Chapter 2

Review of Literature
2.1 Virulence factors of M. tuberculosis

Virulence is described on the basis of microbial characteristics like degree of pathogenicity, capacity of microbe to overcome host defences, severity of disease, death due to infection, invasive power, damage induced and the capacity of the microbe to grow and multiply in a host (Casadevall and Pirofski, 1999). The virulence of Mtb can be measured during the infection of macrophages and animals, using several different assays and making mutations in Mtb genes. The availability of entire Mtb genome sequence (Cole et al., 1998) and advances in gene disruption techniques (Pelicic et al., 1997) coupled with genetic screens have led to the identification of several mycobacterial determinants involved in the virulence (Berthet et al., 1998; McKinney et al., 2000; Cox et al., 1999; Camacho et al., 1999). Researchers have identified several genes that are important for various aspects of Mtb pathogenicity. Some of the virulence factors involved in the mycobacterium survival includes Erp, Eis, Mce, HspX, ESAT6/CFP10, LAM, Sod factor, SigA etc. although their modes of action remain unknown.

2.1.1 Eis (Enhanced Intracellular Survival) protein

Wei et al., (2000) first identified the eis gene encoded by Rv2416c and showed that it enhances the intracellular survival of M. smegmatis in human monocyte like cell line U-937. The eis gene is present in various virulent strains of mycobacterium and M. bovis BCG but absent in M. smegmatis and other nonpathogenic mycobacterial species. Enhanced eis gene expression was observed in the clinical strain M. tuberculosis 210 as compared to the H37Rv strain when grown in a human monocyte cell line, whereas the expression was the same when the strains were grown in vitro (Wu et al., 2005). Cappelli et al., (2001) also reported that eis is differentially expressed in a clinical strain of M. tuberculosis upon infection of activated human macrophages. Forty percent of the sera from pulmonary tuberculosis patients gave positive reactions for anti-Eis antibody in western blot analysis (Dahl et al., 2001). The eis is negatively regulated by the stringent response regulator RelMt so as to maintain constant levels of expression during starvation conditions (Dahl et al., 2005). Morris et al., (2005) demonstrated that eis is a component of the whiB7 regulon, whose expression is upregulated by exposure to sublethal concentrations of antibiotics or fatty acids encountered within the host macrophage during infection.

Over-expression of eis in M. smegmatis produces a unique 42-kDa protein band corresponding to the predicted molecular weight of the eis gene (Dahl et al., 2001). Eis protein appeared not only in the cytoplasmic fraction but also in the membrane, cell wall,
and culture supernatant fractions. Eis was found to be non-glycosylated protein. Samuel et al., (2007) demonstrated that Eis is released into the cytoplasm of M. tuberculosis-infected U-937 macrophages. Eis was also found in the extravesicular fraction and culture supernatant of M. tuberculosis-infected macrophages.

Bioinformatic analysis suggests that Eis is an acetyltransferase of the family of GCN5-related N-acetyltransferases and bears significant similarity to the structures of known members of the GNAT superfamily and also shares the conserved residues that are characteristic of this family (Samuel et al., 2007). The effect of Eis on human macrophage cytokine secretion was also examined and found that Eis modulates the secretion of IL-10 and TNF-α by primary human monocytes in response both to infection with M. tuberculosis and to stimulation with recombinant Eis protein (Samuel et al., 2007). Though a sufficient amount of information is available on Eis protein, precise role in the intracellular survival is still not known. Lella and Sharma (2007), first overexpressed, purified and studied the immunological properties of Eis protein from Mtb H37Rv using T-cell proliferation assay. The purified recombinant Eis protein inhibited the T-cell proliferation under in vitro condition. They also reported an inhibitory effect on the production of TNF-α and IL-4, while an enhanced production of IFN-γ and IL-10 from Eis stimulated T-cells was observed. Thus concluding that the Eis protein from Mtb disturbs the cross regulation of T-cells.

2.1.2 Mce (Macrophage Cell Entry) proteins

The Mce proteins are a family of invasion-like proteins with putative export signal sequences at the N-terminal end and are most likely located at the mycobacterial cell surface (Ahmad et al., 2005; Chitale et al., 2001; Harboe et al., 1999). The Mtb genome contains four dispersed but homologous mce operons (mcel–4) arranged in an identical manner and each encoding six Mce proteins (MceA–F) (Cole et al., 1998). However, the mce3 operon is deleted in the closely related bovine pathogen M. bovis and the vaccine strain M. bovis BCG (Behr et al., 1999; Gordon et al., 1999; Zumarraga et al., 1999). Arruda et al. (1993) initially showed that recombinant expression of Mce1A in E. coli allowed this non-pathogenic bacterium to invade and survive within macrophages. Subsequently, Mce1A-coated latex beads were shown to invade non-phagocytic mammalian (HeLa) cells (Chitale et al., 2001). However, Mce2A-coated beads were not internalized by HeLa cells. The uptake activity of Mce1A is localized in a basic 22 amino acid region, containing motifs that show similarity to other cell penetrating peptides (Lu et al., 2006). The surface-exposed nature of this region predicted from the molecular structure of Mce1A also supports its role
in the invasion of mammalian cells (Das et al., 2003). However, the role of MceB–F encoded by the mce1 and mce2 operons, as well as Mce3A, Mce4A and other Mce proteins encoded by the mce3 and mce4 operons, in mediating the uptake of Mtb inside mammalian cells is not known.

2.1.3 HspX (Rv2031c, hspX)

HspX, also known as Acr, i.e., the α-crystalline protein homolog or the 16-kDa protein, is a major Mtb antigen recognized by the sera of a high proportion of TB patients and is induced under anoxic conditions (Wayne, 1994). This gene is also induced in human THP-1 macrophages, and the growth of hspX gene mutant Mtb was severely attenuated in these macrophages (Yuan et al., 1998). It is postulated that the chaperone-like HspX is an important controlling element in Mtb latency or persistence, since overexpression of this protein inhibits Mtb growth (Yuan et al., 1996).

2.1.4 ESAT-6/CFP-10 (RD1)

The Esat-6 and CFP-10 proteins are members of the Esat6 family of related small secreted proteins found in Mtb culture filtrates. Both proteins are immunodominant antigens that are recognized by the sera in a majority of TB patients (Skjot et al., 2000). Mutation disrupting both closely linked genes in M. bovis caused severe attenuation in a guinea pig model of infection (Wards et al., 2000). Rv3874 and Rv3875 are located in the RD1 deletion region, the first deletion found when comparing the genome of a wild-type M. bovis strain and M. bovis BCG (Mahairas et al., 1996). RD1 contains the structural genes for nine proteins, Rv3971 through Rv23979 (Cole et al., 1998). This region is found in all virulent Mtb and M. bovis strains but is the only deletion found in all M. bovis BCG strains (Brosch et al., 2002), initially suggesting that some of the genes in this region were important for virulence. In one case, using a knockout strategy, a deletion of the RD1 region was made in Mtb H37Rv resulting in attenuated virulence phenotype same as M. bovis BCG strains (Lewis et al., 2003). While in another experiments the RD-1 region was inserted into the chromosome of M. bovis BCG showing that the complemented strain was much more virulent than its attenuated parent (Pym et al., 2002). The Esat6 and CFP-10 genes are cotranscribed in Mtb (Berthet et al., 1998); when co-expressed in E. coli, they form a tight 1:1 complex (Renshaw et al., 2002). The members of the Mtb Esat6 family are frequently found in the same type of genomic arrangement since many of their genes are found in
closely linked pairs in the \textit{Mtb} genome (Cole \textit{et al.}, 1998), suggesting that similar 1:1 complexes might also be formed in \textit{Mtb}.

2.1.5 LAM

LAM is included in the list of virulence factors because of its importance as an immunomodulator. LAM, a complex glycolipid that contains repeating arabinose-mannose disaccharide subunits, is a major component of the \textit{Mtb} cell wall (Hunter \textit{et al.}, 1986). Addition of LAM to murine macrophages depresses IFN-\(\gamma\) production, which, in turn, blocks the expression of IFN-\(\gamma\)-induced genes (Chan \textit{et al.}, 1991). LAM can also scavenge oxygen radicals, \textit{in vitro}, and inhibits the host protein kinase C. These multiple phenotypes suggest that LAM functions to inhibit host responses to \textit{Mtb} infection, protecting the bacterium from potentially lethal mechanisms like the respiratory burst (Chan \textit{et al.}, 1991).

2.1.6 SodA (Rv3846, \textit{sodA}) and SodC (Rv0342, \textit{sodC})

SodA is the iron-factored superoxide dismutase that degrades superoxides, which are normally by-products of aerobic respiration and are also produced by the phagocytic respiratory burst enzyme. It is therefore important for the survival of intracellular pathogens during infections. An antisense approach was used to make a phenotypic \textit{sodA} mutation in \textit{Mtb} H37Rv (Edwards \textit{et al.}, 2001). The phenotypic mutant produced much less SodA protein and was severely attenuated in mice, with a decrease in 5 log units of Mtb CFU counts in lungs and spleens than the wild type. Also, SodA mutant was rapidly cleared.

SodC is the Cu, Zn-factored superoxide dismutase that is responsible for a small part of total Sod activity in \textit{Mtb}. Inactivation of \textit{sodC} in \textit{Mtb} Erdman, using a linear DNA construct resulted in a more sensitive mutant to superoxides than the wild type. This SodC mutant was killed more efficiently than the wild-type parent in activated primary (peritoneal) murine macrophages. But there was no effect on survival ability of SodC mutant \textit{Mtb} in inactivated murine macrophages or activated macrophages from respiratory burst-deficient mice (Piddington \textit{et al.}, 2001). In another study, the \textit{Mtb} H37Rv \textit{sodC} was inactivated by a two-step plasmid procedure, and while this mutant also showed increased sensitivity to superoxides and H2O2, it exhibited wild type growth in activated primary (bone marrow) murine macrophages and in guinea pigs (Dussurget \textit{et al.}, 2001). The reasons for the discrepant results are not known, but here different \textit{Mtb} strains and macrophages were used.
2.1.7 Sigma factors

One of the major strategies used by prokaryotes to radically change their life-style in response to a changing environment is by using RNA polymerase holoenzymes with different promoter specificities. This is obtained by the formation of new holoenzymes containing different sigma factors, which allows the transcription of genes required for the new conditions. Pathogens, including *Mtb*, use this strategy for the transcription of genes that play important role in virulence (Smith, 2003).

**Sigma A (Rv2703, *sigA*):** It is the principal mycobacterial sigma factor presumably necessary for transcription of most mycobacterial housekeeping gene (Gomez et al., 1998, Predich et al., 1995). Hence, suggesting that sigma A might be interacting with a transcriptional activator that is responsible for the expression of these gene(s) necessary for virulence (Gomez et al., 1998). This hypothesis was confirmed by Steyn et al. (2002) who showed that WhiB3 (Rv3416) interacts with sigma A. Hence, the identification of genes in the sigma A regulon that require WhiB3 should ultimately lead to the identification of those genes that are essential for virulence.

**Sigma F (Rv3286c, *sigF*):** The derived amino acid sequence of *Mtb* sigma F is very similar to those of the sigma F of *S. coelicolor* and *B. subtilis*, which are essential for sporulation in these two species, as well as to those of sigma B of *B. subtilis*, which controls responses to environmental stress (DeMaio et al., 1996). It was speculated that the latency of *Mtb* in human TB could be similar to bacterial sporulation, and to provide evidence for this hypothesis, *sigF* in *Mtb* CDC1551 was inactivated (Chen et al., 2000). The mutant *Mtb* had no macrophage phenotype and was attenuated for virulence in mice, using mortality as a criterion.

**Sigma E (Rv1221, *sigE*):** Sigma E is a member of the ECF (for “extracytoplasmic function”) group of sigma factors that control the bacterial response to external stimuli. *sigE* transcripts are induced after exposure of *Mtb* to various environmental stresses such as high temperature and detergent stress (Manganelli et al., 1999). Hybridization-based studies showed that *sigE* mRNA levels increased during *Mtb* growth in human macrophages (Graham and Clark-Curtiss, 1999; Jensen-Cain and Quinn, 2001), hence, these stresses might be found during *Mtb* infections. The *sigE* mutant (Manganelli et al., 2001) is more sensitive to detergent, high temperature and oxidative stress than its wild-type parent *Mtb* H37Rv and grows poorly than the wild type in both mouse and human macrophages.

**Sigma H (Rv3223c, *sigH*):** The *Mtb* *sigH* is another member of ECF family induced after various stresses like heat shock and SDS treatment (Manganelli et al., 1999) and during
macrophage infection (Graham and Clark-Curtiss, 1999). The virulence phenotype of the sigH mutant is subtle in that its growth in macrophages and mice is normal in terms of bacterial load (Kaushal et al., 2002; Manganelli et al., 2002), but there are differences in lung histopathology, including fewer granulomas and a generally delayed pulmonary inflammatory response (Kaushal et al., 2002).

2.2 Exported Repetitive Protein (Erp)

2.2.1 Identification

Cherayil and Young in 1988 first used antibodies from patients with lepromatous leprosy for screening of M. leprae DNA expression library in λ-gt11. They were able to detect a gene for 28kDa M. leprae protein, which was the major target of the humoral immune response in lepromatous leprosy. The sequence of the 28kDa protein suggested that it was targeted for export from the cytoplasmic compartment of the cell with a signal peptide sequence and two hydrophobic patches at amino terminus and carboxyl terminus, respectively (Cherayil and Young; 1988). The corresponding gene was called as iron-regulated gene (irg) since it has a iron box present in the regions controlling its expression (Dale & Patki, 1990; Young et al., 1992). Lim et al. in 1995 cloned genomic library from Mtb fused with PhoA in pJEM11 vector and screened them in M. smegmatis for clones with genes encoding exported proteins with PhoA activity. They found that one of the clones had 77% nucleotide and 68% amino acid sequence similarity to a 28 kDa protein gene from M. leprae (Cherayil and Young; 1988) and a considerable similarity to a 20 amino acid peptide from the Mtb 19kDa antigen found to have a cross reactive T-cell epitope (Harris et al.;1991). Bigi et al. (1995) identified a novel M. bovis antigen and called it P36/34 which was also homologus to the 28 kDa antigen of M. leprae (Cherayil and Young; 1988).

2.2.2 The Protein Erp

The term erp (Exported Repetitive Protein) was first coined by Berthet et al. (1995) for the Mtb gene which was found to be like the M. leprae irg gene. The erp gene was found to be present in a single copy in the genome of Mtb with an ORF of 852 bp starting with GTG as an initiation codon and TAA as stop codon encoding 284 amino acids polypeptide with a calculated molecular mass of 27.6 kDa. The first 22 N-terminal amino acids exhibit structural similarity with signal sequences of exported proteins (Izard & Kendall, 1994; Pugsley, 1993), with a putative cut site between the alanine and serine residues. The central region of Erp protein has twelve tandem repeats of five amino acids with a motif
P(G/A)LTS (Fig. 2). The number and sequence of the erp repeats are conserved in the *Mtb* isolates (Berthet *et al.*, 1995). A homologous protein P36/34 was also detected in *M. bovis* with similar features as that of Erp in *Mtb* suggesting the existence of a new family of mycobacterial proteins (Bigi *et al.*, 1995). Later on De Mendonca-Lima *et al.* (2001) described Erp as a ubiquitous extracellular protein found not only in pathogenic mycobacteria, but also in saprophytic mycobacteria such as *M. smegmatis*, other environmental opportunistic pathogenic mycobacteria such as *M. avium*, *M. marinum* and *M. xenopi*, and in extracellular toxin-producing mycobacteria such as *M. ulcerans*. Erp protein has a modular organization and contain three domains: a highly conserved amino-terminal domain which includes a signal sequence, a central variable region containing repeats based on the motif PGLTS, and a conserved carboxy-terminal domain rich in proline and alanine. The number and fidelity of PGLTS repeats of the central region differ considerably between mycobacterial species. Different Erp orthologues were able to complement the morphological abnormality in the Erp mutant suggesting that this family of protein share similar functions with regards to colony morphology and might, directly or indirectly, contribute to cell-wall structure (Mendonca-Lima *et al.*, 2001). They also observed a pair of proteins at 36 and 34 kDa in *M. bovis* supernatants when probed with anti-P55 serum (sera from infected cattle) while a 38 kDa protein expressed in *E. coli*. These discrepancies were attributed to posttranslational modifications in *M. bovis* or to high proline content of the protein (Cherayil and Young; 1988).

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**Fig. 2:** The predicted domains of *Mtb* erp gene product and Kyte-Doolittle plot (Berthet *et al.*, 1995). Tandem repeats numbered R1 to R12 are represented as black arrow heads.
The Erp-6His protein was first over expressed and purified using nickel-affinity chromatography under denaturing condition. The renatured protein was used to raise polyclonal antibodies in rabbit which were immunoreactive to both the cell associated fraction (36 kDa) and TCA precipitated culture filtrates (36 and 34 kDa) of BCG and Mtb (Berthet et al.; 1998). It was also reported that Erp was a surface exposed molecule and Immunoelectron microscopy of the J774 murine macrophage infected with clinical isolate of Mtb demonstrated that Erp produced in Mtb phagosome might traffic intracellularly. They also generated erp null mutants of Mtb and BCG with similar growth characteristics like wild type bacteria in culture medium, but with impaired multiplication within murine macrophages. The BCG erp::aph mutant was rapidly cleared from the lungs of infected animals, whereas the parental and complemented strains colonized and survived within lungs (Berthet et al.; 1998). In contrast, the H37Rv erp::aph mutant survived but multiplied very slowly in the lungs as compared to the parental and complemented strains (Berthet et al.; 1998). Berthet et al. (1998) reported that BCG erp::aph mutant shows delayed-type hypersensitivity response comparable to that induced by the parental strain, hence BCG erp::aph persists long enough to stimulate antimycobacterial immunity. The Mtb erp gene encodes a surface-exposed protein produced during phagosomal growth and that this protein is required for multiplication of the pathogen within the host cell. Thus, erp may be a good candidate for the rational attenuation of Mtb and the construction of new live-attenuated vaccines against tuberculosis (Berthet et al.; 1998).

In order to purify Erp protein, Bigi et al. (1999) expressed P36 in different expression systems and found that the protein was highly degraded in bacterial system, but M. smegmatis and especially the baculovirus expression system were better choices for the production of Erp from pathogenic mycobacteria for the immune response studies and diagnostic assays. The Erp protein localises to the outer part of the cell wall via carboxyl-terminal domain (Kocincova et al., 2004a). Surprisingly, this domain was not responsible for restoration of full virulence of an erp3 mutant of Mtb. However, due to lack of this domain the cell wall was not properly structured, leading to an enhanced sensitivity to detergents (Kocincova et al., 2004a). In the same year Kocincova et al. (2004b) also reported that the hydrophobic region was not essential for restoration of the virulence and tissue damage by an erp::aph mutant strain of Mtb in mouse model of tuberculosis.

The erp gene of Mtb is functionally exchangeable with the erp genes of M. smegmatis or M. leprae, with similar cfu counts obtained in the spleen (De Mendonca-Lima et al., 2003). However, this was not the case in the lung at early time-points, when the M.
smegmatis orthologue complemented the erp- mutation less efficiently and the M. leprae orthologue more efficiently than the Mtb allele, in terms of bacterial multiplication. Hence, they concluded that the nature of the erp orthologue expressed influences the early phase of infection by affecting levels of bacterial multiplication in the lung. Indeed, very marked histological differences were reported between strains complemented with the M. smegmatis and the M. leprae alleles. The lungs of animals infected by the Mtb erp-/Lp strain contained many large granulomas characterized by a surface approximately seven times larger than those of mice infected with Mtb erp-/Ms; thus, the allelic form of the Erp protein clearly affects lung disease in mice (De Mendonca-Lima et al., 2003). Unlike the BCG mutant strain, the M. bovis ΔP36 and the Mtb mutant were able to survive in mice in spite of their inability to multiply, which is an important attribute to achieve the optimal balance between attenuation and immunogenicity in a live vaccine (Bigi et al., 2005). Hence, they proposed that M. bovis ΔP36 mutant strain represents a potential candidate to be evaluated as a potential live vaccine against bovine tuberculosis. Using zebrafish embryo infection model Cosma et al. (2006) demonstrated that Erp-deficient M. marinum had an early and intrinsic defect in intracellular growth and/or survival in macrophages, where as RD1 mutant bacteria could grow successfully in macrophages but failed to induce subsequent aggregation and intercellular spread (Volkman et al., 2004) (Fig. 3).

Cosma et al. (2008) demonstrated that granulomas, including their caseous centers, are permeable entities and that the concentration of immune elements at these foci fail to protect against native superinfecting bacteria, which persist for a long term inside it. Their findings put a question mark on whether vaccine strategies that use a more persistent or immunogenic strain would be successful. They also showed that Erp was not involved in modulating host cell trafficking and erp- deficient bacteria traffic to granulomas as well as do wild type bacteria. Hence, suggesting a new therapeutic route in which, if properly modified, these strains could rapidly deliver anti-bacterial cargo directly to pre-existing granulomas, including caseum, where the bacilli are thought to be hardest to eradicate (Cosma et al., 2008).

Martinez et al. (2007) studied and compared specific cellular and humoral immune responses to Erp, an antigen of latency, with those to ESAT-6, Ag85B and PPD as comparison in TB patients and in individuals vaccinated (healthy and latent TB) or not vaccinated with BCG. They found that latent TB infection subjects had more Erp-specific IFN-γ-producing cells than TB patients, and Erp-specific IgG3 were present only in infected but non-ill BCG+ subjects corresponding to latent TB infection. Hence, suggesting the utility
of Erp for the diagnosis of tuberculosis disease versus latent infection provided it is being tested in a very large series of patients (Martinez et al., 2007). Betts et al. (2002) in its nutrient starvation model based on Loebel’s work (Loebel et al., 1933b) used microarrays to identify various genes that were up or down regulated under nutrient starvation condition. They found that \textit{pirG} or \textit{erp} was the most highly up regulated cell envelop gene under nutrient starvation condition.

![Diagram of the proposed pathway by Cosma et al. (2006) highlighting the steps impacted by the Erp and RD1 virulence determinants on the early stages of \textit{Mycobacterium} pathogenesis.]

2.3 Host Pathogen interaction

Pathogenicity of mycobacteria not only lies in its ability to survive in host macrophages but also in the intracellular multiplication. In the infected macrophages \textit{Mtb}
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reside within a phagosome and avoids phagolysosome fusion (Vergne et al., 2004), promoting intracellular survival and growth of tubercle bacilli (Sturgill-Koszycki et al., 1996; Vergne et al., 2004 Clemens and Horwitz, 1996) and avoiding immunological detection (Russell et al., 2002). Macrophages infected with live M. bovis BCG surface labeled with fluorescein have revealed a striking release of fluorescent label from the bacterial phagosome which penetrated host subcellular compartments using fluorescence microscopy. The release of labeled bacterial constituents was evident 1hrs postinfection and continued for several days in culture (Beatty and Russell, 2000). Teitelbaum et al. (1999), used real time confocal microscopy to show that viable mycobacteria have the ability to facilitate the transit of macromolecules between the cytosolic and vacuolar compartments of infected macrophages.

Very recently, Kumar et al. (2010) have used genome wide screen to identify host factors that regulate the bacterial load in Mtb infected human macrophages. They have used Small interfering RNA (siRNA) library to target a total of 18,174 host genes to screen out the host molecules involved in stabilization of Mtb infection and to provide insight into the molecular mechanisms that facilitate persistence of pathogen in the host cell. They also found out that some of the host factors predominantly function through the regulation of autophagy and suggested the important role of autophagy in elimination of intracellular mycobacterial subpopulations. The siRNA screen against all known kinases and phosphatases in murine macrophages infected with the virulent strain, H37Rv was employed by Jayaswal et al. (2010) to first identify components of the host signaling machinery that regulate intracellular survival of Mtb. Using this method they were able to identify several validated targets which when silenced led either to a significant decrease, or enhancement in the intracellular mycobacterial load. They found out that silencing of Csnk1d, Adbk1, Prkacb, and TgfβRI yielded a reduced CFU count and showed a marked increase in mycobacterial co-localization with acidified lysozomes, On the other hand, gene silencing of Wee1, Abl1, Dgkz, and Chek1 resulted in an increase in the CFU counts and reduced co-localization with acidified lysozomes. In their study Jayaswal et al. (2010) also reported the inhibitory effect of TGFβRI with pharmacological inhibitors on intracellular survival of mycobacteria. They used D4476 as an inhibitor of TGFβRI and casein kinase 1 (a down stream intermediate to TGFβR signalling pathway) (Rena et al., 2004; Bain et al., 2007). They found a dose-dependent decrease in mycobacterial CFUs (in all the three isolates H37Rv 1934, or JAL2261 tested), with increasing inhibitor concentrations. These facts
make it all the more important to study the host (macrophage) and the pathogen (Mtb) together.

2.3.1 Immune response

Mtb has an extraordinary ability to persist and multiply in extremely hostile environment of alveolar macrophage where most other pathogens would have perished. Immunity against tuberculosis relies mainly on cell mediated immunity rather than humoral immunity (Hingley-Wilson et al., 2003). This is demonstrated by the fact that there is a considerable increase in the risk of tuberculosis in patients with reduced cell mediated immunity, like HIV infected people or individuals under going immunosuppressive therapy when compared with the patients with defective humoral immunity (Barnes et al., 1994). Protective acquired immunity to Mtb is mainly due to CD4+ and CD8+ T cells with T helper type 1 cytokine profile (Flynn and Chan, 2001). Individuals with defects in IL-12, its receptor and IFN-γ receptor show high susceptibility to tuberculosis (Doffinger et al., 2002; Fieschi et al., 2003; Lichtenauer-Kaligis et al., 2003).

Mtb has the capacity to block its transfer to lysosomes in nonactivated macrophages but once macrophages becomes activated following stimulation by cytokines, it is rapidly transferred to lysosomes where they are destroyed by bactericidal activities of reactive oxygen and nitrogen, which are upregulated upon macrophages activation. Macrophage activation can occur in response to several cytokines, of which IFN-γ and TNF-α are probably the most important (Adams and Hamilton, 1984; Bogdan and Schleicher, 2006; Flynn and Chan, 2001). Depression in the IFN-γ or TNF signaling pathways of either animal models or human hosts dramatically enhanced the risk of developing tuberculosis, illustrating the importance of these cytokines (Jouanguy et al., 1999; Stenger, 2005).

Mammalian Toll-like receptor (TLR) proteins have derived their name from the Drosophila Toll protein, with which they share sequence similarity. To date, 10 members of the mammalian TLR family of proteins have been described (Takeuchi and Akira, 2002). A variety of chemically diverse agonists for mammalian TLR proteins have been identified which include lipopolysaccharide (LPS), peptidoglycan, viral coat proteins, lipoproteins, glycolipids, dsRNA, and CpG DNA (Massari et al., 2002; Schwandner et al., 1999; Means et al., 1999; Hirschfeld et al., 2001; Bauer et al., 2001; Matsumoto et al., 2002; Ohashi et al., 2000). Engagement of TLR proteins activates the expression of proinflammatory mediators by macrophages, neutrophils, B cells, endothelial cells, and epithelial cells. In addition, TLR proteins have been shown to regulate host susceptibility to pathogens (Netea
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et al., 2002; Takeuchi et al., 2000). Despite a high degree of similarity between the intracytoplasmic portions of mammalian TLR proteins, activation of different TLR proteins elicits distinct responses from leukocytes. These differences in TLR-mediated macrophage responses in vitro have profound implications on how these proteins contribute in distinct ways to the innate-immune response. Purified 19-kDa lipoprotein (Brightbill et al., 1999), the glycolipids arabinose-capped lipoarabino-mannan (Means et al., 1999) and dimannosylated phosphatidylinositol (Jones et al., 2001) have been found to activate cells in a TLR2-dependent manner. In addition, TLR4 has been shown to mediate cellular activation by live Mtb bacilli (Means et al., 2001), phosphatidylinositol mannosides (Means et al., 1999; Hirschfeld et al., 2001; Bauer et al., 2001) from Mtb (Abel et al., 2002) and by cell-wall preparations purified from M. bovis BCG (Tsuji et al., 2000). Heldwein et al. (2003) found that TLR2 and TLR4 regulate innate and adaptive-immune responses against BCG infection in mice.

Mtb residing within macrophages is kept in check within structures termed granulomas (Cosma et al., 2003; Flynn and Chan, 2003). These structures are beneficial to the host as it helps to contain the infection to a localized region. Although the precise biology of granulomas remain only partially understood, it is believed that granulomas are structured clusters containing Mtb infected macrophages in the center, surrounded by different types of immune cells, in particular macrophages and T lymphocytes (Gordon et al., 1994). Both nonactivated and activated macrophages coexist within granulomas, with the activated macrophages processing and presenting mycobacterial antigens to the surrounding T lymphocytes (Chan and Flynn, 2004). Following presentation of mycobacterial antigens, the T cells become activated through the triggering of T cell receptors (Kaufmann, 1993; Pieters, 1997). Activated T cells secrete cytokines and chemokines keeping the macrophages in an activated state and ensuring the recruitment of other immune cells to the site of infection. Mtb can persist for a long time, even up to the lifetime of the host (Young et al., 2002), within these structures, and as long as host immunity (in the form of activated macrophages and functional T cells) is effective, there is usually no adverse effect of the Mtb on the host’s health (Saunders and Britton, 2007). Thus, the granuloma represents a balance between a potentially dangerous pathogen and the host immune system.

The immunodominant T-cell antigens recognized by Mtb-infected humans or animals are mostly secreted proteins, including well-studied examples like ESAT-6, CFP-10, the Ag85 family of mycolyl transferases, MTB32a, TB9.8, TB8.4, TB10.4 and
lipoproteins such as the 19 and 38 kDa antigens (Mustafa et al., 2006; Skeiky et al., 2004; Dietrich et al., 2006; Young and Garbe, 1991). Many of these antigens are currently being studied as components of new candidate vaccines (Horwitz et al., 2000; Olsen et al., 2004; Reed et al., 2009; Sander et al., 2009), and there is clear evidence for their presentation to T cells by the MHC class I and class II pathways. Mycobacteria produce many unique lipids and glycolipids, and some of these have been found to be specific T-cell antigens that are presented by MHC class I-like CD1 molecules. The ligands presented by CD1b include free and monoglycosylated mycolic acids, phosphatidylinositol [lipoarabinomannan (LAM), PIM2 and PIM6] and diacylated sulfoglycolipids (Bricard and Porcelli, 2007; Layre et al., 2009). Mtb frequently possess multiple highly evolved mechanisms to disrupt antigen processing and presentation (Brodsky et al., 1999; Harding et al., 2003) like infection of antigen-presenting cells by Mtb, use of secreted proteins as immunological decoys and delay the priming of adaptive immunity on Mtb infection (Baena and Porcelli, 2009)

2.3.2 iNos & other Reactive Oxygen Species

Host cells that are protective against TB include macrophages, dendritic cells (DC), T-lymphocytes, and airway epithelial cells. The production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) by innate immune cells is considered to be a relatively effective host-defense mechanism against microbial pathogens like Toxoplasma gondii, Leishmania major, Listeria monocytogenes, Plasmodium species, Ectromelia virus, Coxsackie B3 virus, Mtb and M. leprae (Chan et al., 1992; Nathan and Shiloh, 2000; Fang, 1997; Chan et al., 1995). iNos and NO expression is induced by a wide variety of cytokines and inflammatory mediators such as TNF-α, IFN-γ, LPS, IL-1, hypoxia, and picolinic acid (Chan et al., 1999). However, mycobacterial cell wall components are also capable of eliciting host-inflammatory responses (Chan et al., 2001). In the murine model of TB, NO plays an essential role in the killing of Mtb by mononuclear phagocytes (Chan et al., 1992; Chan et al., 1995; MacMicking et al., 1997). Intratracheal administration of virulent Mtb to rats stimulated iNOS and NO production in alveolar macrophages (Greenberg et al., 1995). Moreover, administration of the NOS inhibitor L-NG monomethylarginine (L-NMMA) intraperitoneally attenuated the Mtb-induced increase in RNI in lung homogenates and bronchoalveolar fluid. It is generally recognized that reactive nitrogen intermediates play an effective role in host defense mechanisms against tuberculosis. In a murine model of tuberculosis, NO plays a crucial role in antimycobacterial activity; however, it is controversial whether NO is critically involved in host defense against Mtb in humans (Yang
et al., 2009). Nevertheless, there is growing evidence that NO produced by TB-infected human macrophages and by epithelial cells are antimycobacterial (Rich et al., 1997; Rockett et al., 1998; Nicholson et al., 1996; Kim et al., 1997; Jagannath et al., 1998).

2.3.3 Phagolysosome fusion

Pathogenicity of mycobacteria is linked to their survival in host macrophages. The pathogenic mycobacteria resists phagosome-lysosome fusion and survive within the macrophages (Armstrong and Hart, 1971; Russell et al., 1997). How mycobacteria interfere with phagosome-lysosome fusion is not understood (Berthet et al., 1998; McKinney et al., 2000; Sambandamurthy et al., 2002). Several lipid moieties arrest phagosomal maturation by interfering with traffic from the Golgi complex (Anes et al., 2003; Fratti et al., 2003), but the mycobacterial proteins responsible for interfering with lysosomal delivery remain unknown (Russell, 2001). Signal transduction molecules expressed by the pathogenic bacteria have the ability to influence the host cellular trafficking pathways. The Mtb genome encodes for eleven eukaryotic-like serine/threonine protein kinases (Armstrong and Hart, 1971). Protein kinase G (PknG) is closely related to the mammalian protein kinase Ca. Unlike most Mtb serine/threonine protein kinases (STPKs), it does not have a transmembrane domain and has been shown to be localized to the bacterial cytosol (Nielsen et al., 1997). The pknG gene was not essential for the mycobacterial growth under normal growth condition but it was found to be crucial for survival of mycobacteria within the macrophages (Walburger et al., 2004). Non-pathogenic mycobacteria M. smegmatis which do not express PknG were readily transferred to lysosomes, while survival of M. smegmatis expressing PknG was significantly enhanced (Walburger et al., 2004). Thus, they were able to show that expression of PknG in M. smegmatis and its kinase activity was sufficient to prevent lysosomal transfer and prolong intracellular survival. PknG is actively secreted by mycobacteria after uptake into macrophages and acquires access to the macrophage cytosol giving selective advantage to the pathogenic mycobacteria by avoiding phagosome-lysosome fusion (Walburger et al., 2004). M. smegmatis normally lack PknG and mice infection studies with M. smegmatis expressing PknG or its three mutants conclusively demonstrated a role for PknG in the persistence of pathogenic mycobacteria in its host. The three PknG mutants included deletion mutant of the N-terminal 73 residues, mutation in T63 residue and deletion mutant of the Trx motifs. These three mutants were individually capable of abrogating PknG-mediated survival of M. smegmatis in host tissues (Tiwari et al., 2009).
An analysis of the protein composition of the mycobacterial phagosome showed the exclusive presence of a protein that was strongly retained on phagosomes harboring viable mycobacteria, but this protein did not associate to any other subcellular organelle (Ferrari et al., 1999; Hasan et al., 1997). This protein, initially termed TACO (Tryptophan aspartate containing coat protein, also known as P57) or now referred to as coronin 1 is recruited to phagosomes containing live bacilli, but rapidly released from phagosomes containing killed mycobacteria. Hence, suggesting that coronin 1 is an important host factor that specifically prevents the lysosomal delivery and death of mycobacteria inside macrophages (Ferrari et al., 1999; Gatfield et al., 2005). Coronin 1 has been reported to prevent phagosome-lysosome fusion by regulating calcium-dependent signaling processes. When macrophages are infected with mycobacteria, they respond with a sustained calcium flux dependent on the presence of coronin 1. The coronin 1-dependent cytosolic influx of calcium activates the calcium-dependent phosphatase calcineurin, while in absence of coronin 1, this influx does not occur and therefore calcineurin is not activated. Strikingly, the coronin 1-dependent activation of calcineurin is required for blocking phagosome-lysosome fusion (Jayachandran et al., 2007). The basic mechanism of how coronin 1 is recruited to the mycobacterial phagosome and the precise activity of calcineurin required to prevent lysosomal delivery of mycobacteria are unknown. However, mycobacterial proliferation inside macrophages is fully blocked by the presence of the calcineurin blockers cyclosporine A and FK506, further providing support for the role of activated calcineurin in inhibiting phagosome-lysosome fusion (Jayachandran et al., 2007).

2.3.4 Escape from phagosome

There are reports of M. marinum escaping from phagosomes in low numbers in an infected macrophage and spread to neighbouring cells in actin based mobility (Stamm et al., 2003; Stamm et al., 2005). Smith et al. (2008) has reported that this escape of M. marinum from mycobacteria containing vacuole, actin polymerisation and spread from cell to cell is ESX-1 dependent phenomenon. Van der Wel et al. (2007) found that the lysosomes rapidly fuse with the virulent Mtb and M. leprae containing phagosomes of human monocyte-derived dendritic cells and macrophages. They found that after 2 days, Mtb and M. leprae progressively translocates from phagolysosomes into the cytosol in non-apoptotic cells, but not the vaccine strains like M. bovis BCG or in heat-killed mycobacteria. They also showed that this escape was dependent upon secretion of the mycobacterial gene products CFP-10 and ESAT-6. The cytosolic bacterial localization and replication are pathogenic features of
virulent mycobacteria, causing significant cell death within a week (Van der Wel et al., 2007).

2.3.5 Granuloma formation

Majority of infected individuals do not develop disease. This raises an important question of how these bacilli are contained by the host immune system. The Mtb residing within macrophages are kept in check within structures termed as granulomas (Cosma et al., 2003; Flynn and Chan, 2003). The granulomas are structured clusters containing macrophages infected with Mtb in the center, surrounded by different types of immune cells, particularly macrophages and T lymphocytes (Gordon et al., 1994). Both non-activated and activated macrophages coexist within granulomas. The activated macrophages process and present mycobacterial antigens to the surrounding T lymphocytes (Chan and Flynn, 2004) which then become activated through the triggering of T cell receptors (Kaufmann, 1993; Pieters, 1997). The activated T cells secrete cytokines and chemokines keeping the macrophages in an activated state and ensuring the recruitment of other immune cells to the site of infection. The Mtb can persist in the granulomas even up to the lifetime of the host (Young et al., 2002). There is no adverse effect of the Mtb on the host’s health as long as host immunity is effective (Saunders and Britton, 2007). Thus, the granuloma represents a balance between a potentially dangerous pathogen and the host immune system. The pathway of granuloma formation and subsequent bacterial dissemination is based upon macrophage responses like recruitment, phagocytosis and apoptosis. The RD-1 competent mycobacteria utilize the granuloma response as an effective tool for pathogenesis (Davis and Ramakrishnan, 2009). They have shown how in the absence of adaptive immunity during the early stages, the bacteria multiply and spread to facilitate the formation of nascent granuloma. The infected macrophages undergo apoptosis and recruit uninfected macrophages to the site. These recruited macrophages phagocytose the remnants of infected cells and their bacterial contents. Some newly infected macrophages egress to seed secondary granuloma formation. When initiation of adaptive immunity eventually occurs, CD4+ and CD8+ effector T lymphocytes are recruited to infected tissue and curtail bacterial growth. Although the adaptive immunity is essential for control of the mycobacterial infection, but it cannot eradicate it. A mature granuloma thus represents equilibrium between virulent mycobacteria and the host immune response (Davis and Ramakrishnan, 2009).
2.3.6 Protein-protein interactions

Pathogenic bacteria have been reported to secrete low-molecular weight tyrosine phosphatases during infection (Bach et al., 2006; Bliska et al., 1992; Fu and Galan, 1998). These bacterial tyrosine phosphatases interfere with the host signalling mechanism. For example, the Salmonella and Yersinia phosphatases, SptP (Fu and Galan, 1998) and YopH (Bliska et al., 1992), were secreted into host cells where they interact with several adhesion proteins. Mtb PtpA (Protein tyrosine phosphatase A) is one such low-molecular weight tyrosine phosphatase (Cowley et al., 2002) secreted by Mtb to promote its survival within the host (Bach et al., 2008). Bach et al. (2008) have clearly demonstrated that secreted PtpA was able to pass through phagosomal membrane, as it was present in the macrophage cytosol where it interacted with the host cytosolic protein VPS33B (Vacuolar protein sorting 33B). They also showed that PtpA impairs phagolysosomal fusion in Mtb-infected macrophages by desphosphorylation of VPS33B. PtpA was the first Mtb enzyme shown to interact directly with an identified host protein substrate providing evidence of an Mtb protein disrupting macrophage signaling. PtpA (Bach et al., 2008) together with PknG (Cowley et al., 2004; Walburger et al., 2004), with unidentified substrate, and the lipid phosphatase SapM (Vergne et al., 2005) have been linked to phagosome maturation arrest. The above information gives us an idea that Mtb utilizes multiple molecular mechanisms in parallel to block phagosome maturation and one such mechanism involves a very well documented protein-protein interaction between PtpA of Mtb and VPS33B protein from macrophage (Bach et al., 2008).

The Yersinia protein tyrosine phosphatase (PTP) YopH is translocated into eukaryotic cells by a type III secretion system requiring bacteria-host cell contact. The YopH protein selectively acts on tyrosine phosphorylated proteins of approximate molecular weight 55 kDa (p55) and 120 kDa (p120) in cultured murine J774A.1 macrophage cells (Bliska et al., 1992; Andersson et al., 1996). Hamid et al. (1999) demonstrated that p120 actually represents two target proteins, Cas and Fyb (Liu et al., 1998). The Cas localized to focal adhesion-like structures in J774A.1 cells and that YopH disrupted these structures during infection (Hamid et al., 1999) thus supporting the fact that Cas was an important integrin-associated target of YopH in macrophages. By protein microsequencing Black et al. (2000) identified p55 as murine SKAP-HOM (Src Kinase Associated Protein Homolog). The direct interaction between SKAP-HOM and a catalytically inactive form of YopH was reported under in vitro condition and in macrophages. In addition, SKAP-HOM was tyrosine phosphorylated in response to macrophage cell adhesion and it formed a signalling complex.
with Fyb (Black et al., 2000). Black et al. (2000) also suggested that dephosphorylation of SKAP-HOM and Fyb by YopH allows Yersinia to interfere with a novel adhesion-regulated signal transduction pathway in macrophages.

In another case of host-pathogen protein-protein interaction, an intracellular pathogen *Salmonella* SipC protein interacts with host TassC and Hook3 proteins inactivating their function. The facultative intracellular pathogen *Salmonella enterica* serovar *typhimurium* (Galan, 2001) harbours two different type III secretion systems (TTSSs). The first being Inv/Spa system encoded in the *Salmonella* pathogenicity island 1 (SPI-1), which mediates bacterial entry into nonphagocytic cells and promotes apoptosis of macrophages (Galan, 2001). While, the second is Spi/Ssa system encoded in the *Salmonella* pathogenicity island 2 (SPI-2), which is essential for proliferation within host cells and intracellular translocation of proteins across the phagosomal membrane into the mammalian cytosol (Ochman et al., 1996; Shea et al., 1996; Hensel et al., 1998). Mutant strains of Spi/Ssa system show high degree of attenuation for virulence in mice and defective growth in host cells (Ochman et al., 1996; Shea et al., 1996; Cirillo et al., 1998; Hensel et al., 1998). Moreover, these mutants are unable to prevent fusion of the phagosome with the lysosome (Uchiya et al., 1999), to recruit actin around the phagosome (Meresse et al., 2001), to avoid the respiratory burst by inhibiting trafficking of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to the phagosome (Vazquez-Torres et al., 2000) and to prevent the localization of the inducible nitric oxide synthase (iNos) to the *Salmonella*-containing vesicles (Chakravortty et al., 2002). The Spi/Ssa system is required for secretion of at least 16 proteins, which are encoded both within and outside the SPI-2 pathogenicity island (Guy et al., 2000; Miao et al., 2000; Hansen-Wester et al., 2002). SpiC is a 127 amino acid protein encoded in the SPI-2 pathogenicity island (Ochman et al., 1996). A spiC mutant cannot replicate within macrophages and is highly attenuated for virulence in mice (Uchiya et al., 1999). The SpiC protein has no known homologues that would suggest function. Mammalian TassC has been identified as a SpiC-interacting protein that determines vesicular trafficking in macrophages and is inactivated by *Salmonella* SpiC (Lee et al., 2002). SpiC also interacts with Hook3 protein (a mammalian protein implicated in cellular trafficking) and targets its function. By inactivating Hook3 function, the SpiC protein might alter the lysosome network and prevent phagosome-lysosome fusion (Shotland et al., 2003).

*Salmonella*’s major virulence protein SifA antagonizes host GTPase Rab9 by binding to the PH domain of SKIP (SifA and Kinesin Interacting Protein) (Jackson et al., 2008). Rab9 is important for LAMP1 (Lysosomal Associated Membrane Protein 1)
distribution in cells (Ganley et al., 2004). Like Rab9, SKIP too can also influence dynamics of the major late endosomal membrane protein LAMP1. Recruitment of LAMP1 to the position of the SCV (Salmonella Containing Vacuole) and formation and/or stability of LAMP1-rich Sifs (Salmonella Induced Filaments) is mediated by the interaction of SifA and SKIP during infection (Boucrot et al., 2005). Jackson et al. (2008) hypothesize that both SKIP and Rab9 are needed to sustain the proper dynamics of LAMP1 but the interaction of SifA with SKIP targets LAMP1 to the SCV during infection.

*Listeria monocytogenes* is an intracellular food-borne pathogen that causes listeriosis, an infection characterised by gastroenteritis, meningitis, encephalitis and maternofetal infections in humans. During infection, it enters, survives and multiplies inside phagocytic and nonphagocytic cells (Khelef et al., 2005). Cabanes et al. (2005) identified Vip as a novel *Listeria* virulence factor present only in pathogenic *Listeria* species which is required for entry into Caco-2 (human enterocyte like) and L2071 (mouse fibroblast) mammalian cells. They also found that Gp96 (an endoplasmic reticulum resident chaperone) was the receptor for Vip and the Vip–Gp96 interaction was critical for the entry of *Listeria* into Caco-2 and L2071 mammalian cells and for *in vivo* infection. In another example of host-pathogen protein-protein interaction it was reported that *Listeria* employs actin and other microfilament-associated proteins to move through the host cell cytoplasm. It was found that *L. monocytogenes* lacking the surface-bound ActA polypeptide were not able to interact with cytoskeletal elements and were rendered non-motile (Domann et al., 1992; Kocks et al., 1992). Pistor et al. (1994) identified that the ActA polypeptide was the nucleator of the actin cytoskeleton and provided the first insights into the molecular nature of such controlling elements in microfilament organization.

### 2.4 Serine Palmitoyl Transferase

#### 2.4.1 The Pathway

Sphingolipids are defined as lipids with a structural backbone made of sphingoid bases (1,3-dihydroxy-2-amino-alkane and its derivatives). Sphingolipid metabolites are known to modulate various cellular events including proliferation, differentiation, and apoptosis (Hannun and Luberto, 2000; Mathias et al., 1998; Spiegel and Merrill, 1996). Sphingolipids, along with cholesterol form detergent-resistant membrane microdomains (London and Brown, 2000; Hanada, 1995; Fukasawa, 2000), which are involved in signal transduction and membrane trafficking (Simons and Ikonen, 1997). There are three major types of sphingoid bases present, namely Sphingosine (principal sphingoid base in
mammalian cells), Dihydrosphingosine (second most abundant) and Phytosphingosine (found in plants, fungi, kidney and stomach cells in mammals). In most of the natural sphingoid bases, the alkyl chain is predominantly made of 18 carbon (Karlsson, 1970). Chemical analysis of metabolically labeled sphingolipids suggested that C-3 to C-18 of sphingosine were derived from palmitic acid (Zabin and Mead, 1953), while C-1 and C-2 were from serine (Sprinson and Coulon, 1954). *De novo* sphingolipid biosynthesis is initiated by the condensation of L-serine with palmitoyl-CoA in presence of pyridoxal 5-phosphate (PLP) to generate 3-ketodihydrosphingosine (Fig. 4). This reaction is catalyzed by the Serine palmitoyl transferase (SPT, EC 2.3.1.50). This is the first committed step in the synthesis of ceramides and sphingolipids. This step is also believed to be rate limiting, making it likely that regulation of SPT controls the rate of sphingolipid synthesis (Gable et al., 2000). SPT is the key enzyme for the regulation of sphingolipid levels in cells. In a mammalian cell ceramide, sphingosine, sphingosine-1-phosphate and glucosylceramide regulate important cellular processes (Fig. 4) (Heung et al., 2006).

**Fig. 4:** *De novo* sphingolipid synthesis pathway (Carton et al., 2003).

### 2.4.2 The enzyme

SPT is believed to be a heterodimer and intracellularly bound to the outer membrane of the endoplasmic reticulum (Hanada, 2003; Han et al., 2004). The two SPT subunits SPTLC1 (55 kDa) and SPTLC2 (65 kDa) are highly conserved among eukaryotes. A new third subunit ‘SPTLC3’ was reported by Hornemann et al. (2006). Both, subunits seem to be
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required for enzyme activity but, SPTLC2 is considered to be the catalytic subunit due to the presence of a PLP binding site (Hanada, 2003). SPT activity was detected in various tissues, including brain, lung, liver, kidney, and muscle (Merrill et al., 1985) and is essential for embryonic development, because homozygous SPTLC1 and SPTLC2 knock-out mice die during embryogenesis (Hojjati et al., 2005). The amino-terminal hydrophobic domain of CHO SPTLC1 that corresponds to TMD1 (Trans membrane domain 1) of yeast Lcb1p (first sub-unit of yeast SPT) is not responsible for membrane association, stabilization of CHO SPTLC2, or enzymatic activity (Han et al., 2004). Horemann et al. (2007), first gave a strong evidence that SPT was not a dimer, but composed of several subunits. They determined the approximate molecular mass of the complex to be 480kDa by size exclusion and native protein electrophoresis. Based on the above findings, Horemann et al. (2007) proposed a model of native SPT enzyme, in which it was said to be an octamer composed of four SPTLC1-SPTLC2 or SPTLC1-SPTLC3 dimers. They also said that the presence of SPTLC2 and SPTLC3 within the complex was dynamically interchangeable, depending on their expression levels in the individual tissues.

The purification of SPT from any eukaryotes has not been successful because of the extremely low content and instability of this enzyme. The purification of the mouse SPT complex in Escherichia coli is also not possible because eukaryotic SPT2 is a heteromeric membrane bound protein enriched in endoplasmic reticulum with the catalytic site facing the cytosol (Ikushiro et al., 2001; Ikushiro et al., 2000). Affinity tagged forms of mouse LCB proteins lacking the membrane-anchor regions were co-expressed in E. coli as partially soluble proteins, but the purified SPT complex did not show enzymatic activity. Hanada et al. (2000) obtained an active SPT complex from the CHO cell mutants expressing a hexahistidine-tagged LCB1 protein. However, sufficient amount of the active enzyme was very difficult to obtain for detailed enzymological analysis.

The SPT is present in all eukaryotes but not in prokaryotic organisms. Although, sphingolipids are not typical membrane constituents in prokaryotes, there are some exceptions. In strict anaerobes such as the genera Bacteroides, Porphyromonas and Prevotella high levels of sphingolipids were found; in some species their contents in the total extractable lipid came out to be 70% (Batrakov et al., 1999; Batrakov et al., 2000). It has been reported that Bacteroides melaninogenicus contains a water-soluble SPT, but the purification of this enzyme was not successful (Lev and Milford, 1981). The Gram-negative
obligatory aerobic bacteria *Sphingomonas* and *Sphingobacterium* are the genera whose lipid composition and structure of their sphingolipids have been investigated most extensively (Yabuuchi *et al.*, 1979; Yano *et al.*, 1982). In contrast to the membrane-bound mammalian isoform a soluble, homodimeric SPT isoform (sSPT) was found in the sphingolipid producing prokaryote *Sphingomonas paucimobilis* (Ikushiro *et al.*, 2001; Ikushiro *et al.*, 2003). The first high resolution crystal structure of homodimeric SPT from *Sphingomonas paucimobilis* (holoenzyme) was reported by Yard *et al.* (2007).

A third 10-kDa subunit Tsc3p was identified in yeast, required for the optimal SPT activity (Gable *et al.*, 2000). Very recently, by genetic screening method two additional mammalian subunits were identified and named as ssSPTa and ssSPTb, for small subunits of SPT (Han *et al.*, 2009). Microsomes prepared from cells transfected with ssSPTa were highly selective for C16-CoA while, little activity was observed with shorter or longer acyl-CoA substrates. However, microsomes prepared from cells transfected with ssSPTb had substantial activity when C18-CoA was presented as the substrate (Han *et al.*, 2009) suggesting distinct Acyl-CoA preference for these subunits.

Overexpression of mouse *LCB2* cDNA in HEK 293 cells enhanced SPT activity in the cells even without co-expression of mouse *LCB1* cDNA (Weiss and Stoffel, 1997). The possible explanation for this discrepancy given by Hanada *et al.* in 1998 was that HEK 293 cells express the human LCB1 protein endogenously; and the overexpressed mouse LCB2 protein might form a functional complex with it. Alternatively, considering that there being about 30% amino acid identity between mammalian LCB1 and LCB2 proteins (Nagiec *et al.*, 1996; Weiss and Stoffel, 1997; Hanada *et al.*, 1997), the overexpressed LCB2 protein might form a homo-oligomer with some degree of SPT activity (Hanada *et al.*, 1992).

### 2.4.3 Inhibitors

*Sphingofungins* (Zweerink *et al.*, 1992), lipoxamycin (Mandala *et al.*, 1994), and myriocin also known as ISP-1/thermozymocidin (Miyake *et al.*, 1995) are potent and highly specific inhibitors of SPT, inhibiting both fungal and mammalian SPT in cell-free preparations with IC50 values in the nanomolar range. *Viridiofungins* are are also potent inhibitors of mammalian SPT (Mandala *et al.*, 1997), but they also inhibit squalene synthase with IC50 values in the micromolar range (Onishi, *et al.*, 1997). L-Cycloserine and β-chloro-L-alanine, are wide-range inhibitors of PLP-dependent enzymes which have sometimes been used to inhibit SPT in intact cells (Sundaram *et al.*, 1984; Medlock *et al.*, 1988), hence caution is needed while using these drugs for the specific inhibition of
sphingolipid synthesis. Most of these potent inhibitors structurally resemble the postulated transient intermediate that forms in the condensation of L-serine and palmitoyl CoA, suggesting that the formation of intermediate-like adducts of these drugs with PLP in the SPT enzyme underlies the strong inhibitory activity. Consistent with this suggestion, the inhibitory activity of sphingofungin B is highly dependent on its stereochemistry (Kobayashi et al., 1998), and myriocin-linked resins bind the LCB1/LCB2 complex tightly (Chen et al., 1999). External sphingosine supplement bypass SPT function and rescues CHO cells from inhibitory effects of sphingofungin B and myriocin (ISP1/thermozymocidin), but not the effects of L-cycloserine and h-chloro-L-alanine (Hanada et al., 2000). Interestingly, ISP-1 was initially discovered as a potent immunosuppressant (Fujita et al., 1994). Very low concentration (~ 50 nM) of ISP-1 is enough to induce apoptosis of mouse CTL-2 cells, but not most other cell types (Miyake et al., 1995; Nakamura et al., 1996).

2.4.4 Mutants

A temperature sensitive CHO cell mutant with thermolabile SPT designated as SPB-1 (Sphingoid base biosynthesis) was isolated by Hanada et al. in 1990. The SPT activity in homogenate of this mutant cultivated at 40° C was distinctly lower than that cultivated at low temperatures. The de novo synthesis of sphingolipids in SPB-1 exhibited a defect depending on culture temperatures. The mutants SPB-1 could grow at lower temperatures, but stopped growing at 40°C (Hanada et al., 1990). Lysenin, a 41-kDa protein derived from the coelomic fluid of the earthworm Eisenia fetida, induces hemolysis of mammalian red cells (Sekizawa et al., 1996; Sekizawa et al., 1997). Lysenin specifically binds to SM among various lipid types in cell-free systems (Yamaji et al., 1998). Hanada et al. in 1998, demonstrated that lysenin requires cell surface SM for its cytolytic activity in CHO cells and isolated SM deficient cell mutant CHO LY-B (where LY stands for lysenin resistant and B stands for clone 16). CHO LY-B cells were defective in de novo synthesis of all sphingolipid species, primarily due to lack of the LCB1 protein. This was the first direct evidence showing complex formation between the LCB1 and LCB2 protein in the SPT enzyme (Hanada et al., 1998). LY-B cells could be maintained in the normal culture medium, F-12 medium containing 10% serum, despite their inability to synthesize sphingolipids de novo, as mammalian sera contain both SM and glycosphingolipids (Svennerholm and Svennerholm, 1963; Yu and Ledeen, 1972; Fishman et al., 1978), and the normal culture medium used had about 20 mM SM (Hanada et al., 1992).
Mice lacking sptlc1 and sptlc2 are embryonic lethal (Hojjati et al., 2005). Recently, for the first time Cre-Lox system was used for disruption of sptlc2 gene in the liver causing loss of SPT activity in the liver without influencing the sptlc1 protein levels (Li et al., 2009). It resulted in significant reduction of plasma sphingomyelin and ceramide levels, along with reduction in liver and hepatocyte plasma membrane sphingomyelin levels. Liver being the major site for plasma lipoprotein biosynthesis, secretion and degradation, hence liver specific knock-out of liver SPT activity caused significant increase of plasma apoE levels without affecting apoA1 and apoB leading to an increase in cholesterol efflux from macrophages into plasma. Thus, liver specific SPT deficiency produces an anti-atherogenic phenotype in mice (Li et al., 2009).

2.4.5 Role in pathogenesis

Microorganisms that do not produce sphingolipids, including most bacteria and viruses, are able to utilize host sphingolipids to promote their virulence. The determination of which sphingolipid(s) (host, microbe, or both) modulate the host-parasite interaction is particularly significant not only because it may provide important insights into the development of new therapeutic strategies but also because the outcome of this sphingolipid interaction may either lead to commensalisms or to host damage/disease (Casadevall and Pirofski, 2003).

2.4.5.1 M. tuberculosis

Lipoarabinomannan (LAM) is a cell wall-associated lipoglycan present in different species of mycobacteria, which exhibits a wide range of immunomodulatory effects (Chan et al., 1991; Hunter et al., 1991; Kaplan et al., 1987; Sibley et al., 1988). LAM alters the signaling in the macrophages (Ghosh et al., 1998). It is reported to inhibit Mtb-induced apoptosis (Rojas et al., 2000). LAM isolated from M. smegmatis causes 60 to 90% inhibition of total protein kinase C (PKC) activity in human PBMC (Ghosh et al., 1998). However, PKC activity has also been found to be inhibited in cells treated with LAM of virulent Mtb (Chan et al., 1991). Treatment of human peripheral blood mononuclear cells (PBMC) with LAM isolated from Mtb resulted in an increase in endogenous cellular level of ceramide (Sirkar and Majumdar, 2002) which is well known to interact with several signaling systems, involving mitogen-activated protein kinase (MAPK) (Modur et al., 1996; Raines et al., 1993), stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (Brenner et al., 1997; Verheij et al., 1996), PKCζ (Bertoloso et al., 1998, Muller et al., 1995), and
phosphatases (Hannun, 1994). Mtb LAM promotes cell survival through ceramide, PI3 kinase, and MAPK (ERK 2) activation (Sirkar and Majumdar, 2002).

Certain effects of LPS might be mediated by ceramide-related pathways (Joseph et al., 1994). LPS does not directly activate sphingomyelinase, but has been reported to trigger the transient accumulation of ceramide in macrophages by augmenting the de novo synthesis of ceramide and other sphingolipids (Balsinde et al., 1997). Furthermore, LPS is also structurally similar to ceramide: the lipid A portion of LPS, responsible for its biological activity, is remarkably similar to the molecular structure of ceramide. Some of the putative downstream targets of ceramide include a ceramide-activated (serine/threonine) protein kinase (CAPK) and phosphatase (CAPP). Similarly, cell-permeable analogs of ceramide provoke TNF secretion by macrophages (Barber et al., 1996). Two cell-permeable ceramide (C2 and C6) analogs up-regulate both TNF and iNos in RAW 264.7 macrophages (Knapp and English, 2000).

Gutierrez et al. (2009), reported that Cer (Ceramide), Sph (Sphingosine) and S1P (Sphingosine 1-phosphate) have the ability to kill mycobacteria independent of NF-kB but SM (Sphingomyelin), AA (Arachidonic acid) and PIP (Phosphatidylinositol-4-phosphate) are potent activators of the pro-inflammatory response via NF-kB. Activation NF-kB pathway leads to an increase in the rate of phagosome maturation and is required for macrophages to kill M. smegmatis (Gutierrez et al., 2008). Consistent with this notion, the mycobactericidal lipids facilitate phagosome maturation (Anes et al., 2003). The lipids EPA (Eicosapentanoic acid), DHA (Docosohexanoic acid), PC (Phosphatidyl choline) and DAG (Diacylglycerol) fail to activate NF-kB. The DAG either has no mycobactericidal effect or enhances mycobacterial growth in macrophages (Anes et al., 2003; Treede et al., 2007). Cer, Sph and S1P fall into a different category of lipids that is unable to activate NF-kB but nevertheless have the ability to enhance killing of mycobacteria by stimulating autophagy (Lavieu et al., 2006; 2008). It is now well established that autophagy is a mechanism by which pathogenic mycobacteria can be eliminated by macrophages (Gutierrez et al., 2004). This demonstrates that lipids can enhance mycobacterial killing via both NF-kB dependent and NF-kB-independent mechanisms.

Greco et al. in 2009 reported that lysophospholipids like lysophosphatidic acid and S1P reduce Mtb-induced cytotoxicity and enhance anti-mycobacterial activity in the A549 cell line (used as a model of type II alveolar epithelial cells). These lysophospholipids induce Ca2+-dependent Phospholipase D (PLD) activation, which, in turn, promotes phagolysosome maturation, intracellular mycobacterial killing and inhibit in vitro
mycobacterial dissemination to macrophages (Greco et al., 2009). The lipid composition of alveolar surfactant can play an important role, both during primary infection and latency, in ensuring the correct activation of the alveolar anti-mycobacterial response at the level of macrophages and alveolar epithelial cells. The local lysophospholipid micro environment may provide protective signals for both ‘conventional’ (e.g. macrophages) (Garg et al., 2004; Garget al., 2006) as well as ‘unconventional’ (e.g. alveolar epithelial cells) immune cells, thereby limiting mycobacterial replication and dissemination, in particular, during the early phases of host–pathogen interaction, before any antigen-specific T helper-1 immune response takes place.

Both, ceramide and S1P are able to induce autophagy in a breast cancer cell line (Lavieu et al., 2006; Scarlatti et al., 2004) and these two sphingolipids have contrasting roles on the response to cellular stress (Ogretmen and Hannun, 2004). Ceramide and sphingosine 1-phosphate (S1P) constitute a rheostat system in which ceramide promotes cell death (Bursch et al., 2000) and S1P may be a mediator of starvation-induced autophagy (Lavieu et al., 2007) which is well established as a survival mechanism (Boya et al., 2005). Apart from this, ceramide is responsible for negative curvature of the membrane (Montes et al., 2002) and it has also been shown that alteration of ceramide biosynthesis results in changes in the vacuole morphology of yeast (Saccharomyces cerevisiae) (Faergeman et al., 2004). SK1 (Sphingosine kinase 1) is usually cytosolic but studies have shown changes in SK1 localization, for example, SK1 is recruited to the nascent phagosome in human macrophages during the phagocytosis of Mtb (Thompson et al., 2005). What is the significance of changes in SK1 enzyme localization and where it is localized during autophagy is still not known (Lavieu et al., 2007).

Increase in endogenous ceramide promotes a robust accumulation of Beclin 1 and an autophagic response associated with cell death. While an increase of S1P level (after starvation) induces a mild accumulation of Beclin 1 and promotes cell survival by inhibiting the induction of apoptosis (Fig. 5). Survival of this pathogen within host macrophages is attributed to its ability to prevent fusion of the phagosome with the lysosomal compartment (Kusner, 2005). Inhibition of SK upon mycobacterial infection suggests that a bacterium-derived component is able to decrease S1P in order to prevent the rise in Ca\(^{2+}\) that would normally lead to maturation and/or acidification of the macrophage phagolysosome and subsequent killing of the internalized bacteria (Malik et al., 2003).
Fig. 5: Hypothetical model proposed by Lavieu et al. (2007) for ceramide and S1P-induced autophagy and its consequences on cell fate.

2.4.5.2 Leishmania donovani

Leishmania donovani is an obligate intracellular protozoan that resides in the mononuclear phagocytes of infected mammalian hosts (Bates, 1994; Chang and Choudhuri, 1990). Host macrophages have specialized mechanisms for killing the invading pathogens and priming its immune response. Leishmania has evolved a range of sophisticated mechanisms to subvert normal macrophage function (Alexander et al., Russell et al., 1992). L. donovani infection induces macrophage dysfunction by causing reduction in respiratory burst, impairment of protein kinase C (PKC) activity and suppression of other proinflammatory molecules (Bhattacharyya et al., 2001a; Bhattacharyya et al., 2001b; Olivier et al., 2005). Recent studies suggest that ceramide is a pleiotropic lipid second messenger which plays an important role in a plethora of cellular functions such as immunomodulation and apoptosis (Hannun, 1996; Hannun and Obeid, 1995; Mathias et al., 1998; Saba et al., 1996). It exerts a diverse array of cellular functions by means of a subtle regulation of downstream kinases and phosphatases. L. donovani infection induces the endogenous ceramide synthesis pathway (Ghosh et al., 2002; Ghosh et al., 2001), which is responsible for the impaired PKC activity and extracellular signal-regulated kinase phosphorylation and activity but also inhibits the Activated Protein-1 and NF-κB activities (Ghosh et al., 2002; Ghosh et al., 2001). Ceramide also induces tyrosine phosphatase that
plays a major role in the deactivation of the mitogen-activated protein kinases in Leishmania-infected macrophages (Ghosh \textit{et al.}, 2002; Ghosh \textit{et al.}, 2001).

Akt has been shown to exert diverse cellular effects depending upon the cell type and stimulus (Chen \textit{et al.}, 2003; Datta \textit{et al.}, 1999). In addition to its well-established role as a cell survival kinase, Akt is implicated in superoxide anion generation and activation of the immune response in different kinds of immune cells (Chen \textit{et al.}, 2003, Kane \textit{et al.}, 2001). Activation of Akt leads to conformational changes in Akt, with subsequent phosphorylation at Thr308 and Ser473 (Andjelkovic \textit{et al.}, 1997). After being phosphorylated, Akt is translocated to the nucleus, where it regulates the synthesis of numerous proteins. It has been reported that Akt phosphorylation was significantly inhibited in \textit{L. donovani} infection, and the elevated level of endogenous ceramide was one of the important factors, since pretreatment with FB-1 (an inhibitor of sphingosine kinase) restored the inhibition of Akt phosphorylation on both sites (Dey \textit{et al.}, 2007). Enhanced level of ceramide not only impairs the nitric oxide generation in \textit{L. donovani} infected macrophages (18), but are also involved in the process of inhibition of TNF-\alpha release (Dey \textit{et al.}, 2007). In leishmaniasis endogenous ceramide is one of the most crucial factors for the dephosphorylation of Akt. These observations strongly suggest that endogenous ceramide generation induced in host cells by the parasite was responsible for the impairment of the normal macrophage antimicrobial machinery (Dey \textit{et al.}, 2007).