Mycobacterial antigens accumulation in foamy macrophages of tuberculosis lesions plays a central role in tuberculosis pathogenesis and tissue destruction in chronic murine pulmonary tuberculosis.

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ABSTRACT

Mycobacterial antigens accumulation in foamy macrophages of tuberculosis lesions plays a central role in tuberculosis pathogenesis and tissue destruction in chronic murine pulmonary tuberculosis

Introduction: Tuberculosis (TB) is the ninth leading cause of death worldwide ranking even higher than HIV/AIDS. Multi-drug resistant TB remains a threat. Post-primary cavitary TB, responsible for 80% of disease burden and 100% transmission, occurs in individuals despite strong immunity to TB, with poorly understood immune pathogenesis. Understanding factors involved in pathogenesis and tissue destruction in cavity formation can help advance the field towards developing more effective vaccine and therapies towards elimination of TB disease.

Aims: The aim was to study the pulmonary pathology, bacterial growth and expression of various mycobacterial antigens in the pulmonary lesions during the course of slowly progressive murine TB and the factors associated with tissue destruction and disease severity.

Methods: B6D2F1 mice were inoculated with Mycobacterium Tuberculosis H37Rv to develop slowly progressive TB. Immunohistochemistry using in-house anti-rabbit polyclonal antibodies was used to investigate the in-situ expression of various mycobacterial antigens. Aperio ScanScope CS® Slide Scanner (Aperio Technologies Inc., Vista, CA, USA) was used to scan slides. For visualization and digital quantification of scanned slides Image Scope software (Aperio Technologies Inc., Vista, CA, USA) and Colour deconvolution algorithm was used.

Results: Until week 12 post-infection, mice were healthy, lesions were small, bacterial colony forming units (CFUs) increased exponentially, little mycobacterial antigens were seen. At week 16-33, mice showed disease signs. The macrophages attained foamy appearance. Mycobacterial antigens were significantly higher (p<0.05), and there was 1.5 log increase in CFUs and approximately 1-fold increase in AFB. At week 37-41, mice started dying. There was a shift in morphology towards necrosis and tremendous increase in mycobacterial antigens was observed which was in sharp contrast to slight increase of less than one log in CFUs and approximately 7-fold increase in AFB. Total secreted mycobacterial antigens and individual secreted mycobacterial antigens were significantly (p<0.05) higher along the course of infection compared to cell-wall mycobacterial antigens. Development of focal areas of necrosis was associated with approximately 40-fold increase in antigen MPT46, functionally active thioredoxin of Mycobacterium tuberculosis, and a significant increase in secreted antigens as compared to cell-wall antigens.

Conclusion: Mycobacterial antigens accumulate in the foamy macrophages in the TB lesions over the course of infection in the slowly progressive murine pulmonary TB. The accumulation of total secreted mycobacterial antigens, and particularly antigen MPT46 correlated with necrosis, tissue destruction, and mortality rather than an increase in CFUs, AFB or the extent of inflammation. Tissue destruction and necrosis is assumed as the precursor of cavitation, thereby implying the role of MT46 in formation of cavities in TB disease.
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ACRONYMS AND ABBREVIATIONS

TB: Tuberculosis
MTB: Mycobacterium tuberculosis
WHO: World Health Organization
HIV: Human Immunodeficiency Virus
MDR: Multi drug resistant
XDR: Extensive drug resistant
NK: Natural killer cells
CMI: Cell-mediated immunity
APCs: Antigen presenting cells
TCR: T cell receptors
MHC: Major histocompatibility complex
TNF: Tumour necrosis factor
TGF-β: Transforming growth factor beta
IFN: Interferon
TLR: Toll like receptors
DTH: Delayed type hypersensitivity
MPT: Proteins purified from Mycobacterium tuberculosis
CF: Culture filtrate
CFUs: Colony forming units
OD: Optic density
CD: Colour deconvolution
IHC: Immunohistochemistry
ZN: Ziehl Neelsen
INTRODUCTION

Tuberculosis (TB) has been known for centuries. The discovery of *Mycobacterium Tuberculosis* (MTB), the causative agent of TB, by Robert Koch in 1882, is regarded as a significant event in the history of medicine (1). MTB is primarily a pulmonary pathogen but can cause extrapulmonary TB in any organ of the body including lymph nodes, pleura, bones, joints, skin, abdomen or meninges (2). Since the discovery of this airborne pathogen scientists are struggling with increasing their understanding of the pathogenesis and immunology of the disease. Even after a century of struggle and advancement in the medical technology, TB remains the ninth leading cause of death worldwide ranking even higher than HIV/AIDS. According to World Health Organization (WHO) annual global TB report of 2017, 1.7 million died and 10.4 million fell ill with TB. Drug-resistant TB remains a threat with 600,000 new cases with resistance to first-line drugs, of which 490,000 had multi-drug resistant TB (MDR-TB) (3). In the WHO European region, which includes all the European Union countries, TB is a major health issue. In 2015, estimated 323000 new cases and estimated 320000 deaths were reported in the region. MDR-TB is very high in the region accounting for almost a quarter of the burden in the world (4). The treatment for MDR-TB and extensive drug resistant (XDR-TB) is long-term and outcome is poor. Currently, only 54% of MDR-TB patients and 30% of XDR-TB patients are successfully treated (3). Bacillus Calmette-Guérin (BCG) is the only available vaccine against TB and has been in use since 1921. It provides protection to children against disseminated disease and but is not efficient to provide protection against pulmonary TB, which is the most common form of the disease (5, 6). The major driving force in TB pandemic is Human Immunodeficiency Virus (HIV) co-infection. The risk of developing active TB disease is 20 to 30 times more in HIV positive individuals then HIV negative individuals. In 2016, about 40% of deaths in HIV positive patients were due to TB. The treatment and diagnosis of TB in HIV patients are difficult. In the absence of proper treatment, nearly all HIV-positive co-infected with TB and 45% of HIV-negative people with TB on average will die (3). The rising rates of MDR-TB and XDR-TB and HIV and TB co-infection are potentially disastrous threat to the global health. One of the targets of sustainable development goals is to end TB epidemic by 2030 (3). This requires a better understanding of mechanisms involved in the pathogenesis and host immune response that can help towards the development of new vaccine and host-directed therapies for TB treatment.
Natural course of tuberculosis:
From a clinical and public health perspective, the natural course of TB could be divided into primary TB, latent TB and post-primary TB

Primary tuberculosis: Primary TB occurs when a person is infected with MTB for the first time without prior immunity to the infection. 10% of the individuals infected with primary TB will progress to develop active TB disease within 1 or 2 years of infection. It may lead to disseminated disease in individuals with suppressed immunity like individuals with HIV, children or individuals on immunosuppressant drugs. However, 90% of immunocompetent individuals are able to mount a protective immune response against MTB and restrict the infection leading to the formation of a granuloma. Granuloma formation is characteristic of primary TB. It symbolizes an effective immune response mediated by adaptive immunity, which is able to control the infection, but seldom eradicates it completely resulting in dormant infection.

Latent tuberculosis: Latent infection occurs when the person harbour MTB in the body but has no signs or symptoms of the clinical disease. This can occur after a primary infection, or after primary disease, where the host adaptive immune response has been activated to control the pathogen and force it into a dormant state. Dormant MTB can remain alive within the human host for years. Individuals infected with latent TB constitute a large reservoir of MTB that can cause an outbreak of the disease in the future (7). However, individuals with latent TB do not transmit the disease to others.

Post-primary tuberculosis: It is also called secondary TB or adult TB. It frequently occurs in the apices of the lungs of a young immunized individual who has developed strong adaptive immunity to control the infection in rest of the body. It can occur as a result of reactivation of dormant MTB or reinfection from the environment. Post-primary TB is characterised by the formation of cavities in the lungs and accounts for 100% of disease transmission and nearly 80% of all clinical disease (8).

Structure of Mycobacterium Tuberculosis
MTB is a non-motile, rod-shaped bacterium. It is an obligate aerobe, gram-positive, non-spore forming, and facultative intracellular pathogen. It has slow replication time of 15-20 hours and requires 3-4 weeks to produce visible colony on a culture medium. The cell wall of MTB is made up of lipids and carbohydrates and accounts for its acid fastness. It is unique and the
major determinant of virulence. More than 60% of MTB cell wall is made up of lipids. It consists of three major components. Mycolic acid, cord factor and wax D. Mycolic acids are an alpha-branched chain and makeup to 50% of dry weight of cell wall. Cord factor also called trehalose 6,6’-dimycolate accounts for serpentine cord like formation of MTB on culture medium (9).

**Secreted and Somatic proteins**

MTB genome has been sequenced and contains 4000 protein-coding genes. The function of 52% of these proteins is known and 376 reported proteins are believed to be unique to MTB since they share no homology with known proteins (10). Viable MTB during growth secrete number of proteins by active secretion, which are called secreted proteins. These proteins lead to the development of efficient acquired resistance against MTB (11). Somatic proteins are cytoplasmic plus cell-wall proteins. Culture filtrate proteins are a mixture of secreted, cytoplasmic and cell-wall proteins, but predominantly contains secreted proteins (fig. 1) (12, 13).

![Diagrammatic representation of somatic and culture filtrate proteins.](image)


Studies have used different approaches to separate proteins that are actively secreted from MTB during growth from intracellular proteins (cytoplasmic and cell-wall). Localization index, which is a measure of secretion efficiency of different MTB proteins, is one approach to group MTB proteins into secreted and cytoplasmic groups. By definition cytoplasmic proteins have
a localization index of zero and the values given are those determined in a particular MTB culture fluid with minimal bacterial lysis (14). Another approach has been using the release of a metabolic enzyme, isocitrate dehydrogenase, from MTB as a marker for bacterial autolysis in early culture filtrates (15). Secreted proteins are believed to play a role in the development of protective immunity and as well as the progression of the disease. However, less is known about the type of proteins that are expressed in tissue destruction and in vivo in lesions during the course of the infection. The nomenclature that is used to describe secreted proteins of MTB is MPT number. The term MPT stands for proteins purified from *Mycobacterium tuberculosis* and the number indicates the relative mobility in 7.7% polyacrylamide gel electrophoresis (PAGE) at a running pH of 9.5 (12).

**Immunity against tuberculosis:**

**Innate Immune response**
The innate and the adaptive host immune responses against MTB are complementary and synergistic (16). Innate immunity is the first line of host immune defence against MTB. MTB enters the lung alveoli through airways in the air droplets coughed by an infected individual with cavities in the lungs. An air droplet may contain up to 3 bacilli (9). Once in the alveoli MTB encounters dendritic cells (DC), alveolar macrophages and pulmonary cells and can infect macrophages, DC or neutrophils. MTB is phagocytosed by alveolar macrophages through receptor-mediated phagocytosis. These receptors include complement receptors, mannose receptors and scavenger receptors. These phagocytic receptors are pattern recognition receptors (PRR) and they detect molecular pattern typical for the pathogens, pathogen-associated molecular pattern (PAMP). The bacterial cell surface proteins activate complement proteins that are present in the alveoli and complement factor C3 opsonize MTB, which then binds to complement receptor and is phagocytosed. Mannose receptor recognises bacterial mannose-capped lipoarabinomannan proteins. Besides phagocytosis another mechanism of recognition of MTB by macrophages is through Toll-like receptors (TLRs). They are also pattern-recognizing receptors and are essential for recognition of MTB by macrophages and dendritic cells. Their activation by MTB leads to secretion of enzymes, cytokines and other proteins involved in antimicrobial function (fig. 2) (16, 17).

Alveolar macrophages stimulated by the phagocytosed MTB and through TLR’s, secrete cytokines including TNF alpha, IL-1alpha and IL-1 beta, IFN-γ and chemokines (CCXL8),
recruiting effectors of innate immunity at the infection site and starting an innate immune response. The main effector cells of the innate immunity are macrophages derived from blood monocytes, neutrophils, natural killer cells and complement proteins (18).

**Figure 2: Phagocytosis and immune recognition of MTB by different macrophage receptors.**


**Macrophages:** After MTB has gained entry into the macrophage, infected macrophage may meet any of the three fates. First, infected macrophages may undergo necrosis resulting in disruption of the plasma membrane and release of MTB to infect other newly recruited blood monocytes/macrophages at the infection site resulting in dissemination of MTB. Second, it may undergo apoptosis resulting in the release of membrane-bounded vesicles called apoptotic bodies. Recruited dendritic cells take up the apoptotic bodies containing MTB, process the antigens and present it to cross-prime the naïve CD 8+ T cells. Hence impeding bacterial replication, making an important link in adaptive immunity activation and augmenting host immune response (fig. 3). The third is the survival of the infected macrophage (19). Infected alveolar macrophages secrete chemokines to recruit blood born monocytes at the site of the infection. In the tissue blood monocytes differentiate into macrophages. These macrophages cannot kill intracellular MTB until activated by IFN-γ secreted by activated CD4+ T cells. Macrophages play a vital role in TB disease pathogenesis both as an effector of innate immunity and after the activation of adaptive immunity under IFN-γ mediated activation. Activated macrophages mediated intracellular killing of MTB is the primary method of elimination of MTB in TB disease (18, 20). Functional activity of
macrophages correlates with their microenvironments and results in one of two activated states of macrophages M1 or M2. Classical macrophage M1 activation is induced by IFN-γ. This is associated with proinflammatory response and increased microbicidal activity. Alternative macrophage M2 activation is induced by IL-4 or IL-13. This is associated with tissue repair, control of inflammation and immune escape of pathogen leading to chronic infection. M1 and M2 macrophages also display different phenotypes and cytokines secretion profile. A recent study has demonstrated that MTB can induce monocyte-derived macrophage polarization into either M1 or M2 phenotypes and drive the fate of the infection (21).

Neutrophils: Neutrophils are commonly first recruited cells at the site of infection and play a dual role in TB disease pathogenesis. They are recruited by IL-8 secreted by MTB infected macrophages. They act against microbes through phagocytosis, intracellular killing and release of neutrophil extracellular traps (18). A study on a murine model of TB has suggested a regulatory non-phagocytic role of neutrophils. They help in generation of adaptive immune response and formation of a granuloma. They are however suggested to contribute to the tissue destruction, disease severity and progression rather than protection of the host (22). It

Figure 3: Fate of macrophages when infected with virulent vs attenuated MTB. Virulent MTB drive the cell death towards necrosis. MTB released by the necrotic macrophages are taken up the newly recruited inactivated macrophages hence spreading the infection. Whereas, attenuated MTB drives cell death towards apoptosis. Apoptosis of infected macrophages can result in the release of apoptotic vesicles that can be taken up by the dendritic cells resulting in cross priming of CD8+ T cells.

is suggested that they play a protective role in early stages of the disease and destructive role at later stages (23).

**Natural Killer (NK) cells:** Natural killer cells are a class of lymphocytes and cells of innate immunity. Their cytotoxic mechanism is the same as used by cytotoxic T lymphocytes (CTL) but they differ from T and B lymphocytes in a way that they do not have clonally distributed T cell receptors (TCR), which recognize specific antigens (17). An *in vitro* study has shown that NK cells can recognize and destroy macrophages infected with MTB. They activate macrophages effector function by secreting IFN-γ during the early phase of the infection (18, 24).

The importance of innate immunity in defence against TB has been underlined by Lurie’s fundamental studies with resistant and susceptible inbred rabbits. The rabbits were infected through inhalation with MTB. It was noted that seven days after the infection the lungs of MTB-susceptible rabbits contained 20- to 30-fold more viable MTB than did the lungs of MTB-resistant rabbits (25). The adaptive immune response mediated by T-cells against MTB is not initiated until eleven to fourteen days after infection (26). This difference in number of the bacilli in susceptible and resistant rabbits suggests an important role of innate immunity in controlling initial stages of the infection (16).

**Adaptive immune response**

Innate immunity plays a vital role in initial stages of TB disease but is not sufficient to control the infection, resulting in accumulation of antigens and activation of adaptive immunity. An adaptive immune response comes into play to control the infection and restrict the growth of MTB but fails to eradicate it completely resulting in latency or chronicity of the infection (18).

The adaptive immune response consists of humoral immunity and cell-mediated immunity (CMI). CMI plays a dominant role in protection against MTB infection. The CMI against MTB is initiated in the lymph nodes and requires transport of MTB or its antigens from the lungs to the draining lymph nodes to prime naïve T lymphocytes. In the lymph nodes, naïve T cells are circulating in search for specific antigens for which they have antigen-specific T cell receptors present (TCR) on the cell membrane. MTB antigens are processed and presented to naïve T cells by antigen presenting cells (APCs) in association with cell surface major
histocompatibility complex (MHC) molecules to activate them. Naïve T cells require three
signals to become fully activated and differentiate into effector T cells. The first signal which
is antigen-specific is provided when APC’s present MTB processed antigens via MHC molecule
that interacts with TCR. The second signal is non-antigen specific activation and is provided
by the interaction between co-stimulatory molecules present on the surface of APCs such as
CD80, CD86 or B7 molecules and CD28 present on the surface of T cells. Activation of T cells
through co-stimulation is important for T cells proliferation, differentiation and survival
otherwise it may result in T cell anergy and development of immune tolerance. The third
signal is cytokines activation provided by cytokines like IL-12 or IL-4. Once differentiated the
effector function of T cells includes differentiation, clonal expansion of antigen-specific T cells,
expression of activation markers, releasing cytokines such as IFN-γ, TNFα and IL-17, activation
of B cells to secrete antibodies and differentiation and maintenance of memory T cells.
Effector T cells, as they express adhesion molecules and chemokines receptors, are then
recruited from the lymph nodes to the site of infection to initiate their effector function (17,
27). The main effectors of adaptive immunity in defence against MTB are CD4+ T cells and
CD8+ T cells.

**CD4+ T cells**: CD4+ T cells, also called T helper (Th) cells, are main effectors of adaptive
immunity against MTB. They recognize antigens presented by APCs in association with MHC-
II molecules. MHC-II molecule presents antigens present in phagosome, vacuoles or endocytic
vesicles of phagocytes. CD4+ T cells have three subsets of effector cells that produce the
distinct set of cytokines and have different functions namely Th-1, Th-2, Th-17 cells. T
regulatory cells (Treg) are also subtype of CD4+ T cells which have immunomodulatory
functions (17).

The differentiation of naïve CD4+ T cells to Th-1 subtype occurs in response to IL-12 and IFN-
γ. Macrophages in response to ingested MTB secrete IL-12 and NK cells produce IFN-γ that
activates transcriptional factor promoting the differentiation to Th-1 subtype. IFN-γ is the
signature cytokine of Th-1. Differentiated Th-1 cells produce IFN-γ to activate macrophages
for intracellular killing which is classical macrophage activation M1 phenotype. Th-1 cells
promote differentiation of more Th-1 cells, secrete TNFα, and inhibit Th-2 and Th-17 subsets
differentiation. Th-1 subsets are associated with protective immunity and elimination of
intracellular MTB (17).
The differentiation of naïve CD4+ T cells to Th-2 subtype occurs in response to IL-4. The signature cytokines of Th-2 subsets are IL-4, IL-5 and IL-13. Th-2 mediated activation of macrophages leads to alternative macrophage M2 phenotype. M2 phenotype inhibits the microbicidal activity of macrophage and enhances its tissue repair function like the synthesis of extracellular matrix proteins and suppresses the activity of Th-1 mediated immunity. Th-2 cells are associated with chronic and progressive disease. The outcome of infection is believed to be dependent on the balance between activation of Th-1 and Th-2 subsets of CD4+ T cells (17).

**T regulatory cells:** Treg cells have immune suppressive properties and are essential in preventing autoimmune diseases. They also play a role in dampening immune response to prevent tissue damage due to excessive inflammation against microbial pathogens including MTB. Chronic exposure to antigens, limited co-stimulation, or presence of inhibitory cytokines induce the production Treg cells (28). They suppress the function of T effector cells and APC’s through diverse mechanisms including upregulating expression of immune check-point inhibitors, decrease expression of co-stimulatory molecules or by producing IL-10 or TGF-β (29). Patients who develop active TB disease with high MTB burden have increase Treg cells in the blood and at the site of infection (30). It has been suggested that Treg cells contribute to suppression of Th-1 response in MTB hence prevent eradication of infection and leading to chronicity.

**CD8+ T cells:** CD8+ T cells are also called cytotoxic T lymphocytes, they recognize antigens presented by APCs in association with MHC-I molecules. MHC-I molecule presents antigens present in the cytosol. Cytotoxic T lymphocytes kill MTB infected macrophages through various cytotoxic mechanisms. They also secrete IFN-γ and TNFα to activate macrophages for bactericidal activity (17).

**Cytokines**

**Pro-inflammatory**

**IFN-γ:** CD4+ T cells in response to IL-12 acquire Th-1 phenotype and secrete IFN-γ to activate macrophages to kill intracellular MTB. This is the main mechanism of elimination of MTB. NK cells produce IFN-γ in response to IL-12 before activation of adaptive immunity (16). IFN-γ also amplifies T cell response by stimulating the expression of MHC-II molecules and B7 costimulatory molecules on macrophages and dendritic cells (17). A study demonstrated that
mice in the absence of IFN-γ were not able to control low dose MTB infection, showed uncontrolled bacterial growth and progressive tissue destruction (31).

**IL-12:** IL-12 has a prominent role in defence against MTB. This is the cytokine that stimulates the production Th-1 phenotype essential for elimination of MTB infection. IL-12 induces its protective effect by stimulating the production of IFN-γ. IL-12 is a regulatory cytokine that connects innate and adaptive host immune response against MTB (16).

**TNFα:** When phagocytes are stimulated by MTB or its antigens they are stimulated to release TNFα. T cells, neutrophils and DC are also involved in TNFα production. TNFα is prototype inflammatory cytokine which induces macrophages activation, granulomatous lesion formation, induces chemokines production, and has immunoregulatory functions (16).

**Anti-inflammatory cytokines**

**IL-10:** IL-10 suppresses an effective Th-1 immune response and downregulates the production of pro-inflammatory cytokines. It inhibits macrophages bactericidal activity, migration and presentation of antigens by DC, and production of chemokines involved in the migration of effector T cells to the site of infection (32).

**TGF-β:** TGF-β synergizes with IL-10 in suppressing the production of IFN-γ by T cells and opposing protective immunity mediated by Th-1 profile. It impedes activation, antigens presentation and production of pro-inflammatory cytokines in macrophages. It promotes production and deposition of macrophage collagenases and collagen matrix and believed to be involved in tissue destruction and fibrosis (16, 33).

**Immune evasion**

MTB is a successful pathogen, despite the elaborative immune responses generated against it, MTB has evolved many ways to subvert them or use them for its own benefit and survival.

Inhibition of phagosome maturation is one of the mechanisms used by MTB to replicate within macrophages and evade immunity. The principal mechanism through which macrophages kill ingested MTB is the fusion of a phagosome with lysosomes. The ingested MTB resides in membrane-bounded vesicle called phagosome that acquires low PH to mature and then fuses with lysosomes to form a phagolysosome. In the phagolysosome, MTB are exposed to bactericidal enzymes of lysosomes, which kills MTB (34). One of the mechanisms that MTB has evolved to inhibit killing inside macrophages is to prevent fusion of phagosomes
with lysosomes, resulting in an intracellular compartment for its survival. One study has shown that MTB containing phagosomes failed to incorporate vacuolated ATPase resulting in failure of phagosomes to acidify and mature (35). Furthermore, another study has shown retention of protein coronin whereas phagosomes that do undergo fusion lose association with this protein (36).

MTB not only manipulates macrophages in its benefit but is also able to withstand stresses that it encounters in activated macrophages. Some mutants of MTB are unable to prevent phagosome lysosome fusion but are still able to survive within phagolysosomes with the help of different resistant mechanisms. Detoxification of reactive oxygen and nitrogen molecules produced by the host and repair of the damage caused by these molecules is one of the survival strategies. In activated macrophages, phagocyte oxidase and inducible nitric oxide synthase generate bactericidal reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), respectively. To mask the effect of these antimicrobials, MTB has the machinery of enzymes which includes catalase peroxide, alkyl hydroperoxide reductases, superoxide dismutase and methionine sulfoxide reductases (37, 38).

Thiols are important cellular compounds that maintain redox cellular balance and repair proteins inactivated by oxidative stress. Thiols bind to proteins and reduce disulphide bonds of deactivated proteins by transferring an electron. MTB lacks conventional glutathione redox system. MTB has a small molecular weight mycothiol and thioredoxin redox system. The thioredoxin system of MTB is comprised of thioredoxin, thioredoxin reductase and NADPH. Thioredoxin(Trx) is a superior thiol reductant and has two redox-active Cys residues in its active site and helps to maintain the reducing intracellular environment in MTB (37).

MTB manipulates macrophage death pathways by inhibiting apoptosis and promoting necrosis to evade immune-mediated killing. In macrophages, it upregulates the production of lipoxin A₄ and inhibits the production of prostaglandins E₂ (PGE₂). PGE₂ enables the repair of plasma membrane and protects mitochondrial inner membrane, hence preventing necrosis and triggering apoptosis. The fate of the infected macrophages contributes in determining the fate of the outcome of infection (fig. 4) (19, 39).
Interference with antigen presentation is a mechanism MTB uses to prevent immune recognition and activation. CD4+ T cells activation by the presentation of antigens in association with MHC-II molecules is critical for MTB control. MTB targets MHC-II pathways to prevent intracellular elimination. MTB products such as 19-kDa lipoproteins inhibit synthesis of new a MHC-II molecule as result of prolonged signalling through TLR-2. MTB infection can induce IL-10 production resulting in suppression of cathepsin S and hence blocks intracellular trafficking of MHC-II molecules. MTB can restrict the generation of peptides antigen for loading onto MHC-II molecule by inhibiting phagosome-lysosome fusion and autophagy (fig. 5) (40).
The activation of adaptive immune response against MTB is delayed both in humans and animal models. A study on mice demonstrated that initiation of adaptive immune response against MTB mediated by antigen-specific T cells after infection is delayed until 9-10 days relative to that of other pathogens (fig. 6) (26).

A study done by Wolf et al suggests that this delay in initiation of adaptive immune response is not attributed to the slow growth of MTB. The study suggests that the probable reason for this delay of initiation could be the localisation of MTB in cells that are not able to migrate from the lung to lymph nodes. The results also demonstrated that MTB is located in cells that hamper the presentation of antigens to naïve T cells for activation in the lymph nodes (41). Furthermore, another study has attributed this delay to inhibition of apoptosis of infected macrophages and neutrophils by MTB resulting in a delay of uptake of apoptotic bodies by dendritic cells and migration to draining lymph nodes (42). Another study has ascribed this delay to MTB inhibiting migration of infected DC from the infection site to the draining lymph nodes (43). The delayed initiation of adaptive immune response against MTB limits its efficacy. This is considered as one of the immune evasion approaches as this gives time to MTB to establish itself in substantial number to prevent complete eradication (18). A study showed that MTB appeared earlier in draining lymph nodes of resistant (C57/BL6) mice as compared to susceptible (C3H/HeJ) mice. This indicates that earlier activation of T cells is related to better control of the infection (44).

Figure 6: Delayed onset of adaptive immune response in TB infection compared with other infections.

Granuloma
The granuloma is the hallmark of the immune response in primary tuberculosis. Immune cells recruited at the site of infection leads to the granuloma formation. A classic TB granuloma contains of infected macrophages in the centre surrounded by differentiated macrophages including epithelioid cells, multinucleated giant cells and foamy macrophages surrounded by a rim of lymphocytes. Other cells that also form the part of granuloma includes neutrophils, dendritic cells, NK cells, B cells, fibroblast and extracellular matrix-producing cells (fig. 7). TNF secreted by the infected macrophages and activated T cells is required for the formation and maintenance of granuloma. The granuloma formation symbolizes an effective immune response mediated by adaptive immunity, which is able to control the infection but may not eradicate it completely. MTB can persist in the granuloma in a latent state for decades (45). Consequently, the debate about the nature of the granuloma being protective, destructive or serving a niche for MTB during latency is still unresolved. During latent infection, MTB and its DNA has been detected more frequently in the normal looking lung parenchyma indicating that granuloma may be able to eradicate MTB quite efficiently and latency is maintained in the “non-professional” phagocytes (46, 47).

Figure 7: Diagrammatic representation of structure and cellular components of tuberculosis granuloma.
Foamy macrophages a niche for survival of MTB:
Foamy macrophages are one of the differentiated cells found in lipid pneumonia of post-primary TB. They are characterized by their high lipid content and vacuolated appearance. A study of *in vitro* granuloma model suggested that oxygenated mycolic acids found in the cell of MTB play a crucial role in the differentiation of macrophages into foamy macrophages. It was found that once macrophages are differentiated into foamy macrophages, they cannot mediate phagocytosis of new MTB and lose their bactericidal activity. MTB already present in them can no longer replicate, acquire intracytoplasmic lipid inclusions derived from host lipid bodies, overexpress dormancy genes and reside in a dormant non-active state. As a part of the study when granuloma from TB patients were stained for lipids with Oil-red stain, they were seen containing foamy macrophages (fig. 8) and they often contained MTB. Furthermore, they were present in only those lesions that had a necrotic centre and were located at the interface of necrosis and granuloma. Foamy macrophages were observed secreting TNF-alpha thus suggesting their strong association with necrosis (48). In another study, it was observed that foamy macrophages accumulated in the alveoli in lipid pneumonia of post-primary TB, and expressed DC marker DEC-205. DC does not have an efficient mechanism of intracellular killing of MTB and can harbour MTB for long intervals, however, the study could not demonstrate the significance of foamy macrophages expressing dendritic cell marker (49). Studies done by Mustafa et al has shown that these foamy macrophages accumulate MTB antigen (50, 51). Many other studies have demonstrated accumulation of MTB antigens in foamy macrophages. Foamy macrophages are resistant to apoptosis. They have reduced antigen processing function by down-regulating expression of MHC II and bacterial antigens are believed to be responsible for this down-regulation (52). These MTB containing foamy macrophages should be eliminated by the cell-mediated immunity or delayed type hypersensitivity (53). However, these antigens containing macrophages seems to evade killing as shown by a study where macrophages containing large amounts of antigens expressed high intensity of Fas Ligand, and anti-apoptotic protein Bcl2 and low intensity of Fas and proapoptotic protein Bax (54, 55). As a consequence of this MTB manipulated macrophages in its favour by evasion of cytotoxicity and apoptosis, thus serving as a nutrient-rich reservoir providing a safe house where MTB can proliferate and accumulate antigens evading the immune response.
Pathogenesis of tuberculosis

Pathogenesis of primary and post-primary TB leading to cavity formation is described in the literature in two contradictory ways based on understanding from pre-antibiotic era studies on human tissues and from post-antibiotic era studies on different animal models.

Pathogenesis of tuberculosis from the pre-antibiotic era:
In 1821 Laennec, using clinical knowledge and by observing gross pathology reported that TB exhibit two distinct type of pathologies. He used the terms “Productive” to describe primary TB and “Exudative” to describe post-primary TB. They refer to tuberculous granuloma and tuberculous pneumonia in modern day terminology (56). These findings were later confirmed by many investigators of the mid-20th century (57-59). After the introduction of microscope Virchow challenged Laennec’s observation of one organism and two pathologies. When viewed under microscope granuloma and pneumonia appeared different and he proposed that they are different diseases caused by different organisms. The tuberculous granuloma is kind of a tumour and pneumonia is an inflammatory process (60). The discovery of MTB in 1881 by Robert Koch proved Virchow wrong. Until the end of the pre-antibiotic era when TB infected human tissues were easily available for study investigators have described two different pathologies of TB (25, 61-63).

After the introduction of antibiotics in 1950’s untreated human tissue was no more readily available. Treatment with antibiotics clears pneumonia and kills the bacteria as a result disease pathology is changed. This forced investigators to develop and use animal models for study. Rabbits infected with Mycobacterium bovis (M. bovis) was most studied model. However, due to some fundamental differences in the M. bovis and MTB, the rabbit model does not mimic the human disease. For example, M. bovis does not become dormant and
does not produce post-primary TB in any species. Post-primary TB occurs only in human lungs and no animal can transmit MTB infection through aerosol production as in humans. Use of animal models and lack of availability of human tissue for confirmation resulted in a new paradigm for TB pathogenesis dating back to the late 20th century. Caseating granuloma is the characteristic lesion of all TB diseases. Cavities occur by expanding granuloma eroding into the bronchi and spilled contents of the granuloma seed pneumonia. This has guided the research on TB since then and unfortunately diverted the focus from the true pathogenesis of post-primary TB (63).

**Pathogenesis of tuberculosis based on contemporary research post-antibiotic era:**

When MTB is inhaled the disease may progress through the following stages of pathogenesis as studied in the lungs of rabbits.

The infection begins when the inhaled MTB is phagocytosed by the alveolar macrophage. The activated macrophage may destroy the MTB before it multiplies without activation of adaptive immunity, in this case, tuberculin skin test is not positive (25).

If alveolar macrophage cannot kill the ingested MTB, MTB can multiply in it and eventually macrophage ruptures releasing the MTB to infect the newly arrived blood monocytes/macrophages to the site of infection. These macrophages are immature, they ingest the MTB but are not activated to kill it, resulting in a symbiotic relationship, where bacilli and macrophages increase in number without evident tissue damage. With time early lesion develops with the accumulation of macrophages and bacilli. This symbiotic stage occurs between 7 to 21 days after infection before the activation of adaptive immunity and there is a logarithmic increase in the number of MTB (25).

This stage marks the activation of MTB specific adaptive immune response constituting of CMI and delayed type hypersensitivity (DTH) acting synergistically with each other. CMI activates the macrophages to kill intracellular MTB. Poorly activated macrophages harbouring more than a few bacilli or containing a high concentration of MTB antigens are killed by DTH along with tissue damage resulting in the production of solid caseous necrosis in which MTB cannot grow. It occurs around 2 to 3 weeks after infection and marks the end of logarithm growth of MTB (25).
Surrounding the solid caesium are activated macrophages produced by CMI as well as non-activated macrophages. In case of strong CMI, MTB escaping from the caesium are ingested by activated macrophages and are readily killed, hence the lesion stabilizes or even regresses at a subclinical stage. In case of susceptible individuals with weak CMI, MTB escaping from the solid caesium are ingested by non-activated macrophages or partially activated macrophages, which fail to kill MTB and in turn, are killed by DTH enlarging solid caesium. As a result, disease progresses and becomes clinically apparent (25).

Liquefaction or softening of solid caesium is a critical event in cavity formation. It allows the extracellular growth of enormous number of MTB. High local concentration of MTB products produced by large number of MTB cause DTH mediated tissue destruction, eroding nearby bronchial wall and resulting in a cavity formation (25). The exact mechanism of liquefaction and cavitation is not known, however, studies suggest the role of hydrolytic enzymes like matrix metalloproteinases, DNases, RNases and lipases in liquefaction (64, 65).

**The new paradigm for the pathogenesis of post-primary TB:**
The new paradigm for the pathogenesis of tuberculosis is based on extensive research of old literature from the pre-antibiotic era and studying human tissue samples. Post-primary TB begins as endogenous lipid pneumonia of foamy macrophages containing MTB antigens and host lipids. It frequently occurs in the apices of the lungs of an immunized individual with acquired immunity to TB. Lipid pneumonia in 90% of the cases heals spontaneously leaving a scar without producing any clinical disease. The lipid pneumonia that fails to heal undergoes necrosis forming caseous pneumonia with the onset of clinical disease (fig. 9). Endobronchial obstruction is an important feature of developing post-primary TB. Bronchial obstruction leading to necrosis and cavitation is also seen in cancer and seems to play a significant role in TB pathogenesis as well. Caseous pneumonia can have one of the two fates. First, it can undergo softening and fragmentation and will be coughed out forming a cavity. With time cavity matures and forms a thin wall of fibrosis supporting the proliferation of large numbers of MTB. Second is fibrocaseous TB, which occurs when ageing caseous pneumonia is surrounded by granulomatous tissue consisting of epithelioid cells, giant cells and lymphocytes but no foamy macrophages. This granulomatous response leads to healing of lesions, and fibrosis. Granuloma in post-primary TB, therefore, are not a precursor of caseous necrosis but are formed as a result of it, and cavities are produced as a result of caseous
necrosis of lipid pneumonia. What causes foamy macrophages in lipid pneumonia to undergo necrosis is not known. It has been hypothesized that an interaction between accumulated MTB antigens and host lipids in the presence of a trigger leads to necrosis of foamy macrophages and spilling of antigens. Released antigens then induce strong perifocal inflammation by interacting with sensitized T cells, already present in the host as a result of prior immunity due to primary infection, resulting in caseous pneumonia and cavitation. A trigger that initiates the necrosis is not known. Some speculate that trigger could be trehalose 6,6-dimycolate, most abundant lipid found in virulent MTB. This can be attributed to its ability to stimulate the release of TNF from macrophages as a result of acquiring highly toxic monolayer configuration when it comes in contact with lipid droplet (8, 61, 63, 66).

Figure 9: Pathogenesis of primary and post-primary TB. Primary-TB leads to the formation of granuloma in most of the individuals, which is an effective immune response. In immunodeficient dissemination of infection can occur. Post-primary TB begins as a lipid pneumonia which resolves in 95% of the cases leaving a scar. In rest of 5% lipid pneumonia can undergo caseous necrosis which clinical manifestation of the disease leading to cavity formation or fibrocaseous necrosis.


Murine Model of Tuberculosis:
We have previously developed a mouse model of slowly progressive TB and characterized it’s clinical, histopathological and bacteriological aspects. Three distinct phases for the course of the disease were observed. During phase 1, mice were healthy, small lesions appeared in the lungs, and number of bacilli in the lungs increased. During phase 2, mice were unwell but
mortality was low. The bacillary colony forming units (CFUs) and lesion size though higher than phase 1, remained stable during this phase. The lesions contained an increasing population of large, vacuolated macrophages. During phase 3, mice became moribund and died rapidly, but the CFUs remained relatively stable. The lesions occupied more than 4/5 of the lung parenchyma (50).

With the revised understanding of the pathogenesis of post-primary TB, the status of foamy macrophages has changed from incidental finding to the main interest (8). Studies have shown that foamy macrophages present in TB pneumonia contain MTB and accumulate MTB antigens with little or no inflammation around them (67). It is hypothesised that MTB antigen accumulation triggers the extensive inflammation leading to tissue destruction and cavitation. However, which types of antigens are accumulated in these macrophages, and what triggers the inflammation has not been studied extensively.
STUDY OBJECTIVES
The main objective was to study the *in vivo* expression of various mycobacterial antigens in the murine lung tissues during the course of slowly progressive TB and its correlation with the disease pathology.

Specific objectives:
1. To study the lesion morphology, CFUs, acid fast bacilli and in situ expression profile of various mycobacterial antigens during the course of infection.
2. To correlate the acid-fast bacilli, CFUs and expression of various mycobacterial antigens with various types of lesion morphology, and disease severity.
3. To compare the mycobacterial antigens expression profile with bacterial load measured by CFUs and acid-fast bacilli.
MATERIAL AND METHODS

Murine Model of slowly progressive TB
The model has been described earlier (50). B6D2F1 hybrid mice used are a cross between C57BL/6 resistant strain and DBA/2 susceptible strain. A total of 260 mice at the age of approximately 12 weeks were inoculated with $1.5 \times 10^6$ CFUs of H37Rv strain of MTB through i.p route. The average lifespan of these mice is 975 days. The relatively low dose of infection was used to allow chronic lung pathology to develop. The advantage of the i.p route is that bacilli would initially reach mediastinal lymph nodes resembling the natural airborne infection. Furthermore, with the aerosol route, bacilli can get trapped in fur, licked and ingested can stimulate gut-associated lymphoid tissue and thus modifying the immune response and the infection dose.

The mice were followed up to 70 weeks post-infection. Average 3 mice were sacrificed at 1, 2, 4 and then 4 weekly intervals up to week 41 and later at 52, 57 and 70 weeks. Until week 24, one lung from each sacrificed mouse was used for histopathology and the other for CFUs. From week 29, lungs were perfused with formalin before immersing in 4% buffered formalin and then embedded in paraffin (50). Lung biopsies from these mice are used in this study.

Antibodies
Antibodies used in the study are listed in table 1. All antibodies used in the study were in-house rabbit polyclonal antibodies except for anti-BCG (68). Rabbit Anti-Mycobacterium bovis (anti-BCG) is a polyclonal antibody available from DAKO Immunoglobulins, Copenhagen, Denmark (code B124; lot 063B).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target antigens</th>
<th>Immunogen</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-BCG</td>
<td>Whole bacteria including secreted and cell wall antigens. It detects heterogenous MTB antigens.</td>
<td>sonicates of <em>M. bovis</em> containing both secreted and cell wall antigens.</td>
<td>1:4000</td>
</tr>
<tr>
<td><strong>Cell wall antigens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-body against cell wall antigens</td>
<td>Cell wall antigens</td>
<td>Cell wall antigens</td>
<td>1:2000</td>
</tr>
<tr>
<td><strong>Total Secreted antigens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-<em>M. Tuberculosis</em> secreted proteins*</td>
<td>Directed against total secreted proteins present in culture filtrate (CF)</td>
<td>5 weeks old culture filtrate of MTB with minimal cell lysis.</td>
<td>1:100</td>
</tr>
</tbody>
</table>
Individual secreted antigens

<table>
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<tr>
<th>Antibody</th>
<th>Target Antigen</th>
<th>Immunogen</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-MPT51</td>
<td>MPT51(Ag85D, fbpD,Rv3803c)</td>
<td>MPT51 antigen found in CF of MTB</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-MPT53</td>
<td>MPT53(DsbE, RV2878c)</td>
<td>MPT53 antigen found in CF of MTB</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-MPT63</td>
<td>MPT63 (Rv1926c)</td>
<td>MPT63 antigen found in CF of MTB</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-MPT64</td>
<td>MPT64 (Rv1980c)</td>
<td>MPT64 antigen found in CF of MTB</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Other antigens

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target Antigen</th>
<th>Immunogen</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-MPT46</td>
<td>MPT46(Thioredoxin TrxC, Rv3914)</td>
<td>MPT46 antigen found in CF of MTB</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Table 1: List of antibodies used in the study, target antigens, immunogens and dilution used for IHC.

Sectioning of mouse biopsy specimens

A series of 5 μm sections were prepared from formalin-fixed paraffin-embedded tissue block using sliding microtome (Leica Biosystems, UK). The sections were mounted on coated glass slides for better fixation (Thermos Scientific Super Frost Plus, USA). All prepared slides were incubated at 60 °C in hot air oven for overnight, then stored at 4 °C for later use.

Haematoxylin and Eosin and Ziehl–Neelsen staining

Haematoxylin and Eosin and Ziehl–Neelsen (ZN) staining was done mechanically in the routine histology laboratory.

MTB has a high content of lipid in its cell wall and is difficult to stain. In ZN staining, Carbol fuchsin which is a lipid soluble compound is used as a primary stain. When treated with acid, MTB retains the dye hence the name acid-fast bacilli (AFB) is given to it. For background staining of tissue, methylene blue is used to provide colour contrast to visualized red AFB (fig. 10) (69)

Figure 10: Diagrammatic representation of steps involved in ZN staining.

**Colony Forming Units**

Tissue homogenates were plated on Middlebrook 7H10 medium (Difco Laboratories, Detroit, MI, USA) after tenfold serial dilution. CFU per organ for the number of MTB was calculated and expressed as log₁₀ per organ. After sealing the culture plates with adhesive tape, they were incubated at 37 °C and 5% of CO₂ in an incubator for 3 weeks followed by incubation in air at 37°C. When CFU counts were constant for three consecutive weeks readings were stopped. Culture plates were kept for maximum 3 months after inoculation (50, 70).

**Immunohistochemistry**

Immunohistochemistry (IHC) is used for in situ detection of antigens in a tissue section and is based on the principle of antibodies binding to the specific antigens (fig. 11). The advantage of IHC is that it characterizes protein expression in the context of the tissue histology and gives valuable information about localization and identification of the antigens.

All the reagents were used from the company Dako Agilent Pathology Solutions. All steps of IHC were performed manually as described previously (51). Deparaffinisation was performed using xylene and then sections were placed in a series of decreasing concentration of alcohol to remove xylene and finally in distilled water for rehydration. Since tertiary protein structures are altered as a result of cross-linking during the formalin fixation of tissues, retrieval process was done to increase the access to antigens epitopes. Sections were boiled for 20 minutes in citrate buffer (PH 6) in a microwave oven. Tris Buffered Saline (TBS) was used for washing tissue sections. PAP pen (Dako A/S) was used to encircle the region of tissues to keep the reagents within the prescribed area. Peroxidase block (Dako A/S) was applied for 15 minutes to block the activity of endogenous peroxidase. The sections were washed again with TBS and then serum-free protein block (Dako A/S) was applied for 12 minutes to prevent non-specific binding of the antibody to tissues. Primary antibody was applied for 60 minutes. The sections were washed with TBS and secondary antibody, labelled polymer HRP anti-rabbit (Dako A/S), was applied for 40 minutes. The
sections were washed, and 3-amino-9-ethylcarbazole (AEC) substrate chromogen was applied for 15 minutes. AEC gives a rust red colour on a positive tissue sample. The sections were washed again and were counterstained with Mayer’s Haematoxylin (Dako 3309). Finally, slides were covered with coverslip using an immuno-mount medium ((Dako A/S).

Immunohistochemistry Control: IHC staining was monitored by using 2 negative controls and 1 positive control.

1. Negative control 1: No primary antibody was used
2. Negative control 2: Full protocol on a known non-TB lymph node biopsy
3. Positive control: Known TB biopsy with high bacterial and MTB antigen load

Analysis

Histopathological evaluation
Histopathological evaluation of haematoxylin and eosin stained slides was done by light microscopy (Leica Microsystems).

Digital scanning
IHC and ZN stained slides were digitally scanned at X40 magnification using Aperio ScanScope CS® Slide Scanner (Aperio Technologies Inc., Vista, CA, USA). For visualization and quantification of scanned slides Image Scope software (Aperio Technologies Inc., Vista, CA, USA) was used.

Annotation and staining quantification
All the scanned slides were annotated manually and only the area containing the lesion was annotated for quantification of IHC and ZN staining (fig. 12).

Figure 12 showing annotation of lesion area for IHC staining. The green colour outline of the lesion area shows the marked annotation. (b)Diagram showing annotation of lesion area for ZN staining. The green colour outline of lesion area shows the marked annotation.
Colour deconvolution (CD) algorithm, provided commercially from Aperio Technologies, was used for quantification of slides. The reason behind using this algorithm was, in it, we were able to set the Red, Brown, Green (RGB) value for the positive stain. The algorithm was optimized separately for quantification of ZN and IHC staining.

**Optimization for immunohistochemistry quantification**

CD algorithm allows to measure and differentiate maximum three colours in a stained slide. In our study, we were interested in two colours; red for AEC, designated as colour 1 and positive stain, blue for haematoxylin counterstain; colour 2 designated as the negative stain. We adjusted the algorithm parameters accordingly. RGB values were adjusted for colour 1 and colour 2 and threshold levels were set for weak, medium and strong intensities. To obtain the RGB values for positive colour (red), a small region of red colour was annotated (fig. 13), and default CD algorithm was run and optic density (OD) values of RGB for the red colour were obtained. To adjust the input parameters to detect red colour as positive, OD values of RGB obtained were copied and added to our algorithm settings as input values for Colour 1. The similar procedure was repeated for negative stain.

![Image of diagram showing annotation area for red color and OD values of RGB obtained](image)

**Figure 13:** (a) The diagram shows the annotation area for red color that is selected to find the RGB value of positive colour of our study (b) shows the OD values of RGB that were obtained and used as input values for positive colour.
The threshold value for weak, medium and strong intensity is the required saturation of the positive colour. It is between the range of 0-255, where 0 is the darkest pixel and 255 is the brightest pixel. Greater the value of threshold intensity, the brighter pixel it detects. We have set the value for weak positive threshold very low (towards 0) to avoid false reading for background or non-specific staining. The threshold values were adjusted and tested to make sure non-specific staining is not read as positive. The input values we used for IHC quantification in this study are shown in the diagram (fig. 14).

**Understanding the results**

The results we used in our analysis are explained as following (fig. 15)

- **Total Stained Area** is the cumulative total area of combined positive and negative pixels.
- **To amplify the strong intensity pixels. Score** is used which is calculated by the formula, \( \text{Score} = 1.0\%\text{Weak} + 2.0\%\text{Medium} + 3.0\%\text{Strong} \).
- **The Percent Strong, Medium, Weak Positive** is the percent of positive pixels that are strongly, moderately, and weakly stained, as defined by our input parameters for strong, medium and weak positive thresholds, in the total stained area.
- Percent Total Positive is the sum of strong, medium and weak pixels in the total stained area.

Figure 16 shows the mark-up image produced when the slide is analysed, where red is strong positive, orange is medium positive, yellow is weak positive and blue is negative. The intensity of positivity is taken as a proxy measure for amount of antigens expression. Strong positive is taken as increased accumulation of antigens marked by rusty red granular staining (fig. 17).

Figure 17: (a) Magnified image of the same slide (b) Mark-up image showing different colours reading different staining intensities, red for strong, orange for medium and yellow for weak intensity.
Optimization for Ziehl–Neelsen quantification

For ZN quantification, we followed the same steps, as described above, for obtaining OD values of RGB for positive stain, which in this case, is the pink stain of acid-fast bacilli. For the threshold values, since we were not interested in intensities, but one colour, we kept it same for weak, medium and strong intensities. The RGB values for background negative stain were also adjusted (fig. 18a). For the results, we got percent strong positive, which in this case, is the same as percent total positive (fig. 18b). Counting AFB manually is very subjective and difficult, also we did not have same size tissue in all sections. We took percent total positive as a proxy for AFB count to avoid error due to different tissue size.
Statistical Analysis
Non-parametric statistical analysis methods were used. Mann-Whitney test was used for comparison of two independent groups. Wilcoxon signed rank test was used for comparison of two related samples. Spearman’s rho was used for correlation analysis. All the analysis was performed using SPSS.

Ethics
The mouse model has been developed previously with permission taken from Norwegian Experimental Animal Board (50).
RESULTS:

Histopathology:

**Early small diffuse lesions week 8 – 12**

During week 8 and 12 small lesions were visible in the lungs. Lesion formation seemed to start with the appearance of macrophage and increasing lymphocytic infiltration (fig. 20a). The peribronchiolar region was infiltrated by lymphocytes and macrophages. There was interstitial pneumonia evident by thick alveolar septa containing inflammatory cells mainly monocytes and lymphocytes, and few neutrophils (fig. 20b, c). Usually, inflammatory cells were not present in alveolar spaces. Lesions were not demarcated from the normal lung tissue.

![Figure 20: (a) Peribronchiolar infiltration of cells with lymphocytes mixed with macrophages. (b) Interstitial pneumonia of lymphocytes and monocytes, no inflammatory cells were seen in alveolar space, (c) enlarged image showing interstitial pneumonia.](image)

**Well demarcated focal lesions week 16 – 33**

With the course of infection, the amount, morphology and organization of macrophages changed. Focally organized lesions, with more defined distribution of macrophages separated from lymphocytes, were seen (fig. 21a). Lymphocytes formed tightly packed islets surrounded by foamy macrophages (fig. 21b). With the progression of infection, the macrophage predominant area increased in size compared to lymphocyte area. Macrophages with large light staining nuclei with vacuolated eosinophilic cytoplasm were seen (fig. 21d). Macrophages became increasingly vacuolated with increase in size of the cell, called as foamy macrophages. From week 33, early destructive changes could be seen in the macrophages evident by cell swelling, pyknotic bodies and karyorrhexis (fig. 21e, f). Neutrophils were seen adjacent to dying macrophages (fig. 21g). There was a sharp demarcation between lesion area and normal lung tissue. Parenchyma surrounding the lesion had interstitial pneumonia with
lymphocytes and very few neutrophils, but no foamy macrophages. Hyperplasia of bronchus-associated lymphoid tissue was also observed.

From week 37, a shift in disease severity was observed with an increase in mortality. The lesions expanded to occupy more than 80% of lung parenchyma. The expansive lesions contained more foamy macrophages and smaller number and size of lymphocytes aggregate as compared to the earlier time points. Very sick mice were seen containing more areas of focal necrosis in the lesions as compared to the less sick mice. Overall, mice in these weeks showed two types of morphological lesions, and were grouped into two based on the presence of focal necrosis.

Figure 21: (a) well-demarcated lesion shown by the arrow, (b) Tightly packed lymphocytes islets (black arrow) with foamy macrophages aggregate (red arrow) nearby could be seen, (c) Foamy macrophage aggregates separated from lymphocytes islets (d) clusters of foamy macrophage attaining increasingly vacuolated foamy appearance as the disease progresses, (e) pyknotic nuclei (black arrow) and karyorrhexis (red arrows). (f) early destructive changes shown by the arrow, (g) neutrophils surrounding the dying macrophages (shown by the arrow) and macrophages in alveoli with destructive changes (red arrow). (h) showing early cell destruction evident by karyorrhexis (black arrow) and neutrophils adjacent to dying macrophages (red arrow). (i) foamy macrophages with early destructive changes are present in alveoli.
**Expansive lesions with no necrosis week 37-41**

Lesions in these mice occupied most of the lung parenchyma but did not show extensive destructive changes or areas of focal necrosis (fig. 22a, c). The bronchial epithelium in most cases was intact (fig. 22b).

![Images](image1.png)

*Figure 22: (a) Diffuse lesion with no destructive changes or focal necrosis, (b) Note intact bronchial epithelium, (c) No destructive changes seen in the diffuse lesion*

**Expansive lesions with necrosis week 37-52**

These lesions showed regions of focal necrosis (fig. 23c, d). Destructive changes involved most of the lesion area. Completely sloughed off bronchial epithelium with cellular debris and proteinous material in the lumen could be seen. 2 mice had complete bronchial occlusion (fig. 23b). Normal looking parenchyma surrounding the necrotic foci was infiltrated by neutrophils.

![Images](image2.png)

*Figure 23: (a) lymphocytes islets small and loosely aggregated, (b) complete bronchial obstruction (black arrow) surrounded by necrosis (red arrow) (c) Focal necrosis (d) tissue with early destructive changes containing foci of necrosis.*

**Lesions in surviving mice week 57-71**

By this time most of the sick mice have died or sacrificed and relatively healthier mice survived. The lesions were expansive, diffuse as mentioned in the previous phase, and involved about 80% of lung parenchyma (fig. 25a). The inflammatory cells consisted mainly of foamy macrophages and lymphocyte islets. As compared to previous phase, the lymphocyte islets were larger in size and were tightly packed, and the lymphocyte area was more or equal...
to macrophage area. Macrophages had a foamy appearance, the necrotic foci were visible, however these areas were smaller than in the very sick mice mentioned above (fig. 24b). Bronchial occlusion was not seen, and bronchial epithelium did not exhibit destructive change (fig. 24c).

![Figure 24: (a) Diffuse lesion with intact bronchial epithelium. (b) Foci of necrosis could also be seen in survival phase mice, (c) note intact epithelium.](image)

Bacterial burden measured by Colony Forming Units (CFUs)

CFUs in the lungs of mice increased progressively until week 20 (fig. 25). From week 33 onwards, the CFUs were rather stable with a significant increase (p<0.05) at week 41, when there was a sudden change in histopathology and mortality. CFUs were significantly less in mice at week 57 and 71.

![Figure 25: Colony forming unit of MTB in lungs over the course of slowly progressive TB in weeks. The error bar indicates standard error of the mean.](image)
Acid Fast Bacilli:

Figure 26: The acid-fast bacilli (AFB) count measured as percent total positive pixels count in the lesion area of mice during the course of infection. Error bars indicate confidence interval of the median.

Weeks after infection: 8-12, 16, 20, 24, 33, 37, 41, 57, 71

- Week 8–12, when lesions were small, and lesions were not organized, AFB count was very small.
- Week 16, when well-demarcated focal lesions begin to appear, the AFB increased as well.
- Week 24 and 33, AFB counts decreased until week 33. From week 37 an increase was observed corresponding to an increase in mortality and expansion of lesions. In the surviving mice at weeks 57 and 71, AFB count was noticeably low.

Figure 27: The acid-fast count measured as percent total positive pixels counts in various types of lesion grouped according to lesion morphology. Error bars indicate confidence interval of the median.

- Week 8-12, 16-33, 37-41, 57-71

Figure 26, shows the counts of acid fast bacilli (AFB) in the pulmonary lesions of mice during the course of infection. In the week 8 – 12, when lesions were small, and lesions were not organized, AFB count was very small. In the week 16, when well-demarcated focal lesions begin to appear, the AFB increased as well. However, at week 24 and 33, AFB counts decreased until week 33. From week 37 an increase was observed corresponding to an increase in mortality and expansion of lesions. In the surviving mice at weeks 57 and 71, AFB count was noticeably low.

Figure 27, shows the counts of AFB in various types of lesion grouped according to morphology. The AFB increased as the lesions matured into well demarcated lesions with macrophage and lymphocyte aggregates. AFB were seen mainly in the macrophages. Cells present in alveolar spaces also had AFB. Expansive lesion without foci of necrosis contained very small amount of AFB. An increase by 9 folds was observed in lesions containing necrotic foci. Figure 28 (f, g) shows necrotic foci with tremendous AFB.
Figure 28: Few AFB were detected (arrow) in small diffuse lesions (b) increase in AFB count was observed when granulomas were organized in well-demarcated lesions, (c) macrophages in alveoli were seen containing AFB in well-demarcated lesions, (d) AFB detected in macrophages in alveoli in expansive lesion with no necrosis, (b) comparatively few AFB detected in expansive lesions with no necrosis (f) one mouse with necrosis involving the whole parenchyma had very high AFB count in expansive lesions with necrosis (g) foci of necrosis full of AFB (h) total bronchial obstruction with cellular debris containing AFB (arrow) seen in expansive lesions with necrosis (i) foci of necrosis in the tissue also had more AFB as compared to necrotic area of survival mice. (j) surviving mice containing very less AFB, (k) necrotic foci in surviving mice had less AFB when compared mice with necrosis mice.
Mycobacterial antigens

Pattern of staining
MTB antigens were seen as present only in foamy macrophages. The pattern of staining was mainly granular in the cytoplasm (fig. 29a). Clusters of macrophages staining positive were seen in the alveolar space (fig 29b). Within lesions, macrophages in close vicinity of lymphocytes were seen containing more antigens (fig. 29c). Very often macrophages residing in alveoli of normal looking parenchyma were also seen containing the antigens. Different staining intensities were measured as strong, medium and weak positive pixels. Macrophages staining positive for antigens contained variable amount and different intensities of antigens. Some macrophages showed diffused granular staining completely filling the cytoplasm and some have lesser focal staining in parts of the cytoplasm which could be indicative of antigens contained in the sub cytoplasmic vacuoles (fig. 29d). Macrophages staining for different intensities of antigens were present at all time points but with varying frequencies. Larger lesions had comparatively more antigens containing macrophages compared to smaller lesions. Macrophages in vicinity of larger islets of lymphocytes seem to contain more antigens.

![Figure 29: (a) granular staining seen in macrophages completely filling the cytoplasm, (b) clusters of macrophages seen in alveolar space, (c) macrophages in vicinity of lymphocytes contained more stain, (d) different amount and intensity of stain present in different macrophages.](image)

Mycobacterial antigens during the course of the disease
Figure 30 shows the various mycobacterial antigens during the course of infection. During week 8-12, all antigens were very low. From week 16 all antigen increased. The expression of BCG and cell-wall antigens was stable throughout the course of the infection. The total secreted antigens increased significantly at week 41, followed by decline at later time points. However, the four individual secreted antigens, MPT51, MPT53, MPT63, MPT64 did not follow this pattern. MPT64 was expressed stably throughout the course after week 16 with no peaks in expression. The expression of MPT46 was high in the later course of the disease at week 33 and showed a peak expression at week 41.
Figure 30: Expression of various mycobacterial antigens during the course of the disease shown in weeks and measure in score. Error bars indicate confidence of interval for the median.
**Mycobacterial antigens according to lesion morphology**

Figure 33-36 shows the mycobacterial antigens expression according to the lesion morphology. The expression of all antigens had significant positive correlation ($r < 0.05$) (table 2). All antigens increased during the course of infection and in various lesions but the magnitude of increase was different. There was significant difference ($p < 0.05$) in expression of cell-wall, total secreted antigens, MPT51, MPT53, MPT63 and MPT46 with a change in the lesion morphology. BCG and MPT64 did not show significant difference (fig. 32). BCG, total secreted, individual secreted and MPT46 antigens were higher compared to cell wall antigens. Throughout the course of infection strong-intensity secreted antigens were higher as compared to the strong-intensity cell wall antigens (fig. 31).

Expression of all MTB antigens was little in the early lesions of the infection at week 8 and 12. Among well-demarcated focal lesions between week 16-33, all the antigens were detectable. There was significant increase in the levels of all antigens as compared to early lesions ($p<0.00$). Levels of secreted antigens was 6-fold more than cell-wall antigens. Among individual antigens, MPT46 was expressed at the highest level followed by MPT64.

Among expansive lesions without necrosis during week 37-52, expression of all antigens was significantly less as compared to the lesions with necrosis ($p<0.05$) except for MPT64, MPT46 and MPT53. The secreted antigens were approximately 10-fold higher than cell-wall antigens. The expression of total secreted antigens was higher than cell-wall antigens. MPT46 expression was highest among all antigens.

Among expansive lesions with necrosis during week 37-52, the level of expression of total secreted antigens was significantly higher than the cell-wall antigens. The total secreted antigens increased to more than 20-folds whereas cell-wall antigens increased to < 10-folds. The pattern of intensities of staining for cell-wall and total secreted antigens was reverse. For total secreted antigens, strong-intensity antigens were significantly higher than weak-intensity antigens, while for cell-wall antigens, weak-intensity antigens were higher than the strong-intensity indicating more accumulation and higher amount of secreted antigens as compared to cell-wall antigens (fig. 31). The expression of BCG was the same as expression of cell-wall antigens. Among individual secreted antigens the expression of MPT63 was highest. Among all the antigens the expression of MPT46 was highest in these lesions. The expression of MPT46 was also highest for strong-intensity antigens.
Among the surviving mice at week 57-71, the expression of all antigens was significantly lower in the lesions as compared to the necrotic lesions (p<0.05) except for MPT64. MPT64 seemed to be expressed at more or less the same level throughout the course of the infection.

Figure 31: Expression of strong, medium and weak intensity antigens for total secreted and cell-wall antigens according to lesion morphology
Figure 32: The comparison of differential expression pattern of various mycobacterial antigens according to lesion morphology.

Figure 33: Expression pattern of various mycobacterial antigens in well demarcated lesions.
Figure 34: The expression pattern of various mycobacterial antigens in expansive lesions with no necrosis.

Figure 35: The expression pattern of various mycobacterial antigens in expansive lesion with necrosis.

Figure 36: The expression pattern of various mycobacterial antigens in lesions of surviving mice.
Comparison of mycobacterial antigens with CFUs and AFB

From week 8-24, one lung from each sacrificed mouse was used for histopathology and the other for CFUs. From week 29 onwards, separate mice from the same time point were used for histology and CFUs, therefore direct correlation of CFUs with necrosis was not possible. Mortality was generally high from week 37, we have merged the phase of necrosis and diffuse lesion for description of CFU.

In the early small diffuse lesions, CFU increased but there was no increase in AFB and antigens. With progression of infection when the lesions attained well-demarcated focal morphology, there was an increase in the levels antigens, 1.5 log increase in CFU and approximately 1-fold increase in AFB.

With the shift in lesion morphology towards necrosis at week 37 and increased mortality, the tremendous increase in mycobacterial antigens and approximately 7-fold increase in AFB was in sharp contrast to slight increase of less than one log in CFU. Total secreted antigens increased significantly (p<0.05) along the course of infection with appearance of necrosis in the lesions while CFUs did not show similar high increase. There was no significant correlation between CFU and total secreted antigens (r >0.05). Suggesting that increase in antigens level was not parallel to viable MTB in lung and mycobacterial antigens might be accumulating in the foamy macrophages leading to their increased amount. This implies the critical role of the accumulation of secreted mycobacterial antigens in the foamy macrophages rather than an increase in the number of live bacilli in tissue destruction, necrosis, and disease severity.

Table 2: Correlation between various mycobacterial antigens, AFB and CFU during the course of the disease.
DISCUSSION
While a substantial information is available on the immune response involved in primary TB leading to granulomatous immune response, little information is available on the immune mechanisms involved in the development of cavitary lesions, a hallmark of human post-primary TB.

Animal models are important to study in vivo immune responses. They give flexibility to change components of host response in physiological settings that are hypothesized to be involved in disease pathogenesis. It is believed that immune response in murine TB disease is similar in many regards to that in human making it a useful model to study specific questions about the human TB disease (71). Murine models of slowly progressive TB and reactivation TB mimics stages of post-primary TB in humans in some aspects. Inflammation pattern is not granulomatous, it is a pneumonia of MTB infected foamy macrophages and the infection is restricted to the lungs. The alveolar septae remains intact with macrophages and other mononuclear cells distributed among air sacs giving lung a honeycomb appearance. The disease progresses for many months without clinical symptoms, then rapidly undergoes necrosis and kills its host like in humans. (8, 71). These similarities make it a useful animal model to study the pathology of post-primary TB.

We have previously developed a mouse model of slowly progressive TB by low dose infection (50). In this model the disease begins as pneumonic infiltration of macrophages and lymphocytes and during the course of the disease, foamy macrophages accumulate MTB antigens resulting in extensive inflammation and tissue destruction leading to necrosis. Necrosis is believed to be the precursor of cavitation in the lesions. In this study, we re-analysed the biopsies to study the expression pattern of various mycobacterial antigens during the course of the slowly progressive TB and correlated with the clinical course of the disease, lesion morphology and tissue destruction. The course of the disease was divided into five distinct stages based on the detailed morphological pattern with the focus on the tissue destruction. When lesions were early and diffuse until week 12, mice were clinically healthy. Early inflammatory changes were seen in lung tissue. Macrophages were seen intermixed with lymphocytes, there was little or no accumulation of antigens in macrophages. There was an exponential increase in CFUs, little or no AFB were detected in macrophages. When lesions become well-demarcated from week 16 to 33, mice started developing signs of illness, but
mortality was low. Lesions became organized, well-demarcated, lymphocytes and macrophages form separate aggregates. Antigens started to accumulate in foamy macrophages, with marked accumulation of MPT46 and secreted antigens. There was an approximately 1.5 log increase in CFUs and less than 2-fold increase AFB, and a 10-fold increase in total secreted antigens and a 4-fold increase in cell-wall antigens. From week 37, there was a sudden increase in mortality and morbidity. There was spread of inflammation which occupied almost 80% of lung parenchyma. Between week 37-41, mice showed two type of lesion morphology and were grouped into two based on tissue destruction and necrosis. In one group large regions of focal necrosis, destructive changes and bronchial obstruction was seen. These were very sick pre-terminal mice. In these mice, antigens accumulation, CFUs, and acid-fast bacilli were highest as compared to earlier time points and as compared to mice with expansive lesions with no necrosis. However, increase in mycobacterial antigens was many folds in these mice (secreted antigens increased by more than 20 folds and cell-wall antigens by approximately 5 folds) as compared to rather modest increase in CFUs, and a moderate increase in AFB approximately 8 folds. Comparatively healthier surviving mice of week 57 and 71 also had the diffuse lesion and focal areas of necrosis but overall lesion did not show destructive changes. The levels of mycobacterial antigens accumulation in these lesions were less as compared to lesions with necrosis supporting the role accumulated MTB antigens in tissue destruction.

Progressive destruction in pulmonary TB lesions does not occur due to increase number of viable bacilli but as a result of immune response towards MTB antigens (72). The tremendous increase in secreted mycobacterial antigens exclusively in necrotic lesions suggests a relation between accumulation of secreted mycobacterial antigen and tissue destruction and disease severity. In a previous study, on human material, similar findings have been observed. Severe pulmonary disease with pneumonic lesions contain higher levels of secreted antigens, while mild lymphadenitis disease with granulomatous lesions contain very little antigens. Granuloma formation is believed to be a successful immune response that contains the infection and controls bacillary growth. Furthermore, secreted antigens are expressed in the necrotic centre and not in the granulomas cells (51). Secreted antigens of mycobacteria are most immunogenic proteins and are linked to the bacterial virulence (73, 74). In our study, secreted antigens seem to accumulated over the course of the disease, as indicated by an
increase in the levels of strong-intensity secreted antigens. The cause of necrosis in pneumonic infiltration of foamy macrophages in post-primary TB has been hypothesized to be an interaction of accumulated MTB antigens with host lipids (66). From this study it can be hypothesised that accumulation of secreted antigens in the foamy macrophages reaches a threshold where it triggers the immune response towards tissue destruction.

Mice at week 52 and 71 was an interesting group as they had survived the infection and even though the inflammation had involved more than 4/5 of lung parenchyma, the extent of necrosis was less. The secreted antigens did not accumulate to the same extent as in the high mortality phase of infection. These findings support the role of mycobacterial antigen accumulation, rather than extent of inflammation, in the tissue destruction and disease severity.

Among all the antigens, MPT46, was the most abundantly expressed antigen throughout the course of the disease. Interestingly, there was a marked increase when necrosis occurs in the lesions. MPT46 is found in the culture filtrate of Mycobacterium tuberculosis. Localization index of individual proteins and two-dimensional electrophoresis, which are used to classify proteins as secreted or non-secreted could not categorize MPT46 in either of the two categories (14, 68). A study has demonstrated that MPT46 is the functionally active thioredoxin (Trx) of MTB and function as active disulphide reductases (75, 76). Trx plays an important role in cellular redox balance. Trx can switch between oxidized disulfide and the reduced dithiol forms. Trx is a natural substrate for thioredoxin reductase, reduces the oxidized form of Trx in NADPH dependent manner. The reduced dithiol form of Trx has a variety of physiological functions including cellular DNA synthesis, protein repair, cellular protein disulphide reduction, detoxification of reactive oxygen species, regulation of transcription factor and cell death pathways. Trx acts as a substrate for many enzymes including ribonucleotide reductases, thioredoxin peroxidases and methionine sulfoxide reductases (77). Thioredoxin system is involved in many important physiological functions and for these reasons has been an attractive target for anti-tuberculous drugs (78). The absence of glutathione reductase systems, an antioxidant system, in MTB makes thioredoxin system essential for survival under oxidative stress (37, 79). The genome of MTB can encode for one copy of thioredoxin reductase and three thioredoxins namely, Trx A (Rv 1470), Trx B (Rv 1471) and Trx C (Rv 3914). MPT 49 is Trx C of MTB. When tested for expression of Trx genes in
various growth conditions, TrxC was expressed in all applied oxidative stress conditions (76). Human Trx has shown to exhibit immune modulatory actions. Human thioredoxin is a potent inducer of IFN-γ gene expression and drives the immune response towards Th1 immunity (80). It has been shown to increase the upregulation of IL-12 production by monocytes. Human peripheral blood mononuclear cells have shown to increase the production of IFN-γ when treated with thioredoxin in the presence of IL-2 (81). Immune modulatory role of MTB thioredoxin is not known. Based on our findings, it can be hypothesised that MTB overexpresses thioredoxin in the stressful conditions of host immune response and is essential for bacterial survival and persistence and host tissue destruction. This enzyme may have a central role in the formation of cavities in human lesions leading to bacterial transmission.

Among individual secreted antigens, MPT63, was highly secreted in necrotic lesions. MPT63 is among the three most abundantly secreted antigens in culture fluid of MTB. The other two are parts of antigens 85 complex, MPT 59 (85B) and MPT45 (85C). The gene coding for MPT63 is found only in mycobacteria of *M. tuberculosis* complex and no homologs in non-tuberculous mycobacteria were found. The function of MPT63 is not known. However, it is an immunogenic protein as it is shown to elicit humoral immune responses and DTH response in guinea pigs infected with *M. tuberculosis* (82, 83). MPT63 induces immune response during TB and its high expression in necrotic lesions suggests its role in MTB virulence.

MPT64 (Rv 1980c) is an actively secreted, immunogenic protein, found in culture filtrate of MTB (84). The expression pattern of MPT64 was different than other MTB antigens. MPT64 antigens started to express from week 16 and was constantly expressed throughout the course of the infection with no significant changes related to lesion morphology. In our previous study on MTB antigens expression, MPT64 was the only secreted antigen that was detected in granulomatous lesions of lymph nodes, suggesting that it is accumulated in granuloma structures (51). It is not found in mycobacterium other than tuberculosis and is highly specific for *Mycobacterium tuberculosis complex* (85, 86). The exact physiological function of MPT64 is not known as MPT 64 and its homologs share no evident sequence homology to other proteins of known structure or function. It is expressed by actively dividing MTB, is one of the predominant proteins among more than 33 actively secreted proteins, and accounts for 8% of the total protein found in culture filtrate (87-90). This is highly
immunogenic, and shows strong reactivity in sensitized guinea pigs, it showed strong reactivity (68). As MPT64 is signal peptide secreted from the cell it is not likely to be involved in cell haemostasis (90). Loss of gene region encoding for MPT64 from BCG progenitor has been correlated to drop in its virulence (91). The accumulation of MPT 64 over disease course and in granulomas suggests a role in the persistence of chronic infection and its role in MTB virulence, but probably not a major role in tissue destruction.

In-house rabbit polyclonal antibodies were used in the study. Immunogens for these antibodies were bacterial sonicate for cell-wall antigens and for other antigens it was 5 weeks old culture fluid of MTB with minimal lysis and MTB antigens extracted and purified from this culture fluid (68). Immunogens while separation from culture fluid, preparation and deliverance might have acquired minor impurities. However, polyclonal antibodies produced by the immune system are largely directed towards dominant injected immunogen. Using this knowledge, we can assuredly rely on the specificity of these antibodies.

We have developed a new method to quantify Ziehl Neelsen stained slides by digital image analysis using colour deconvolution algorithm. The algorithm is provided with the advantage that it can read pixels of any range of given RGB values. Reading AFB stained slides digitally is believed to be more accurate, precise and reproducible and less subjective to human error. We have maximized the accuracy of the algorithm by comparing the mark-up image under an optical microscope. In the revolutionary world of pathology, this can help in automation of reading Ziehl Neelsen stained slides and will increase capacity and reproducibility.
CONCLUSION
This study shows that mycobacterial antigens accumulate in the foamy macrophages in the TB lesions over the course of infection in the slowly progressive murine pulmonary TB. The accumulation of total secreted mycobacterial antigens, and particularly antigen MPT46, are correlated with necrosis, tissue destruction, and mortality rather than an increase in CFUs, AFB or the extent of inflammation. Tissue destruction and necrosis is assumed as the precursor of cavitation, thereby implying the role of MT46 in formation of cavities in TB disease.
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