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In silico selected peptide antigens from mycobacterium tuberculosis: New perspectives for diagnosis and vaccine development

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ABSTRACT

Tuberculosis (TB) remains a global health problem and effective control of TB is dependent on the availability of efficient vaccines and diagnostic tests. However, the above require the identification of highly specific antigens of *Mycobacterium tuberculosis* (*M.tb*), that are safe enough to be used *in vivo* and structurally stable for broad technical application. However, the purified or recombinant antigens of *M.tb* are immunologically quite complex. The potential use of these reagents is also limited by the technical problems related to their production. HLA-promiscuous T-cell multi-epitopic peptides (HLA-p.T-c.m-EP) are designed on the basis of the prediction of sequences that bind to MHC molecules and their interaction with T-cell receptors in stimulating the immune system. The gain in time, cost and facile investigation provided by selection of such HLA-p.T-c.m-EP using bioinformatics, makes possible the analysis of the immunological aspect of a high number of *M.tb* gene products with the aim of better understanding their involvement in immune host defense against *M.tb*. This approach could also provide a rational basis for the development of a subunit vaccine for TB. Furthermore, the ability of these epitopic peptides to induce differential responses specifically to distinct stages of the disease might offer a relevant perspective for better diagnosis of TB. This review describes the structural and functional characteristics of HLA-p.T-c.m-EP and emphasizes their use as novel agents for development of diagnostics and vaccines.

KEYWORDS

Tuberculosis;
Diagnosis;
Vaccine;
Bioinformatics;
Epitope;
Peptides;
Antigens.

INTRODUCTION

Tuberculosis (TB) remains a global health problem with one-third of the world's population being latently infected with Mycobacterium tuberculosis (*M.tb*) and approximately nine million cases of active disease occurring each year[1]. It is responsible for more human deaths than any other single infectious agent, representing 26% of all preventable deaths and 7% of all deaths[1]. Effective control and future eradication of TB is dependent upon the availability of efficient vaccines and diagnostic tests. However, this necessitates the identifica-

tion of highly specific antigens of *M.tb* that are: (i) capable of inducing a detectable and functional response of the host immune system, (ii) safe enough to be used *in vivo* and (iii) structurally stable for broad technical application. In this context, recent findings in the domains of the genomics of *M.tb* and of human immunogenetics as well as the development of bioinformatic tools have made way for the design of HLA-p T-c m-EP derived from *M.tb* antigens. The successful use of these antigenic products in some recent studies predicts promising perspectives for their exploitation as biomarkers and targets for new diagnostic tests and vaccines against TB. The present article will review some of these aspects.

Interaction between *M.tuberculosis* and the host

1. *M.tb* and host cells: virulence factors

M.tb has evolved to avoid killing by the innate and the adaptive mechanisms of immune response by inducing chronic immunopathology. The later begins with the accumulation of macrophages at sites of bacterial multiplication to form compact granulomas that contain the pathogen[3]. A key aspect of granuloma formation is the development of fibrosis within the granuloma and in surrounding parenchyma, which produces macroscopic nodules (tubercles). In adults, the disease advances as a necrotizing pneumonic process that can involve bronchioles and results in the spread of infection to other areas of the lungs[4].

The ability of mycobacteria to survive within macrophages is the main strategy developed to circumvent the major killing mechanisms employed by macrophages and takes advantage of the enclosed environment within its host cell to avoid the antibody and complement mediated humoral immune response. In fact, mycobacteria not only have the ability to adapt to a changing host environment[5], but also actively interfere with the signaling machinery within the host cell to counteract or inhibit parts of the killing apparatus employed by the macrophage[6,7,8,9,10].

The critical point remains the identification of mycobacterial antigens selectively expressed by virulent mycobacteria and, in particular, those expressed during different steps of the infectious phases and pathogenesis.

2. Host response to natural infection by *M.tuberculosis*: correlate of protection

Mycobacteria primarily infect host macrophages, which represents the first line of cellular defense against microbial invasion. The first step in the encounter between the human host immune system and mycobacteria is the binding and uptake of pathogens by dendritic cells and macrophages via innate pattern recognition receptors (some of toll-like receptors, DC-SIGN and mannose receptors). In fact, activation of such receptors induces the early production of Interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) from specific phagocyte-subsets[11]. IL-12 then provokes the production of interferon-γ (IFN-γ) from natural killer (NK) cells[12,13,14,15,16]. Thus, the latter plays a key role in the activation, differentiation and expansion of antigen specific T helper-1 cells, in the early phase of the immune response. During the adaptive immune response, T helper-1 cells are the major source of IFN-γ that activates, in synergy with TNF-α, infected macrophages, thereby initiating a major effector mechanism of the cell-mediated immune response to control infections with mycobacteria. This mechanism is also necessary to control the chronic phase of infection[15]. Indeed, an effective cell-mediated immune response protects the host against a disseminating infection by containing mycobacteria locally inside well-organized granulomatous lesions[17,18,19].

In general, infection by *M.tb* is initially controlled by host defenses, and the infection remains latent. However, latent TB infection (LTBI) has the potential to develop into active TB (ATB) within 1-2 years in about 5% of infected cases and another 5% can develop active disease at any time during their life.

Diagnosis of tuberculosis

Because active TB is infectious and leads to the spread of M.tb, despite available efficacious treatment, the rapid diagnosis of ATB and LTBI is the most important component of TB control programs.

The gold standard for diagnosis of TB is the demonstration of the presence of mycobacteria in various body fluids. However, the sensitivity and the specificity of the two standard microbiological tests for the diagnosis of TB, i.e. the acid fast bacilli (AFB) stain and the culture techniques, are not satisfactory; corresponding to 20% and 80% for AFB and 60% and 99% for culture, respectively for sensitivity and specificity. In this scenario, diagnosis is often based on clinical signs and

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symptoms, X ray chest radiograph, tuberculin testing and history of contact with adult patients. However, this leads the under-diagnosis of TB.

On the other hand, significant improvement in the understanding of the molecular biology of *M.tb* obtained from the sequencing of its genome, has led to development of new diagnostic techniques for TB. In fact, because of the ability to detect even a single copy of *M.tb*, nucleic acid amplification tests (NAAT) and their modifications, have been welcomed as a revolutionary diagnostic tool, capable of reducing the time to diagnosis from weeks to hours of patients with suspected TB. Consequently they are being adopted in clinical practice[20]. Nevertheless, a statement from the U.S. Centers for Disease Control (CDC)[22] indicates that commercial NAAT should be used in addition to microscopy as tests in parallel to improve diagnostic certainty, pending culture results and/or patient's response to therapy. In addition, the CDC recommendations are limited to the two U.S. Food and Drug Administration-approved commercial NAAT[23]. Furthermore, the CDC strongly advises physicians to rely upon clinical evaluation in the interpretation of the NAAT laboratory data[24].

On the other hand, immune diagnosis is still a valuable way of detecting *M.tb* infection and TB disease. In fact, the host immune response to *M.tb* infection is an early reaction that can be induced and detected via peripheral immune pathways. Thus, the immunological approach could offer reagents for specific diagnosis of ATB and LTBI. The immune test commonly used for the evaluation of the immune response against *M.tb* is the Mantoux or tuberculin skin test, which consists in the measurement of the delayed type hypersensitivity (DHT) to the purified protein derivative (PPD). However, it represents the only medical tool used for the diagnosis of both ATB and LTBI and has significant limitations. In fact, the PPD used for the tuberculin skin test is an antigen-complex prepared from a crude precipitate of a filtered *M.tb* culture containing more than 200 antigens, most of which are widely shared among *M.tb*, and the *M.bovis* Bacillus Calmette Guerin (BCG) vaccine strain and other environmental mycobacteria. Thus, a positive PPD test may not distinguish between active disease, vaccination and exposure of healthy individuals to *M.tb*, environmental mycobacteria or other mycobacterial species. Further, in patients with ATB, the tuberculin skin test is 75%-90% sensitive, but among those with disseminated disease, this sensitivity falls to 50%, and is even lower in human immunodefeciency virus (HIV) positive patients with only mild degrees of immunosuppression.

Moreover, PPD is a crude preparation and its antigenic components are not standardized. The commercially available PPDs from different sources may vary in the antigenic content and therefore result in differences in the skin test response.

Nevertheless, a successful alternative has been provided by newly developed in vitro diagnostic blood tests. In fact the ability of *M.tb* of evoking a strong type-1 immune response, hence allowing the rapid detection of *M.tb*-specific IFNγ producing T-cells, has been exploited in the design of diagnostic tests for latent *M.tb* infections. In fact, since 2001, a new test (Quanti FERON-TB) that measures the release of IFN-γ in whole blood in response to stimulation by PPD was approved by the U.S. FDA[27]. The use of this test was comparable with the tuberculin skin test (TST) in its ability to detect LTBI. Advances in the area of genomics have led to the identification of antigens such as the early secreted antigenic target 6 protein (ESAT-6) and culture filtrate protein 10 (CFP-10). These proteins, encoded within the region of difference 1 (RD1) of the *M.tb* genome, are significantly more specific to *M.tb* than PPD, as they are not shared with BCG substrains or most environmental mycobacteria (with the exception of *M.kansasii*, *M.szulgai*, *M.flavescens*, *M.marinum*) [88]. Current evidence suggests that IFN-γ based assays using cocktails of RD1 antigens, have the potential to become useful diagnostic tools in clinical and public-health settings. In fact, peripheral blood mononuclear Cells (PBMC) of patients with TB and of household contacts of TB patients release IFN-γ when exposed in vitro to ESAT-6 and CFP-10 intact proteins, and to overlapping peptides spanning the length of these antigens. These studies resulted in the development of two commercially available tests (QuantiFERON-TB Gold-Cellestis Limited, Carnagie, Victoria, Australia and T SPOT-TB, Oxford Immunotec, Oxford, UK) approved for TB infection diagnosis[25,26,27,23]. Both tests are based on ESAT-6 and CFP-10 proteins and/or those overlapping peptides, and employ ELISA and ELISpot techniques, respectively. By distinguishing BCG vaccination and exposure to non-tuberculous mycobacteria within both HIV negative and HIV positive individuals,

these tests represent a successful alternative to PPD tests for the screening of LTBI in healthy individuals. Nevertheless, although these commercial assays provide an accurate diagnosis of *M.tb* infections, they do not discriminate between ATB and LTBI. However, this distinction is required for better global control of TB in subjects from countries with high rates of exposure to *M.tb*[25,28,29]. Further, the potential impact of these tests in practice remains to be confirmed in large, well-designed trials and long-term follow-up studies. With respect to assay methods, future research should attempt to enhance the sensitivity of RD1-based IFN-γ assays, without compromising the specificity. Current evidence suggests that the addition of more specific antigens and use of them in combination may be effective.

Anti-tuberculous vaccine

BCG is the only currently available TB vaccine for use in humans and is among the world's most widely used vaccine. This vaccine has been given more than 3 billion times with an extraordinarily high safety record. Nevertheless, it is the most controversial vaccine in current use. Although BCG offers protection against miliary TB (86%) and meningitis (75%) and reduces the risk of pulmonary TB by 50% in children[30], it failed to protect against the highly prevalent pulmonary TB in adults. Furthermore, the BCG suffers from the lack of consistent protective efficacy in different parts of the world. In fact, estimates of protection imparted by BCG against pulmonary disease range from 0 to 80%[31]. This is probably due to the fact that all of the BCG strains in current use lack some of the DNA segments present in pathogenic M.tb and M. bovis, which encode antigens important for inducing protective immunity[32]. This could be due also to the differences between M.tb and BCG at the level of expression of some immuno-dominant antigens. In addition, there are differences between different strains of BCG with respect to DNA content and growth in animals[33]. Moreover, BCG vaccination induces a DHT response to PPD that cannot be distinguished from exposure to M.tb, and therefore it compromises the diagnostic efficacy of the PPD test.

Immunogenetics as new tools for development of vaccines and diagnostics

Globally, the lack of efficiency of the immune approach-for both diagnostic and vaccinal development-is due to the insufficient specificity of antigens used to induce a protective immune response or to detect specific and early infection phase(s) TB. Thus, to identify candidates for improved vaccines and standardized preparations for specific diagnosis of TB, it is necessary to characterize other antigens and epitopes of M.tb that are less well studied.

From genes to antigens

The current available vaccine against TB, the attenuated *M.bovis* BCG, has been shown to be protective against TB only in some conditions, and although it shares more than 98% identity with the virulent M.tb $H_{37}R_v$ reference strain, very little is known about the critical differences that confer pathogenic behavior to *M.tb* in respect to BCG[34]. The identification of mycobacterial antigens selectively expressed by virulent mycobacteria like the region designated as RD1 which includes some relevant immuno-dominant antigens of M.tb, such as ESAT-6[36] and CFP-10[37], could represent, together with other proteins in the same region, potential antigen targets[28,39,40,41,42, 43,44]. Further, other differences between *M.tb* and BCG could also be due to the different level of expression of some immunodominant antigens, as described for the 38 kDa *M.tb* -complex specific protein that is expressed at a 10 times higher level by *M.tb* $H_{37}R_v$ than by BCG. Moreover, it also appears fundamental to exploit the differences determined by the physiological conditions in which *M.tb* interacts with the host immune system[45]. In this context, it has been found that M.tb regulates gene transcription in different ways according to the growth conditions (in synthetic medium or inside human macrophages)[46]. As a consequence *M.tb* gene products with possible immune potentialities have not yet been identified. In fact, a recent study analyzing the expression profile of *M.tb* genes in human macrophages[47] showed that only 5.5% of the whole *M.tb* genome is expressed and that 32.5% of the expressed gene products are still classified as hypothetical proteins. Such data indicate that the differential expression of *M.tb* genes in different environments represent a potential system for the identification of proteins of vaccinal and diagnostic interest.

Concerning the immuno-genicity of such products, current evidence shows indeed that these antigens provoke antigen-specific T-cells reactions upon challenge. In particular, ESAT-6 and CFP-10 were identified as

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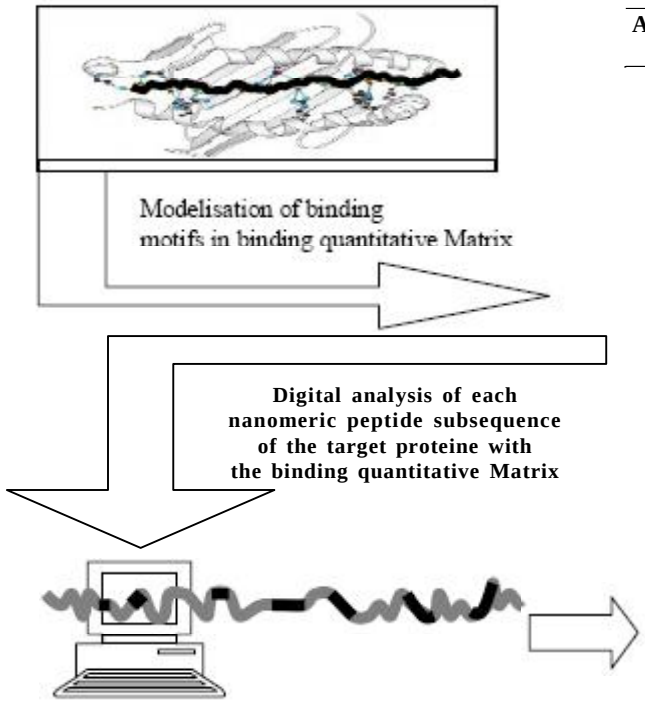

Amino	Position								
acid	P1	P2	P3	P4	P5	P6	P7	P8	P9
A:	-999.00	0.00	0.00	0.00	-	0.00	0.00	-	0.00
D:	-999.00	-1.30	-1.30	-2.10	-	-2.70	-2.00	-	-1.90
E:	-999.00	0.10	-1.20	-0.40	-	-2.40	-0.60	-	-1.90
F:	0.00	0.80	0.80	0.80	-	-2.10	0.30	-	-0.40
G:	-999.00	0.50	0.20	0.20	-	-0.30	-1.10	-	-0.80
H:	-999.00	0.80	0.20	0.20	-	-2.20	0.10	-	-1.10
I:	-1.00	1.10	1.50	1.50	-	-1.90	0.60	-	0.70
K:	-999.00	1.10	0.00	0.00	-	-2.00	-0.20	-	-1.70
L:	-1.00	1.00	1.00	0.90	-	-2.00	0.30	-	0.50
M:	-1.00	1.10	1.40	0.80	-	-1.80	0.09	-	0.08
N:	-999.00	0.80	0.50	0.04	-	-1.10	0.10	-	-1.20
P:	-999.00	-0.50	0.30	-1.90	-	-0.20	0.07	-	-1.10
Q:	-999.00	1.20	0.00	0.10	-	-1.80	0.20	-	-1.60
R:	-999.00	2.20	0.70	-2.10	-	-1.80	0.09	-	-1.00
S:	-999.00	-0.30	0.20	-0.70	-	-0.60	-0.20	-	-0.30
T:	-999.00	0.00	0.00	-1.00	-	-1.20	0.09	-	-0.20
V:	-1.00	2.10	0.50	-0.05	-	-1.10	0.70	-	0.30
W:	0.00	-0.10	0.00	-1.80	-	-2.40	-0.08	-	-1.40
Y:	0.00	0.90	0.80	-1.10	-	-2.00	0.50	-	-0.90

Figure 1: Computer assisted quantitative peptide binding motif analysis

potent RD1-encoded T-cell antigens that induce IFN-γ production in mice[48, 49], cattle[50], and humans[51,52] infected with *M.tb* or pathogenic *M.bovis* but not when vaccinated with BCG. Such responses were observed both in patients with clinically ATB and in infected but healthy tuberculin skin test-positive individuals[28]. RD1-encoded antigens are, therefore, currently used as antigens in the IFN-γ derived immuno-assay for the diagnosis of TB infections[23,26,27]. Furthermore, other members of RD1 and other RD regions of *M.tb* as well as proteins belonging to PPE family[40] have been described as potential immuno-dominant targets[53,54]. However, the immune potentiality of all these protein antigens together has not yet been investigated in detail.

From whole protein antigens to peptidic antigens

The whole purified or recombinant antigens of *M.tb* are immunologically quite complex and the potential use of these reagents is limited by the technical problems related to the production of these proteins in recombinant and in stable batch to batch form. In fact, many of these antigens may have several hundred amino acids. Since the length of T-cell epitopes usually ranges between 9 and 20 amino acids, the alternative would be to use just the immunologically functional parts of these antigens which represent a potential perspective for the development of subunit antigens for vaccinal and diagnostic use.

The identification of T-cell epitopes within protein antigens has traditionally been accomplished using a variety of methods, including the use of whole and fragmented native or recombinant antigenic protein, as well as the more commonly employed overlapping peptide method figure 1. The latter method for the identification of T-cell epitopes within protein antigens involves the synthesis of overlapping peptides which span the entire sequence of a given protein antigen. These peptides are then tested for their capacity to stimulate T-cell responses in vitro. Nevertheless, the number of overlapping peptides covering the sequences of proteins with several hundred amino acids would be very high and, proportionally, the identification of promiscuous peptides via the synthesis and the ex vivo analysis of such products would be very laborious and costly figure 1.

To bypass these technical constraints, the reverse immunogenetic approach, in particular its recent development of quantitative implemented HLA peptide-binding motifs algorithms (see box I)[55], has been successfully used to define T-cell epitopes and to identify new peptide epitopes that can be used for assessing a T-cell response.

TABLE 1: World Web Wide resources for the prediction of T-cell epitopes

Database	WWW site	Tips
HIV-immunology database	http://hiv-web.lanl.gov/immunology/	Human and murine MHC class I and II qualitative prediction by peptide binding motif analysis
SYFPEITHI	http://www.syfpeithi.de	Human and Murine MHC class I and II qualitative and qualitative prediction by peptide binding motif analysis and quantitative implemented peptide binding motif analysis (additive algorithm)
BIMAS	http://bimas.dctr.nih.gov/molbio/hla_bind	HLA class I quantitative prediction by quantitative implemented peptide binding motif algorithm (multiplicative algorithm)
ProPred	http://www.imtech.res.in/raghava/propred/	HLA class II quantitative implemented peptide binding motif algorithm (additive algorithm) based on Hammer & Sturniolo binding matrix
ProPred2	http://www.imtech.res.in/raghava/propred2/	HLA class I quantitative implemented peptide binding motif algorithm (additive algorithm)
EpiPredict	http://www.epipredict.de	HLA class II quantitative prediction by quantitative implemented peptide binding motif algorithm (available for few HLA-DR alleles)
JenPep	http://www.jenner.ac.uk/jenpep	Human, Murine and Primates MHC class I and II quantitative peptid binding prediction by quantitative implemented peptide binding motif analysis
PAProC	http://www.paproc.de	Algorithm for prediction of ImmuneProteasome cleavage pattern
FIMM	http://sdmc.krdl.org.sg:8080/fimm	Site with multiple algorithms for ImmuneProteasome and peptide binding prediction by Artificial Neuronal Network system
Predep	http://bioinfo.md.huji.ac.il/marg/Teppred/mhc-bind/index.html	HLA class I quantitative peptide binding prediction (available for few alleles)

3. Quantitative implemented HLA peptide-binding motif analysis: the road to epitopic peptides

The goal of T-cell epitope prediction is to accurately identify peptide sequences within any protein that, in the context of a defined HLA molecule, will elicit desired T-cell responses.

Recently, the rapid expansion of information on the structure of HLA molecules and the characterization of a large pool of peptides able to bind most of the HLA class I and II alleles have led to the evolution of a new class of computer-driven algorithms based on HLA binding motifs.

The quantitative implemented HLA peptide-binding motif approach is based on the results of studies that identify the effect on binding of different amino acids in different positions along the HLA bound peptide, enabling the extraction of quantitative coefficients for each amino acid in each peptide position for a number of HLA class I and II alleles[56]. Consequently, predictive schemes for a number of HLA alleles could be extended to include sequence dependent coefficients in the calculation of a peptide score, enabling the prediction of the binding hierarchy of different peptides based on the estimated binding probability figure 1.

The quantitative implemented HLA peptide-binding motif approach is based on the experimentally evaluated value of each amino acid in different positions (P1 to P9) along the HLA bound peptide. These values are organized in HLA allele-dependent binding quantitative matrices and digitalized with software able to analyze the whole sequence of the target protein and to calculate the resultant binding score for each peptidic combination. Putative epitopic peptides are identified by selecting the peptides containing a high number of epitopes having high binding scores.

Furthermore, the most recent implementation of the systems available for the prediction of the T-cell epitopes is based on the complementation of HLA structural data within HLA binding data. In fact, the finding that HLA molecules share the same pocket conformation i.e., composed of the same polymorphic residues, presenting the same binding specificities for that pocket, led to determine putative binding properties for HLA alleles in which binding data are poor or not available. As a consequence, the creation of a database of the conformation of HLA binding pockets has allowed the generation of virtual binding matrix for HLA molecules in which binding data are not available simply by comparing the primary structure of the given HLA molecule with the primary protein structure of the pocket in the database[57]. This approach has determined expansion of the number of HLA alleles that could be considered for the screening of putative T-cell epitopes. In fact, large sets of binding data are now available for HLA class I and II alleles, that together now allow coverage of more than 90% of the worldwide allelic variants present in the

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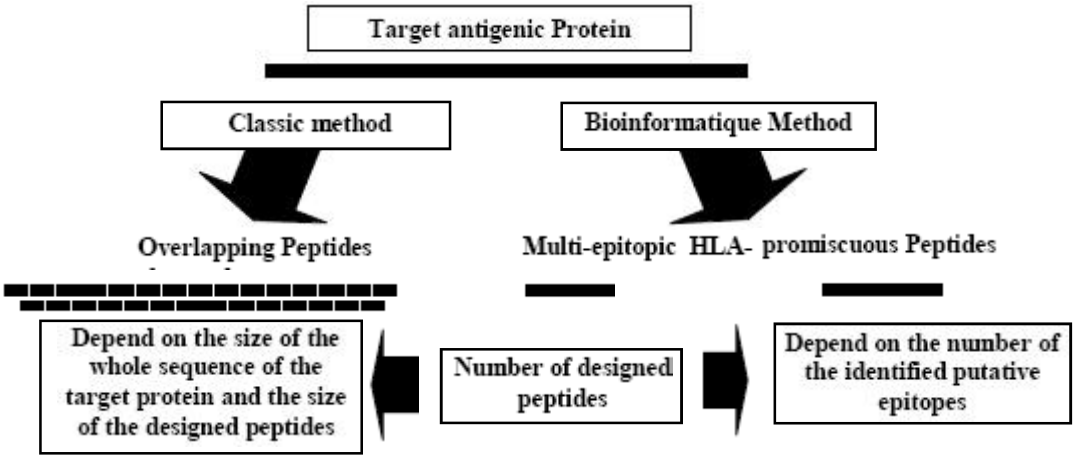

Figure 2: Outline of the overall approaches in classical and computer assisted T-cell epitope prediction

different human populations[56,57,58,59, 60,61,62,63]. The huge amount of information and the dynamic changes of the structural and functional data need to be managed in automatic systems. World web wide based systems (TABLE 1) as well as specific offline software are typically used to this aim.

Many databases and on-line resources are available for T-cell epitope prediction via the internet allowing direct testing of a protein sequence. Most of the available free facilities are listed below together with some tip. As most of these databases evaluated the prediction of T-cell epitopes with different approaches, the direct use of the results obtained using these databases have to be carefully evaluated in the context of the method used. Moreover, the comparison of results obtained with more than one prediction method for each HLA allele has also to be considered.

Consequently, in recent years the development of mathematical models for the prediction of T-cell epitopes based on specific peptide binding motifs of the various HLA class I and II alleles have been developed. These models consist in determining mathematical formula for the structural and functional experimental results collected for HLA molecules and their interaction with the antigenic peptides. The final goal of this approach is to determine the digital exploration of a target protein in the structural data in a manageable database.

The force of this bioinformatic approach comes from the speed with which a large number of proteins can be screened in a short period of time for assessing putative peptide epitopes. In addition, in silico screening usually allows for a reduction of about 95% in the number of peptides to be screened by using standard methods for epitope prediction[56] (Figure 2).

The selection of T-cell epitopes within protein antigens using a classical approach requires the synthesis of overlapping peptides which span the entire sequence and provide a generally high number of peptides (depending on the size of the target protein). In contrast, the use of a bioinformatic approach involves the synthesis of a reduced number of multi-epitopic peptides (reduction of about 95%). Thus, this later method allows a gain in time, reduced cost and facility in both peptide synthesis and in in vitro evaluation of the effective capacity to stimulate T-cell responses.

Consequently, although the dominant T-cell epitopes vary in patients of different geographical location, probably due to differences in the genetic backgrounds[51,64,65,66], the application of this technique could allow the selection of peptides presenting more than one peptide binding motif (i.e. HLA-promiscuous) and more than one epitope from the protein sequence. In this way, with few defined antigens, it is possible to cover almost all the HLA-variability in a population as well as to have a sufficiently large panel of epitopes specific for each HLA type to measure and/or to induce the immune response at the single subject level.

Nevertheless, it is important to keep in mind that what can be determined by this analysis is the putative HLA binding ability of a protein portion which is a characteristic necessary but not sufficient for a peptide to be antigenic. In fact, a complete protein antigen may have several T-cell epitopes, some may have an activator phenotype and others a suppressor phenotype. In addition, the peptide-MHC complex must still interact with the TCR of a neighboring cell allowing the induc-

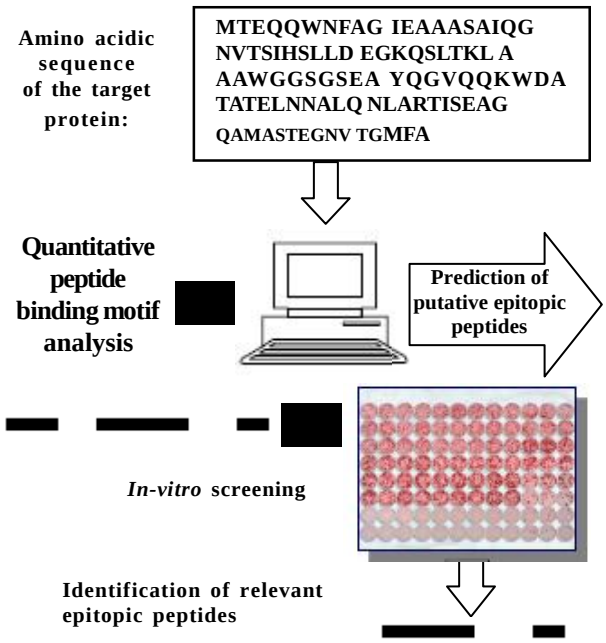

Figure 3: T-cell epitope prediction and in vitro verification of the "real" functional quality

tion of a cellular immune response. Thus, the use of an *in vivo* or in vitro system to determine the capability of the in silico predicted epitopes to be recognized by specific T-cells, remains fundamental to check the "real" functional quality of these in silico selected products figure 3.

The prediction of putative epitopic peptides using the quantitative implemented HLA peptide-binding motif approach is based on the selection of peptides with a high probability to contain the more relevant epitopes. Thus, the use of an in vitro system to determine the capability of the in silico predicted epitopes to be recognized by specific T-cells, remains fundamental to verify the "real" functional quality of these in silico selected products.

This option has practical advantages over whole antigen, in terms of immunological specificity, chemical definition, stability, cost, and lack of potentially mutagenic material. Furthermore, synthetically produced, these peptides offer distinct advantages in that no ingredients of animal origin are used for production. In addition, if an antigen common to more than one mycobacterial species is selected, peptides of species-specific sequences can be chosen. Consequently, if the antigen is of compromised specificity, such as the 19-kDa protein, it is possible to select epitopes from regions of the protein, which are *M.tb* -specific.

With regard to the technical aspect, designed peptides, with sizes usually ranging between 13 and 25 amino acids, could be synthesized as free amino acid termini, sufficiently stable in lyophylized form, and purified using classical chemical techniques. In addition, such synthetic peptidic antigens could be successfully tested using rapid and easily automated systems working on all individuals infected with *M.tb*. In fact, specific CTL and/or Th cells could be assessed by ELISA, ELISpot and Intracellular Cytokine Staining (ICS). Moreover, these techniques could be easily automated and performed in routine by using analytical instruments already available in most clinical laboratories.

In the context of the infection with *M.tb*, protective immunity is mediated by epitopes of *M.tb* antigens recognized by human T-cells of the Th1 type. With the aim to detect such epitopes, Al-Attiyah et al.[67] and Vincenti, et al.[68] reported some dominant epitopes recognized by T-cells from most of the tested individuals in association with frequently expressed and multiple HLA-DR molecules. Such HLA-promiscuous T-cell multi-epitopic peptides (HLA-p.T-c.m-EP) may be exploited in designing peptide-based vaccines and diagnostic reagents against TB. However, for future development of the clinical use of peptidic antigens, the identification and better definition of the immunological proprieties of HLA-p.T-c.m-EP is still need. On the other hand, these particular proprieties could be exploited in an original way to explore the interaction between the host and *M.tb*.

Perspectives for the clinical use of HLA-p.T-c.m-EP in tuberculosis

Interests in such novel approaches is growing due to continuous improvements in the better understanding of the physiology of immune responses and also in the determination of the complete genome sequences of the principal microbial species involved in human pathology[69]. In this context, the use of epitopes to design peptide fragments or DNA encoding epitopic sequences to activate T-cells capable of recognizing and neutralizing microbial particles could be a valuable alternative to current diagnostic tests and conventional vaccines.

1. *M.tb* HLA-p.T-c.m-EP as biomarkers

The gain in time, cost and facile investigation provided by bioinformatic selection of HLA-p.T-c.m-EP, make possible the analysis of the immunologic charac-

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teristics of a high number of *M.tb* gene products with the aim to clarify their involvement in the immune host defense against *M.tb*.

In this perspective, considering that *M.tb* is capable of expressing differential transcriptional programs in response to different growth conditions, attention was paid to results of studies[46,47] into the regulation of *M.tb* gene transcription inside human macrophages. Our group put forward the hypothesis that, inside macrophages, in contrast to RD1 proteins expressed at a very low level [46, 47,70,71,72], proteins expressed at the highest level could induce a stronger T-cell response in *M.tb* infected subjects. Nevertheless, none of the products of these identified genes have been immunologically characterized or have been cloned or expressed yet. In this context, we, in a recent study[73], used this approach to examine the immunological characteristics of antigens expressed by *M.tb* genes in activated and non activated human macrophages.

Thus, seventy-five HLA-p.T-c.m-EP[74] were designed from twenty-five different products of *M.tb* genes, particularly up-regulated inside activated or non activated human macrophages, among with the RD1 genomic region and house keeping genes. When considering the pivotal role of CD4 T in the anti-tubercular immune response, these cells were exclusively targeted by designing these peptides on the basis of HLA class II epitopes. The type 1 response to each peptide was evaluated by measuring the number of the human peripheral blood T-cells producing IFN-γ These in-vitro results, while confirming the immunogenicity of the RD1 proteins in ATB and LTBI, identified a group of peptides derived from 19 other proteins that elicit an equally strong T-cell response in LTBI. In particular, a group of peptides derived from a panel of proteins selected for their expression in human macrophage cultures is highly immunogenic, as it was capable of eliciting responses in subjects with LTBI that are as high as those elicited by RD1 derived peptides used in up to date widely implemented diagnostic tests. Further, comparison of the time courses of responses elicited by peptides, subsequent to treatment of ATB, showed that the response to RD1 peptides declined with the rapid decrease in the mycobacterial load achieved by standard chemotherapy[75,76]. Interestingly, the response to HLA-p.T-c.m-EP derived from genes expressed in activated macrophages seems to increase with time, suggesting a possible inhibitory effect of *M.tb* on the presentation of such antigens during the active phase of infection.

In addition to the relevance and the originality of the information obtained with such a panel of *M.tb* HLA-p.T-c.m-EP, many other aspects of the anti- *M.tb* immune response can be explored by using this approach with peptide antigens.

In particularly, along the same line as the study described above, the possibility of producing and testing a large number of such antigen markers makes possible immunological analysis of a panel of *M.tb* antigens that is large enough to cover the significant diversity regarding their function, their quantitative level of production and the phases of their exposition to the host immune system. Such analysis will undoubtedly provide new information concerning the host-pathogen interaction.

1.1. Induction of an adaptive immune response to a large panel of mycobacterial antigens

In general, biomarkers assessed by the measurement of IFN-γ produced by T-cells should be good indicators of the immune response status against *M.tb*[27] and hence, correlate with protection or pathogenesis. Nevertheless, this approach is based on the prerequisite that peripheral blood, at least in part, reflects the status of the interaction between *M.tb* and the host in the lung[77]. However, the gene expression profile of *M.tb* contained within pulmonary lesions could not be automatically assessed in the periphery. In fact, a recent study[78] showed that, for ATB patients, the response induced by a peptide from the Ag85 antigen concerns T-cells with a central memory phenotype (T_{CM}) when in the peripheral blood whereas it concerns T-cells with a memory effector phenotype (T_{EM}) when in cerebrospinal liquid. Similarly, the effector response of T-cells to ESAT-6 and CFP-10 in the lung was found to be associated with ATB.

Thus, to assess the different T-cell phenotypes involved in the immune response against *M.tb* and hence to analyze their eventual contribution in protective or suppressive mechanisms in host defense, measurement of the activity of cell should be done in peripheral blood as well as at the site of infection. Furthermore, measurement should concern not only cells specific to a particular category of *M.tb* antigens because a given antigen could induce different responses in peripheral blood and at the site of infection. In this context, a large

panel of HLA-p.T-c.m-EP selected from a panel of antigens with high diversity represents an opportunity to study the responses of different T-cells clones to antigens differentially exposed, thus providing a better understanding of interactions between the site of lesions and the peripheral immune system. Furthermore, characterization of these responses will clarify the perspectives for employment of multi-epitopic peptides as a subunit boosting vaccine.

1.2. Discrimination between adaptive and innate immune responses

The small size of HLA-p.T-c.m-EPs peptide structures may be exploited in understanding the mechanisms of host defense to *M.tb*. In fact, this may lead to bypass the stimulation of the innate immunity actors induced by the processing of the entire protein antigen. Thus, this feature provides an original way to study the interaction between innate and adaptive immunity against *M.tb* infections.

Cytokines are the pivotal factor being exploited to measure the anti- *M.tb* immune host defense. However, not only cells of adaptive immunity but also cells of innate immunity could produce the same cytokines in response to induction of Antigen Presenting Cells (APC) after up-take of *M.tb* antigens. This innate production of cytokines as a result of antigen processing is undoubtedly influenced by the structure of the processed antigen. IFN-γ is an example. This cytokine is secreted by specific T-cell clones in response to *M.tb* specific antigens. However, it was shown, in the non-infected young calf, that IFN-γ can also be secreted by NK cells when the peripheral blood mononuclear cells are stimulated by antigens specific to *M.tb* such as ESAT-6[79,80,81]. In fact, mycobacteria infected macrophages[82,83] and Dendritic cells (DC)[84,85] secrete IL-12 and IL-18. The latter have a synergistic effect on the production of IFN-γ by NK cells[82]. Consequently, IFN-γ is simultaneously produced by NK cells through the innate pathway as well as by the T-cell specific response to *M.tb* antigens when the two cells are conjointly stimulated within peripheral whole blood[82]. Furthermore, although secreted by a relatively reduced number of cells, the quantity of IFN-γ produced by the NK cells remains considerable[86].

To determine the precise involvement of innate or adaptive immunity in the anti-*M.tb* immune process, discriminative biomarkers with differential responses are still needed. In this context, the secretion of cytokines by cells of innate immunity is initiated by activated APCs following the capture and the processing of antigens. This processing should be influenced by the structural features of the internalized antigen. Considering their size, the peptidic antigen forms are certainly differently taken-in-charge by APCs compared to the entire protein forms of these antigens. Thus peptidic antigens should induce different levels of activation than the entire protein antigens and hence, of cytokines production, by adaptive immune cells.

Taken together, the cytokines produced by innate immunity could work in a synergic or an antagonist way with those produced by adaptive immunity in increasing the performance of the peptides based vaccine or diagnosis tests. In this context, the precise evaluation of the amount of cytokine produced by each immune branch will allow exploration of the involvement of the innate immune reactivity in the performance of vaccine and diagnostic tests based on these subunit antigens. Moreover, HLA-p.T-c.m-EP from *M.tb* represent, thus, a perspective of promising antigens capable of inducing stimulation of single adaptive immunity allowing hence an innovative way to study the precise involvement and the cooperation between innate and adaptive immunity in TB.

2. Perspective of the HLA-p.T-c.m-EP use in the diagnosis of tuberculosis

In the context of a lack of antigens specific enough to allow the discrimination between ATB and a LTBI, HLA-p.T-c.m-EP selected by quantitative implemented peptide-binding motif algorithms from *M.tb* proteins might offer a relevant perspective for diagnostic use.

The interest in HLA-p.T-c.m-EP selected peptides in diagnosis comes from their capacity to induce specific responses. This could be due to the fact that the frequency of T-cells specific to single epitopes contained in the selected peptides might change as a function of the antigens still exposed to the immune system along a given phase of *M.tb* infection. In fact, it was demonstrated that lymphocytes with immediate effector memory function circulate for a limited time, until the antigen has been cleared. On the other hand, the expression levels of *M.tb* antigens are modulated by interactions between pathogen and human host defense.

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Consequently, during infection, some antigenic components are continuously exposed while others are present in a small load. For these down-expressed antigens, the number of T-cells recognizing HLA-p.T-c.m-EP in peripheral blood could not be high enough to be detected, while the number of T-cells recognizing the entire antigenic protein form or the overlapping peptides covering the whole proteins, could remain high enough to be detected[87]. This could explain, in particular, the presence at the high frequency of IFN-γ-producing T-cells specific to selected epitopes in response to HLA-p.T-c.m-EP from ESAT-6 and CFP-10 secreted by metabolically active and viable bacilli during ATB[88]. When *M.tb* is in a period of dormancy, during a LTBI or after efficacious treatment, these RD1 antigens are down-regulated and the response to epitopic peptides derived from these antigens is decreased. in contrast, the response to the same antigens, presented in the entire antigenic protein form or as overlapping peptides covering the whole proteins, is still increased in LTBI.

This difference in specificity between HLA-p.T-c.m-EP and original entire antigens could, also, be due to the non-specific part of IFN-γ produced by activated innate immunity actors. In theory these diagnostic tests are based on the measurement of IFN-γ exclusively secreted by sensitized T-lymphocytes, which are stimulated following specific recognition of presented antigen. However, as described above, the NK cells, which constitute a system of recognition at the interface between innate and adaptive immunity, are also reported to be one of the major IFN-γ producers following an interaction with antigen presenting cells. Furthermore, NK cells are able to produce a considerable amount of IFN-γ when mononuclear cells in the peripheral blood are stimulated by antigens specific to *M.tb* such as ESAT-6[81]. Consequently, the measured IFN-γ is the result of simultaneous non-specific production by NK cells and specific production by T-cells in response to *M.tb* antigens when the two cells are conjointly stimulated ex vivo in peripheral whole blood[82]. Thus, this non-specific IFN-γ production by NK cells could alter the specificity of diagnostic tests supposed to measure the IFN-γ exclusively produced by sensitized T-cells, even when the antigens used are highly specific to *M.tb*[79,80]. Thus, it is hypothesized that the small size of HLA-p.T-c.m-EP could induce a lower level of activation and hence less production of IFN-γ by NK cells than entire protein antigens. The IFN-γ measured by HLA-p.T-c.m-EP-based tests is less affected by the non-specific IFN-γ produced by NK cells. Thus, this could explain the high level of specificity of the tests based on peptidic antigens in comparison with the entire antigen-based tests in the detection of an infection with *M.tb*.

2.1. *In vitro* immune diagnostic assay based on the T-cell-response to HLA-p.T-c.m-EP

A few studies showed that peptides from ESAT-6 induce reactivity in *M.tb* infected guinea pig but not in M. bovis (BCG) or M. avium sensitized guinea pigs[50]. In humans, RD1 protein-derived HLA-p.T-c.m-EP are used as antigens to perform an immune-diagnostic assay for ATB using an ex vivo ELISpot assay to assess for specific IFN-γ -secreting CD4+ T-cells[51]. The positive response with this assay presented the highest TB diagnostic sensitivity in patients with ATB and even in HIV-positive patients. Furthermore, in contrast to the whole protein, the response to selected peptides reduced significantly under effective anti-TB therapy and no response was observed in PPD-positive individuals[68]. Such data suggest that the in vitro immune diagnostic assay based on the T-cell-response to HLA-p.T-c.m-EP could be used to discriminate between ATB and LTBI and also to monitor the efficacy of anti-TB therapy[75].

In comparison to overlapping peptides from the same original proteins, a recent study[89] showed that an immune assay based on multi-epitopic selected peptides has a higher diagnostic accuracy for ATB in a clinical setting compared with commercially available assays.

2.2. *In vivo* immune diagnostic assay based on the T-cell-response to HLA-p.T-c.m-EP

Another perspective for the diagnosis of TB consists in the use of HLA-p.T-c.m-EP from *M.tb* as antigens to develop a new skin test. In fact, the immunogenicity of the multi-epitopic peptides as well as the specificity and the rapidity of the immune response induced, as described above, support investigation of options to exploit the DHT reaction to such peptidic antigens to identify infection by *M.tb*. Several studies showed that the addition of a lipoid portion to a synthetic peptides provided it with the ability to provoke a positive DHT[90], required in particular for skin test reactivity.

In addition, simple lipopeptides have been shown to induce cellular and humoral immune responses in

mice[91,92], in primates[93,94], and in humans[95,96]. The frequency and duration of the cytotoxic T-cell (CTL) response are directly influenced by the presence of potent CD4 expressing T-cell epitopes[94,95,97].

Furthermore, it was also reported that lipopeptides containing short epitopes are endocytosed and presented to specific T lymphocytes by DCs. Moreover, lipopeptides have been shown to stimulate the release of the pro-inflammatory cytokine TNFα from bovine macrophages and DCs in vitro, thereby providing a possible mechanism for its DTH-enhancing properties. On the other hand, the synthetic lipopeptides comprising the epitopes of bacterial lipoproteins are being increasingly used in the immunization of animal models and have been shown to stimulate immunity to Plasmodium falciparium and to hepatitis B virus. In a phase I trial of a human vaccine for HIV infection[98], such products have been demonstrated to be safe and to be able to stimulate helper CD4-T-cells as well as specific cytotoxic CD8-T-cells which recognize naturally processed viral proteins. In the context of TB, an ESAT-6 based skin test showed to have a promising diagnostic potential in animal models with a higher specificity than the PPD test[50]. It was also possible to stimulate antigen-specific bovine skin-DTH responses using ESAT-6 in combination with a synthetic bacterial lipopeptides. Of greater interest, a study showed a DTH of peptides from ESAT-6 in guinea pigs[99].

3. Perspective of HLA-p.T-c.m-EP use in vaccines against tuberculosis

To meet the ever-growing need for improving existing vaccines against TB in terms of efficacy and safety, several antigen discovery programs were undertaken in the 1980s in an attempt to find new immunogens that could constitute subunit or recombinant vaccines to replace the live BCG vaccine[100]. The vaccine candidates against TB are designed to evoke an immune response that is able to control subsequent infection more efficaciously than the immune response stimulated during natural infection[101]. New technological possibilities, combined with increased knowledge in related fields, such as immunology and molecular biology, open the way to new vaccination strategies. Two general types of vaccines are currently being pursued[76]: (i) recombinant viable vaccines should have a superior protective effect than the BCG to replace conventional vaccination in newborns, which is generally based on the BCG but use different strategies in evoking improved protection[102,103], (ii) subunit vaccines developed from one or a few antigens. Due to decreased immunogenicity compared to the BCG vaccine, the latter are conceived as a booster vaccine following a conventional BCG prime vaccination in the newborn[80,104,105]. Since it is widely believed that protection against the disease requires the induction of Th1-type immune responses against secreted or surface-exposed polypeptides, the approach based on the identification and selection of immunogens containing T-cell epitopes can be used, together with epitope-enhancement strategies, to increase binding to MHC, or to improve recognition by T-cell receptor complexes. This approach represents, besides the classical whole-cell vaccines consisting of killed or attenuated pathogens, a new prophylactic and therapeutic treatment by vaccines against TB.

In this context, the reverse immunogenetic approach in identifying the most relevant T-cell epitopes could provide a rational basis for the development of subunit vaccine strategies against TB as already suggested in part in different reports in this field[106,107,108].

In fact, the use of entire relevant immuno-dominant antigens of *M.tb*, such as ESAT-6 and CFP-10, together with other proteins of the same region, as potential subunit vaccine targets for improving the BCG vaccine has already been reported extensively [28,35,38,39,40,41,42,43,44]. However, the use of these recently developed recombinant BCG vaccines is limited by the risk of regeneration of *M.bovis* pathogenicity consequent to reinsertion of such whole proteins[39]. Thus, the approach to determine by reverse immunogenetics the most relevant T-cell epitopes from immuno-dominant whole proteins may allow insertion into BCG of a chimeric protein containing only the immunogenetic portions[109]. Furthermore, these HLA-p.T-c.m-EPs are safe, they can be designed to induce defined immune responses and they can be synthesized in large quantities in high purity. This approach is under extensive evaluation against, not only TB, but also some of the most relevant infectious diseases such as malaria and AIDS[110].

Concluding remarks

Epitopic peptides are able to activate T-cells capable of recognizing and neutralizing microbial particles, they are safe and suitable for synthesis in large quanti-

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ties in high purity. Given all these features, epitope-driven diagnostic tools and vaccines for infectious diseases of worldwide impact such as TB are now within reach.

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Abbreviations

AFB : Acid Fast Bacilli; APC : Antigen Presenting Cells ; ATB : Active Tuberculosis; BCG : Bacillus Calmette Guerin ; M. bovis vaccine strain ; CDC : Centers for Disease Control; CFP : Culture Filtrate Protein 10 (-10) ; CTL : Cytotoxic T Lymphocytes ; DC : Dendritic Cells; DHT : Delayed Type Hypersensitivity; ESAT-6 : Early Secreted Antigenic Target 6 protein; FDA : Food and Drug Administration; HIV : Human Immunodefeciency Virus; HLA : Human Leucocyte Antigens; HLA-p T-c m-EP : HLA-promiscuous T-cell multi-epitopic peptides ; IFN-γ : Interferon-γ ; IL-12: Interleukin-12 ; IL-18 : Interleukin-18 ; LTBI : Latent Tuberculosis Infection ; *M.tb* : Mycobacterium tuberculosis ; NAAT : Nucleic Acid Amplification Tests ; NK : Natural killer cells; PBMC : Peripheral Blood Mononuclear Cells; PPD : Purified Protein Derivative; TB : Tuberculosis ; T_{CM} : Central Memory Phenotype T-cells; TCR: T Cell Receptor; T_{EM} : Memory Effector Phenotype T-cells; TNF-α : Tumor Necrosis Factor-α ; TST: Tuberculin Skin Test

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