and critical function of DCs both in vitro and in vivo. DCs are equally important in priming naïve CD8+ T cells. One important factor that contributes to this unique feature of a DC is the retention of MHC peptide complexes that are 10- to 100-fold higher on DCs than on other APCs like B cells and monocytes (Inaba et al., 1997). Recognition of MHC-peptide complexes on DCs by Antigen-specific TCRs constitutes "signal one" in DC–T cell interaction. DC–T cell clustering is mediated by several adhesion molecules, like integrins b1 and b2 and members of the immunoglobulin superfamily (CD2, CD50, CD54, and CD58) (Bell et al., 1999; Hart, 1997).

The crucial factor, that constitutes "signal two," required to sustain T cell activation, is the interaction between costimulatory molecules expressed by DCs and their ligands expressed by T cells. CD86 on DCs is so far the most critical molecule for amplification of T cell responses (Caux et al., 1994; Inaba et al., 1994). T cells can activate DCs via CD40 ligand (CD40-L)-CD40 signaling leading to increased expression of CD80/CD86 and cytokine release (IL-1, TNF, many chemokines, and IL-12) (Sallusto et al., 1994; Ridge et al., 1998; Bennett et al., 1998; Schoenberger et
al., 1998; Caux et al., 1994). Triggering of CD40 on DCs results in upregulation of OX40 ligand (OX40-L) (Stuber et al., 1995), which then signals naive T cells to express IL-4 (Flynn et al., 1998) and upregulates the chemokine receptor CXCR-5, whose ligand directs B lymphocytes into follicles (Brocker et al., 1999). Accordingly, expression of the OX40-L transgene into DCs leads to accumulation of CD4+ T cells in B follicles. Mature DCs also express 4–1BB ligand (DeBenedette et al., 1997), which complements the function of OX40-L. 4–1BB is a costimulator expressed primarily on activated CD4+ and CD8+ T cells (Saoulli et al., 1998). 4–1BB costimulatory signals preferentially induce CD8+ T cell proliferation and production of IFNγ (but not of IL-4) (Kim et al., 1998), leading to the amplification of in vivo cytotoxic T cell responses in graft-vs-host disease as well as allograft rejection (Shuford et al., 1997). Engagement of RANK, a member of the TNFR family, by its ligand (RANKL/TRANCE) expressed on activated T cells, stimulates the secretion of cytokines like IL-1, IL-6, and IL-12 by DCs. This results in increased DC survival, by inhibition of DC apoptosis and, in turn, in enhanced proliferative T cell responses in mixed lymphocyte reactions. The demonstration that TRANCE is responsible for the CD40-L–independent T-helper cell activation during viral infection suggests an important and specific role for this molecule during infection (Anderson et al., 1997; Wong et al., 1997; Josien et al., 1999).

Activation of T cells is mediated by positive signals, those that decide whether recognition of antigen will lead to full T cell activation or anergy/death, and negative signals, that are complimentary to the signals provided by TCR receptor and prevent unnecessary activation of T cells and thus autoimmunity. One such signal is provided by the PD-1:PD-L-1 (Program death-1:Program death ligand-1) pathway. PD-1 expression is induced by T cell activation. The ligands for PD-1 are PD-L1 (B7H1) and PD-L2 (B7-DC). These ligands are members of the B7 family of receptors and are constitutively expressed by DCs. Activation of human T cells by anti-CD3 is inhibited by PD-L1 engagement of PD-1 on T cells (Freeman et al., 2000). In addition, PD-L1:PD-1 interactions can counterbalance CD3-mediated activation when CD28-mediated costimulation is suboptimal (Freeman et al., 2000). PD-1 expression on activated T cells is highest with a weak TCR signal, so that weakly activated T cells are most readily downregulated by PD-L engagement (Freeman et al., 2000). Gene chip analyses have demonstrated that PD-1 mRNA is highly
expressed in CD4\(^-\)CD25\(^+\) regulatory T cells and anergic T cells, suggesting several means by which PD-1 may be involved in regulating T cell tolerance (Gavin et al., 2002; Lechner et al., 2001). Engagement of PD-1 by PD-L2 also inhibits T cell TCR-mediated proliferation and cytokine production by CD4\(^+\) T cells (Latchman et al., 2001). At low antigen concentrations, PD-L2:PD-1 interactions inhibit strong B7-CD28 signals. In contrast, at high antigen concentrations, PD-L2:PD-1 interactions reduce cytokine production but do not inhibit T cell proliferation. Inhibition of T cell proliferation and cytokine production by both resting and previously activated CD4\(^+\) and CD8\(^+\) T cells, and even naive T cells from cord blood have been observed using PD-L1Ig or PD-L2Ig fusion proteins together with anti-CD3-mAb (Carter et al., 2002; Gao et al., 2003). In that setting, the proliferative responses of wild-type, but not PD-1\(-/-\), T cells were inhibited by anti-CD3 plus PD-L1-Ig, indicating that the inhibitory signal was transduced by PD-1. Thus PD-1:PD-L-1 pathway represents an effective means of controlling heightened T cell activation and thus preventing autoimmunity.

Once committed to the first division, T cells proliferate rapidly in response to IL-2, which is produced by activated T cells and can act in autocrine and paracrine fashion. Since IL-2 production is induced by antigenic stimulation and is greatly enhanced by costimulation, its availability in T cell areas varies widely as a function of the extent and duration of T cell activation.

There is growing evidence that the duration of TCR stimulation, together with polarizing cytokines, determines the progressive differentiation of CD4\(^+\) T cells, leading to the generation of terminally differentiated effector cells as well as intermediates (Lanzavecchia et al., 2000). CD4\(^+\) T cells that receive a short TCR stimulation in the absence of IL-12 proliferate but do not differentiate to effector cells. Upon in vivo transfer, they home to the lymph nodes and, following secondary antigenic challenge, differentiate to effector cells. In contrast, T cells that receive a prolonged TCR stimulation in the presence of IL-12 or IL-4 terminally differentiate to Th1 cells, producing IFN-\(\gamma\), or to Th2 cells, producing IL-4, IL-5, and IL-13. As part of their differentiation program, Th1 and Th2 cells lose the lymph node homing receptors and acquire the capacity to migrate to inflamed nonlymphoid tissues to execute their effector functions. The progressive differentiation model is supported by
the existence of a distinct population of central memory T cells that lack immediate effector function and carry the lymph node homing receptor CCR7.

DCs therefore provide the adaptive immune system with the essential function of context discrimination. Within individual DCs, multiple stimuli from pathogens and inflammatory cytokines are integrated into distinct outputs in terms of antigen presentation, costimulation, and cytokine production. The same stimuli also recruit large numbers of DC precursors, thus leading to sustained antigen sampling in peripheral tissue and presentation to T cells in lymph nodes. The T cell activation and differentiation program is tailored to discriminate antigen concentration, cytokine and costimulatory molecule composition, and DC density, leading to the generation of appropriate T cell responses ranging from tolerance to inflammation, cytotoxicity and memory.

**Role of Dendritic cells in Mycobacterial infection**

DCs are distributed throughout the respiratory tract, from the nasal mucosa to the lung pleura. The most prominent populations are localized within the epithelium of the conducting airways, in which they form a rich network comparable to the Langerhans cells (LC) population in the epidermis and within the lung parenchyma, in particular at the interseptal junctions between adjacent alveolar units. In the lungs, DCs represent only <1% of the total cells but an increase in the recruitment of blood DC precursor to the lungs has been noted during inflammation. These DCs recognize and capture antigens including those of mycobacterium. Immature DCs screen for pathogen entry using conserved pattern recognition receptors, which recognize characteristic molecular patterns in microbial cell wall components such as carbohydrate structures, or lipids or nucleic acids. These receptors include the Toll like Receptors (TLRs) and the C type lectins. Each TLR recognizes specific pathogenic component, such as lipoprotein, lipopolysaccharide (LPS) or bacterial DNA. TLRs relay information about the interacting pathogen to DCs through intracellular signaling cascades, thereby eliciting appropriate cellular processes that lead to DC maturation and the induction of inflammatory cytokines. By contrast, C type lectins recognize specific carbohydrate structures that are present on cell-wall components of pathogens, and internalize pathogens for degradation in lysosomal compartments to enhance antigen processing and presentation by DCs. C-type lectins
also recognize carbohydrate structures on self glycoproteins to allow tolerance to self antigens and to mediate cellular processes, such as cell signaling, cell adhesion and migration. Endocytosis of mycobacterium occurs via a specialized C-type lectin known as DC specific ICAM-3 grabbing nonintegrin or DC-SIGN (Geijtenbeek et al., 2003; Tailleux et al., 2003). DC-SIGN interacts with mannose-capped lipoarabinomannan (Man-LAM) of the *M. tuberculosis* cell wall, specifically binding dimeric and trimeric mannose residues (Geijtenbeek et al., 2003; Tailleux et al., 2003). For DCs, DC-SIGN is the main receptor for mycobacteria (Geijtenbeek et al., 2003). Targeting of DC-SIGN by ManLAM results in an altered immune response through signaling between C-type lectins and TLRs (Nigou et al., 2001). Pathogens might exploit this function of C-type lectins to interfere with TLR signaling, thereby modulating DC-dependent immune response (Engering et al., 2002). Binding of immature DCs to the mycobacterial component ManLAM blocks LPS-induced secretion of IL-12 (Nigou et al., 2001). The study indicates that binding of ManLAM to immature DCs interferes with TLR4 signaling, as LPS signaling is mediated through TLR4 (Akira et al., 2001). ManLAM inhibits LPS-induced DC maturation by interacting with DC-SIGN, as LPS induced DC maturation in the presence of ManLAM is fully restored by inhibiting the DC-SIGN ManLAM interaction with specific antibodies. Furthermore the binding of ManLAM to DC-SIGN induces the production of the anti-inflammatory cytokine IL-10 by LPS activated DCs (Geijtenbeek et al., 2003). The inhibition of DC maturation and the induction of IL-10 might contribute to the virulence of mycobacteria. The fact that DCs do not support the growth of mycobacteria due to IL-10 induced reversion of DC maturation indicates that pathogen recognition by DC-SIGN might modulate DC-induced immune responses, shifting the balance from immune activation towards impairment of immune responses, which would be beneficial to pathogen survival. Mycobacterial binding of DC-SIGN is associated with targeting of the pathogen to lysosome associated membrane protein 1 (LAMP-1) containing compartments within the cell (Geijtenbeek et al., 2003; Tailleux et al., 2003). LAMP-1 compartments within DCs can mature to late endosomes/Lysosomes, facilitating antigen processing and presentation, as is evidenced by ManLAM presentation to T cells via CD1b (Prigozy et al., 1997). Following uptake by murine bone marrow-derived DCs, virulent *M. tuberculosis* appears to replicate within the cells. However, the extent of replication is dependent on the maturation status of the cell (Bodnar et al., 2001). Infection of DC
with live *M. tuberculosis* resulted in increased APC surface expression of the costimulatory molecules CD54, CD40, and B7.1, as well as MHC class I molecules. In addition, infected DC secreted elevated levels of inflammatory cytokines, including TNF-α, IL-1, and IL-12 (Henderson et al., 1997). When DCs are first activated by IFN-γ and lipopolysaccharide, mycobacterial growth is inhibited, although the organism is probably not killed. *M. tuberculosis* also downregulates the surface expression of CD1 expression, thereby regulating the ability of DCs to present mycobacterial lipid antigens (Stenger et al., 1998). It has also been observed that *M. tuberculosis* H37Rv, in the presence of a maturation cocktail of cytokines that induce full DC maturation, inhibit the expression of phenotypic markers of DC maturation. These DCs failed to induce alloreactive T-cell proliferation (Hanekom et al., 2003). In an effort to globally characterize cells harboring *M. tuberculosis*, Wolf et al. (2007) have performed temporal and quantitative characterization of the cells following aerosol infection of mice by using GFP-expressing bacteria and flow cytometry. It was discovered that *M. tuberculosis* infects phagocytic cells of diverse phenotypes, that the predominant infected cell population change with time and that myeloid DCs are the major cell population infected with *M. tuberculosis* in the lungs and lymph nodes. They also found that the bacteria in the lung-draining lymph node are transported there from the lungs by a CCL19/21-dependent mechanism and that the transport of bacteria to the lymph node is a transient phenomenon despite chronic infection. Further, when the antigen presentation capacity of DCs isolated from lungs were monitored they were found to be less effective in stimulating *M. tuberculosis* Ag85B-specific CD4+ T cells than were lymph node cells, which could be because of a defective maturation program that limits their capacity to migrate to lymph nodes as well as to present peptide antigens by the class II pathway. These results indicate that *M. tuberculosis* targets DC migration and antigen presentation in vivo to promote persistent infection.

All the above observation reveals that since DCs are at the forefront of induction of immunity it is pertinent that some of their functions will be modulated by *M. tuberculosis* or other pathogens to shift the balance of immune response where it is beneficial for the pathogen
Dendritic cells as Immunotherapeutic Agents

DCs are an attractive target for therapeutic manipulation of the immune system, to enhance insufficient immune responses, in infectious diseases and cancer, or attenuate excessive immune responses, in allergy and autoimmunity (Steinman et al., 2007). However, the complexity of the DC system brings about the necessity for its rational manipulation to achieve protective or therapeutic immunity. Immunization with ex vivo generated DCs has proven feasible, and permits the enhancement as well as dampening of antigen-specific immune responses in man. These ex vivo strategies should help identify the parameters for DC targeting in vivo. Inaba and colleagues demonstrated that the injection of DCs, charged with antigen ex vivo, could sensitize normal mice to protein antigens (Steinman, 1991). This seminal work also suggested that using DCs directly as a vaccine might best circumvent the problem of variable in vivo DC targeting. The immunogenicity of antigens delivered on DCs has now been demonstrated in human studies. Indeed, single subcutaneous immunization of healthy volunteers with 2-4x10^6 antigen-loaded mature monocyte-derived DCs rapidly expanded CD8+ and CD4+ T cell immunity. A single boost several months later led to expansion of CTL with increased affinity against viral peptide, an observation never made with any other vaccination strategy so far (Dhodapkar et al., 2000). Because DCs play such a pivotal role in the initiation of adaptive immune responses, targeting endogenous DC populations in vivo with cytokine adjuvants may amplify the level of immune activation. Moreover, the state of maturation of the DCs is critical, as a DC that is not fully matured can lead to T-cell tolerance.

Since it is becoming increasingly difficult to treat TB infections caused by multidrug-resistant strains of *M. tuberculosis* and many patients who complete drug therapy are at increased risk of re-infection, there is now an urgent need to develop a therapeutic vaccine for these individuals. In this regard, DC-based TB vaccines possess the power of antigen presentation and do not trigger an overwhelming acute inflammatory response thus serving as an ideal candidate for therapeutic TB vaccines. DC-based TB vaccines have been examined to a limited extent in murine models. One of the first approaches to manipulating DCs ex vivo to induce immunity against pulmonary *M. tuberculosis* challenge was carried out by Demangel et al. (1999). They reported that ex vivo-generated DCs infected with BCG delivered intratracheally induced only a short-lived protective immune response against pulmonary *M. tuberculosis* challenge.
However, it was also reported that the ex vivo DCs infected with BCG were able to harbour viable bacilli (Demangel et al., 1999). Delivering viable bacilli to the respiratory tract is not a safe approach to inducing TB immunity in TB patients, particularly those with immune compromised conditions.

Other strategies to using DC-based vaccines in more recent years have focused on pulsing ex vivo derived DCs with whole proteins or immunodominant peptides (McShane et al., 2002; Gonzalez-Juarrero et al., 2002). Ag85A is a major secreted protein found in all clinically isolated strains of M. tuberculosis. DCs pulsed with whole Ag85A protein delivered intranasally gave rise to increased numbers of IFN-γ - secreting CD4+ and CD8+ T-cells in the lung (Gonzalez-Juarrero et al., 2002). However, these cells were not able to confer protection against pulmonary M. tuberculosis challenge. Another demonstration showed that DCs pulsed with Ag85A immunodominant CD4+ and CD8+ peptides induced slightly better immune responses than DCs pulsed with whole protein when delivered intramuscularly or intravenously (Malowany et al., 2005). Malowany et al. developed a novel approach to using DC-based vaccines in anti-TB vaccinology. They infected bone marrow derived DCs with a recombinant adenovirus expressing Ag85A (AdAg85A), which is capable of expressing Ag85A fused to a signal peptide sequence, so that Ag85A can be secreted from infected mammalian cells. They found that DCAdAg85A (DCs virally transduced with AdAg85A) induced significantly greater CD4+ and CD8+ IFN-γ + responses than peptide- or protein-pulsed DCs when delivered intramuscularly. This enhanced immune activation is probably due to the increased antigen presentation, by the infected DC on MHC I as well as antigen presentation of secreted Ag85A by endogenous DCs on MHC II. Upon infection with AdAg85A, DCs expressed enhanced co-stimulatory molecules CD80, CD86 and enhanced production of IL-6 and IL-12 (Malowany et al., 2005), thus having greater type 1 immune-activating capabilities. In order to better target lung-specific immunity, DCAdAg85A was given intranasally. Mucosally administered DCAdAg85A resulted in recruitment of antigen specific CD4+ IFN-γ + and CD8+ IFN-γ + T-cells to the airway lumen, which conferred protection against pulmonary M. tuberculosis infection. Such respiratory mucosal immunization with DCAdAg85A may represent an effective way to trigger anti-TB memory T cell responses without causing an unwanted inflammatory response. This strategy can be used to treat pulmonary TB in mouse models.
To enhance further the antigen-presenting power of the DC, DCs transduced with AdAg85A co-expressing GM-CSF can further potentiate the antigen-specific T-cell responses. This is probably due to GM-CSF secreted by the virally transduced DCs recruiting endogenous DCs to the vaccination location and site of antigen deposition. The secreted GM-CSF may act on infected DCs, as well as acting on the endogenous DCs to provide full maturation signals. The uninfected DCs can then pick up the secreted Ag85A from the vaccine DCs to potentiate the number of DCs activated by a single immunization event. Thus, DCs manipulated *ex vivo* to express an antigen of interest may recruit and expand the endogenous DCs, leading to a robust anti-TB immune response. DCs are critical players in the initiation of an effective immune response against intracellular infections such as *M. tuberculosis*. Efforts that have focused on expanding endogenous populations of DCs *in vivo* have proven to be very effective in increasing the immunogenicity of vaccines and such cytokine adjuvants as GM-CSF would be prime candidates to be applied in conjunction with current BCG vaccination strategies. More recent efforts have focused on manipulating DCs *ex vivo* to generate cell-based vaccines that can be used *in vivo* to trigger type 1 immunity against intracellular infections. While *ex vivo*-derived DCs pulsed with antigenic peptides or proteins have resulted in mediocre induction of protective immunity, virally transduced DCs are capable of much greater protective immunity. Such virally transduced DC vaccines may also serve as therapeutic vaccines to treat intracellular infections without causing tissue damage and virus-neutralizing antibodies. Dendritic cells are an important innate immune cell type which act as the bridge between innate and adaptive immunity.

Mounting experimental evidence suggests that manipulating DCs represents a powerful means to enhance host defence against intracellular infectious diseases. Several strategies have been developed to manipulate DCs either *in vivo* or *in vitro* for the purpose of enhancing the effect of vaccination or immunotherapeutics. *In vivo* delivery of transgene encoding GM-CSF (granulocyte/macrophage colony stimulating factor), a DC-activating cytokine, increases the number and activation status of DCs at various tissue sites and enhances antimicrobial immune responses in murine models. Co-expression or co-delivery of GM-CSF gene transfer vector with an antimicrobial vaccine enhances microbial antigen-specific T-cell responses and immune protection. Murine bone marrow-derived DCs are being manipulated *in vitro*
and exploited as a vaccine delivery system. Transduction of DCs with a virus-vectored tuberculosis vaccine is a powerful way to activate T-cells in vivo. Such genetically modified DC vaccines can be administered either parenterally or mucosally via the respiratory tract and present an alternative option to enhance the efficacy of present vaccine candidates.

Comparative Account of DC vs Macrophage on Their Interaction with M. tuberculosis

The primary target cells of M. tuberculosis infection are pulmonary macrophages which capture the bacteria on its entry through the respiratory tract; DCs too, are present in airways and capture foreign particles. In addition to their function in antigen presentation, macrophages and DCs play an important role in sensing pathogens and delivering both start-up and class differentiation signals to the adaptive immune system. Although the ability of both cells to produce IL-12 makes them capable of directing Th1 responses, the ability of macrophages to initiate an inflammatory response to infectious agents through the production of proinflammatory molecules such as IL-8, TNF-α, and IL-1β and their microbicidal effector functions distinguishes them from DCs. In contrast, DCs have the unique capacity to capture antigen from the periphery and deliver it to secondary lymphoid organs. Precursor DCs that encounter pathogenic organisms induce the production and release of chemokines and cytokines that, in turn, can attract and/or activate other cell types such as eosinophils, macrophages, and natural killer cells. Moreover, DCs themselves are important producers of type I interferons, TNF-α, and IL-1β, mediators that can enhance DC activity in an autocrine manner. Triggering of both DC and macrophages function by pathogens is thought to occur as a consequence of ligation of distinct pattern recognition receptors on the surface of these cells.

DCs infected with M. tuberculosis express high levels of costimulatory molecules as compared to macrophages which upon infection with M. tuberculosis results only in moderate upregulation of CD40 and CD54. Moreover DCs show a significant induction in the level of MHC class II DR and DQ while constitutive expression of MHC class II molecules show a downregulation in macrophages on M. tuberculosis infection. The up-regulation of these surface markers in infected DCs underlines the capacity of DCs to mature following M. tuberculosis infection, which correlates with
the acquired ability to present antigens to T lymphocytes, indicating that while *M. tuberculosis* infection results in the direct activation and maturation of DCs followed by enhanced presentation of antigen and capacity to stimulate T cells, it impairs the ability of macrophages to process and/or present soluble antigen and in turn, to serve as accessory cells in T cell activation (Giacomini *et al.*, 2001).

In terms of cytokine secretion also, the two cell types respond differently; while macrophages induce a rapid, sustained and prolific secretion of TNF-α, IL-1, IL-6 and IL-18, DCs on the other hand produced very low levels of these cytokines on *M. tuberculosis* stimulation suggesting that a stronger stimulus is required to induce the expression of these inflammatory cytokines. However, while macrophages induce the secretion of IL-10, a suppressive cytokine which may have a bearing on the final outcome of the immune response, infected DCs on the other hand respond by producing elevated levels of IL-12 which is a Th1 type cytokine (Giacomini *et al.*, 2001). This discrepancy in the secretion of IL-12 by macrophages and DCs is because of the fact that *M. tuberculosis* interaction with macrophages and DCs leads to the generation of different sets of TLR ligands. Alternatively, the ligands are generated in different intracytoplasmic compartments. This differential handling of *M. tuberculosis* results in subsequent activation of either TLR2 signaling in macrophages or both TLR9 and TLR2 signaling in DCs. The TLR9 pathway activated in DCs leads to faster remodeling at the p40 promoter and robust release of IL-12. In contrast, the TLR2 pathway activated by *M. tuberculosis* in macrophages results in slower remodeling of the p40 promoter and reduced IL-12 release. Robust TLR2-dependent IL-12 production from macrophages is seen only under conditions where IL-10 levels are lowered, for example, with the addition of IFN-γ, but without enhancing remodeling. It is because of the fact that DCs are the priming APCs and therefore need to rapidly secrete IL-12 for initiating Th1 immunity. DCs are not long-lived and their IL-12 secretion is thus terminated quickly. In contrast, because *M. tuberculosis* reside and replicate within macrophages, the regulated expression of IL-12 from macrophages may be part of a developmental program to prevent excessive Th1 activation in the lung (Pompei *et al.*, 2007).

There are two key differences in the way they respond following *M. tuberculosis* infection. First, only *M. tuberculosis*-infected DCs support Th1 priming because of
their unique ability to make IL-12 in response to *M. tuberculosis* infection, and, second, mechanistically the failure of *M. tuberculosis*-infected macrophages to synthesize IL-12 is due to IL-10. The fact that DCs are able to synthesize sufficient bioactive IL-12 for Th1 priming despite the production of IL-10 underscores the complexity of the molecular mechanisms regulating IL-12 gene expression in the two APC types. The differences in response of the two types of APCs to *M. tuberculosis* could be a reflection of their respective roles in immune initiation to *M. tuberculosis* and granuloma regulation (Hickman et al., 2002).

It is generally accepted that *M. tuberculosis* enters the macrophages and stays in the phagosomes, without allowing fusion of lysosome with the phagosome, while DCs on other hand actively engulf *M. tuberculosis* which remains in endosomal compartment leading to presentation and processing of mycobacterial antigen through endosomal pathway. However, a recent study contradicts this well established paradigm of *M. tuberculosis* infection in DCs. Van der wal et al. (Van der wal et al., 2007) have made their observations on human monocyte derived DCs where they observe that *M. tuberculosis* containing phagosome fuses with lysosome as early as 2 hours after infection which was evident by the presence of lysosomal markers like LAMP-1, LAMP-2, CD63 and major lysosomal aspartic proteinase cathepsin D. They also observed the intracellular survival of bacteria and found that during initial 48 hours of infection, the titer of *M. tuberculosis* remains constant, indicating no net growth, however following this period, the titer of *M. tuberculosis* increases steadily over the next 48 hr. of culture, suggesting that alteration occurs to the phagolysosome that create a more favorable growth environment. Further they observed that *M. tuberculosis* present in the phagolysosome translocate to the cytosol following 4 and 7 days of infection, on the other hand heat killed bacteria resides exclusively in the phagolysosomes indicating that translocation to the cytosol requires an active process of mycobacteria. To further investigate the role of RD1 region in translocation they used a *M. tuberculosis* strain containing a transposon insertion in *cfp-10* which prevents the synthesis of CFP-10 and ESAT-6 and found that this mutant failed to enter the host cytosol over the course of 7 days of infection and resided in LAMP-1 positive compartments suggesting a role for RD1 region encoded protein CFP-10 and ESAT-6 in the translocation of mycobacteria from the host endocytic system. Above
finding contradict established facts of mycobacterial infection in DCs and establishes a role for the RD1 region encoded protein in the infection process.

DCs and macrophages very clearly have distinct role in *M. tuberculosis* infection. DCs capture antigens of *M. tuberculosis* and transport it to the lymph nodes for T cell priming and Th1 polarization, because they are the primary secretors of IL-12 following *M. tuberculosis* infection. In contrast, macrophages are important for microbicidal function in the granuloma because they are more efficient in killing intracellular *M. tuberculosis* and for sustaining Th1 polarity.

**Calcium**

Calcium and PKC are the two second messengers that influence the development of Th subsets. It has been reported that a critical balance between PKC and calcium influence the development of Th subsets, such that a strong PKC stimulation (with PMA) of T cells led to the preferential development of Type 2 effectors, while a strong calcium signal (with ionomycin) in contrast led to the development of Type 1 effectors (Noble *et al.*, 2000). In the above study the authors stimulated T cells with high concentrations of PMA and ionomycin to increase PKC activation and intracellular calcium concentrations, respectively, and looked at their effects on Th1 or Th2 responses. Increased intracellular calcium in peripheral blood mononuclear cells along with high Th1 responses have been reported in BCG-immunized and healthy controls as compared to tuberculosis patients (Talreja *et al.*, 2003), indicating the importance of calcium in governing Th1 responses in tuberculosis. Sinha *et al.* (Sinha *et al.* 2006) have shown that CFP-10 induced increased activation of PKC both during DC differentiation and subsequent activation by *M. tuberculosis* WCL and live *M. bovis* BCG, while GM-CSF-DCs induced a poor activation of PKC. On the other hand GM-CSF-DCs mounted good intracellular mobilization of calcium to *M. tuberculosis* WCL stimulation, while CFP10-DCs induced a poor calcium influx. Inhibiting PKC in CFP10-DCs induced Th1 responses to WCL, while activating PKC in GM-CSF-DCs with phorbol esters induced Th2 responses. Interestingly, this differential activation of PKC and calcium in the two DCs was regulated by PI 3-K. Inhibiting PI 3-K in CFP10-DCs, prevented PKC activation. In contrast, inhibiting PI 3-K in GM-CSF-DCs increased PKC activation. Similarly, inhibiting PI 3-K in CFP10-DCs induced Th1 responses while inhibiting PI 3-K in GM-CSF-DCs induced
Th2 responses. The authors have also shown that calcium influx in CFP10-DCs is compromised that results in increased survival of mycobacteria in CFP10-DCs. Mycobacteria interfere with the signaling pathways of the host, as they inhibit the increase in Ca^{2+} normally caused by phagocytosis. In addition, macrophages that were infected with live \textit{M. tuberculosis} showed a significant reduction in the amounts of Ca^{2+} bound calmodulin and phosphorylated CaMKII that were associated with the cytosolic face of the phagosomal membrane compared with phagosomes containing dead bacteria (Malik \textit{et al.}, 2001). The delivery of lysosomal components to mycobacterial phagosomes can be blocked by using inhibitors of CaMKII or by chelating cytosolic Ca^{2+}. These data indicate that pathogenic mycobacteria are able to suppress the increase in cytosolic Ca^{2+} that results from host cell interaction, and thereby inhibit Ca^{2+} signaling pathways, which would otherwise lead to phagosomal maturation. Inhibition of the increase in cytosolic Ca^{2+} concentration by \textit{M. tuberculosis} is mediated by the lipid effector molecule mannose bound lipoarabinomaman (Man-LAM), which is able to inhibit ionophore-induced increase in Ca^{2+} concentration in macrophages (Rojas \textit{et al.}, 2002). This effect is specific for LAM from pathogenic mycobacteria, as LAM from \textit{M. smegmatis} does not block this increase.

**Discovery of Calcium as signaling molecule**

An experiment performed in London nearly 125 years ago, which by today’s standards would be considered unacceptably sloppy, marked the beginning of the calcium (Ca^{2+}) signaling saga. Sidney Ringer (Ringer, 1883) was studying the contraction of isolated rat hearts. In earlier experiments, Ringer had suspended them in a saline medium for which he admitted to having used London tap water, which is hard: The hearts contracted beautifully. When he proceeded to replace the tap water with distilled water, he made a startling finding: The beating of the hearts became progressively weaker, and stopped altogether after about 20 min. To maintain contraction, he found it necessary to add Ca^{2+} salts to the suspension medium. Thus, Ringer had serendipitously discovered that Ca^{2+}, hitherto exclusively considered as a structural element, was active in a tissue that has nothing to do with bone or teeth, and performed there a completely novel function: It carried the signal that initiated heart contraction. It was a landmark observation, which has paved the way for the acceptance of what is now called the calcium concept.
Thanks to some seminal discoveries, interest in the signaling role of Ca\(^{2+}\) started to increase, slowly at first and then more rapidly, eventually reaching today’s explosive phase. The element is now recognized as an essential messenger that accompanies cells throughout their entire lifespan, from their origin at fertilization, to their eventual demise at the end of the life cycle. At first glance, then, Ca\(^{2+}\) could be considered both an essential mediator of activity during cell life and as a conveyor of doom at the moment of cell death. Such a view, however, would be simplistic. The Ca\(^{2+}\)-mediated death of cells exposed to toxic insults has an obvious negative connotation, but the processing of Ca\(^{2+}\) signals to terminate the cell life by apoptosis is instead a positive and necessary way to decode the Ca\(^{2+}\) signal.

Once Ca\(^{2+}\) was recognized as a carrier of signals, it became important to understand how its concentration within cells was regulated. Reversible complexation to specific ligands soon emerged as the only reasonable means to perform the task. A number of small cell ligands bind Ca\(^{2+}\) with low affinity, but the process needed complex ligands able to complex Ca\(^{2+}\) with the specificity and affinity demanded by the intracellular ambient. A breakthrough in this direction was the solution of the crystal structure of parvalbumin by Kretsinger (Kretsinger, 1972) in 1972. This still functionally mysterious Ca\(^{2+}\) binding protein was to become the progenitor of a family of proteins known as EF hand proteins, which has now grown to nearly 600 members. EF hand proteins do buffer Ca\(^{2+}\) but also play another important role: They decode the information carried by Ca\(^{2+}\) and pass it on to targets. They do so by changing conformation after binding Ca\(^{2+}\) and after interacting with targets. Essentially, EF hand proteins become more hydrophobic on the surface after complexing Ca\(^{2+}\), approach the target, and collapse around its binding domain. Thus, these proteins are better defined as Ca\(^{2+}\) modulated proteins, or Ca\(^{2+}\) sensors.

Other proteins also decipher Ca\(^{2+}\) signals, e.g., the annexins, gelsolin, and proteins containing C2 domains, but the EF hand proteins are the most important. They may function as a committed separate subunit of a single (enzyme) protein or as a subunit that associates reversibly with different proteins (e.g., CaM). They may even be an integral portion of the sequence of enzymes (e.g., calpain). The control of Ca\(^{2+}\) concentration in the cytoplasm and organelles is instead the sole role of proteins that, as a rule, are intrinsic to the plasma membrane and to the membranes of organelles.
(the rule has exceptions, e.g., the reticular luminal protein calsequestrin) and transport \( \text{Ca}^{2+} \) across them. These proteins have no direct role in the processing of the \( \text{Ca}^{2+} \) signal, but may also be targets of \( \text{Ca}^{2+} \) regulation as is the case of \( \text{Ca}^{2+} \) channels.

These proteins belong to various classes: \( \text{Ca}^{2+} \) channels in the plasma membrane are gated by voltage, by ligands, or by the emptying of internal \( \text{Ca}^{2+} \) stores. In the endo(sarco)plasmic reticulum (ER/SR), they are instead activated by the second "messengers," inositol 1,4,5-trisphosphate (InsP3) and cyclic ADP ribose (cADPr). cADPr is assumed to act on channels that are also called ryanodine receptors and that are sensitive to the agonist caffeine. Accessory (protein) factors, among them CaM, may be required for the \( \text{Ca}^{2+} \)-releasing effect of cADPr. ATPases (pumps) are found in the plasma membrane (PMCA), in the ER/SR \( \text{Ca}^{2+} \) pump (SERCA), in the Golgi, and in the nuclear envelope (in yeasts, they are also found in other organelles). They export \( \text{Ca}^{2+} \) to the ER/SR lumen or to the extracellular spaces (Carafoli, 2002).

**Calcium channels:**

In lymphocytes, crosslinking of antigen receptors typically activates phosphoinositide-specific phospholipase C. Phospholipase C breaks down phosphatidylinositol-4,5-bisphosphate to generate inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol. Ins(1,4,5)P3 binds its receptor located on the surface of internal \( \text{Ca}^{2+} \) stores, mainly the endoplasmic reticulum, and activates the release of \( \text{Ca}^{2+} \) into the cytoplasm. This event, known as 'store depletion', in turn activates store-operated calcium (SOC) channels in the plasma membrane to recruit \( \text{Ca}^{2+} \). Lymphocytes are believed to use SOC entry (SOCE) as the main mode of \( \text{Ca}^{2+} \) influx. The best characterized SOC channels in lymphocytes are known as 'calcium release-calcium activated channel' (CRAC channels) (Parekh *et al.*, 2005). CRAC channels are highly \( \text{Ca}^{2+} \)-selective, low-conductance channels with a characteristic inwardly rectifying current-voltage relationship. The past few years have shown substantial progress in understanding the molecular composition of the CRAC signaling complex. High-throughput screens based on RNA-mediated interference have identified STIM1 (stromal interaction molecule 1) as the endoplasmic reticulum–resident \( \text{Ca}^{2+} \) sensor and CRACM1 (calcium release-activated calcium modulator 1; also called Orai1) as the pore-forming subunit of CRAC channels (Liou *et al.*, 2005; Zhang *et al.*, 2005; Vig *et al.*, 2006; Feske *et al.*, 2006; Zhang *et al.*, 2006). STIM1 has one homolog, STIM2, whereas CRACM1 has two homologous
proteins, CRACM2 and CRACM3, in mice and humans. Diacylglycerol can accomplish several other functions, including directly activating non-store-operated Ca\(^{2+}\) influx through certain TRPC channels and stimulating the GTPase Ras-mitogen-activated protein kinase signaling cascade, which subsequently activates the AP-1 transcriptional complex (Venkatachalam et al., 2001; Putney et al., 2005; Yuan et al., 2007; Villereal et al., 2006; Hardie et al., 2007). In lymphocytes, AP-1 acts together with the transcription factors NFAT and NF-kB to modulate gene expression (Malek et al., 2008). Ca\(^{2+}\) influx mediated by CRAC channels may also directly activate the Ras-mitogen-activated protein kinase cascade and thus also promote AP-1 activation (Baba et al., 2008). Other transcriptional regulators that depend on Ca\(^{2+}\) signaling for lymphocyte activation include NFAT, NF-kB, the kinase Jnk and calmodulin-dependent kinase, although the duration and amplitude of cytosolic Ca\(^{2+}\) flux required for activation of each transcription factor varies (Dolmetsch et al., 1997; Ho et al., 1996). For example, a prolonged increase in cytosolic Ca\(^{2+}\) concentration via the CRAC channels is crucial for the activation of calcineurin, a serine-threonine phosphatase needed for the dephosphorylation and nuclear localization of NFAT. Even after its translocation into the nucleus, NFAT requires a sustained increase in Ca\(^{2+}\) concentration to prevent its ejection (Crabtree et al., 2002).

The canonical transient receptor potential (TRPC) channels have also been reported to increase intracellular Ca\(^{2+}\) concentrations either directly through coupled plasma membrane receptor stimulation or, arguably, through 'store depletion', in different cell types (Philipp et al., 2003; Venkatachalam et al., 2001; Putney et al., 2005). Of the seven mammalian TRPC channels (TRPC1–TRPC7), TRPC1 has most often been reported to form diverse channels, ranging from relatively Ca\(^{2+}\) selective to nonselective, in a variety of cell types by selective homomeric or heteromeric interactions with TRPC3, TRPC4 and TRPC7 (Yuan et al., 2007; Villereal et al., 2006). In their non-store-operated mode, TRPC3, TRPC6 and TRPC7 can also be activated by diacylglycerol (Venkatachalam et al., 2001; Hardie et al., 2007; Rao et al., 2006). Direct involvement of TRPCs in SOCE remains controversial with no conclusive reports of store-operated TRPC Ca\(^{2+}\) currents in lymphocytes.
STIM proteins

STIM1 is a 77-kilodalton single-spanning transmembrane protein that resides mainly in the endoplasmic reticulum and to some extent also in the plasma membrane. STIM1 has been proposed to sense the depletion of Ca\(^{2+}\) stores through its amino-terminal Ca\(^{2+}\)-binding EF hand domain. Store depletion triggers the formation of oligomers of STIM1 in the endoplasmic reticulum through the EF-SAM region and subsequent translocation to discrete ‘puncta’ at endoplasmic reticulum–plasma membrane junctions (Stathopulos et al., 2006; Liou et al., 2007). Structural insights into the mechanism of the formation of oligomers have shown that in resting, Ca\(^{2+}\)-replete conditions, the EF-SAM domain exists as a well-folded monomer. Ca\(^{2+}\) depletion induces partial unfolding and exposure of hydrophobic residues in the EF-SAM region, which results in its formation of oligomers (Stathopulos et al., 2008). Consistent with those findings, forced formation of heterodimers of STIM1 results in Ca\(^{2+}\)-independent punctae formation and activation of CRAC (Luik et al., 2006; Luik et al., 2008). The coiled-coil domains of STIM1, which mediate constitutive homotypic interactions, may further stabilize the EF-SAM-triggered oligomers and promote the translocation of STIM1 to endoplasmic reticulum–plasma membrane junctions (Baba et al., 2006). The exact mechanism by which STIM1 oligomers activate CRAC channels once located in the endoplasmic reticulum–plasma membrane junctional region still remains to be determined. STIM1 has a closely related homolog, STIM2. The function of STIM2 has remained somewhat controversial, with some early reports assigning it a positive function in SOCE and others suggesting a negative regulatory function (Soboloff et al., 2006). A subsequent report has confirmed that STIM1 and STIM2 are activators of SOCE in HeLa cervical cancer cells, human embryonic kidney 293 cells and human umbilical vein endothelial cells (Brandman et al., 2007). More notably, knockdown of STIM1 or STIM2 expression mediated by small interfering RNA has shown that STIM2, but not STIM1, regulates mainly basal cytosolic and endoplasmic reticulum Ca\(^{2+}\) concentrations in these cells (Brandman et al., 2007). The authors suggest that to maintain basal Ca\(^{2+}\) concentrations, STIM2 has a Ca\(^{2+}\)-binding EF hand domain with a lower affinity for Ca\(^{2+}\) than that of STIM1. As a result, even a small decrease in the endoplasmic reticulum Ca\(^{2+}\) concentration results in the translocation of STIM2 to endoplasmic reticulum–plasma membrane junctions to activate CRAC channels. In contrast to that report (Brandman et al., 2007), no obvious changes in the basal
cytosolic Ca\(^{2+}\) or endoplasmic reticulum Ca\(^{2+}\) pools have been detected in \textit{ex vivo} naive or \textit{in vitro}-differentiated T cells isolated from Stim2\(^{-/-}\) mice, whereas naive Stim1\(^{-/-}\) T cells seem to have less release of endoplasmic reticulum Ca\(^{2+}\) induced by antibody to CD3, which indicates that Stim1\(^{-/-}\) cells have depleted resting endoplasmic reticulum Ca\(^{2+}\) pools (Oh-Hora \textit{et al}., 2008). Similarly, mast cells derived from Stim1\(^{-/-}\) mice have much less antigen-or thapsigargin-stimulated release of endoplasmic reticulum Ca\(^{2+}\) than do Stim1\(^{+/+}\) mast cells (Baba \textit{et al}., 2008). Although the size of resting endoplasmic reticulum Ca\(^{2+}\) pools in Stim1\(^{-/-}\) and Stim1\(^{+/+}\) mast cells was not specifically assessed in that study (Baba \textit{et al}., 2008), as mentioned above, it seems likely that STIM1 is crucial for the replenishment of endoplasmic reticulum Ca\(^{2+}\) during the resting state in mast cells and T cells. Thus, although it is clear that both STIM1 and STIM2 contribute to the maintenance of intracellular Ca\(^{2+}\) concentrations, their exact physiological functions seem to be complex and may differ depending on the cell type and mechanism of activation.

**CRACM proteins**

CRACM1 is a small protein of 32.7 kilodaltons with four transmembrane domains and amino and carboxyl ends that face the cytosol (Vig \textit{et al}., 2006, Feske \textit{et al}., 2006, Zhang \textit{et al}., 2006). One of the first experiments to test whether CRACM1 could form the CRAC channel involved expressing CRACM1 together with STIM1 in human embryonic kidney and Jurkat cell lines. This resulted in an enormous amplification of 50-to 100-fold in CRAC currents (Peinelt \textit{et al}., 2006; Mercer \textit{et al}., 2006; Soboloff \textit{et al}., 2006). Further biochemical analysis, including coimmunoprecipitation and site-directed mutagenesis of negatively charged transmembrane glutamate residues, has shown that CRACM1 creates the pore of the CRAC channel by forming homo-oligomers (Vig \textit{et al}., 2006; Yeromin \textit{et al}., 2006; Prakriya \textit{et al}., 2006). In experiments with heterologous expression systems, CRACM1 can also form heteropolymers with CRACM2 (Orai2) and CRACM3 (Orai3) and possibly some TRPC channel subunits (Lis \textit{et al}., 2007; Ong \textit{et al}., 2007). A functional CRAC channel pore requires the tetrameric assembly of CRACM subunits. This has been shown by coexpression of preassembled tandem CRACM1 multimers composed of varying numbers of subunits along with a dominant negative CRACM1 mutant (Mignen \textit{et al}., 2008; Ji \textit{et al}., 2008).
Communication between STIM1 and CRACM1

A strong functional interaction between STIM1 and CRACM1 was demonstrated by many laboratories (Peinelt et al., 2006; Mercer et al., 2006; Soboloff et al., 2006). Such studies suggest that STIM1 and CRACM1 are necessary and sufficient to generate CRAC-like currents in vitro. Additional interacting molecules are either not required or are not limiting for the in vitro reconstitution of CRAC currents. After store depletion, STIM1 forms oligomers, moves to the endoplasmic reticulum–plasma membrane junctions and localizes within 10–25 nm of the plasma membrane (Wu et al., 2006). Furthermore, the STIM1 clusters in the endoplasmic reticulum–plasma membrane junctions are present near the regions of Ca²⁺ influx from the plasma membrane. Although much insight has been gained from heterologous expression studies of CRACM1 and STIM1, many basic questions remain unanswered.

Notably, CRACM1-knockout and Stim1⁻/⁻ mast cells show no defects in proliferation or differentiation in vitro, and tissue mast cell numbers in CRACM1-knockout and Stim1⁻/⁻ mice are similar to those in wild-type mice. These data suggest that a non-store-operated mode of Ca²⁺ entry may be crucial for the differentiation and proliferation of mast cells, but SOCE is not (Baba et al., 2008; Vig et al., 2008).

Non-store-operated Ca²⁺ signaling in T cells

T lymphocytes also express voltage-gated Ca²⁺ channels (Caᵥ channels), but whether these channels are functional in lymphocytes remains debatable (Kotturi et al., 2006; Badou et al., 2006). Caᵥ channels are expressed and functional mainly in excitable cells such as neurons and muscle cells, where they are activated in response to membrane depolarization. CD4⁺ T cells lacking the Caᵥ regulatory β4 or β3 subunits show impaired Ca²⁺ responses in response to stimulation of the T cell antigen receptor (Badou et al., 2006). These Ca²⁺ responses seemed to be independent of the release of Ca²⁺ from endoplasmic reticulum stores, as no defect is found in response to thapsigargin, which mediates passive release of Ca²⁺ from the endoplasmic reticulum and activates CRAC channels. Nuclear translocation of both NFAT subtypes, NFATc2 and NFATc1, is inhibited in both β-subunit-deficient T cell strains, which shows that the entire calcium-calcineurin-NFAT pathway is affected. Although the proliferation of β4-mutant CD4 T lymphocytes is intact, cytokine secretion from these cells is lower. These data collectively suggest that Caᵥ channels are necessary for a
normal T cell antigen receptor–mediated Ca\(^{2+}\) response in CD4\(^+\) T cells. However, membrane depolarization does not result in the activation of Ca\(_v\) channels in T lymphocytes, which suggests the existence of an alternative gating mechanism for these channels (Badou et al., 2006). An independent study has shown that a carboxy-terminal fragment of Ca\(_{1.2}\) (also called the ‘L-type voltage-gated calcium channel’) can translocate to the nucleus and regulate transcription (Gomez-Ospina et al., 2006). This calcium channel–associated transcription regulator can bind to a nuclear protein and an endogenous promoter and to regulate the expression of a wide variety of genes essential for signaling and excitability in neurons. The nuclear localization of the calcium channel–associated transcription regulator is regulated by changes in intracellular Ca\(^{2+}\) concentrations, among other parameters.

**Native Voltage-Gated Calcium Channels**

Early electrophysiological recordings from neurons, muscle and endocrine cells revealed voltage-activated calcium (Ca\(^{2+}\)) currents with distinct characteristics, suggesting the existence of two major classes of Ca\(^{2+}\) channels based upon the membrane potentials at which they first open; low-voltage activated (LVA) and high-voltage activated (HVA). The LVA (or T-type) channels, typically have a small conductance (8-12 pico Siemens (pS)), open in response to small changes from the resting membrane potential and inactivate rapidly. In contrast, the HVA currents generally possess larger conductances (15-25 pS), are activated by stronger depolarizations and display variable inactivation kinetics. To date, multiple types of HVA Ca\(^{2+}\) channels (L-, N-, P/Q- and R-type) have been categorized on the basis of a number of criteria including, single channel conductance, kinetics, pharmacology, and cellular distribution (Nowycky et al., 1985; Bean, 1989; Tsien et al., 1991).

**L-Type Calcium Channels**

L-type Ca\(^{2+}\) channels were initially described in peripheral neurons and cardiac cells, but appear to be present in all excitable as well as many types of non-excitable cells (Tsien et al., 1991). In certain cells, L-type channels have been shown to be preferentially localized to specific sub-cellular regions. For example, the L-type channels responsible for skeletal muscle contraction are concentrated on the transverse tubule membrane (Tanabe et al., 1987), while neuronal L-type channels are located primarily on cell bodies and proximal dendrites (Hell et al., 1993). The L-type
channel is the primary route for Ca\(^{2+}\) entry into cardiac, skeletal, and smooth muscles (Bean, 1989). The skeletal muscle L-type channel acts as a voltage sensor for excitation-contraction (E-C) coupling in skeletal muscle, presumably linking membrane depolarization to Ca\(^{2+}\) release from intracellular stores. While Ca\(^{2+}\) entry through this channel is not required for the initiation of contraction in skeletal muscle, it may provide a source of Ca\(^{2+}\) to replenish internal stores (Bean, 1989; Tsien et al., 1991; Tanabe et al., 1988; Miller et al., 1992). There is some evidence that L-type channels are involved in exocytotic release from endocrine cells and some neurons (Perney et al., 1986; Rane et al., 1987; Wang et al., 1994) and the localization of L-type channels on the cell soma (Hell et al., 1993) has also implicated these channels in the regulation of gene expression (Murphy et al., 1991; Sutton et al., 1999; Dolmetsch et al., 2001).

Much is known about the pharmacological properties of L-type Ca\(^{2+}\) channels. The three main classes of organic L-type channel blockers are the phenylalkylamines (verapamil), benzothiazapines (diltiazem), and 1,4-dihydropyridines (DHPs) (e.g., nitrendipine, nifedipine, nimodipine). The DHP antagonists bind preferentially to channels in the active conformation, a state favored by depolarization (producing more potent inhibition at depolarized potentials). A number of DHP agonists have also been developed, the most highly utilized of which is (-)-Bay K 8644 which increases both the open time and the single channel conductance (see chapter by Striessnig for more detail). L-type channels are also blocked by certain native peptide toxins such as ω-agatoxin IIIA (ω-Aga IIIA), isolated from the venom of the funnel web spider Agelenopsis aperta (Mintz et al., 1991; Cohen et al., 1992). ω-Aga IIIA reduces the current amplitude without affecting the time course and unlike the DHPs, ω-Aga IIIA inhibition is voltage-independent and blocks L-type channels at all potentials (Bean, 1989).

L-type Voltage-gated Ca\(^{2+}\) channels are composed of a pore-forming \(\alpha_1\) unit and accessory subunits \(\beta, \alpha_2\delta \) and sometimes \(\gamma\). The \(\alpha_1\) subunit confers voltage dependence, whereas the accessory subunits modulate biophysical properties as well as expression and trafficking of the channel. The pore-forming \(\alpha_1\) subunit defines the nature of the four L-type Ca\(^{2+}\) channels: \(\alpha_{1S}, \alpha_{1C}, \alpha_{1D}\) and \(\alpha_{1F}\), encoded by \(\text{Cacnalg}, \text{Cacnalc}, \text{Cacna1d}\) and \(\text{Cacnalf}\), respectively (Ertel et al., 2000). In excitable cells,
voltage-gated Ca\(^{2+}\) channels open after depolarization to allow Ca\(^{2+}\) influx. In neurons, this depolarization-dependent influx of Ca\(^{2+}\) rapidly activates NFATc4 translocation and gene expression (Genazzani et al., 1999; Graef et al., 1999). Membrane depolarization does not initiate lymphocyte activation and in fact seems to have a detrimental effect (Freedman et al., 1992; Price et al., 1989). Consequently, the function of voltage-gated Ca\(^{2+}\) channels in lymphocytes has been unclear.

Several classes of Ca\(^{2+}\) channel antagonists, including dihydropyridine derivatives, have been used to indicate the existence of channels with L-type, Ca\(^{2+}\) channel properties in lymphocytes (Gomes et al., 2004). However, some of these compounds, such as verapamil, dilitazem and nifedipine (Chandy et al., 1984), can also inhibit membrane repolarization by acting on voltage-gated K\(^{+}\) channels and hence have an indirect detrimental effect on Ca\(^{2+}\) entry (Chandy et al., 2004). In B cells, nicardipine and calciseptine block the Ca\(^{2+}\) influx induced by antibody to immunoglobulin D (IgD) or to IgM, whereas the dihydropyridine agonist Bay K 8644 prolongs the increase in [Ca\(^{2+}\)]\(_i\), induced by antibody to immunoglobulin (Sadighi Akha et al., 1996; Grafton et al., 2003). BCR activation induced by antibody to IgD can cause an additional Ca\(^{2+}\) influx in cells whose Ca\(^{2+}\) intracellular stores have been depleted by thapsigargin treatment (Sadighi Akha et al., 1996). That result suggests that independently of CRAC channels, an additional mechanism is involved in IgD-induced Ca\(^{2+}\) influx across the cell membrane. However, similar experiments done with crosslinking by antibody to IgM have not reproduced this effect (Grafton et al., 2003). Thus, it remains to be determined whether CRAC channels and L-type Ca\(^{2+}\) channels function in the same or in parallel pathways during physiological B cell activation. Similar experiments with inhibitors of L-type Ca\(^{2+}\) channels have been done in T cells (Kotturi et al., 2003; Kotturi et al., 2005; Stokes et al., 2004). However, in those experiments, Ca\(^{2+}\) influx was only partially inhibited.

Despite partial inhibition of Ca\(^{2+}\) influx, the L-type Ca\(^{2+}\) channel antagonist nifedipine has substantial 'downstream' effects, including decreased phosphorylation of extracellular signal–regulated kinase, decreased NFAT-dependent transcription and decreased IL-2 production in human T cells (Kotturi et al., 2003). Truncated α\(_{1C}\) and α\(_{1D}\), as well as the full-length B\(_1\) and B\(_3\) subunits, have been detected in human T cells, confirming the existence of partial L-type Ca\(^{2+}\) channels in lymphocytes (Stokes...
et al., 2004). The identification of two previously unknown α₁F splice variants in human T cells missing the voltage-sensor domain or containing an altered voltage-sensor domain (Kotturi et al., 2005) supports the belief that altered L-type Ca²⁺ channels are expressed and possibly activated by some process independent of membrane depolarization in nonexcitable cells. Nevertheless, the physiological function of these channels in T cell and B cell activation remains highly controversial.

N-Type Channels

In addition to L- and T-type Ca²⁺ channels, recordings from chick dorsal root ganglion (DRG) cells revealed a third type of single channel Ca²⁺ conductance of 13 pS (in 110 mM Ba²⁺), intermediate between that of the T- (8 pS) and L- (25 pS) type channels (Nowycky et al., 1985; Bean, 1989; Fox et al., 1987). Although this conductance shares some general electrophysiological characteristics with currents through both T- and L-type channels, it could not be attributed to either. Consequently, the corresponding channel was designated as N (neither)-type. N-type channels appear to be expressed only in neuronal tissues (Plummer et al., 1989; Plummer et al., 1991), although an N-type current has been reported in rat thyroid C-cell line (Biagi et al., 1991). Electrophysiologically, N-type channels are most easily distinguished from L-type channels by their inactivation properties. Unlike L-type channels, N-type channels display time-dependent inactivation (with Ba²⁺ as the charge carrier). Theoretically, the different inactivation properties of N- and L-type channels provides two parameters that can be used to dissect the relative contributions of the two channel types to the whole cell HVA current (Bean, 1989; Fox et al., 1987). One method takes advantage of the different inactivation rates. The component of whole cell current that decays during a prolonged depolarization can be attributed to the inactivating N-type channels, while the non-inactivating portion is identified as L-type current. The second approach exploits the different ranges over which voltage-dependent inactivation takes place. Pharmacologically, N-type channels are sensitive to inhibition by a class of native peptide toxins called the ω-conotoxins, which are a family of small (13-29 amino acid) peptides found in the venom of predatory marine snails of the genus Conus (Olivera et al., 1985; Olivera et al., 1994). All known ω-conotoxins inhibit N-type Ca²⁺ channels, although their specificities and blocking affinities for this particular channel vary significantly. To date, ω-conotoxin GVIA (ω-CgTx), a 27-amino acid peptide from Conus geographus (Olivera et al., 1984) is
the most specific ω-conotoxin peptide for N-type channel inhibition. ω-CgTx produces complete and irreversible inhibition of N-type currents in DRG, hippocampal, sympathetic, and sensory neurons at concentrations of approximately 100 nM to 1 μM (Kasai et al., 1987).

R-Type Channels
A component of the HVA current in cerebellar granule cells remains even after the application of nimodipine, ω-CgTx, ω-Aga VIA, and ω-CgTx MVIIC. This current, categorized as R (residual or resistant) – type (Zhang et al., 1993), comprises approximately 15% of the HVA current in these cells. R-type current may not necessarily reflect a single channel type, but a family of molecularly distinct channels with similar pharmacological and electrophysiological characteristics. R-type currents begin to activate around -40 mV and reach peak amplitude at 0 mV. The current inactivates rapidly, and the increased rate of inactivation with Ca as the charge carrier suggests that the channels supporting the R-type current inactivate in a Ca$^{2+}$-dependent manner. R-type channels are equally sensitive to block by Cd$^{2+}$ and Ni$^{2+}$ ions. The exact nature of the channels supporting this current is currently unknown.

HVA Ca$^{2+}$ Channels Are Multi-Subunit Complexes
Biochemical studies have established that high threshold voltage-gated Ca$^{2+}$ channels are multi-subunit complexes. Taking advantage of the high-affinity binding of organic antagonists, several groups purified the L-type channel from skeletal muscle. Four distinct polypeptides, designated $\alpha_1$ (175-kDa), $\alpha_2\delta$ (170-kDa), $\beta$ (52-kDa), and $\gamma$ (32-kDa), co-migrate with the ligand-binding activity (Curtis et al., 1983; Flockerzi et al., 1986; Takahashi et al., 1987; De Waard et al., 1996). A minor 212-kDa band also co-purified and was shown to represent a larger, much less abundant form of the skeletal muscle $\alpha_1$ subunit (De Jongh et al., 1989). Similar approaches have been used to isolate the cardiac L-type (Norman et al., 1994) and brain N-type channels (Witcher et al., 1993). These complexes also consist of a $\alpha_1$ subunit associated with $\beta$ and $\alpha_2\delta$ subunits. The $\beta$ and $\alpha_2\delta$ are highly similar, if not identical to, the subunits associated with the skeletal muscle $\alpha_1$ (Ahlijanian et al., 1991; Sakamoto et al., 1991). However, unlike the skeletal muscle L-type channel, no $\gamma$ subunits appeared as part of either complex. A novel 95-kDa polypeptide was found to co-migrate with the N-type
channel although it is unclear whether this represents a bona fide channel subunit or a proteolytic fragment (Witcher et al., 1993).

Figure: Composition of a VGCC complex and structure of the \( \alpha_1 \) subunit. A) Diagram of a high-voltage-activated VGCC complex, indicating the \( \alpha_1 \), \( \alpha_2/\delta \), \( \beta \), and \( \gamma \) subunits. The \( \alpha_1 \) subunit forms the channel proper, comprising the voltage-sensing mechanism, the \( \text{Ca}^{2+} \) selective pore, and target of identified pharmacological agents. B) Predicted structure and transmembrane topology of the \( \alpha_1 \) subunit. Each domain possesses six putative membrane-spanning segments (1-6) and pore-forming P-loop (SS1-SS2). (Figure adapted from Snutch et al., 2004)

While subunit composition differs slightly depending on channel type, a general model has been proposed for HVA channels in which four to five proteins form a multi-subunit complex as shown in the figure. In this model, the \( \alpha_1 \) subunit forms the
channel proper, comprising both the voltage-sensing mechanism and the Ca\(^{2+}\) selective pore, and the remaining proteins interact with the \(\alpha_1\) subunit to modulate activity.

**Primary Structure and Properties of Ca\(^{2+}\) Channel \(\alpha_1\) Subunits**

The calcium channels that have been characterized biochemically are complex proteins composed of four or five distinct subunits that are encoded by multiple genes (Catterall, 2000). The \(\alpha_1\) subunit of 190 to 250 kDa is the largest subunit, and it incorporates the conduction pore, the voltage sensor and gating apparatus, and most of the known sites of channel regulation by second messengers, drugs, and toxins. The first cDNAs encoding Ca\(^{2+}\) channel \(\alpha_1\) subunits were isolated from rabbit skeletal muscle (Tanabe et al., 1987; Ellis et al., 1988). The \(\alpha_{1S}\) L-type subunit is an 1873-residue protein that bears a high degree of amino acid similarity to the voltage-gated Na\(^+\) and potassium (K\(^+\)) channels. Like the \(\alpha\) subunits of sodium channels, the \(\alpha_1\) subunit of voltage-gated calcium channels is organized in four homologous domains (I–IV), with six transmembrane segments (S1–S6) in each. The S4 segment serves as the voltage sensor. The S4 segment in each domain contains positively-charged residues every third or fourth position and is believed to form part of the voltage-sensing mechanism of the channel. The pore loop between transmembrane segments S5 and S6 in each domain determines ion conductance and selectivity, and changes of only three amino acids in the pore loops in domains I, III, and IV will convert a sodium channel to calcium selectivity. Between the S5 and S6 segments of each domain are two hydrophobic segments, SS1 and SS2, which are predicted to form the channel pore. An intracellular \(\beta\) subunit and a transmembrane, disulfide-linked \(\alpha_2\delta\) subunit complex are components of most types of calcium channels. A \(\gamma\) subunit has also been found in skeletal muscle calcium channels, and related subunits are expressed in heart and brain. Although these auxiliary subunits modulate the properties of the channel complex, the pharmacological and electrophysiological diversity of calcium channels arises primarily from the existence of multiple \(\alpha_1\) subunits (Hofmann et al., 1994).

Based upon similarity to voltage-gated Na\(^+\) channels, Tanabe and co-workers (Tanabe et al., 1987) speculated that the \(\alpha_1\) subunit may form both the Ca\(^{2+}\)-selective pore and
the voltage sensor of the channel complex. This hypothesis was supported by studies demonstrating that expression of the \( \alpha_{1S} \) in myotubes from dysgenic mice restored normal skeletal muscle-type E-C coupling and the slow \( \text{Ca}^{2+} \) current absent in these cells (Tanabe et al., 1988). In addition, \( \alpha_{1S} \) expression in dysgenic myotubes restored the charge movement observed in normal myotubes upon membrane depolarization (Adams et al., 1990). These results indicated that the skeletal muscle \( \alpha_{1S} \) subunit acts both as a voltage-sensor, providing a physical connection between membrane depolarization and \( \text{Ca}^{2+} \)-release from intracellular stores for the initiation of muscle contraction, and is also part of a functional VGCC.

Calcium channels were named using the chemical symbol of the principal permeating ion (Ca) with the principal physiological regulator (voltage) indicated as a subscript (Cav). The numerical identifier corresponds to the Cav channel \( \alpha_1 \) subunit gene subfamily (1 to 3 at present) and the order of discovery of the \( \alpha_1 \) subunit within that subfamily (1 through \( n \)). According to this nomenclature, the Cav1 subfamily (Cav1.1–Cav1.4) includes channels containing \( \alpha_{1S}, \alpha_{1C}, \alpha_{1D}, \) and \( \alpha_{1F} \), which mediate L-type \( \text{Ca}^{2+} \) currents. The Cav2 subfamily (Cav2.1–Cav2.3) includes channels containing \( \alpha_{1A}, \alpha_{1B}, \) and \( \alpha_{1E} \), which mediate P/Q-type, N-type, and R-type \( \text{Ca}^{2+} \) currents, respectively. The Cav3 subfamily (Cav3.1–Cav3.3) includes channels containing \( \alpha_{1G}, \alpha_{1H}, \) and \( \alpha_{1I} \), which mediate T-type \( \text{Ca}^{2+} \) currents.

**β Subunits**

The β subunit is the most extensively studied of the auxiliary subunits and appears to have the most profound effects on the functional properties of the \( \alpha_1 \) subunit. In mammals, there are at least four different β subunits (\( \beta_1, \beta_2, \beta_3, \) and \( \beta_4 \)) which are encoded by distinct genes. The transcripts of at least two of these genes, the \( \beta_1 \) and \( \beta_2 \), are alternatively spliced to give rise to \( \beta_{1a}, \beta_{1b}, \) and \( \beta_{1c} \) and \( \beta_{2a} \) and \( \beta_{2b} \).

Biochemical and primary sequence analyses indicate that the β subunits are hydrophilic with no transmembrane segments or glycosylation sites (Ruth et al., 1989; Pragnell et al., 1991; Hullin et al., 1992; Perez-Reyes et al., 1992). The β subunits contain potential phosphorylation sites for both protein kinase C and cAMP-dependent protein kinase. The modulatory effects of these enzymes on VGCC
function may, in part, be the result of their actions on this auxiliary subunit (Isom et al., 1994).

Although the specific effects of channel modulation depend upon the β subunit isoform, all β subunits appear to have the same general impact on the properties of HVA α1 subunits. The β subunits exhibit homology with the Src homology 3-guanylate kinase domain of membrane associated guanylate kinases and this region appears to regulate inactivation of HVA VGCCs (McGee et al., 2004).

The role of L-type VGCC in CD4+ T cells has recently been shown in the context of *Leishmania* infection wherein despite being non-excitable, these T cells express functional L-type VGCC (Hsu et al., 2001). VGCC in these T cells played a major role in inducing calcium influx with their association with the scaffold protein AHNAK-1 (Matza et al., 2008). Therefore, the data on T cells add support to our results, wherein these channels directly influence functional outcomes in non-excitable cells (like DCs). Further, these channels could be active during an infection, such as HIV (Hsu et al., 2001) and *Leishmania* (Matza et al., 2008). A negative role for L-type VGCC has been previously shown when knockdown of the beta subunit resulted in increased frequency of calcium oscillations leading to increased insulin secretion in beta cells (Berggren et al., 2004).

Keeping the above in view the work embodied in this thesis was initiated on deciphering the role of calcium in modulating immune responses to *M. tuberculosis* with emphasis on the role of VGCC in regulating calcium homeostasis in dendritic cells and the effects thereof on mediating the priming of T cells, the activation of macrophages and the survival of mycobacteria in vivo in a mouse model of tuberculosis.