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*Mycobacterium tuberculosis* has been present in the human population since antiquity. Fragments of the spinal column from Egyptian mummies from 2400 BC show definite pathological signs of tubercular decay. In 1882, Robert Koch discovered a staining technique that enabled him to see *M. tuberculosis*. What excited the world was not so much the scientific brilliance of Koch's discovery, but the accompanying certainty that now the fight against humanity's deadliest enemy could really begin. Another important development was provided by the French bacteriologist Calmette, who, together with Guerin, used specific culture media to lower the virulence of the bovine tuberculosis (TB) bacterium, creating the basis for the BCG vaccine which is still in widespread use. In the middle of World War II came the final breakthrough against TB: the chemotherapy.

Tuberculosis (TB) is a major global health problem causing infection to ~8 million people and death of ~2 million people every year. The incidence of disease is the highest among the 20-45 year age group, thereby seriously affecting both social and economic development. India has the largest number of TB cases, with an estimated 1.8 million (20% of the global TB burden) followed by Indonesia (627 000), Bangladesh (361 000), Thailand (89 000) and Myanmar (85 000) (Fig.1).

![Fig. 1: TB incidence, country and region-wise, 2004](source: WHO Stop TB Department, website: www.who.int/tb)
Although long term chemotherapy is available and disease is curable, the breakdown in health services, spread of HIV/AIDS and the emergence of multi-drug resistant tuberculosis (MDR-TB) are contributing to the worsening impact of TB. This has led WHO to declare TB as a 'global health emergency' in the year 1993. It is estimated that between years 2000 to 2020 nearly one billion people will be newly infected, 200 million people will be sick and 35 million people will die (WHO, 2006).

*Mycobacterium tuberculosis* (*M. tuberculosis* or *M. tb*) is a facultative intracellular bacillus and primarily resides in the macrophages of the host lung (in cases of pulmonary TB) as well as other parts of the body such as lymph nodes, plural space, the joints, the bones, the genito-urinary tract, the central nervous system, the adrenal glands and pericardium (extra-pulmonary TB accounts for nearly 15% of all TB patients). TB is transmitted aerially, by exposure of germs present in saliva and pulmonary expectorations (e.g. by coughing and sneezing) from a person carrying the disease. Various distinct disease stages observed during the course of tuberculosis infection are products of multifaceted dialogue between host and *M. tuberculosis*. In a naïve host, following initial infection of macrophage in the lung, the bacteria undergo a period of rapid expansion in the absence of specific, limiting immune response. Once the host becomes aware of the infection (typically within 2-3 weeks), the cell-mediated immune system responds, with the influx of lymphocytes and activated macrophage into lesion and limits dissemination of infection by developing granuloma or tubercles at the infection foci. In this dormant state, the bacterium persists for longer time but fails to transmit disease. Reactivation of the endogenously persisting infection and the onset of the active disease are required for successful spread to a new host. Thus, though one third of the world population is infected with TB, only about 10% of the infected people develop overt disease during their lifetime. All others generate an effective immune response.

4.1. **Chronological events in pathogenesis of tuberculosis**

TB is primarily a disease of the lung, which serves as both the port of entry for pathogen as well as the major site of disease manifestation. Pulmonary tuberculosis accounts for
the vast majority of cases in adults and is transmitted by the aerogenic route (Kaufmann, 2000). An individual with active pulmonary tuberculosis expels small droplets containing tubercle bacilli, which can be inhaled by another individual in the vicinity (Fig. 2). If these small droplets enter the alveolar space, the pulmonary dendritic cells and macrophages engulf the microorganisms. Some infected macrophages remain in the lung tissue while some infected dendritic cells migrate to the draining lymph nodes. T cells in the draining lymph nodes get activated and migrate to recognize the mycobacterial foci in the lung. Granulomatous lesions are formed and contain the bacteria thereby preventing development of active disease (Kaufmann, 2001) in immuno-competent individuals. Infection is arrested at this stage. This is illustrated by the fact that out of two billion people infected with *M. tuberculosis* only eight to nine million develop disease annually. Hence, the immune response stimulated during infection is highly effective in the vast majority of infected individuals. However, control of infection is incomplete and the pathogen is not eradicated so that the risk of reactivation, even decades after infection, remains. Although it rarely happens, reinfection can also occur (Kaufmann, 2001). If the balance between the host’s defense and the persisting mycobacteria is tipped in favor of the pathogen, active disease occurs (Kaufmann, 2000; Stewart et al., 2003).

![TB pathology overview](image)

Fig. 2: TB pathology overview
4.1.1. Receptors involved in phagocytosis of *M. tuberculosis* into macrophage

Pathogenic mycobacteria have evolved numerous mechanisms that allow them to rapidly invade monocytes and macrophages. Recent research has unveiled cellular and molecular mechanisms that are part of complex interaction between mycobacteria and host cells. The mode of entry into macrophages has been considered to determine the subsequent intracellular fate of mycobacteria. *M. tuberculosis* uses two distinct mechanisms for getting opsonized with complement (the alternative pathway and capture of C2a), two distinct ligands for binding of different domains of complement receptors (CR3) and a minimum of seven to nine distinct macrophage receptors for recognition (CR1, CR3, CR4, MR, scavenger receptors, CD14, and up to three different Sp-A receptors) (Ernst, 1998) (Fig. 3).

![Fig. 3 Receptors involved in phagocytosis process](image)

4.1.1.a. Complement receptors:

Complement proteins in the serum can opsonize microbes through antibody- dependant and antibody-independent mechanisms. Complement opsonized particles are recognized and internalized via specific complement receptors. Phagocytic complement receptors include the CR1 expressed on erythrocytes, B-cells, monocytes, neutrophils, eosionophils, and dendritic cells; CR3 (αMβ2 integrin, CD11c/CD18, or Mac1) found on monocytes, macrophages, neutrophils, granulocyte, dendritic cells, and NK cells; and CR4
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(αβ₂, integrin, CD11c/CD18, or gp150/95) which has not been as well characterized as CR3 (Ross, 2000). For many years, the complement receptors CR1, CR3 and CR4 were considered the main macrophage receptors for mycobacteria (Hirsch et al., 1994; Schlesinger, 1993; Schlesinger et al., 1990). CR1 is monomeric transmembrane protein that binds C1q, C3b, C4b as well as mannan binding lectin, but not C3bi (Ghiran et al., 2000; Klickstein et al., 1997). CR1 possesses complement regulatory activity and can mediate phagocytosis of bound particles. CR3 and CR4 are heterodimeric proteins of integrin super-family and bind C3bi. CR3 also contains a glycan binding site (Thornton et al., 1996). Like many other bacteria, M. tuberculosis can trigger alternative pathway of complement activation resulting in opsonization with C3b and C3bi (Schlesinger et al., 1990). Pathogenic mycobacteria can uniquely recruit the complement fragment 2a to form C3 convertase and generate opsonically active C3b in the absence of the early activation components of the alternative or classical pathways (Schorey et al., 1997). It is speculated that the predominant opsonin generated by scavenging C2a is C3b, rather than C3bi, as mycobacteria that are opsonized by this mechanism bind predominantly to CR1 rather than CR3 or CR4. M. tuberculosis can bind to CR3 at two distinct sites on the receptor. Opsonized M. tuberculosis binds to CR3 at its C3bi binding domain, and the nonopsonized M. tuberculosis uses its endogenous capsular polysaccharides to interact with β-glucan binding site near C terminus of CD11b (Cywes et al., 1996; Cywes et al., 1997).

Experiments using human monocytes and murine macrophages have strongly implied that there is more than one mode of interaction between M. tuberculosis and CR3 (Schlesinger et al., 1990; Stokes et al., 1993), but the unambiguous evidence that non-opsonic (C3bi independent) interactions also occur was obtained in studies in which CR3 was expressed on non-macrophage background, so that synthesis of C3 by macrophage could not interfere. Chinese Hamster Overy (CHO) cells stably transfected with CD18 and CD11b bind a strain of M. tuberculosis H37Rv (“CC”) in serum independent manner, and binding of this strain is not enhanced by fresh human or bovine serum (Cywes et al., 1996). A monoclonal antibody that blocks the C3bi binding site in the I domain of CD11b does not block binding of H37Rv-CC to the transfected CHO cells, whereas an
antibody to an alternative site within I domain and an antibody to C terminal domain do block CR3 expressed on CHO cells. Further analysis also revealed that strains and sub-strains of *M. tuberculosis* vary in their predominant mode of interaction with CR3. Nonopsonic binding of *M. tuberculosis* to CR3 is inhibited by laminarin (a seaweed-derived b-glucan), N-acetyl-D-glucosamine, or by purified *M. tuberculosis* capsular glucan or mannan; but not by capsular arabinomannan or yeast mannan. Moreover, mild mechanical extraction of capsular polysaccharides or treatment with amyloglucosidase markedly reduces nonopsonic binding, implying that the bacterial ligands for this domain of CR3 are peripherally located capsular carbohydrate residues. These studies clearly show that individual strains of *M. tuberculosis* can vary in their modes of interaction with CR3 and that they interact with distinct domains of the receptor. These results are consistent with the results of studies of the polysaccharide specificity of the b-glucan binding site(s) of CR3 (Thornton et al., 1996).

Internalization signaled by CR3 requires second activation step that increase the number of receptors at cell surface (Berger et al., 1984; Sengelov et al., 1993), and the affinity of receptor (Jones et al., 1998). Inflammatory cytokines (TNF-α), microbial products (LPS) and adhesion (fibronectin) stimulate phagocytosis through CR3, demonstrating that heterogeneous cellular processes influence phagocytosis. Thus *M. tuberculosis* can exploit complement receptors through multiple mechanisms to bind and enter the macrophage. The mechanism and consequence that predominate *in vivo* may be determined by features of individual bacterial strain (Complement dependant or independent), the environment of macrophage (availability of complement proteins) and state of differentiation and activation of macrophage.

4.1.1.b. Mannose receptor::

The macrophage mannose receptor is monomeric transmembrane protein, with an extracellular domain containing eight carbohydrate-recognition domains characteristic of C-type (calcium dependant) lectins (Taylor and Drickamer, 1993). Human monocyte derived macrophage bind and internalize virulent *M. tuberculosis* primarily via mannose
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receptor (Schlesinger, 1993). The quantitatively important role for mannose receptor in binding and phagocytosis was demonstrated by competitive inhibition, downregulation of mannose receptors by adherence of macrophage of mannan coated coverslips and blocking with anti-mannose receptor antibody (Schlesinger, 1993). Mannose receptors bind the virulent Erdmann and H37Rv but not avirulent H37Ra strain. A prominent mannose containing mannose-capped lipoarabinomannan (ManLAM) (Brennan and Nikaido, 1995) has been directly implicated in the regulation of these processes (Strohmeier and Fenton, 1999). The presence of ManLAM on the mycobacterial (Hunter and Brennan, 1990) surface places this molecule in an ideal position to mediate the initial interactions between the M. tuberculosis and macrophages. ManLAM has been shown to be a critical regulator of phagosome maturation in murine macrophages and a human monocytic cell line (Chua and Deretic, 2004; Hmama et al., 2004). ManLAM blocks the increase of macrophage cytosolic Ca2+ and thereby inhibits interaction of the phosphatidylinositol 3 kinase (PI3K), hVPS34 with calmodulin, a step necessary for the production of PI3K-phosphate involved in the recruitment of the Rab5 effectors with early endosomal antigen 1. These steps are necessary for the delivery of lysosomal components from the trans-golgi network to phagosome and regulate fusion with vesicles of the endosomal-lysosomal pathway (Fratti et al., 2003b). Mannose receptors are expressed on mature macrophages but not on fresh monocytes. The expression of mannose receptors is down-regulated by gamma interferon (Schreiber et al., 1993), therefore the role of these receptors is important in individuals with compromised cellular immunity.

4.1.1.c. Surfactant protein A receptors:

Surfactant protein A (Sp-A) is a member of collectin family protein which includes serum mannose binding protein (MBP) and complement component C1q. Sp-A, like other collectins, possesses a collagenase like domain and has a domain resembling C-type of lectins such as that in MBP. Sp-A enhances macrophage binding and uptake of M. tuberculosis although the mechanisms of these phenomena are not clear (Downing et al., 1995). Sp-A can operate as an opsonin, binding to M. tuberculosis by N-linked
polysaccharides of Sp-A and to macrophages by collagen-like domain. In addition to opsonin, there is also evidence that Sp-A enhances ingestion of particles including *M. tuberculosis* by other mechanisms. Attachment of macrophages to Sp-A coated surfaces enhances phagocytosis by Fc- and Complement-receptors (Turner et al., 2002) and probably by mannose receptors (Gaynor et al., 1995). It is more likely that this effect of Sp-A is exerted at a step in phagocytosis that is common to and downstream of various receptors.

4.1.1.d. CD14 receptor:

CD14, a phosphatidylinositol glycan linked membrane protein, is best known and characterized as high affinity receptor for lipopolysaccharides of gram negative bacteria. However, CD14 can also bind LAM of *M. tuberculosis* (H37Ra), and this binding induces macrophage to secret interleukin-8 (Pugin et al., 1994). Microglial cells are derived from monocyte precursors and exhibit many macrophage-like functions, including phagocytosis. These cells have been shown to utilize CD14 to recognize whole *M. tuberculosis* (H37Rv) (Peterson et al., 1995). These findings were contradicted by other authors concluding that CD14 does not mediate cellular entry by *M. tuberculosis*. *M. tuberculosis* infection up regulates CD14 expression on mononuclear phagocytes and this may facilitate the pathogen's capacity to modulate the immune response (Shams et al., 2003)

4.1.1.e. Scavenger receptor:

Macrophage scavenger receptors bind polyanionic macromolecules and particles, including lipo-polysaccharides of gram-negative bacteria and lipo-teichoic acid of gram-positive bacteria (Dunne et al., 1994). Scavenger receptors additionally bind to lipo-proteins, polyribonucleotides and silica particles. Experiments using competitive inhibitors have implicated class A scavenger receptors as quantitatively important receptors for *M. tuberculosis* (Erdman) on human monocyte-derived macrophages (Zimmerli et al., 1996).
It is not yet known whether scavenger receptors can activate the cytoskeleton to internalize bacteria or, alternatively, whether scavenger receptors act to bind bacteria but phagocytosis is executed by other receptors.

4.1.1.f. Fc-γ receptor:

Particles opsonized by IgG-antibody are recognized by several surface receptors that bind the Fc region of IgG (FcγRs; Daeron, 1997; Ravetch and Bolland, 2001). Phagocytes such as macrophages or neutrophils express different combinations of FcγRs; thus recognition of IgG-opsonized particles occurs simultaneously through several receptors. FcγRs fall into two classes: (a) receptors that contain immune tyrosine activation motif (ITAM) motifs in their intracellular domains that recruit kinases and activate phosphorylation cascades, and (b) receptors that contain immune tyrosine inhibitory motif (ITIM) motifs that recruit phosphatases that inhibit signaling (Daeron, 1997; Ravetch and Bolland, 2001). Receptors with high (FcγRI) and low affinities (FcγRIIA and FcγR IIIA) bind IgG opsonized particles and trigger internalization through actin polymerization beneath the particle and membrane recruitment to the site of particle contact, membrane extension outword to surround particle and particle engulfment. The efficiency of the process is regulated by inhibitory FcγR (FcγRIIB) that recruits the SH2-containing inositol-5-phosphatase (SHIP) that blocks phoshoinisitide signaling (Ravetch and Bolland, 2001). Thus, the relative expression of activating and inhibiting FcγRs determines the threshold for phagocytosis and inflammatory responses to IgG-opsonized particles.

Even the latently infected individuals have circulating antibodies to M. tuberculosis (Laal et al., 1997), and one study has shown that the intracellular trafficking of M. tuberculosis (H37Rv) opsonized with immune serum is different from that of non-opsonized bacteria. IgG-coated mycobacteria were ingested by human macrophages in vesicles that readily fused with ferritin-loaded lysosomes, whereas un-opsonized mycobacteria resided in phagosomes that did not acquire ferritin from labeled lysosomes (Armstrong and Hart, 1975).
4.1.1.g. Role of different receptors in intracellular survival/killing of pathogen:

Certain intracellular pathogens exploit specific macrophage receptors to ensure their own survival. *Leishmania major* activates the alternative complement pathway to deposit C3b on its surface (Mosser and Edelson, 1987). When opsonized metacyclic promastigotes bind to CR1, they survive and replicate intracellularly. When promastigotes (non-infective forms) enter macrophages through the lectin-like domain of CR3, they are killed (Da Silva et al., 1989). *Salmonella typhi* that enters murine macrophages through CR3 is phagocytosed in a vesicle that fuses with lysosomes, while entry via CR1 allows *S. typhi* to survive in a phagosome that does not acquire lysosomal markers (Ishibashi and Arai, 1990). These observations suggest that one way that successful pathogens can survive within phagocytes is by entering by a receptor-mediated pathway that is not coupled to the activation of macrophage antimicrobial mechanisms such as production of reactive oxygen or nitrogen intermediates. So far, there has been only limited examination of whether *M. tuberculosis* uses such a mechanism to favor its survival in macrophages. By using monoclonal antibodies or competitive ligands to block CR1, CR3/4, mannose receptors, and class A scavenger receptors during initial entry of *M. tuberculosis* into human macrophages, no apparent difference in the extent of survival or rate of intracellular growth of one virulent strain (Erdman) was observed (Zimmerli et al., 1996).

4.1.2. Events following entry of bacilli:

4.1.2.a. Phago-lysoosome fusion and phagosome maturation:

Immediately after phagosome formation, the membrane of phagosome resembles the plasma membrane and its fluid contents are a sample of extracellular medium. As such phagosome does not exert bactericidal activity. However shortly after sealing, the vacuole undergoes a complete overhaul resulting in massive change in its composition due to progressive maturation process that ultimately yields a hybrid organelle- the phagolysosome (Pitt et al., 1992). After internalization of invading microorganism,
macrophage gets activated and results in a series of events specially designed to induce killing of engulfed micro-organism. These include: Gradual acidification of the phagosome due to the activity of a proton-ATPase pump located in the phagosomal membrane; phagosome-lysosome fusion which leads the enriching the vesicle with proteolytic enzymes, defensins and other bactericidal peptides; induction of reactive oxygen and nitrogen intermediates; and degradation of invading organism and antigen processing.

The maturation process that endows the phagosomes with lytic activity depends critically on the interactions of nascent vacuole with endocytic pathway. The endocytic pathway is organized as a continuum of organelles ranging from early endosomes to lysosomes. Following formation, endocytic vesicles are targeted to sorting/early endosome. Early endosomes are molecularly equipped to differentiate, organize, and re-route the assortment of internalized molecules. These organelles are often tuberovesicular and can be typically recognized by presence of Rab5 or Early Endosome Antigen (EEA).

The lumen of sorting endosomes is relatively poor in proteases and is midly acidic with pH of ~6. During sorting, enzyme cargo can be directed towards recycling endosomes, which are morphologically and biochemically distinct from sorting endosomes. Late endosomes are more acidic than sorting endosomes, reaching pH of 5.5, and are comparatively enriched in hydrolytic enzymes. They can be identified by their multivesicular nature (i.e. they contain small intraluminal vesicles) and by the presence of Rab7, Rab9, lysobisphosphatidic acid, mannose 6-phosphate receptors and lysosome associated membrane proteins (LAMPs) (Mukherjee et al., 1997; Somsel Rodman and Wandinger-Ness, 2000). Sorting to late endosomes traffic occurs by one of two known mechanisms: vesicle shuttle model or the maturation model. According to the vesicle shuttle model, sorting endosomes are stable organelles from which transport intermediates, or multivesicular bodies (MVBs) are derived and subsequently targeted to late endosomes. The maturation model proposes instead that sorting endosomes are transient organelles that mature into MVBs via a series of poorly characterized fusion and
fission events, ultimately generating late endosomes (Gruenberg, 2001; Gruenberg and Maxfield, 1995; Gu and Gruenberg, 1999; Thilo et al., 1995). (Fig 4).

Fig. 4: Changing composition during phagosome maturation

Regardless of the precise mechanism leading to the creation of late endosomes, it is agreed that lysosomes are the final step in the endocytic sequence. These organelles contain the bulk of active proteases and lipases, and are extremely acidic (pH ~5.5). Lysosomes characteristically contain LAMPs and hydrolytic enzymes such as cathepsin D; but, while these proteins were thought to be unique to lysosomes, it is now apparent that they are also found in late endosomes. The resulting acidic and otherwise lethal environment is effectively designed to eliminate the invader and present the immune system with its antigenic determinants on the surface of the macrophage.

More than 30 years ago, pioneering work by Armstrong and Hart established that *M. tuberculosis* resides in a macrophage vesicular compartment distinct from phago-lysosome that is the natural destination of any ingested particle or microbe (Armstrong, 1971; Armstrong 1975). Phago-lysosome fusion is central to tuberculosis infection,
latency, activation, spread and suppression of immunological detection by the host (Flynn and Chan, 2003; Hanekom et al., 2003; Hmama et al., 1998; Russell et al., 2002; Stenger et al., 1998). Phagosomes containing an inert particle interact with the endosomal pathway through transient contacts, described as 'kiss and run' mechanism where fusion and fission events facilitate the delivery of contents and membrane components between endosomes and phagosomes (Duclos et al., 2003). The phagosome thereby acquires a membrane composition, which is more or less mirrored by the endosomal compartment with which it interacts at a given time.

Purification of endocytic organelles and analysis of coat proteins has highlighted differences in the membrane composition of mycobacterial phagosomes compared to that of early and late endosomes (Table 1) and helped to better understand the interaction between these compartments.

Phagosomes containing viable, virulent mycobacteria show presence of early endosomal markers such as transferrin receptor, major histocompatibility complex (MHC) class-II molecules and ganglioside GM1 (Clemens and Horwitz, 1995; Russell et al., 1996). However, in contrast to phagosomes containing killed mycobacteria or nonpathogenic strain M. smegmatis, those

Table I: Selected list of membrane marker present in different endocytic compartments

<table>
<thead>
<tr>
<th>Protein</th>
<th>Early endosomes</th>
<th>Late endosomes</th>
<th>Mycobacterial phagosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase proton pump</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>EEA1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ganglioside GM1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LAMP</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M6PR</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Procathepsin D</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rab5</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rab7</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Syntaxin 6</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TACO</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
containing virulent live organisms exclude late endosomal markers such as proton ATPase, mannose 6-P receptor and the lysosomal protease cathepsin D. These observations lead to understanding that mycobacterial phagosomes interact with early endosomal compartment while resisting fusion with later stages. The absence of proton ATPase is thought to account for the reduced acidicification of mycobacterial phagosome (Sturgill-Koszycki et al., 1994), which equilibrate to a pH 6.2-6.3 compared to pH of 5.3-5.4 normally associated with endosomal compartments.

Analysis of BCG phagosome has shown that coronin-I (named as TACO) is actively retained on phagosomes containing live but not killed bacteria (Ferrari et al., 1999). Since Coronin-I is normally released prior to phagosome maturation into phagolysosome (Grogan et al., 1997), its retention on phagosome was suggested as a marker of phagosome arrest. Contradicting to this finding, it was shown that the retention of TACO is associated with only early stages of mycobacterial phagosome and not in phagosome maintenance (Schuller et al., 2001). Coronin was not sufficient to protect mycobacteria from the bacteriostatic or cidal mechanisms (Tailleux et al., 2003). Studies with coronin mutants (Solomon et al., 2003) showed no effects on intracellular replication of mycobacteria. In addition, a number of reports suggest that mycobacteria inhibit actin polymerization (Anes et al., 2003; Guerin and de Chastellier, 2000) which appears incompatible with the proposed accumulation of TACO because coronin is actin binding protein.

Mechanistic aspects of phagosomal maturation studies started with analysis of Rab5 - small GTP binding proteins. An early work has indicated that block occurs between the maturation stages controlled by Rab5 (early endosomal) and Rab 7 (late endosomal) (Via et al., 1998), which directs trafficking and maintains the identity of endocytic organelles. Rab5 accumulates on mycobacterial phagosome and Rab 7 is not detected at the usual times of its recruitment. Recently a role for Rab5 in facilitating acquisition of iron by intracellular mycobacteria has been reported (Kelley and Schorey, 2003). Follow-up studies to further refine and narrowing down the exact target causing the block were
focused on Rab5-interacting components (Fratti et al., 2001). It was observed that majority of Rab5-effectors were recruited both to the latex bead and mycobacterial phagosomes with the notable exception of EEA-1 (early endosomal antigen-1). EEA-1, a Rab5 effector, serves as an organelle tethering molecule by bridging membranes destined for fusion (Christoforidis et al., 1999a; Simonsen et al., 1998). EEA-1 interacts not only with Rab5 but its recruitment and association with endosomal membrane is strengthened by the binding of its FYVE domain to PI3. PI3P is generated on the endosomal membrane by another Rab5 effector hVPS34 in the mycobacterial phagosome maturation block (Christoforidis et al., 1999b).

In addition to hVPS34 interaction with Rab-5, its recruitment to endosomal membranes is controlled in macrophages by Ca++, Calmodulin (CaM) and Ca++/Calmodulin kinase II (Vergne et al., 2003). Calcium is an important regulator of signaling pathways involved in phagosome-lysosome fusion. Macrophages infected with M. tuberculosis demonstrated a decrease in the elevation of calcium normally associated with phagocytic uptake (Malik et al., 2000). It has been reported that M. tuberculosis lipoarabinomannan inhibits cytosolic Ca++ rise during phagocytosis (Rojas et al., 2000; Vergne et al., 2003).

The reduced or altered recruitment of EEA1 to mycobacterial phagosomes has implicated PI3P, PI3K hVPS34 and a number of PI3K binding proteins in the mycobacterial phagosome maturation block (Chua and Deretic, 2004; Fratti et al., 2001; Fratti et al., 2003a; Fratti et al., 2003b; Vieira et al., 2004). PI3P affects localization and functions of proteins containing the PI3P binding domains (FYVE, PH and PX) (Lemmon, 2003). These proteins in turn execute various steps in membrane trafficking, endosomal protein sorting and multi-subunit enzyme assembly at the membrane, including phagosomal maturation (Fratti et al., 2001; Vieira et al., 2001), early endosomal homotypic fusion (Simonsen et al., 1998), delivery of internalized plasma membrane receptors to late endosomes (Siddhanta et al., 1998), formation of internal vesicles within late endosomal multivesicular bodies involved in termination of signaling events (Gruenberg and Stenmark, 2004; Katzmann et al., 2002) and phagocyte NADPH oxidase assembly at the membrane (Kanai et al., 2001). PI3P is also important for execution of process of
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autophagy, which has recently been shown to restrict *M. tuberculosis* growth as a defense mechanism downstream to macrophage activation by IFN-γ (Gutierrez et al., 2004).

### 4.1.3. Intracellular killing of bacilli

Following phagocytosis, phagosome typically fuses with lysosome, where ingested mycobacterium is destroyed by a combination of mechanisms including vesicle acidification, enzymatic digestion and oxidative burst (Babior, 1999), in which the NADPH oxidase generates reactive oxygen and nitrogen intermediates (ROI, RNI). These may arise through the by-products of aerobic metabolism; hydrogen peroxide, hydroxyl radicals and superoxide or from environmental agents such as UV irradiation or redox-cycling agents (Fig. 5). ROIs and RNIs exert their cidal effects in a nonspecific manner and as such are also dangerous to the host.

#### 4.1.3.a Reactive oxygen intermediates (ROIs):

ROIs and their downstream products are highly toxic molecules that control spectrum of infectious disease due to their damaging effects on proteins, RNA and DNA (Miller and Britigan, 1997). ROIs are bactericidal or bacteriostatic *in vitro*, but their concentration in the human macrophage *in vivo* is still uncertain. The genes *katG*, which encodes a catalase-peroxidase protein, and *aphC*, an alkyl hydroperoxide reductase protein, are believed to be involved in protecting mycobacteria against millimolar concentrations of hydrogen peroxide.

Hydrogen peroxide is one of ROIs generated by macrophage via oxidative burst. It was the first identified effector molecule that mediated mycobactericidal effects of activated mononuclear phagocytes (Walker and Lowrie, 1981). Alveolar macrophages in tuberculosis patients possess higher capacity of oxidant metabolism. The magnitude of 2',7' dichlorofluorescein oxidation was found to be higher in TB patients than in normal subjects (Kuo et al., 1996). However, increased H₂O₂ production is not specific to
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tuberculosis and alveolar macrophages produces less H$_2$O$_2$ compared to blood monocytes (Selvaraj et al., 1988).

4.1.3.b. Reactive Nitrogen Intermediates (RNIs)

Cytokines produced by macrophages such as TNF-α and IL-β, along with IFN-γ produced by T-lymphocytes, can induce nitric oxide productions in the macrophages. It is produced via the action of inducible form of the enzyme nitric oxide synthetase. RNIs include NO and species resulting from its rapid oxidation, reduction, or adduction in physiologic milieu, e.g., NO$_2$, NO$_2^-$, N$_2$O$_3$, N$_2$O$_4$, S-nitrosothiols, and peroxynitrite (OONO$_2^-$). In mammals, there is a rough correspondence between toxic and homeostatic functions of NO and its production in large and small quantities, respectively. There is evidence that the production of NO and related RNIs is well correlated with anti-mycobacterial effect of murine macrophages. There are, however, few reports on the anti-mycobacterial effect of the human macrophage and its correlation with production of NO. Human macrophages have been reported to produce RNI sufficient for killing of $M$. avium in vitro. The results of efforts to demonstrate NO production in human macrophages have been inconsistent. ROI and their downstream products are highly toxic molecules that control spectrum of infectious disease due to their damaging effects on proteins, RNA and DNA (Miller and Britigan, 1997). ROIs are bactericidal or bacteriostatic in vitro, but their concentration in the human macrophage in vivo is still uncertain. The genes katG, which encodes a catalase-peroxidase protein, and aphC, an alkyl hydroperoxide reductase protein, are believed to be involved in protecting mycobacteria against millimolar concentrations of hydrogen peroxide.

There is however evidence suggesting that human macrophages have potential to produce NO. Individuals experiencing sepsis (Ochoa et al., 1991) or undergoing cytokine chemotherapy for tumor (Hibbs et al., 1992), generate large amounts of NO. When cultured peripheral blood monocytes were stimulated with low numbers of tubercule bacilli (5 x 10$^5$ bacilli/ml) nitrite concentration in supernatant was minimal. However, when they were stimulated with higher numbers of bacilli (5 x 10$^7$ bacilli/ml), nitrite concentration in supernatant increased significantly (Kim et al., 1997).
NO concentration in supernatants of cultured monocytes stimulated with *M. tuberculosis* H37Ra (avirulent strain) was found to be significantly higher than those stimulated with *M. tuberculosis* H37Rv (virulent strain). This result supports the theory that virulence of *M. tuberculosis* may be determined by whether or not appropriate immune response including NO production is present in host. However, there was no difference in NO production in cultured monocytes from healthy volunteers and patients with tuberculosis (Kim et al., 1997).

Another group has reported that the magnitude of iNOS expression was significantly increased in both alveolar as well as peripheral blood monocytes from tuberculosis patients compared to the normal subjects. They also found that both alveolar as well as peripheral blood monocytes from active tuberculosis patients produce more nitrite than those from normal subjects (Wang et al., 1997).

4.1.3.c. Lytic enzymes produced by macrophages

Lysosomes within macrophages play very important role in degradation and lysis of the invading microbe. They are morphologically heterogeneous, often resembling other organelles of the endocytic and secretory pathways. Therefore, they are currently distinguished from other organelles on the basis of an operational definition, which describes them as membrane-bound acidic organelles that contain mature acid-dependent
hydrolases and LAMPs but lack mannose 6-phosphate receptors (MPRs). They serve as major degradative compartment in eukaryotic cells. Both endogenous and exogenous molecules can be delivered to lysosomes through biosynthetic and endocytic pathway respectively. The degradative function of these organelles is carried out by more than 50 acid dependant hydrolases (e.g. proteases, lipases and glycosidases). These hydrolases are active only at low pH, hence in case of rupture or leakage these enzymes are not active. Proteases of cathepsin family are among the best studied lysosomal hydrolases. Cahtepsin can be subdivided into three subgroups according to their active site amino acids i.e., cysteine (B, C, H, F, K, L, O, S, V, W, and X/Z), aspartate (D and E), and serine (G) cathepsins (Zavasnik-Bergant and Turk, 2006). Cathepsins are synthesized as proenzymes that are processed to their active and mature forms by other hydrolases in acidic environments. Cathepsin D is synthesized as 51-55 kDa proenzyme that is cleaved to yield 49 kDa immature form in early endosomes and subsequently processed to a two chain 31/17kDa forms of enzyme in dense lysosome. Mycobacterial phagosome show limited acidification and hydrolytic activity despite possession of known lysosomal constituents such as Cathepsin-D, B and L, and LAMP1.

4.1.4. Cytokine response and tuberculosis:

Early cytokine response by innate immune system has a decisive influence on host response against the infectious agents (Fearon and Locksley, 1996). Following infection with M. tuberculosi, host cytokine response influences disease manifestation. Differences in cytokine expression are likely to determine whether the infection progresses, resolves or becomes latent. The early cytokines fulfill two major functions; first they mobilize innate immune system to achieve rapid control of infectious agent and, second, they perform instructive role in the acquired immune response by promoting appropriate expressions of T-cell functions. In adaptive immunity, CD4+ T cells are divided into two principal types based on pattern of cytokine expression by the cells. Th1 cells secrete interleukin-2 (IL-2), lymphotoxin α and interferon-γ; TH2 cells secret IL-4, IL-5, IL6, and IL-10. Two clinical manifestations of TB may mirror a predominance of either Th1 or Th2 phenotypes: Tuberculous pleural effusion, generally seen in primary
TB, is typically associated with Th1 cellular responses. In contrast, milliary TB, a sign of uncontrolled disseminated infection, is more commonly linked to Th2 phenotype (Sharma et al., 2002). Th1 cytokines as well as TGF-β is increased while Th2 cytokines are decreased in well formed pulmonary granulomas of TB patients compared to control (Bai et al., 2004). An effective host response against TB involves the differentiation of specific T cells to secrete an appropriate Th1 cytokine profile and the development of granuloma in which activated epithelioid macrophages restrict mycobacterial growth.

4.1.4.a: Tumor necrosis factor-α

TNF is produced primarily by macrophages in response to stimuli activating ‘toll like receptors’, but can also be expressed by activated T-cells, B-cells, and NK cells (Old, 1988). TNF is multipotent cytokine which plays a part in apoptosis, cell activation, cell recruitment and cell differentiation (Old, 1988; Sedgwick et al., 2000). The pleiotropic effects of TNF have been elucidated in a number of studies (Papadakis and Targan, 2000; Wajant et al., 2003). (Fig. 6)

**Fig. 6:** TNF is involved at multiple steps in antibacterial and inflammatory responses to *M tuberculosis* infection. It is a macrophage activating cytokine and is necessary for the sustained recruitment of inflammatory cells into granulomatous lesions.
TNF increases the phagocytic ability of macrophages and enhances the killing of mycobacteria, particularly in concert with interferon γ (Bekker et al., 2001). It may also deprive mycobacteria of their intracellular sanctuary by inducing apoptosis of permissive macrophages (Fratazzi et al., 1999; Keane et al., 2000). Virulent *M. tuberculosis* prevents apoptosis via suppression of TNF (Balcewicz-Sablinska et al., 1998; Keane et al., 2000). Neutralization of TNF in cultures of avirulent mycobacterium restores the ability to induce apoptosis in host cells (Spira et al., 2003). To date there is no clear consensus on whether apoptosis of infected macrophages is beneficial or harmful for the infected host. The induction of apoptosis could contribute to protection by directly killing the mycobacteria (Fratazzi et al., 1997; Molloy et al., 1994; Oddo et al., 1998), or by strengthening adaptive immunity by inducing the cross-presentation of mycobacterial antigens by dendritic cells (DCs) (Schaible et al., 2003). On the other hand, TNF, by virtue of stimulating chemokine production (such as CCL-2, -3, -4, -5, -8) as well as the expression of endothelial cell adhesion molecules (such as CD54), is crucial in inflammatory cell recruitment, leading to the focused accumulation of mononuclear cells (Roach et al., 2002). Thus, in TNF or TNFRp55 deficient mice, granuloma formation is significantly delayed (Bean et al., 1999; Ehlers et al., 1999). Even incipient granulomas cannot be maintained in the absence of TNF signaling and they rapidly disintegrate causing the death of infected mice (Benini et al., 1999). In this situation TNF presumably regulates the inflammatory response by maintaining the viability of activated macrophages at the site of infection. In addition, TNFRp55 signaling is required for modulation of the T cell response because in its absence hyper-inflammatory T cell mediated tissue destruction becomes evident (Ehlers et al., 2000). Since both antibacterial mechanisms and demarcation of the infectious focus are seriously impaired in the absence of TNF signaling, lesions present as disorganized, diffused, necrotizing infiltrations of mixed cellularity in TNF and TNFR deficient mice (Flynn et al., 1995a). Taken together, these reports suggest that TNF supports apoptosis of macrophages infected with *M. tuberculosis*, thereby contributing to the clearance of the pathogen.

Granulomatous inflammation is a highly dynamic process, and continuous recruitment of inflammatory cells into the lesion is necessary to maintain antibacterial vigilance.
Therefore, even during the chronic phase of infection, when compact granulomas have already been established to wall-off the infectious focus, wild-type mice given antibodies effectively neutralizing TNF can no longer contain mycobacterial growth within the lesions, and granuloma breakdown is followed by dissemination of mycobacteria (Kindler et al., 1989; Mohan et al., 2001). Thus, TNF is needed for maintenance of the state of dormancy in mice and humans (Carmona et al., 2003; Gomez-Reino et al., 2003; Keane et al., 2001; Mohan et al., 2001; Wallis et al., 2004a; Wallis et al., 2004b).

4.1.4.b. Interferon-γ:

The protective role of IFN-γ in tuberculosis is well established (Flynn et al., 1993), primarily in context of antigen specific T cell immunity (Andersen, 1997). IFN-γ is key cytokine in the protective immune response against *M. tuberculosis* in mice (Cooper et al., 1993; Fenton et al., 1997; Flynn et al., 1993). In humans, individuals with various defects in IFN-γ signaling are more predisposed to mycobacterium infection, including TB (Fieschi et al., 2003). Exogenous IFN-γ also appears promising as an adjunctive treatment for *M. avium* complex induced pulmonary disease, leprosy and multi-drug resistant tuberculosis (Condos et al., 1997). IFN-γ may contribute to granuloma formation by inducing chemokines such as IP-10, MIG (monokine induced by IFN-γ) and ITAC (IFNγ inducible T-cell alpha chemoattractant) (Bergeron et al., 1997; Fenhalls et al., 2002). Contrary to these findings, Aung and colleagues were unable to detect INF-γ and TNF-α in active pulmonary cases (Aung et al., 2000).

4.1.4.c. Interleukin-1 β:

A second proinflammatory cytokine involved in the host response to *M. tuberculosis* is IL-1β. Like TNF-α, IL-1β is produced by monocytes, macrophages and dendritic cells (Dahl et al., 1996; Roach et al., 1993). In tuberculosis patients IL-1β is expressed in excess (Schauf et al., 1993) and at the site of infection (Bergeron et al., 1997; Law et al., 1996). Studies with mice suggest an important role of IL-1β in tuberculosis. IL-1α and IL-1β double knockout mice (Yoshimura et al., 1999) and IL-1R deficient mice display an
increased outgrowth and also defective granuloma formation after infection with *M. tuberculosis* (Juffermans et al., 2000).

### 4.1.4.d. Interleukin-4

IL-4 is required for differentiation of monocytes into dendritic cells, an important antigen presenting cell in TB. On the other hand, IL-4 blocks production of several cytokines and chemokines including IL-1, IL-6, IL-8, IL-10, IL-12, IP-10 and TNF-α. The pleiotropic functions of IL-4 are likely to account for different effects seen in experimental models of TB. IL-4 has been shown to have protective, detrimental or neutral roles (Jung et al., 2002; Sugawara et al., 2000). Although serum levels for IL-4 were not different for patients with active TB and normal controls (Verbon et al., 1999), a higher frequency of circulating CD4 and CD8 cells expressing IL-4 was found in individuals with cavitary TB, a form associated with a high burden of bacilli (van Crevel et al., 2000).

### 4.1.4.e. Interleukin -6

IL-6, which has both pro- and anti-inflammatory properties, is produced early during mycobacterial infection (VanHeyningen et al., 1997) and at the site of infection (Hoheisel et al., 1998; Ladel et al., 1997; Okamura et al., 1998). IL-6 may be harmful in mycobacterial infection as it inhibits production of TNF-α and IL-1β (Schindler et al., 1990) and promotes *in vitro* growth of *M. avium* (Shiratsuchi et al., 1991). Other reports support protective role for IL-6; IL-6 deficient mice display increased susceptibility to *M. tuberculosis* infection (Ladel et al., 1997), which seems to be related to a deficient production IFN-γ early in the infection, before adaptive T-cell immunity develops (Saunders et al., 2000).

### 4.1.4.f. Interleukin-10

IL-10 is produced by activated macrophages and T-cells in response to mycobacteria and mycobacterial products such as lipoarabinomannans (Roach et al., 1995) and inhibits
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IFN-γ production. Exogenous IL-10 antagonized the reduction of mycobacterial growth by IFN-γ and TNF-α (Flesch et al., 1994). This ability of IL-10 to inhibit host defense against mycobacteria varies depending on the mycobacterial species. For example, whereas IL-10 suppressed the host response against *M. bovis* and *M. avium*, it did not affect long-term control of *M. tuberculosis* in the IL-10 knockout mice (Roach et al., 2001). Over expression of IL-10 in transgenic mice showed no increased susceptibility to TB during the early stages of infection, though there was an increased risk of TB reactivation (Turner et al., 2002). The decreased IL-10 expression in the tuberculous lungs of the patients is in contrast to the elevated IL-10 levels seen in *M. tuberculosis*-infected mouse lungs (Jung et al., 2003), and in the sera of TB-infected subjects (Vankayalapati et al., 2003).

4.1.4.g. Interleukin-12

IL-12 is a key player in host defense against *M. tuberculosis*. It is a heterodimeric cytokine secreted by macrophages, dendritic cells, and neutrophils in response to infection with *M. tuberculosis*, lipoarabinomannan of the tubercle bacilli, and IL-12 itself (Fieschi et al., 2003; Fulton et al., 1996; Ladel et al., 1997). Genetic defect in the production or signaling of IL-12 predisposes to disseminated atypical mycobacterial infection or extrapulmonary TB. IL-12 may have functional role in granuloma formation by virtue of its reciprocal stimulatory relationship with IFN-γ (O'Neill and Greene, 1998). This is also supported by observation that IL-12 in increased in granulomatous lesion of TB (Bergeron et al., 1997; Flynn et al., 1995b). It is also detected in lung infiltrates (Taha et al., 1997), in pleurisy (Zhang et al., 1999), 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 mouse are highly susceptible to mycobacterial infection (Cooper et al., 1997; 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., 1998a; Altare et al., 1998b; de Jong et al., 1998; Frucht and Holland, 1996). These patients display
reduced capacity to produce IFN-γ (Ottenhoff et al., 1998). Apparently IL-12 is a regulatory cytokine which connects the innate and adaptive immune host response to mycobacteria (O'Neill and Greene, 1998; Sieling et al., 1994; Trinchieri, 1995) and exerts positive effects mainly through the induction of IFN-γ (Cooper et al., 1997).

4.1.5. Humoral immune response

A considerable effort has been made during last few years to understand the infectious process of tuberculosis at the cellular level and a large amount of data has been accumulated, whose significance in infection process in vivo is not immediately evident. In the mean time, scant attention has been given to the humoral antibody response of patients and BCG-vaccinated healthy people. The most important investigations in this aspect were made with the proteinaceous antigen complex-60 (A60). A60 is an immunodominant antigen in human tuberculosis and has been recognized 40 years ago (Harboe et al., 1977), as well as isolated (Cocito and Vanlinden, 1986). It was applied as a tuberculin (Benoit et al., 1989) and as a capture antigen for humoral antibodies (Benoit et al., 1989) and its usefulness as vaccine was assessed (Hubbard et al., 1992). An evaluation of results obtained in these studies led to development of new concept (Maes, 1991) that evolved and ripened into the realization that mycobacteria are weakly immunosuppressive, which allows their multiplication in immunologically compromised hosts (Maes et al., 1996). The humoral immune response can be followed with an ELISA test based either on protein antigens dominant during TB disease, or else with non-protein antigens such as cell wall associated glycolipids of M. tuberculosis.