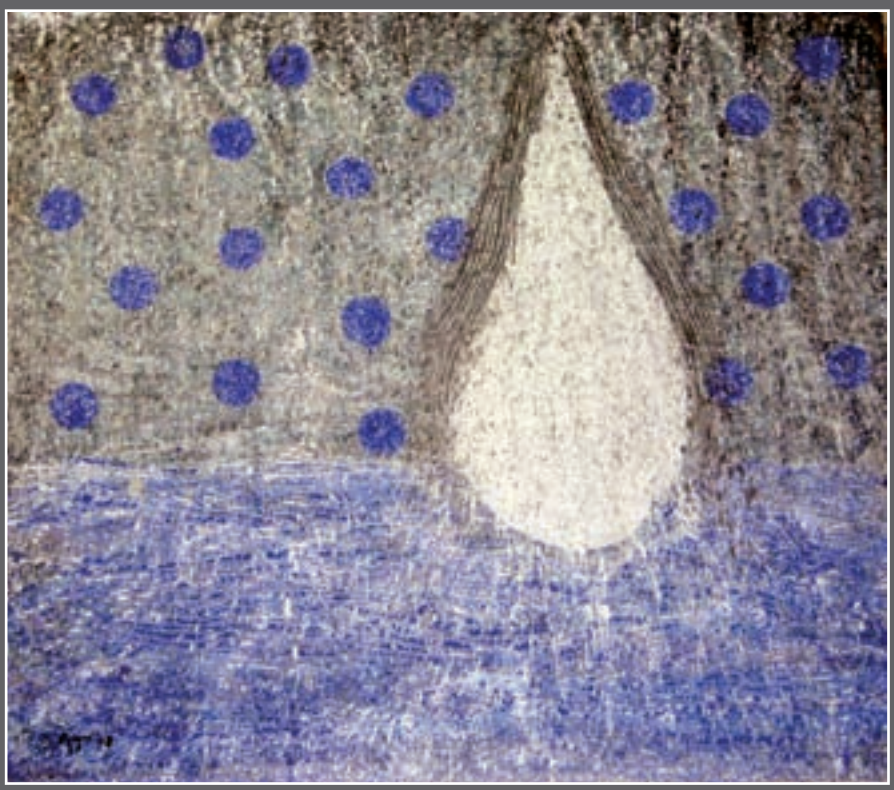
The mechanism used by MTB to evade the immune response is as yet unknown, thus the development of a protective TB vaccine is still a challenge



Mystery – Aziyadé Ruiz Vallejo
Oil/canvas
(165 x 210 cm)

'Apart from the known and the unknown,
what else is there?'

The Homecoming **(Play)– Harold Pinter**

Aziyadé Ruiz Vallejo
Camaguey, Cuba

Aziyadé Ruiz Vallejo graduated from the School of Art (Camaguey) and from the Higher Institute of Art (La Habana), both in Cuba. She has conducted solo exhibitions and taken part in group exhibitions held in various countries around the world. For the International Workshop of Tuberculosis Vaccines held at Varadero, Cuba in 2007, she created a mural. She is a member of UNEAC.

CHAPTER 4

Proteomics Studies and Antigen Discovery

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Introduction

The identification of antigens and other immunogenic components that have a fairly similar expression in different clinical isolates of *Mycobacterium tuberculosis* (MTB) is a key component of the development of new and better vaccines and diagnostic tests for tuberculosis (TB).

Proteomics studies on clinical and laboratory strains of MTB represent a good approach to identify potential antigens that are well represented within different isolates and also allow the identification of the most abundant proteins expressed by this bacillus. This is one of the first steps to identify immunogenic proteins that are recognized for the majority of people infected with MTB and can be selected to be tested for protective immunity.

Comparison of MTB and BCG Proteomes

The identification of virulent MTB specific proteins is also important since it has been hypothesized that proteins present only in MTB but not in Bacillus Calmette-Guerin (BCG) or other avirulent strains are good targets for vaccine development (1).

In this respect, proteomes of MTB H37Rv and BCG have been compared by 2D gel analysis and spots unique to MTB have been identified (1, 2).

MTB specific proteins were identified as L-alanine dehydrogenase (40 kDa antigen, ald, Rv2780), isopropyl malate synthase (Rv3710), nicotinate–nucleotide pyrophosphatase (Rv1596), Mpt64 (Rv1980c), and two conserved hypothetical proteins (Rv2449c and Rv0036c) (1).

Esat-6, Cfp10, and other Esat-6 like proteins were also identified only in MTB (2). In addition, other proteins that are not predicted to be absent in the BCG genome were identified only in MTB and include the chaperone GroES, elongation factor EF-Tu (Tuf), Rv3269, and seven hypothetical proteins (Rv0020c, Rv1684, Rv1893, Rv3046c, Rv3881c, Rv1198, and Rv1793).

Proteomes of MTB H37Rv and *M. bovis* BCG have also been compared using a complementary approach of 2D gels and Isotope-coded Affinity Tag Technology (ICAT) (3). In that study some of the proteins predicted by genomic analysis to be unique to H37Rv were confirmed (i.e. Rv0223 and Rv1513). In addition, Rv0570 was also absent in the BCG proteome. These three proteins represent potential vaccine candidates; however, they have not been characterized in the context of immune response.

Interestingly, levels of L-alanine dehydrogenase (Ald) and elongation factor EF-Tu have been shown to be higher under low oxygen conditions suggesting that this protein is being expressed during infection (4, 5). However, a recombinant BCG expressing the Ald gene did not improve the protective response observed with BCG only (6).

On the other hand, the identification of Mpt64 as a unique MTB protein has led to the development of new recombinant BCG vaccines that are currently being evaluated for protection (7, 8). Mpt64 is also considered one of the proteins with the most potential as a protective antigen, in addition to Ag85B, the 38kDa antigen, Esat-6 and Mtb8.4 (7, 9–12).

Particularly, a recombinant BCG vaccine overexpressing Ag85B, Mtb8.4, and the peptide 190–198 of Mpt64 was found to have protective levels in the mouse model similar or better than BCG or recombinant BCG overexpressing only Ag85B (7, 13).

GroES is also an immunodominant protein and has been proposed as a good vaccine candidate (14). In a recent study however, this protein—used as a single antigen—failed to induce both IFN-γ and TNF-α, thus questioning its use as a potential vaccine antigen (15).

Proteins Expressed during MTB Infection

Identification of proteins induced or repressed under different conditions mimicking the host cell environment has also been achieved using proteomic approaches. Recognition of proteins that are being expressed by MTB in vivo,

and thus have a major potential for being recognized by the host, is important for the development of vaccines and immunodiagnostic tests.

Proteins such as the 45kDa antigen (ModD), Mpt64, and Tig, a protein similar to the trigger factor, are less abundant during starvation, suggesting that their expression during natural infection might be low. Meanwhile, two hypothetical proteins—Rv2557 and Rv2558—in addition to HspX, were found to be induced under nutrient starvation conditions (16).

In another study, proteins induced by either superoxide or nitric oxide radicals were detected. Reactive oxygen species and nitrogen intermediates are both believed to have a role in the immune response against TB. DnaK and other heat shock proteins were induced after exposure to menadione, a compound that increases intracellular superoxide levels (17).

However, DnaK as well as GroEL—both chaperone proteins—induce TNF-α in PBMCs (Peripheral Blood Mononuclear Cells) from TB patients but failed to stimulate IFN-γ. In addition, they induce the production of IL-10 suggesting that these chaperone proteins, although immunodominant during natural infection, might not be good vaccine candidates. In contrast, the same study showed that Ag85B, Mpt64, and Esat-6 induced high amounts of IFN-γ and failed to induce IL-10 (15).

Recently, proteomes from intracellularly growing mycobacteria were obtained and compared to in vitro growing MTB. From this study, 11 proteins were found only in intracellularly growing MTB including several enzymes involved in cell metabolism such as Cystathionine (beta)-synthase (CysM2), GMP synthase (GuaA), malate dehydrogenase (Mdh),and phosphoglycerate mutase I (Gpm) among others. A hypothetical protein, Rv1130, was also detected in eight different spots unique to intracellular mycobacteria (18).

In another study, proteins induced upon phagocytosis of BCG by THP-1 cells were detected. These included HspX, chaperonines GroEL1 and 2, and elongation factor EF-Tu (19).

Some of the proteins that are recognized during in vivo growth or during growth in conditions mimicking the host environment correspond to heat shock proteins. Overexpression of these proteins has also been shown to reduce survival of the mycobacteria during the chronic phase of infection. In particular, the reduction of bacterial load in the spleen of infected mice was similar to what is obtained after immunization with BCG (20). IFN-γ was also induced in splenocytes from BCG-vaccinated mice after stimulation with the heat shock protein Hsp70, which directed the authors to conclude that heat shock proteins could be used to complement novel vaccines against TB. There is also an excellent review on HSPs as potential vaccine antigens (14).

Secreted Proteins of Laboratory and Clinical MTB Isolates

For many years proteomics studies have been focused on the characterization of the proteome of different subcellular fractions of MTB, specifically of the laboratory strain H37Rv secreted proteins (2, 21–25).

Secreted proteins are considered to be important T cell antigens (26). Therefore, the identification of the most abundant secreted proteins by MTB is very relevant. Ag85 complex, Mpt63, Mpt64, Mtc28, GroES, SodA, DnaK, and members of the Esat6 family are within the most abundant proteins in the secreted fraction of H37Rv (24, 25).

Using a shotgun strategy we determined that Esat-6 is also the most abundant protein released from the cell wall after a solubilization treatment, while GroES seems to be the most abundant protein in both secreted and cytosolic fractions. Interestingly, an acyl carrier protein (AcpP, Rv2244) was found within the most abundant proteins in all three subcellular fractions (soluble cell wall, cytosol, and secreted) (Unpublished data).

Mpt63 (Rv1926c) induces antibody responses in the guinea pig model (27). This antigen also shows Th1 cell reactivity in BCG-vaccinated and TB-infected individuals. In addition, an '*in silico*' analysis indicated that Mpt63 is a promiscuous antigen in terms of its binding to different HLA-DR types and therefore has potential for vaccine development (28).

Comparison of protein levels in clinical isolates is important in the identification of possible vaccine and diagnostic antigens. The K-strain is the most prevalent clinical isolate in Korea and belongs to the Beijing family (29); therefore, proteins expressed in high quantity by this and other strains represent potential antigens for vaccine and immunodiagnosis.

Three proteins (Cfp10 (Rv3874), Rv0560c, and Rv3648c) have been shown to be more abundant in the clinical isolates CDC1551 and K-strain when compared to H37Rv (30).

Rv0560c is a possible methyltransferase and has also been shown to be more abundant during anaerobic conditions (5); however, its role in the immune response against TB has not been evaluated.

Rv3648c corresponds to the cold shock protein CspA which has been shown to induce DTH response in guinea pigs and induction of IFN-γ from TB infected splenocytes in mice (31).

Cfp10 has been shown to be recognized by sera from TB patients and has also been used in the development of new diagnostic tools and recombinant vaccines (32). A DNA vaccine that overexpresses Cfp10 has been used alone and in a cocktail including Ag85B and Cfp21 to immunize mice. The cocktail preparation showed potential as vaccine development in terms of T cell

proliferation and colony-forming unit (CFU) reduction; however, the levels of protection were similar to that of BCG (32).

More recently, using a CD4+ T cell expression cloning strategy, Cfp10 was recognized by T cell clones after exposure to dendritic cells (DC) from mice that were previously fed with rCFP10 *E.coli*. Using this same strategy, hundreds of pools corresponding to TB recombinant proteins were analysed, and a novel antigen (Mtb9.8)—in addition to previously characterized TB antigens (i.e. Ag85B, Esat-6, Mpt83, 19KDa, and HspX)—was identified (33). Mtb9.8 corresponds to Rv0287c (EsxG), a member of the Esat-6 like proteins.

Secreted proteins of H37Rv and CDC1551 had been studied previously showing that only 13 proteins, from over 1,500, were found to be differentially expressed between these strains. Interestingly, most of the differences were due to absolute absence/presence of specific proteins rather than differences in quantity (23). This is consistent with the comparison of MTB H37Rv and Erdman which revealed 16 differential proteins (1). This is of importance because proteins that are present in only some virulent strains might not represent good candidates for diagnosis and vaccine development.

Proteomes of strains that belong to the Beijing and family 23 (F23) lineages have been analysed and compared to H37Rv. In this study, Pheiffer and co-workers (34) found eight protein spots that were more abundant in the whole cell lysates of clinical isolates (Beijing and F23) in relation to H37Rv. Interestingly, seven of these were identified as α-crystallin (HspX). The remaining spot contained two proteins: Rv2005c and the 35kDa antigen Rv2744c.

Hypothetical protein Rv2005c, a protein showing similarity to Universal Stress Proteins (USP), has also been shown to be expressed specifically in anaerobic conditions, suggesting that it is expressed during natural infection and could play a role in the virulence and survival of the mycobacteria (5).

Three spots identified as Ag85A had decreased intensity in the culture filtrates of both clinical isolates as compared to H37Rv. In addition, levels of PstS1 were lower in the Beijing strain and presented the most marked difference between Beijing and F23 strains. Interestingly, lower expression of this protein correlated to decrease recognition by sera from Beijing-infected patients (34).

This correlates to previous findings in which reactivity of TB patients sera towards PstS1 (38KDa antigen) was very variable (22). In addition, we have found that this protein is abundant in CDC1551 strain, but is present in lower levels in a cluster of closely related clinical isolates (Unpublished data).

This suggests that this antigen is either not consistently expressed during infection or it is not a good immunoreactive protein.

Variable gene expression in clinical isolates has been noted previously, specially in genes associated with T cell antigens, PE and PPE family, and genes involved in lipid metabolism, all of which are thought to be important

for host–pathogen interactions (35, 36).

Due to its variability and relatively high number of genes in the TB genome, members of the PE and PPE families have been postulated to be involved in antigen variation as a mechanism to evade immune responses (37, 38).

One of the PPE genes (i.e. PPE44) bears nucleotide substitutions and has a significant higher expression in Beijing isolates as compared to non-Beijing strains (39).

PPE44 has also been evaluated as a potential vaccine candidate and it has shown to induce strong cellular and humoral responses in mice. PPE44 was able to induce high levels of IFN-γ in both acute and latently infected mice, in comparison to Ag85B and Esat-6 specific IFN-γ which decreased during latency. PPE44 alone induced similar protection against the MTB challenge when compared to BCG. These data suggest this protein could be a good candidate for a subunit vaccine against TB (40).

In a comprehensive study, Bertholet et al. (41) evaluated the protective response of 49 potential antigens in the mouse model. The selection of antigens was based on previous information about genes that were required for growth in macrophages, genes that were differentially expressed in response to oxygen and carbon limitation, members of immunogenic families such as EsX and PE/PPE, and finally those identified as secreted proteins by proteomic analysis. Twenty-eight of these antigens were recognized for all PPD+ donors used in the study, suggesting that these proteins are consistently immunodominant. Interestingly, seven of these were PE/PPE or EsX proteins, six were hypoxia-associated, five were secreted/membrane proteins, and four were associated with survival in macrophages. Some of these antigens are already under evaluation as novel vaccines, including Ag85B, Mpt64, and Mpt83 from the secreted fraction and Esat-6 from the EsX proteins.

Using a different strategy, one of the EsX proteins (Rv0287, EsxG) was recently shown to be recognized by different T cell clones (33).

In addition, other members of the EsX family (Rv2364c, Rv2347c, Rv3619, and Rv3620) have shown Th1 cell reactivity when an overlapping synthetic peptide approach was used (42).

Two PE/PPE (Rv3478, Rv2608) proteins presented the highest reduction in bacterial load after aerosol challenge. Rv3478 was also within the proteins that were recognized by all PPD+ donors, indicating that proteins from this family are promising antigens for vaccine development.

One of these proteins, PPE68 (Rv3873), is encoded in the RD1 region. As with other members of RD1, such as Cfp10 and Esat-6, PPE68 has been shown to induce high levels of IFN-γ in PBMC from TB patients and PPD+ donors. (43, 44) In addition, Mustafa et al. (45) showed that part of the most

immunodominant peptide of PPE68 is also present in several other PPE proteins, suggesting that this peptide could be a good candidate for a subunit vaccine against TB.

In this regard, other PE/PPE proteins have been analysed in terms of their role in immune responses. In addition to PPE44 that was mentioned above, PPE46 and PE_PGRS62 have also been studied.

Peptides from these two proteins show not only high affinity to MHC-I, but they also stabilized the complex on the cell surface significantly. In addition, they were able to stimulate splenocyte proliferation and IFN-γ production (46).

Rv1168c, another PPE protein, has been shown to be recognized by active TB patients with a higher immunoreactivity than Esat-6. In addition, this protein, called PPE17, was able to induce higher IFN-γ levels in TB-infected patients as compared to BCG-vaccinated individuals. This not only indicates that this protein is a good T cell antigen, but it also shows that PPE17 is an immunodominant protein that is recognized by the immune system during natural TB infection (47).

Studies on PE/PPE proteins and their role in the immune response during TB infection are very recent; however, this group of proteins seems to have potential for future developments towards the control of TB.

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