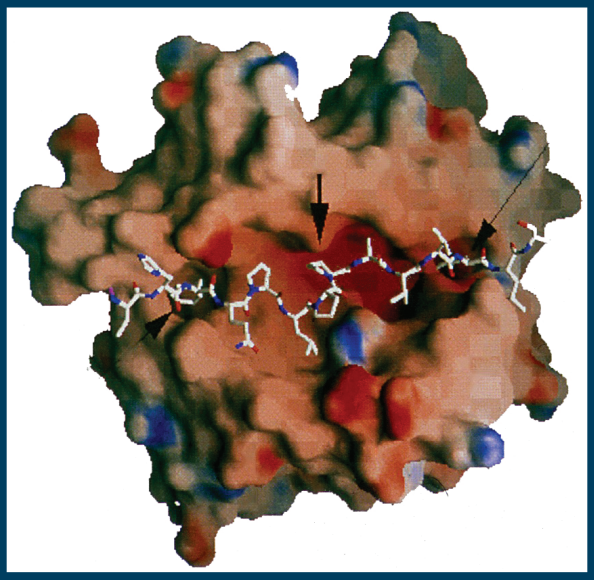
frontiers

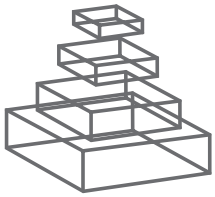
RESEARCH TOPICS



SIGNIFICANCE OF ANTIGEN AND EPITOPE SPECIFICITY IN TUBERCULOSIS

Topic Editors
Juraj Ivanyi and Tom H.M. Ottenhoff

frontiers in IMMUNOLOGY



frontiers

ISSN 1664-8714
ISBN 978-2-88919-451-3
DOI 10.3389/978-2-88919-451-3

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

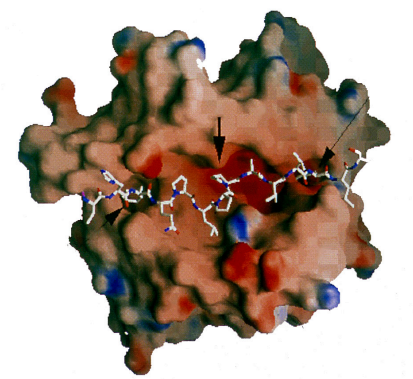
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

SIGNIFICANCE OF ANTIGEN AND EPITOPE SPECIFICITY IN TUBERCULOSIS

Topic Editors:
Juraj Ivanyi, Kings College London, United Kingdom
Tom H.M. Ottenhoff, Leiden University Medical Center, Netherlands



Molecular model of epitope binding to an HLA.DRB molecule. The colour reflects the surface potential (red for acidic and blue for basic charge); the arrows show the N- and C-terminal anchors and a 'kink' in the middle of the epitope. From Jurcevic et al Mol. Immunology 8: 1807-14, 1996.

Dissection of the specificity of host immune responses following infection with Mycobacterium tuberculosis is essential for designing effective vaccination and diagnostic biomarkers as well as for better understanding of immunopathogenesis of active tuberculosis. The articles in this volume of the Topics in Microbial Immunology review the significance of this area of research from both experimental models and clinical surveys. This includes T cell recognition of MHC permissive epitopes, use of algorithms for genome-based prediction of immunodominant epitopes, evaluation of candidate antigens/epitopes and adjuvants for vaccination and immunodiagnosis. Future research strategies indicate the need for better understanding of the relationship between epitope specificity and the phenotype of responding T cells and search for biomarkers with a capacity to discriminate and predict the change from latent infection to active disease. These research avenues have important potentials for improving the prevention and control of tuberculosis.

Table of Contents

05 ***Significance of antigen and epitope specificity in tuberculosis***
Juraj Ivanyi and Tom H. M. Ottenhoff

07 ***Function and potentials of M. tuberculosis epitopes***
Juraj Ivanyi

18 ***Definition of CD4 immunosignatures associated with MTB***
Cecilia S. Lindestam Arlehamn and Alessandro Sette

25 ***Innovative strategies to identify M. tuberculosis antigens and epitopes using genome-wide analyses***
Annemieke Geluk, Krista E. van Meijgaarden, Simone A. Joosten, Susanna Commandeur and Tom H. M. Ottenhoff

33 ***Functional signatures of human CD4 and CD8 T cell responses to Mycobacterium tuberculosis***
Teresa Prezzemolo, Giuliana Guggino, Marco Pio La Manna, Diana Di Liberto, Francesco Dieli and Nadia Caccamo

46 ***Epitope-specific antibody levels in tuberculosis: biomarkers of protection, disease, and response to treatment***
Graham H. Bothamley

50 ***The immunodominant T-cell epitopes of the mycolyl-transferases of the antigen 85 complex of M. tuberculosis***
Kris Huygen

61 ***Role of fused Mycobacterium tuberculosis immunogens and adjuvants in modern tuberculosis vaccines***
Ana Paula Junqueira-Kipnis, Lázaro Moreira Marques Neto and André Kipnis

70 ***Recombinant BCG: innovations on an old vaccine. Scope of BCG strains and strategies to improve long-lasting memory***
Adeliane Castro da Costa, Sarah Veloso Nogueira, André Kipnis and Ana Paula Junqueira-Kipnis

79 ***The T-cell response to lipid antigens of Mycobacterium tuberculosis***
Gennaro De Libero and Lucia Mori

88 ***Toward understanding the essence of post-translational modifications for the Mycobacterium tuberculosis immunoproteome***
Cécile A. C. M. van Els, Véronique Corbière, Kaat Smits, Jacqueline A. M. van Gaans-van den Brink, Martien C. M. Poelen, Francoise Mascart, Hugo D. Meiring and Camille Locht

98 ***The PGRS domain from PE_PGRS33 of Mycobacterium tuberculosis is target of humoral immune response in mice and humans***
Ingrid Cohen, Cristina Parada, Enrique Acosta-Gío and Clara Espitia

107 Immunogenicity of 60 novel latency-related antigens of Mycobacterium tuberculosis

Mªdel Mar Serra-Vidal, Irene Latorre, Kees L. C. M. Franken, Jéssica Díaz, Maria Luiza de Souza-Galvão, Irma Casas, José Maldonado, Cèlia Milà, Jordi Solsona, M. Ángeles Jimenez-Fuentes, Neus Altet, Alícia Lacoma, Juan Ruiz-Manzano, Vicente Ausina, Cristina Prat, Tom H. M.Ottenhoff and José Domínguez

frontiers in
IMMUNOLOGY

EDITORIAL
published: 23 October 2014
doi: 10.3389/fimmu.2014.00524

Significance of antigen and epitope specificity in tuberculosis

Juraj Ivanyi*[1] *and* ***Tom H. M. Ottenhoff*[2]***

[1] *Guy's Hospital Campus of Kings College London, London, UK*
[2] *Department of Infectious Diseases, Leiden University Medical Center, Leiden, Netherlands*
**Correspondence: juraj.ivanyi@kcl.ac.uk; t.h.m.ottenhoff@lumc.nl*

Edited and reviewed by:
Ian Marriott, University of North Carolina at Charlotte, USA

Keywords: tuberculosis, antigens, bacterial, T-lymphocyte, epitopes, MHC restriction, vaccine adjuvant, TB diagnosis

Tuberculosis (TB) remains a major global health problem, because (i) diagnosis is usually made too late to avoid spread of infection to contacts; (ii) vaccination with bacillus Calmette–Guérin (BCG) does not prevent the most prevalent pulmonary disease; and (iii) defaulting from lengthy chemotherapy leads to an increase in drug resistant strains. The continued impact of human immunodeficiency virus (HIV) co-infections remains a major aggravating factor in TB resurgence. Intensive research on the specificity and function of immunological responses is of major importance since protective host defense is critically dependent on T cells, which selectively recognize only certain antigens and epitopes of the tubercle bacillus. Such knowledge is therefore necessary for designing a novel effective vaccine and better diagnostic tools. The specificity of the host immune response may also help to explain how the intracellular tubercle bacilli evade host resistance, probably by decoy pro-inflammatory actions of some of their antigens and/or immunomodulatory constituents, which lead to chronic infection and lung pathology.

The 12 articles in this Research Topic in the section Microbial Immunology of the journal Frontiers in Immunology review current knowledge, as well as gaps in our understanding of the mechanisms and functions of T and B cell recognition of antigens and constituent epitopes of *Mycobacterium tuberculosis* (Mtb). The abundant occurrence of major histocompatibility complex (MHC) class II-permissive epitopes in tubercle bacilli has important implications for the development of both subunit vaccines and diagnostic tests (1, 2). Moreover, an evolutionary interpretation has been that selection of Mtb strains carrying protective MHC-permissive epitopes could have extended the survival of infectious individuals and hence was advantageous for the protracted aerosol transmission of the pathogen (1).

Better understanding of the "antigenome" of Mtb has been advancing with the aid of new powerful strategies for the identification of antigens and its epitope determinants. These methods include bioinformatic approaches toward genome wide predictive algorithms for HLA binding and high-throughput tetramer generation (2). Hypothesis driven approaches or hypothesis free searches of the whole Mtb genome and functional screening algorithms led to the evaluation of candidate antigens, which are recognized well by T cells from latently infected individuals. They involve antigens expressed *in vivo*, encoded by the *DosR* regulon, resuscitation promoting factor (Rpf) proteins, and new HLA-class Ia or Ib (HLA-E) restricted Mtb epitopes, recognized by classical and non-classical CD8 T cells (3).

Mycobacterium tuberculosis-specific epitopes of immunodominant and HLA-permissive nature have been used extensively in IFNγ release assays (IGRAs). Several test kits can detect latent Mtb infection with better specificity than the tuberculin based skin test. However, these kits still need improving on their sensitivity and fail to distinguish active TB from latent infection. Moreover, biomarkers for predicting the risk of latent TB progressing into active TB are yet to be found (1, 3, 4). Further research on possible associations between epitope specificity and the phenotype of responding T cells could be an area of potential importance. Polyfunctional T cells have been associated with protective immunity on the grounds that the number of T cells producing IFNγ, IL-2, and/or tumor necrosis factor-α (TNFα) is correlated with vaccine induced protection in models of infectious diseases (4). However, the role of these cells is a subject of debate, since they are readily detectable also in patients with active or past TB. Although these cytokines are produced by several cell types [CD4, CD8, TCRγδ, mucosa-associated invariant T cells (MAIT), CD1-restricted T cells, and natural killer (NK) cells], it is significant that CD4 T cell depletion cannot be compensated with cell types other than CD4 T cells. Epitope-specific serum antibody levels in TB patients have been found to be influenced by the pulmonary bacterial load (associated with HLA-DR15), recent exposure to infection, and response to chemotherapy (5).

Identifying those antigenic determinants, which lead to host protection, is mandatory for designing more effective vaccination strategies. A recently failed vaccine trial in children employed Ag85A, which is highly immunogenic, but changes in its expression in infected cells could influence the susceptibility of infected cells to host immunity (6). Hence, there is a need to select suitable candidate antigens by more rigorous comparison of their protective capacity in animal vaccination models, before proceeding toward evaluation in human trials. In addition to antigen specificity, the success of a subunit vaccine may lie in its presentation, i.e., in the adjuvant formulation. To this effect, the fusion of antigens with interleukins, lipids, lipoproteins, and immune stimulatory peptides has been employed (7). Continued efforts to obtain better protection using recombinant strains of BCG engage over-expression of either Mtb-specific antigens (which had been lost during the attenuation

process of BCG) or of some cytokines (IL-2, IL-12, IL-15, and GM-CSF) (8).

Although classical CD4 and CD8 T cells recognize peptide epitopes bound to MHC, molecules with different chemical structures could be of potential importance. Thus, T cells recognizing lipid antigens may contribute to natural host protection and might potentially be exploited for subunit based vaccination (9). Another structural aspect is the role of post-translational modifications of proteins identified using mass spectrometry-based proteomics (10). Recent attention to the proline–glutamic acid (PE) family of cell surface expressed proteins has been due to their immunomodulatory properties and possible evasion from host immunity by antigenic variation. Immunogenicity was attributed to the PE domain, while the specific epitopes were localized within the polymorphic GC-rich sequence (PGRS) domain. However, sensitization in human beings was found to be associated with BCG vaccination, rather than latent Mtb infection (11). Apparently, still other families of antigens need to be evaluated in the search for biomarkers, which could distinguish between stable protection and a tendency for recrudescence in latently infected populations and also for monitoring the efficacy of protection following prophylactic vaccination (12).

In conclusion, further research on Mtb antigen and epitope specificities seems mandatory for realizing the crucially important aims of both prophylactic and post exposure vaccination against TB. Advancing the knowledge of antigenic determinants is essential also for differentiating patients with active TB from latently infected healthy subjects. There is potential in the ambitious search for specific immunological biomarkers for predicting the reactivation of TB in populations, both without and with HIV infection. These endeavors will undoubtedly need to be combined with better knowledge of the functional phenotypes of the respective T cell subsets. Other potential avenues are the construction of fusion proteins with improved vaccine adjuvanticity (7) and the proposed construction of T cell receptor (TCR)-like ligands for immunotherapy (1). Future research may benefit also from advances in computer algorithm based analysis of Mtb epitopes and host cytokine signatures, as well as from reduced costs of DNA and RNA sequencing and synthetic peptide libraries.

REFERENCES

1. Ivanyi J. Function and potentials of *M. tuberculosis* epitopes. *Front Immunol* (2014) **5**:107. doi:10.3389/fimmu.2014.00107
2. Lindestam Arlehamn CS, Sette A. Definition of CD4 immunosignatures associated with MTB. *Front Immunol* (2014) **5**:124. doi:10.3389/fimmu.2014.00124
3. Geluk A, van Meijgaarden KE, Joosten SA, Commandeur S, Ottenhoff THM. Innovative strategies to identify *M. tuberculosis* antigens and epitopes using genome-wide analyses. *Front Immunol* (2014) **5**:256. doi:10.3389/fimmu.2014.00256
4. Prezzemolo T, Guggino G, La Manna MP, Di Liberto D, Dieli F, Caccamo N. Functional signatures of human CD4 and CD8 T cell responses to *Mycobacterium tuberculosis*. *Front Immunol* (2014) **5**:180. doi:10.3389/fimmu.2014.00180
5. Bothamley GH. Epitope-specific antibody levels in tuberculosis: biomarkers of protection, disease, and response to treatment. *Front Immunol* (2014) **5**:243. doi:10.3389/fimmu.2014.00243
6. Huygen K. The immunodominant T-cell epitopes of the mycolyl-transferases of the antigen 85 complex of *M. tuberculosis*. *Front Immunol* (2014) **5**:321. doi:10.3389/fimmu.2014.00321
7. Junqueira-Kipnis AP, Marques Neto LM, Kipnis A. Role of fused *Mycobacterium tuberculosis* immunogens and adjuvants in modern tuberculosis vaccines. *Front Immunol* (2014) **5**:188. doi:10.3389/fimmu.2014.00188
8. da Costa AC, Nogueira SV, Kipnis A, Junqueira-Kipnis AP. Recombinant BCG: innovations on an old vaccine. Scope of BCG strains and strategies to improve long-lasting memory. *Front Immunol* (2014) **5**:152. doi:10.3389/fimmu.2014.00152
9. De Libero G, Mori L. The T-cell response to lipid antigens of *Mycobacterium tuberculosis*. *Front Immunol* (2014) **5**:219. doi:10.3389/fimmu.2014.00219
10. van Els CACM, Corbière V, Smits K, van Gaans-van den Brink JAM, Poelen MCM, Mascart F, et al. Toward understanding the essence of post-translational modifications for the *Mycobacterium tuberculosis* immunoproteome. *Front Immunol* (2014) **5**:361. doi:10.3389/fimmu.2014.00361
11. Cohen I, Parada C, Acosta-Gío E, Espitia C. The PGRS domain from PE_PGRS33 of *Mycobacterium tuberculosis* is target of humoral immune response in mice and humans. *Front Immunol* (2014) **5**:236. doi:10.3389/fimmu.2014.00236
12. Serra-Vidal M, Latorre I, Franken KLCM, Díaz J, de Souza-Galvão ML, Casas I, et al. Immunogenicity of 60 novel latency-related antigens of *Mycobacterium tuberculosis*. *Front Microbiol* (2014) **5**:517. doi:10.3389/fmicb.2014.00517

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 September 2014; accepted: 06 October 2014; published online: 23 October 2014.

*Citation: Ivanyi J and Ottenhoff THM (2014) Significance of antigen and epitope specificity in tuberculosis. Front. Immunol. **5**:524. doi: 10.3389/fimmu.2014.00524*

This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 24 March 2014
doi: 10.3389/fimmu.2014.00107

Function and potentials of *M. tuberculosis* epitopes

Juraj Ivanyi*

Guy's Hospital, Kings College London, London, UK

Edited by:
Tom H. M. Ottenhoff, Leiden University Medical Center, Netherlands

Reviewed by:
Tom H. M. Ottenhoff, Leiden University Medical Center, Netherlands
Annemieke Geluk, Leiden University Medical Center, Netherlands

***Correspondence:**
Juraj Ivanyi, Guy's Hospital, Kings College London, Hodgkin Building, London SE1 1UL, UK
e-mail: juraj.ivanyi@kcl.ac.uk

Study of the function of epitopes of *Mycobacterium tuberculosis* antigens contributed significantly toward better understanding of the immunopathogenesis and to efforts for improving infection and disease control. Characterization of genetically permissively presented immunodominant epitopes has implications for the evolution of the host–parasite relationship, development of immunodiagnostic tests, and subunit prophylactic vaccines. Knowledge of the determinants of cross-sensitization, relevant to other pathogenic or environmental mycobacteria and to host constituents has advanced. Epitope-defined IFNγ assay kits became established for the specific detection of infection with tubercle bacilli both in humans and cattle. The CD4 T-cell epitope repertoire was found to be more narrow in patients with active disease than in latently infected subjects. However, differential diagnosis of active TB could not be made reliably merely on the basis of epitope recognition. The mechanisms by which HLA polymorphism can influence the development of multibacillary tuberculosis (TB) need further analysis of epitopes, recognized by Th2 helper cells for B-cell responses. Future vaccine development would benefit from better definition of protective epitopes and from improved construction and formulation of subunits with enhanced immunogenicity. Epitope-defined serology, due to its operational advantages is suitable for active case finding in selected high disease incidence populations, aiming for an early detection of infectious cases and hence for reducing the transmission of infection. The existing knowledge of HLA class I binding epitopes could be the basis for the construction of T-cell receptor-like ligands for immunotherapeutic application. Continued analysis of the functions of mycobacterial epitopes, recognized by T cells and antibodies, remains a fertile avenue in TB research.

Keywords: tuberculosis, antigenic structure, epitope mapping, immunodominant epitopes, immunodiagnosis, immunotherapy, immunopathogenesis

INTRODUCTION

Pathogenic bacteria produce a wide range of constituents, which determine their virulence and host responses following infection. In the case of *Mycobacterium tuberculosis* (Mtb), antigenic and immunomodulatory constituents may be considered as virulence factors, because they can act as "decoys," triggering excessive immune responses which can lead to pathology of the lungs in active tuberculosis (TB), instead of host protection (1). Hence, immunological research has been essential for the study of pathogenesis as well as for the development of prophylactic vaccination and for the detection of latent infection. Detailed analysis of the specificity and of the phenotype of immune responses is mandatory in the desire to discover biomarkers for protective immunity, for predicting the risk of reactivation from latent infection and for developing immunotherapies, adjunct to chemotherapy.

Dissection of the antigenic structure of Mtb to its epitope constituents has been driven by the newly developed technologies, starting with hybridoma-produced monoclonal antibodies (2, 3), followed by recombinant DNA expression libraries (4), T-cell cloning and hybridomas (5), and DNA sequencing. More recently, new epitopes predicted within the whole Mtb genome on the basis of algorithms ("silico mapping") (6) have a useful rate of empirical confirmation (7). The location of discontinuous and conformational epitopes recognized by antibodies can be predicted by integrated analysis of the dynamical and energetic properties of proteins (8). Mapping of T-cell epitopes within the known protein sequence used synthetic peptides with overlapping sequence ("pepscan") and single-residue substitutions identified epitope cores, flanks, and key residues involved in binding to major histocompatibility complex (MHC) or T cell receptor (TcR) molecules.

Characterization of the membrane markers and cytokine profiles of responding T cells identified the existence of T-cell subsets and their regulatory networks. Emphasis on the T-cell phenotype and recently on the transcriptomic signature of T cells is currently expanding (9), but without full attention to antigen and epitope specificity. However, restoring the balance of knowledge between the functional phenotype and recognition specificities of T cells seems compelling.

The extensive knowledge on the mapping of antigenic epitopes has been cataloged and made accessible by the NIH IEDB database http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2228276/; http://www.iedb.org/; http://help.iedb.org/entries/19150-user-documentation-iedb-version-2.

This inventory contains more than 1000 epitopes, mostly derived from only about 30 of the most immunogenic antigens,

representing a very small fraction from the about 4000 known open reading frame proteins of the Mtb genome (10). Recently combined analysis of epitope predictions, high throughput ELISPOT, and T-cell libraries from latently Mtb-infected subjects, categorized the epitopes into prominent "antigenic islands" (11). The wider significance of epitope specificity of immune responses of Mtb-infected hosts has been reviewed recently (12).

This chapter points out the role of individual epitope specificities and aims to integrate this knowledge with different functions, relevant for the host–parasite relationship. Potentials for future research are targeted at improving the control of TB, particularly for vaccination, immunotherapy, detection of latent infection, and early diagnosis of infectious forms of active disease.

MHC-PERMISSIVE EPITOPES

Immunodominant epitopes were originally thought to be recognized in the context of only one or a few MHC class II alleles, though a number of genetically permissive epitopes were found in microbial pathogens. Initial mapping of CD4 T-cell stimulatory epitopes showed two hsp65 peptides recognized by several H-2-disparate mouse strains, one of them presented by both I-A and I-E molecules (13). Subsequent analysis of two glyco-lipoproteins and the α-crystallin (Acr) antigen identified the same few epitopes as immunodominant in a number of inbred strains of mice, carrying different H-2A alleles (14–16). H2I-A promiscuous recognition of p350–369 was demonstrated using CD4 T-cell hybridomas, though with allele-specific binding of IA polymorphic critical residues (17). Moreover, pepscan analysis revealed separate patterns of recognition by T hybridomas of the same H-2 haplotype, whereby every core residue was critical for at least one hybridoma, with only one substitution (74 Val → Ala) common to all hybridomas (18). While core residues were critical for both MHC and TcR binding, T-cell recognition was influenced also by the substitution of flanking residues (19).

The apparently abundant occurrence of MHC-permissive epitopes in tubercle bacilli may be an evolutionary consequence of selection of mutants carrying protective MHC-permissive epitopes. These organisms would have been advantageous to the pathogen, by being conducive to the longer survival of individuals, who were capable of aerosol transmission of the infection. This evolutionary concept is supported by the finding that T hybridomas from H-2Ab/d heterozygous mice had a higher frequency of IA-promiscuous recognition than hybridomas from each of the parental H-2 homozygous hybridomas (17). The IA-promiscuous hybridomas could also be stimulated with lower peptide concentrations, indicating TcR recognition of higher TcR affinity. Selection of MHC-permissive epitopes by low antigen concentrations in chronically Mtb-infected outbred populations would have had the advantage for the protection and survival of the infected hosts.

Analysis of HLA-DR heterozygous T-cell lines against the permissively recognized 91–110 epitope of the Acr antigen also showed superior stimulation in the context of heterozygous antigen presenting cells (APCs) (20). Moreover, stimulation in the context of DR-homozygous APCs showed that the HLA-DR haplotype influenced not only the magnitude, but also the IFNγ/IL-4 secretion profiles of the T-cell lines. The demography and evolution of Mtb lineages, virulence, and selection of epitopes of conserved structure were suggested also as an adaptation to other genetically diverse constituents in human macrophages, but without due consideration of the selective role of the HLA system for the selection of permissive epitopes (21).

The definition of MHC-permissive epitopes is mandatory for further development of both diagnostics and vaccines with a potential to function in large sections of genetically diverse human populations. HLA class II-permissive epitopes have been identified in a number of antigens of different structure, such as PstS1 glycolipoprotein (22), heat shock proteins hsp65 (23), and GroES (24), Acr (25), ESX proteins (26, 27), secreted proteins Ag85B (28) and MPB70 (29), and PE/PPE proteins (30) (**Table 1**). The abundance of human CD4 and CD8 T-cell responses to the respective epitopes was explained by their permissive binding to several HLA-DR (15, 22, 31, 32) and also HLA class I (26, 33, 34) molecules.

Detailed analysis of the p350–369 epitope of PstS1 (35) showed a range of binding affinities to different DR molecules and identified the epitope core to be of 9–11 residues. Binding to both DR1 and DRB5*0101 shared F-354 as the common primary contact residue. Molecular modeling suggested that the peptide bound to DR1 in the elongated conformation as usual for MHC class II molecules, but in a "kink," when bound to DRB5*0101, which is common for peptides bound to MHC class I complexes. The possible influence of different conformations imposed on the same peptide by distinct HLA alleles on T-cell responses has yet not been elucidated.

Substitution of single amino acids in the epitope core has been employed to identify both HLA-DR and TcR-binding contact residues within the DR17 restricted p3–13 epitope from the hsp65 antigen. Using this approach, TcR V gene families of human CD4 T-cell clones were analyzed in respect of the most immunodominant, HLA-DR promiscuous 91–110 epitope of Acr (25). The HLA-DR-binding and TcR-binding cores and contact residues were identified within 9-mer or 13-mer cores, which differed between the DR haplotypes. Notably, preferential TcR usage was demonstrated by the finding that the majority of clones used the BV2 TcR and contained a common R-L/V-G/S-Y/W-E/D sequence motif in the CDR3 region (36). These data may be useful to design peptides with altered HLA anchor residues or TcR interaction sites to increase their immunogenicity.

The "in silico" algorithms (ProPred1) predicted a number of HLA class I-permissive epitopes in histone or proteins of undefined function from Mtb (37). ProPred prediction of *Mycobacterium leprae* epitopes identified a number of both class I and II, HLA-permissive T-cell reactivity, in leprosy endemic populations in Brazil, Ethiopia, and Nepal (38). Comparing algorithms for HLA-binding promiscuity between ecologically diverse human microbial pathogens, found the promiscuity of Mtb epitopes to be of a similar degree as in HIV, *S. pyogenes*, or even higher for *B. anthracis* and *C. tetani* (39). However, these epitope predictions need empirical confirmation and should take into account that HLA binding affinities may not always associate with the magnitude of T-cell responses.

CROSS-REACTIVITY OF MYCOBACTERIAL EPITOPES

The antigens of Mtb are related to a number of proteins from non-tuberculous mycobacterial pathogens or commensal

Table 1 | Mtb antigens with identified HLA-DR-permissive CD4+ T-cell stimulatory epitopes.

Protein group	Antigen name	Gene accession no.	kDa	Epitope sequence	Reference
Glyco-lipoprotein	PstS1	Rv0934	38	1–20; 350–369	Jurcevic et al. (22)
Chaperonin stress proteins	α-Crystallin, Acr	Rv2031	16	91–110	Caccamo et al. (25)
	GroEL2, hsp65	Rv0440	65	61–75; 141–155	Mustafa et al. (23)
	GroES	Rv3418c	10	25–40	Chua-Intra et al. (24)
RD-1, ESX family	ESAT-6, EsxA	Rv3875	6	1–20	Tully et al. (27)
	CFP-10, EsxB	Rv3874	10	71–88	Shams et al. (26)
Secreted proteins	Ag85B mycolyl transferase	Rv1886c	30–32	91–108	Valle et al. (28)
	MPB70, mpt70	Rv2875	22	106–130, 166–190	Al-Attiyah et al. (29)
Cell surface	PPE family	Eight genes	3–316	Eight epitopes	Wang et al. (30)

non-pathogenic mycobacterial species and more rarely with human proteins. These relationships are of interest, because environmental priming can influence resistance to Mtb, it can interfere or enhance the protective immune response to vaccination and may exclude from vaccine development, any molecules that can lead to autoimmunity. The latter category includes the chaperonins hsp65 and hsp71 with highly conserved sequences between prokaryotic and eukaryotic species and consequently extensive cross-reactivity between mycobacteria and humans (40). An hsp65 epitope at sequence 285–295, detected by mAb ML30 is strongly expressed on the surface of human cells with abundant mitochondria (40). Elevated expression was observed on monocyte-derived cells in different inflammatory diseases, including rheumatoid arthritis (41), atherosclerosis (42–44), and multiple sclerosis (45).

Analysis of antigen homologs from different species of mycobacteria showed that cross-recognition by T cells requires sharing fewer amino acids than cross-reaction by antibodies. Thus, polyclonal and monoclonal antibodies to ESAT-6 from Mtb and *M. leprae,* which share only 36% amino acids, are all strictly species-specific (46). In contrast, recombinant ESAT-6 from both species are similarly recognized by T cells from individuals, who were exposed to either tuberculous or leprosy infection (46). Cross-reactivity at the level of T-cell, but not B-cell recognition can lead to the recall of antibody production with specificity for the initial antigen (termed: "original antigenic sin") (47). This interpretation was given to the finding of elevated antibody levels against Mtb-specific epitopes in lepromatous leprosy patients from TB endemic areas (48). However, *M. leprae*-specific antibodies in patients with active TB were not raised, since these patients were probably not exposed to *M. leprae* infection.

Cross-reactivity without sequence homology, i.e., mimicry, has been observed with a broad range of molecules of different structure, such as lactoferrin, transferrin, and proteoglycan. Another example of mimicry is the cross-recognition by CD4 T cells of an octamer epitope on two unrelated mycobacterial proteins, which is immunodominant for the 19-kDa protein of Mtb and cryptic for the 28-kDa protein of *M. leprae* (49, 50). Assumptions that epitope-based mimicry between proteins could lead to unsuspected cross-sensitization, maintain T-cell memory, or lead to autoimmunity, need further study.

Despite wide sequence homologies, heat shock proteins contain also species-specific epitopes. Increased Mtb-specific antibody levels were reported for hsp65, hsp71 (51, 52) in patients with active TB, including patients with smear-negative disease, which remains a diagnostic obstacle. Elevated serum antibodies to mycobacterial, but not to human hsp65 in Crohn's disease, implied a pathogenic role of mycobacteria, whereas antibodies in ulcerative colitis bound to human hsp65 (53). Further support for the mycobacterial pathogenesis of Crohn's disease came from the finding of elevated antibody levels against three different antigens derived from *Mycobacterium paratuberculosis* (54).

Immunodominant species-specific T- and B-cell epitopes can be found in a mycobacterial 10-kDa GroEL heat shock protein despite its highly conserved amino acid sequence. Despite a 90% sequence identity with Mtb, studies in mice identified two *M. leprae*-specific closely overlapping CD4 T-cell epitope cores (24–34 and 28–34), restricted by H-2Ad and H-2Ed, respectively and overlapping with an *M. leprae*-specific mouse mAb (ML6 and 10) epitope at residues 25–31 (55). The lack of antibody response to this epitope in lepromatous leprosy patients was suggested to be due to the T-cell epitope overlap.

The CD4 T-cell epitope repertoire of GroES was investigated also in TB and leprosy patients. The N-terminal (1–16) peptide (residues 1–16) was specifically stimulatory in the majority of active TB patients (56), while none of the other peptides was discriminatory. On the other hand, peptide 25–40 (29–37 core) of *M. leprae*, but not of Mtb sequence was specifically stimulatory in tuberculoid leprosy patients; this peptide bound to a number of HLA-DR molecules, of which HLA-DRB5*0101 had the strongest affinity (24). Four other leprosy-specific epitopes were identified on the 35-kDa protein of *M. leprae*, which has a homologous constituent in *M. avium*, but not in Mtb (57). Analysis of epitope specificities explained the phenomenon of split leprosin/tuberculin anergy of skin hypersensitivity in a proportion of leprosy patients. Thus, blood T-cell proliferative responses were found to be diminished to cross-reactive antigens, but elevated toward the predominantly Mtb-expressed PstS1 antigen and the Acr epitope 71–91 (58).

A special case of cross-reactivity is the induction of epitope-specific immune responses by anti-idiotype (Id) antibodies, acting

as the epitope's "internal image." This was demonstrated for both mouse and human CD4 T-cell responses, using rabbit anti-Ids raised against PstS1-specific mouse mAbs (59, 60). However, the corresponding structural determinants remained undefined and the approach has been overtaken by the more exact recombinant DNA and synthetic peptide technologies. Id specificities have also been identified on anti-DNA autoantibodies stimulated probably by bacterial polyclonal B-cell activation in patients with TB and leprosy (61, 62).

T-CELL EPITOPE ANALYSIS IN LATENT Mtb INFECTION

Mycobacterium tuberculosis infection is routinely being monitored by skin delayed type hypersensitivity (DTH) reactions against tuberculin (PPD), a crude extract from *M. tuberculosis*. This test has poor specificity, due to cross-reaction with environmental mycobacteria and vaccination by Bacillus Calmette-Guerrin (BCG). The discovery of Mtb-specific, immunodominant, HLA-permissive epitopes, led to the use of synthetic peptide for the *in vitro* stimulation of blood T-cell responses. The numerous candidate peptides described (not reviewed here) have all been selected from several antigens on the grounds of better specificity than PPD, but they are performing at lower sensitivity than PPD. Improvements to sensitivity by using peptide pools has however had a limited impact, because of overlapping, rather than complementary recognition by the T-cell repertoire (22). The increase in stimulation by a pool of eight different peptides over the best single peptide (p91–110 from Acr) has been merely marginal. This outcome was attributed to HLA permissiveness and to competition between peptides for a limited number of binding sites on the HLA class II molecules of the APCs. This latter explanation has been supported by the finding of a declining response trend to higher concentrations of pools, but not of single peptides.

Nevertheless, commercially available IFNγ detection kits (IGRA), e.g., QuantiFERON-TB Gold (QTF-G), T-SPOT.TB etc. have been widely used for the specific detection of latent Mtb detection. They usually contain a mixture of several epitopes, predicted by algorithms of the Mtb-specific ESAT-6 and CFP-10 RD-1 antigens (absent from BCG and environmental mycobacteria). However, the use of these kits in areas highly endemic for TB is not of great added value for diagnosis. Recent side-by-side analysis of constituent antigens indicated potentials for further improvement of the test kits (63). Peptide cocktails have also been useful for the diagnosis of bovine TB in cattle, using either skin test or blood assays (64, 65). The blood INFγ assay (BOVIGRAM) readout has been further enhanced by adsorbing the peptides onto a range of microparticulate and nanoparticulate substrates (66). Detection IFNγ-induced protein (IP-10), which is produced in 100-fold greater amounts than IFNγ, has been developed for a simplified and more robust lateral flow test than IGRA (67). Notably, satisfactory results were obtained using dried plasma spots, amenable for conventional postal transport (68).

In view of the possibility that sequence variations in epitopic regions between clinical Mtb isolates might affect the results of IFNγ assays, human clinical samples were sequenced to identify substitutions that may have an impact on immunogenicity. A number of sequence polymorphisms (SNPs) have been revealed in the epitope regions of EsxB and EsxH genes (69), with evidence for recombination events, which may truncate the corresponding protein. Even single-residue differences altered the responder frequencies to these antigens from *M. bovis* isolates (70). Hence, immune variation may influence the diagnostic performance of kits, which contain epitopes from the ESX proteins.

T-CELL EPITOPE REPERTOIRE IN ACTIVE TB

Commercially available IGRA assays routinely used in clinical practice do not distinguish reliably between active TB and latent infection and have limited value for predicting the risk of developing active TB (71). Therefore, it has been of interest to search, if fine analysis of epitope specificities could improve the diagnosis. Proliferation assays of blood T cells from patients with active TB recognize a smaller number of Acr epitopes than sensitized healthy subjects (**Table 2**) (32). Similarly, patients with leprosy recognize fewer GroEL epitopes than healthy contacts (24). These findings corroborate with the previously known skin DTH anergy to PPD in a fraction of active TB patients and with the development of leprosin anergy in multibacillary leprosy. The search for epitopes, which would distinguish patients from latent infection, yielded a promising result only for the amino-terminal peptide of GroES (1–16) (56). The selective power of this peptide is surprising, considering its overlap, except for one residue, with the *M. leprae* sequence and is in need of confirmation with more clinical samples.

A reciprocal approach to the differential diagnosis has come from the finding of selective T-cell anergy in active TB in respect of the carboxy-terminal epitope p350–369 of the PstS1 antigen, which is strongly immunogenic in latently infected subjects (31). The lack of blood T-cell response can be due to sequestration to the site of disease (e.g., pleural fluid) and the ratio of T cells between these compartments is influenced by chemotherapy (72, 73). The post-chemotherapy recovery of blood response seemed more pronounced for T cells reacting with the Acr peptides, than those responding to the PstS1 peptides, which could be explained by differences in expression between replicating and

Table 2 | Recognition of fewer epitopes in active disease than in latently infected subjects.

Subjects (no. tested)	Peptides from	Frequency (%) of responders[a], no. of test peptides (p)		
From London[b]	Acr	1–3 p	4–6 p	>6 p
Healthy PPD+ skin test (25)		24	52	24
Active TB (38)		52	30	18
From Bangkok[c]	GroES	1–4 p	5–10 p	>10 p
Healthy family contacts (12)		<1	58	42
Tuberculoid leprosy (18)		33	56	11

[a] *Blood mononuclear cells of responders had at least threefold elevated ^{3}H-thymidine uptake in cultures containing the test peptide over medium alone.Data from:*

[b] *Friscia et al. (32);*

[c] *Chua-Intra et al. (24).*

chemotherapy-generated persister organisms. While dissecting of active from latent TB T-cell repertoire merely on the grounds of epitope specificity had failed to reach consensus (72), significant differences in their cytokine secretion are represented by elevated number of polyfunctional T cells (secreting IL-2, IFNγ, and TNFα) in active TB (74).

HIV-infected subjects have a high risk of reactivating their latent Mtb infection. Predicting this outcome better than just on the grounds of declining CD4 counts, would be of prime interest. The ratio of IFNγ ELISPOT counts in response to RD-1 peptides over CD4+ T-cell counts, greater than 0.21, showed 100% sensitivity and 80% specificity for active TB (75). However, the finding of 19% non-responder TB patients limited the diagnostic scope of this study. PPD-stimulated T cells carry higher levels of HIV DNA and depleted sooner than T cells of other specificity (76). This could explain the frequent reactivation of latent Mtb infection in HIV+ subjects. The antigen and epitope specificities involved have not been studied much beyond the crude PPD extract and the mechanism, which renders Mtb-reactive cells more permissive to HIV infection, is not understood. One possibility could be the lower stimulatory dose of the HLA-permissive epitopes for T cells with high affinity TcRs. This mechanism would imply selective recognition of certain epitopes, which would be rewarding to identify in the future. So far however, attention has been directed toward the study of the CD4 T-cell phenotype, characterized as CXCR3+ CCR4+ CCR6+ CD57− IFNg+ IL-17+ IL-2^{hi} MIP1b^{low} for the highly HIV-permissive PPD-reactive and CXCR3+ CCR4− CCR6− CD57+ IFNg+ IL-2^{low} MIP1b^{high} for low HIV-permissive CMV and other virus-reactive T cells (76).

ASSOCIATION OF HLA-DR AND ANTIBODY EPITOPE SPECIFICITY WITH TB

Susceptibility to TB is considered to be under the influence of multiple genetic loci, including HLA alleles. HLA-DR2 was found inherited more frequently in offspring with pulmonary TB, from both diseased and healthy parents (77) and associated with sputum-positive, but not with sputum-negative active pulmonary TB (78). DR2 alleles in sputum-positive TB associated also with elevated antibody levels to two epitopes of the PstS1 lipoglycoprotein antigen (79), but not with the similarly elevated antibody levels to epitopes of three other antigens of diverse nature (Acr, 19-kDa lipoglycoprotein, and lipoarabinomannan). The intriguing aspect of this finding is that both the genetic and immunological specificities were identified.

To explain the DR2 gene control of TB susceptibility, it has been proposed (80) that T-cell recognition of DR2-restricted PstS1 epitopes may lead to a Th2 response, producing IL-4 and IL-10 cytokines, which can lead to the development of lung pathology, rather than host protection. This hypothesis is supported by the finding, that selection of epitopes presented by B cells, rather than dendritic cells, diverted T cells from protection toward pathogenicity in *Leishmania* infection (81). Thus, the specificity of antibody responses during active TB could guide toward antigens, containing potentially pathogenic Th2 recognized epitopes. The search for such T-cell epitopes on the PstS1 antigen so far did not yield supportive data. Epitope specificity was determined only for Th1 cell clones which were mostly HLA-DR promiscuous (82) with all immunodominant epitopes of PstS1 binding to several HLA-DR molecules (22). However, these assays may not be suitable to reveal a DR2-restricted presentation of the same epitope to Th2 T cells. Development of assays for the mapping of Th2 cell stimulatory epitopes will be important to explain the mechanism of HLA class II-mediated influences on the development of multibacillary TB.

Analysis of some of the above raised aspects had been approached in mouse experimental models. Notably, influence of H-2 genes (Db or lack of I-E expression) was observed on the late progression of intraperitoneally delivered infection and pathology in the lungs, when spleen and liver bacillary counts remained stationary (83). Though this model of selective multibacillary lung disease seems relevant, the antigen specificity of the underlying immune responses was not identified. Although antibody responses to different antigens and epitopes is under H-2A control following immunization with soluble antigens in adjuvants (84, 85), there is no clear corresponding evidence following Mtb infection. On the other hand, antibody responses to hsp65 and hsp71 antigens (86) and liver granuloma formation (87) following Mtb infection were associated with non-H-2 genes.

Further analysis of the Th2 (T-helpers for B-cell responses) epitope repertoire also needs further studies in mouse models, addressing the topographical relationship between CD4 T-cell and B-cell stimulatory epitopes (88). Though using merely proliferation assays, it appeared, that PstS1 antigen immunized mice produced CD4 T cells, but not antibodies against the p65–83 peptide, which contains a non-overlapping cryptic B and an immunodominant T epitope core, while T-cell help was "delegated" probably to distantly located linear or conformational B-cell epitopes (**Table 3**) (89). A functional association between topographically distinct epitopes was suggested also by the finding that a single amino acid mutation of epitope core of the 19-kDa antigen abrogated T-cell, but not the B-cell immunogenicity (90).

PEPTIDE EPITOPE-BASED VACCINATION AGAINST TB

Most research toward a better vaccine against TB has been based on boosting immunity after BCG priming. The choice of antigen for this purpose has been to some extent subjective, though usually targeting proteins which appeared as most immunogenic in Mtb-infected individuals or experimental animals. Further breakdown of the immune repertoire to individual epitope specificities showed that both immune recognition was influenced by the nature of the immunogen. Thus, CD4 T cells recognized different peptides, when mice were vaccinated with either PstS1 antigen or heat-killed Mtb or infected with H37Rv bacilli (**Table 3**) (89). Antibodies reacted to different epitopes following vaccination, but bound only to conformational epitopes following infection. CD8 T cells also recognized different peptides following vaccination or Mtb infection (91, 92). These results indicate that the nature of the immunogen could influence antigen processing, which may deviate T-cell help from one to another B-cell epitope. Substantial differences in protection, cytokine profile, and recognition of T-cell epitopes of Ag85A or the Ag85B–ESAT-6 fusion protein were observed after its presentation either expressed in adenovirus vector or with an adjuvant (93). Disparities were observed also in response to Ag85A and its immunogenic peptides, when inoculated intranasally or parenterally (94).

Table 3 | Differences in epitope recognition between immunized and infected C57Bl/10 mice.

Foot pad injection	T-cell proliferation to peptides				Antibody level		
	44–64	65–83	123–143	350–368	p1–20	p201–220	TB71
Recombinant PstS1[a]	–	++	++	–	–	+++	++
Heat-killed H37Rv[a]	–	–	+	+	+++	–	+
H37Rv infection	+	+	–	–	–	–	++

Magnitude of the immune response: stimulation indices: –, <1; +, 1–10; ++, 10–20 of spleen and lymph node cells 7 days after immunization.

ELISA peptide binding or TB71 mAb competition titers: –, <10; +, 10–100; ++, 100–1000; +++, >1000 in sera harvested 12 weeks after first inoculation.

[a]*Antigen in incomplete Freund's adjuvant followed by three boosters without adjuvant. Data from Vordermeier et al. (89).*

DNA gun bombardment has been used for the mapping of T-cell epitopes and the effect of self-adjuvanting domains. Several CD4+ and one CD8+ T-cell epitopes were identified on the DNA-binding protein 1 (MDP1) antigen (95), while spleen CD4 T cells from HLA-DRB1*0401 transgenic mice recognized only the p191–210 epitope on the MPT51 antigen (96). A fusion DNA vaccine incorporating the HSP70 C-terminal domain (as adjuvant) and MPT51 (as target antigen) stimulated CD4, but not the CD8 T-cell response (97). Though documenting immunogenicity, these results need to be extended for protection against challenge.

Vaccine design could benefit from modifying the structure of peptides to increase their immunogenicity, while conjugation of a MHC-permissive peptide could abrogate genetic restriction for another MHC-restricted epitope. Studies in this direction showed that orientation between two epitopes within a synthetic peptide dimer can profoundly influence immunogenicity (98). Orientation of peptides played a role also for chimeric peptides constructed by recombinant DNA technology (99). Immunogenicity can be increased also by extension of an epitope core with non-native flanking residues (100) or by covalent attachment to biodegradable amphoteric branched chain polypeptides (101). Lipoylation of the MHC-promiscuous 91–110 peptide of Acr inoculated without any adjuvants was reported to enhance the immunogenicity and imparted protection against aerosol Mtb challenge in both mice and guinea pigs to an even better extent than BCG (102).

EPITOPE-SPECIFIC SERODIAGNOSIS

Mycobacterium tuberculosis species-specific mAbs had been used in a competition serodiagnostic test for TB and leprosy, preceding the purification of target antigens (103, 104). The mAb competition test has the advantage of higher sensitivity due to its low background values, which allowed the use of 20 times lower serum dilutions (i.e., 1/5) than standard ELISA tests (1/100). The competition assay also discriminated species-specific from cross-reactive epitopes on a number of antigens and identified several associations between antibody specificity and clinical aspects of TB (105, 106) (see also chapter by G. Bothamley). Epitope-specific antibody levels can be representative for both specificity and sensitivity of the whole antigen (e.g., PstS1) (51). However, the mAb competition test was more specific than binding to the whole lipoarabinomann, by targeting a Mtb-specific epitope or avoiding detection of contaminants. Epitope-specific titers also reflected the clinical form of TB, when related to titers against the whole 19-kDa lipoprotein (107).

Several serological surveys showed that serum antibody levels are consistently elevated in the great majority of sputum-positive TB, but not in sputum-negative disease (108). Though the latter aspect is greatly limiting the diagnostic application of serology, this hindrance was not acknowledged during the uncontrolled marketing of commercial kits. On the other hand, detection of antibodies in the cerebrospinal fluid is of particular value for the diagnosis of TB meningitis (109), where rapid detection can be life saving. Due to the high sensitivity, low cost, and operational advantages, serological screening has been suggested for active case finding in high-risk populations for multibacillary infectious patients (108, 110). Their early diagnosis could reduce the transmission of Mtb infection and therefore represents an important epidemiological, rather than clinical objective. A similar rationale could apply to leprosy, where antibody levels are elevated in the infectious multibacillary lepromatous, rather than the paucibacillary tuberculoid form (3).

A recent advance in epitope screening has been the high-content peptide microarray chip technology, involving the testing of thousands of different peptides. This approach showed that several epitopes are differentially recognized by IgG antibodies in pulmonary TB sera (111) and identified epitope "hotspots" within a number of protein antigens with similar patterns for patients of different genetic background. These linear epitopes are likely to detect a different repertoire than serology based on whole protein molecules, which detects mostly antibodies against conformational epitopes. Further clinical evaluation, particularly comparing multi- and paucibacillary forms of active TB, seems warranted.

TcR-LIKE LIGANDS

Tubercle bacilli multiply and persist predominantly in macrophages, which display on their surface HLA-bound antigenic mycobacterial peptides, which are recognized by T-cell receptors (TcR). T cells can impart protection, but their excessive reactions can lead also to inflammatory pathology, characteristic of active TB. To avoid the latter outcome, suitable T-cell receptor-like constructs could be protective, without the accompanying undesirable T-cell-mediated inflammation. Immunotherapy using soluble TcR ligands could kill macrophages infected with both replicating and dormant Mtb organisms. Based on this hypothesis, TcR-based immunotherapy could be used as an adjunct to chemotherapy and be particularly useful to HIV-infected TB patients, many of whom being immunocompromised, cannot be protected by active vaccination. Therefore, TcR immunotherapy might be better than "therapeutic" active vaccination and also more efficient than passive antibody therapy, which can target probably only extracellular Mtb bacilli (112, 113). Unlike antibodies to B-cell epitopes [including those directed against overlapping T and B epitopes (114)], TcR-like ligands would be directed against epitopes, displayed in complex with MHC molecules on the surface of Mtb-infected cells.

Development of immunotherapeutic agents with TcR specificity has become feasible with the introduction of two technologies: (1) single chain antibody fragments (scFv) with TcR specificity can be selected from phage antibody libraries (115–118); (2) monomeric high affinity soluble human TcRs (mTcRs) have been expressed from cloned CD8 T cells and produced within the cytoplasm of trxB gor mutant *E. coli* strains (119). TcR-like mAbs against HLA class I presented epitopes of tumors (120) and virus-infected cells are being developed as novel immunotherapeutics with epitope-specific killing potentials, as well as diagnostic reagents (118). Moreover, genetic fusion of *Pseudomonas* exotoxin with TcR-like mAbs amplified the killing tumor cells (121, 122).

These advances, particularly in epitope-specific cancer immunotherapy, seem attractive for the development of TB immunotherapy. Production of mAb and mTcR ligands needs to target some of the empirically identified HLA class I immunodominant epitopes, which have been mapped for a number of Mtb antigens (26, 33, 34, 91, 123, 124). However, a similar approach to MHC class II-presented epitopes could be much more difficult, because their expression is impaired in infected macrophages (125) and because the procedures for producing the corresponding TcR-like ligands are yet underdeveloped. However, some concerns need to be addressed: immunodominance of the currently known MHC class I epitopes may have resulted from peptide presentation by dendritic cells or by cross-presentation, while a TcR-based immunotherapy would need to be targeted against epitopes expressed by infected macrophages. Therefore, it is a prerequisite to confirm the specificity and density of epitope expression on Mtb-infected macrophages. This needs to be ascertained by their capacity to stimulate CD8 T-cell clones or better by direct detection of the levels of epitope expression by antibody staining (126), or by tandem mass spectrometry (127). Notably, the latter technique showed that epitope abundance does not necessarily associate with the immunodominance hierarchy of epitopes.

Since apoptosis of macrophages is known to be the key mechanism for the killing of intracellular mycobacteria (128, 129), conjugation of TcR-specific ligands with apoptosis-inducing agents may amplify the therapeutic effect. Suitable candidate compounds for this purpose, with proven apoptosis-inducing capacity are *Pseudomonas* exotoxin A (130), granzyme B (131, 132), or BH3 peptide (133, 134).

Further synergistic benefit may come from recombinant INFγ treatment, which enhances the surface expression of MHC-bound epitopes and has even alone been therapeutically beneficial in TB patients (135).

TREGITOPES

Tregitopes are epitopes, mostly in the Fc and constant Fab region of IgG, with highly conserved structure between mammalian species. They bind promiscuously to HLA class II molecules and stimulate and expand CD25(+) FoxP3(+) natural regulatory T cells (nTreg) (136). Tregitopes were shown to inhibit CD8 T-cell responses to co-administered antigens, with potentials to prevent or treat autoimmune disease, e.g., Type 1 diabetes or suppress allo-specific responses in mouse models. Co-administration of Tregitopes and auto-antigens reduced diabetes in NOD mice, while the *in vitro* response of T cells from diabetic patients to GAD65 epitopes was found suppressed by Tregitopes (137). Tregitopes might also prevent immune responses against hyper-variable Ig Ids, generated by somatic mutations.

The long-known therapeutic effects of intravenous human gamma globulin therapy (ivG) in autoimmune or allergic diseases, organ transplantation, and graft-versus-host disease have been attributed to the presence of Tregitopes in IgG (136, 138). The proposed mechanism as ivG-induced immunological tolerance has been supported by the increase of Treg cells and IL-10 production after ivG treatment. This concept is relevant to TB, considering the finding that intranasal or intraperitoneal inoculations of human gamma globulin inhibited the BCG viable counts in the lungs of intranasally infected mice (139, 140). Although the authors attributed this effect to the action of specific antibodies, an alternative possible explanation could involve the role of Tregitopes.

Increased Treg numbers in patients with active TB depress the IFNγ-secreting T-cell response to a protective antigen, such as the heparin binding hemagglutinin (141). Mycobacterium-activated human CD8 Treg cells co-express CD39, which is involved in the suppression of CD4 Th1 cell proliferation, lymphocyte activation, and express also LAG-3 and CCL4 (142). Impairing the function of Tregs in mice reduced Mtb infection (143), but antibody inactivation of Tregs, which increased the immune response did not affect the bacterial load after infection (144) and did not influence protection by BCG vaccination (145). In view of these discrepancies about the possible Treg function in TB and the lack of knowledge about their specificity, the possible role of Tregitope recognition deserves further study.

CONCLUSION

Epitope specificity of immune responses of Mtb-infected hosts is significant for the immunopathogenesis of TB and its knowledge is mandatory for the development of new approaches toward TB control. HLA-permissive epitopes may have evolved in the tubercle bacilli due to the advantage from immune reactions, which lead to protracted transmission of the infection. Further research needs to expand knowledge on associations between epitope specificity with different effector and regulatory T-cell populations. It is proposed that combining of the biosignature of the T-cell phenotype with epitope specificity might lead to the discovery of protection and disease-associated biomarkers. There are important potentials toward the future development of epitope-defined diagnostics, prophylactic vaccines, and immunotherapies.

ACKNOWLEDGMENTS

I thank for collaboration all co-authors, quoted in the several joint publications.

REFERENCES

1. Ivanyi J. Pathogenic and protective interactions in mycobacterial infections. *Clin Immunol* (1986) **6**:127–57.
2. Ivanyi J, Morris JA, Keen M. Studies with monoclonal antibodies to mycobacteria. In: Macario AJL, Macario EC, editors. *Monoclonal Antibodies Against Bacteria*. New York: Academic Press (1985). p. 59–90.
3. Ivanyi J, Sharp K, Jackett P, Bothamley G. Immunological study of the defined constituents of mycobacteria. *Springer Semin Immunopathol* (1988) **10**:279–300. doi:10.1007/BF02053841

4. Young RA, Bloom BR, Grosskinsky CM, Ivanyi J, Thomas D, Davis RW. Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA. *Proc Natl Acad Sci U S A* (1985) **82**:2583–7. doi:10.1073/pnas.82.9.2583
5. Lamb JR, Ivanyi J, Rees AD, Rothbard JB, Howland K, Young RA, et al. Mapping of T cell epitopes using recombinant antigens and synthetic peptides. *EMBO J* (1987) **6**:1245–9.
6. McMurry JA, Kimball S, Lee JH, Rivera D, Martin W, Weiner DB, et al. Epitope-driven TB vaccine development: a streamlined approach using immunoinformatics, ELISpot assays, and HLA transgenic mice. *Curr Mol Med* (2007) **7**:351–68. doi:10.2174/156652407780831584
7. Vordermeier M, Whelan AO, Hewinson RG. Recognition of mycobacterial epitopes by T cells across mammalian species and use of a program that predicts human HLA-DR binding peptides to predict bovine epitopes. *Infect Immun* (2003) **71**:1980–7. doi:10.1128/IAI.71.4.1980-1987.2003
8. Scarabelli G, Morra G, Colombo G. Predicting interaction sites from the energetics of isolated proteins: a new approach to epitope mapping. *Biophys J* (2010) **98**:1966–75. doi:10.1016/j.bpj.2010.01.014
9. Berry MP, Blankley S, Graham CM, Bloom CI, O'Garra A. Systems approaches to studying the immune response in tuberculosis. *Curr Opin Immunol* (2013) **25**:579–87. doi:10.1016/j.coi.2013.08.003
10. Ernst JD, Lewinsohn DM, Behar S, Blythe M, Schlesinger LS, Kornfeld H, et al. Meeting report: NIH workshop on the tuberculosis immune epitope database. *Tuberculosis (Edinb)* (2008) **88**:366–70. doi:10.1016/j.tube.2007.11.002
11. Lindestam Arlehamn CS, Gerasimova A, Mele F, Henderson R, Swann J, Greenbaum JA, et al. Memory T cells in latent *Mycobacterium tuberculosis* infection are directed against three antigenic islands and largely contained in a CXCR3+CCR6+ Th1 subset. *PLoS Pathog* (2013) **9**:e1003130. doi:10.1371/journal.ppat.1003130
12. Axelsson-Robertson R, Magalhaes I, Parida SK, Zumla A, Maeurer M. The immunological footprint of *Mycobacterium tuberculosis* T-cell epitope recognition. *J Infect Dis* (2012) **205**(Suppl 2):S301–15. doi:10.1093/infdis/jis198
13. Brett SJ, Lamb JR, Cox JH, Rothbard JB, Mehlert A, Ivanyi J. Differential pattern of T cell recognition of the 65-kDa mycobacterial antigen following immunization with the whole protein or peptides. *Eur J Immunol* (1989) **19**:1303–10. doi:10.1002/eji.1830190723
14. Vordemeier HM, Harris DP, Roman E, Lathigra R, Moreno C, Ivanyi J. Identification of T cell stimulatory peptides from the 38-kDa protein of *Mycobacterium tuberculosis. J Immunol* (1991) **147**:1023–9.
15. Harris DP, Vordermeier HM, Friscia G, Roman E, Surcel HM, Pasvol G, et al. Genetically permissive recognition of adjacent epitopes from the 19-kDa antigen of *Mycobacterium tuberculosis* by human and murine T cells. *J Immunol* (1993) **150**:5041–50.
16. Vordermeier HM, Harris DP, Lathigra R, Roman E, Moreno C, Ivanyi J. Recognition of peptide epitopes of the 16,000 MW antigen of *Mycobacterium tuberculosis* by murine T cells. *Immunology* (1993) **80**:6–12.
17. Vordermeier HM, Arya A, Harris DP, Moreno C, Ivanyi J. Abundance of H-2 promiscuous T cells specific for mycobacterial determinants in H-2b/d F1 hybrid mice. *Eur J Immunol* (1995) **25**:2770–4. doi:10.1002/eji.1830251009
18. Harris DP, Vordermeier HM, Arya A, Moreno C, Ivanyi J. Permissive recognition of a mycobacterial T-cell epitope: localization of overlapping epitope core sequences recognized in association with multiple major histocompatibility complex class II I-A molecules. *Immunology* (1995) **84**:555–61.
19. Roman E, Harris DP, Jurcevic S, Ivanyi J, Moreno C. H-2-associated effects of flanking residues on the recognition of a permissive mycobacterial T-cell epitope. *Immunology* (1995) **86**:183–9.
20. Agrewala JN, Wilkinson RJ. Influence of HLA-DR on the phenotype of CD4+ T lymphocytes specific for an epitope of the 16-kDa alpha-crystallin antigen of *Mycobacterium tuberculosis. Eur J Immunol* (1999) **29**:1753–61. doi:10.1002/(SICI)1521-4141(199906)29:06<753::AID-IMMU1753>3.0.CO;2-B
21. Comas I, Chakravartti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of *Mycobacterium tuberculosis* are evolutionarily hyperconserved. *Nat Genet* (2010) **42**:498–503. doi:10.1038/ng.590
22. Jurcevic S, Hills A, Pasvol G, Davidson RN, Ivanyi J, Wilkinson RJ. T cell responses to a mixture of *Mycobacterium tuberculosis* peptides with complementary HLA-DR binding profiles. *Clin Exp Immunol* (1996) **105**:416–21. doi:10.1046/j.1365-2249.1996.d01-791.x
23. Mustafa AS, Lundin KE, Meloen RH, Shinnick TM, Oftung F. Identification of promiscuous epitopes from the mycobacterial 65-kilodalton heat shock protein recognized by human CD4(+) T cells of the *Mycobacterium leprae* memory repertoire. *Infect Immun* (1999) **67**:5683–9.
24. Chua-Intra B, Peerapakorn S, Davey N, Jurcevic S, Busson M, Vordermeier HM, et al. T-cell recognition of mycobacterial GroES peptides in Thai leprosy patients and contacts. *Infect Immun* (1998) **66**:4903–9.
25. Caccamo N, Barera A, Di Sano C, Meraviglia S, Ivanyi J, Hudecz F, et al. Cytokine profile, HLA restriction and TCR sequence analysis of human CD4+ T clones specific for an immunodominant epitope of *Mycobacterium tuberculosis* 16-kDa protein. *Clin Exp Immunol* (2003) **133**:260–6. doi:10.1046/j.1365-2249.2003.02201.x
26. Shams H, Klucar P, Weis SE, Lalvani A, Moonan PK, Safi H, et al. Characterization of a *Mycobacterium tuberculosis* peptide that is recognized by human CD4+ and CD8+ T cells in the context of multiple HLA alleles. *J Immunol* (2004) **173**:1966–77.
27. Tully G, Kortsik C, Hohn H, Zehbe I, Hitzler WE, Neukirch C, et al. Highly focused T cell responses in latent human pulmonary *Mycobacterium tuberculosis* infection. *J Immunol* (2005) **174**:2174–84.
28. Valle MT, Megiovanni AM, Merlo A, Li Pira G, Bottone L, Angelini G, et al. Epitope focus, clonal composition and Th1 phenotype of the human CD4 response to the secretory mycobacterial antigen Ag85. *Clin Exp Immunol* (2001) **123**:226–32. doi:10.1046/j.1365-2249.2001.01450.x
29. Al-Attiyah R, Shaban FA, Wiker HG, Oftung F, Mustafa AS. Synthetic peptides identify promiscuous human Th1 cell epitopes of the secreted mycobacterial antigen MPB70. *Infect Immun* (2003) **71**:1953–60. doi:10.1128/IAI.71.4.1953-1960.2003
30. Wang M, Tang ST, Stryhn A, Justesen S, Larsen MV, Dziegiel MH, et al. Identification of MHC class II restricted T-cell-mediated reactivity against MHC class I binding *Mycobacterium tuberculosis* peptides. *Immunology* (2011) **132**:482–91. doi:10.1111/j.1365-2567.2010.03383.x
31. Vordermeier HM, Harris DP, Friscia G, Roman E, Surcel HM, Moreno C, et al. T cell repertoire in tuberculosis: selective anergy to an immunodominant epitope of the 38-kDa antigen in patients with active disease. *Eur J Immunol* (1992) **22**:2631–7. doi:10.1002/eji.1830221024
32. Friscia G, Vordermeier HM, Pasvol G, Harris DP, Moreno C, Ivanyi J. Human T cell responses to peptide epitopes of the 16-kD antigen in tuberculosis. *Clin Exp Immunol* (1995) **102**:53–7. doi:10.1111/j.1365-2249.1995.tb06635.x
33. Weichold FF, Mueller S, Kortsik C, Hitzler WE, Wulf MJ, Hone DM, et al. Impact of MHC class I alleles on the *M. tuberculosis* antigen-specific CD8+ T-cell response in patients with pulmonary tuberculosis. *Genes Immun* (2007) **8**:334–43. doi:10.1038/sj.gene.6364392
34. Axelsson-Robertson R, Weichold F, Sizemore D, Wulf M, Skeiky YA, Sadoff J, et al. Extensive major histocompatibility complex class I binding promiscuity for *Mycobacterium tuberculosis* TB10.4 peptides and immune dominance of human leucocyte antigen (HLA)-B*0702 and HLA-B*0801 alleles in TB10.4 CD8 T-cell responses. *Immunology* (2010) **129**:496–505. doi:10.1111/j.1365-2567.2009.03201.x
35. Jurcevic S, Travers PJ, Hills A, Agrewala JN, Moreno C, Ivanyi J. Distinct conformations of a peptide bound to HLA-DR1 or DRB5*0101 suggested by molecular modelling. *Int Immunol* (1996) **8**:1807–14. doi:10.1093/intimm/8.11.1807
36. Caccamo N, Meraviglia S, La Mendola C, Bosze S, Hudecz F, Ivanyi J, et al. Characterization of HLA-DR- and TCR-binding residues of an immunodominant and genetically permissive peptide of the 16-kDa protein of *Mycobacterium tuberculosis. Eur J Immunol* (2004) **34**:2220–9. doi:10.1002/eji.200425090
37. Sundaramurthi JC, Brindha S, Shobitha SR, Swathi A, Ramanandan P, Hanna LE. In silico identification of potential antigenic proteins and promiscuous CTL epitopes in *Mycobacterium tuberculosis. Infect Genet Evol* (2012) **12**:1312–8. doi:10.1016/j.meegid.2012.03.023
38. Bobosha K, Tang ST, van der Ploeg-van Schip JJ, Bekele Y, Martins MV, Lund O, et al. *Mycobacterium leprae* virulence-associated peptides are indicators of exposure to *M. leprae* in Brazil, Ethiopia and Nepal. *Mem Inst Oswaldo Cruz* (2012) **107**(Suppl 1):112–23. doi:10.1590/S0074-02762012000900018
39. Wiens KE, Swaminathan H, Copin R, Lun DS, Ernst JD. Equivalent T cell epitope promiscuity in ecologically diverse human pathogens. *PLoS One* (2013) **8**:e73124. doi:10.1371/journal.pone.0073124
40. Ivanyi J, Norton PM, Matsuzaki G. Immune responses to stress proteins in mycobacterial infections. In: van Eden W, Young DB, editors. *Stress Proteins in Medicine.* New York: Marcel Dekker, Inc. (1996). p. 265–285.

41. Kiessling R, Gronberg A, Ivanyi J, Soderstrom K, Ferm M, Kleinau S, et al. Role of hsp60 during autoimmune and bacterial inflammation. *Immunol Rev* (1991) **121**:91–111. doi:10.1111/j.1600-065X.1991.tb00824.x
42. Wick G, Kleindienst R, Schett G, Amberger A, Xu Q. Role of heat shock protein 65/60 in the pathogenesis of atherosclerosis. *Int Arch Allergy Immunol* (1995) **107**:130–1. doi:10.1159/000236952
43. Van Eden W, Wick G, Albani S, Cohen I. Stress, heat shock proteins, and autoimmunity: how immune responses to heat shock proteins are to be used for the control of chronic inflammatory diseases. *Ann N Y Acad Sci* (2007) **1113**:217–37. doi:10.1196/annals.1391.020
44. Grundtman C, Kreutmayer SB, Almanzar G, Wick MC, Wick G. Heat shock protein 60 and immune inflammatory responses in atherosclerosis. *Arterioscler Thromb Vasc Biol* (2011) **31**:960–8. doi:10.1161/ATVBAHA.110.217877
45. Raine CS, Wu E, Ivanyi J, Katz D, Brosnan CF. Multiple sclerosis: a protective or a pathogenic role for heat shock protein 60 in the central nervous system? *Lab Invest* (1996) **75**:109–23.
46. Spencer JS, Marques MA, Lima MC, Junqueira-Kipnis AP, Gregory BC, Truman RW, et al. Antigenic specificity of the *Mycobacterium leprae* homologue of ESAT-6. *Infect Immun* (2002) **70**:1010–3. doi:10.1128/IAI.70.2.1010-1013.2002
47. Ivanyi J. Recall of antibody synthesis to the primary antigen following successive immunization with heterologous albumins. A two-cell theory of the original antigenic sin. *Eur J Immunol* (1972) **2**:354–9. doi:10.1002/eji.1830020411
48. Bothamley G, Beck JS, Britton W, Elsaghier A, Ivanyi J. Antibodies to *Mycobacterium tuberculosis*-specific epitopes in lepromatous leprosy. *Clin Exp Immunol* (1991) **86**:426–32. doi:10.1111/j.1365-2249.1991.tb02948.x
49. Harris DP, Vordermeier HM, Singh M, Moreno C, Jurcevic S, Ivanyi J. Cross-recognition by T cells of an epitope shared by two unrelated mycobacterial antigens. *Eur J Immunol* (1995) **25**:3173–9. doi:10.1002/eji.1830251128
50. Harris DP, Vordermeier HM, Roman E, Lathigra R, Brett SJ, Moreno C, et al. Murine T cell-stimulatory peptides from the 19-kDa antigen of *Mycobacterium tuberculosis*. Epitope-restricted homology with the 28-kDa protein of *Mycobacterium leprae*. *J Immunol* (1991) **147**:2706–12.
51. Jackett PS, Bothamley GH, Batra HV, Mistry A, Young DB, Ivanyi J. Specificity of antibodies to immunodominant mycobacterial antigens in pulmonary tuberculosis. *J Clin Microbiol* (1988) **26**:2313–8.
52. Elsaghier A, Lathigra R, Ivanyi J. Localisation of linear epitopes at the carboxy-terminal end of the mycobacterial 71 kDa heat shock protein. *Mol Immunol* (1992) **29**:1153–6. doi:10.1016/0161-5890(92)90049-4
53. Elsaghier A, Prantera C, Bothamley G, Wilkins E, Jindal S, Ivanyi J. Disease association of antibodies to human and mycobacterial hsp70 and hsp60 stress proteins. *Clin Exp Immunol* (1992) **89**:305–9. doi:10.1111/j.1365-2249.1992.tb06950.x
54. Elsaghier A, Prantera C, Moreno C, Ivanyi J. Antibodies to *Mycobacterium paratuberculosis*-specific protein antigens in Crohn's disease. *Clin Exp Immunol* (1992) **90**:503–8. doi:10.1111/j.1365-2249.1992.tb05874.x
55. Chua-Intra B, Ivanyi J, Hills A, Thole J, Moreno C, Vordermeier HM. Predominant recognition of species-specific determinants of the GroES homologues from *Mycobacterium leprae* and *M. tuberculosis*. *Immunology* (1998) **93**:64–72. doi:10.1046/j.1365-2567.1998.00400.x
56. Chua-Intra B, Wilkinson RJ, Ivanyi J. Selective T-cell recognition of the N-terminal peptide of GroES in tuberculosis. *Infect Immun* (2002) **70**:1645–7. doi:10.1128/IAI.70.3.1645-1647.2002
57. Chua-Intra B, Wattanapokayakit S, Srisungngam S, Srisungngam T, Mahotarn K, Brennan PJ, et al. T-cell recognition of peptides from the *Mycobacterium leprae* 35 kDa protein in Thai leprosy patients, healthy contacts, and non-contacts. *Immunol Lett* (2003) **88**:71–6. doi:10.1016/S0165-2478(03)00065-8
58. Kaleab B, Wondimu A, Likassa R, Woldehawariat N, Ivanyi J. Sustained T-cell reactivity to *Mycobacterium tuberculosis* specific antigens in "split-anergic" leprosy. *Lepr Rev* (1995) **66**:19–25.
59. Praputpittaya K, Ivanyi J. Stimulation by anti-idiotype antibody of murine T cell responses to the 38 kD antigen of *Mycobacterium tuberculosis*. *Clin Exp Immunol* (1987) **70**:307–15.
60. Rees AD, Scoging A, Dobson N, Praputpittaya K, Young D, Ivanyi J, et al. T cell activation by anti-idiotypic antibody: mechanism of interaction with antigen-reactive T cells. *Eur J Immunol* (1987) **17**:197–201. doi:10.1002/eji.1830170208
61. Mackworth-Young CG, Cairns E, Sabbaga J, Massicotte H, Diamond B, Bell DA, et al. Comparative study of idiotypes on monoclonal antibodies derived from patients with lupus and leprosy and from normal individuals. *J Autoimmun* (1990) **3**:415–29. doi:10.1016/S0896-8411(05)80009-5
62. Zumla A, Williams W, Mudd D, Locniskar M, Behrens R, Isenberg D, et al. Expression of a common idiotype PR4 in the sera of patients with leprosy. *Clin Exp Immunol* (1991) **84**:522–6.
63. Arlehamn CS, Sidney J, Henderson R, Greenbaum JA, James EA, Moutaftsi M, et al. Dissecting mechanisms of immunodominance to the common tuberculosis antigens ESAT-6, CFP10, Rv2031c (hspX), Rv2654c (TB7.7), and Rv1038c (EsxJ). *J Immunol* (2012) **188**:5020–31. doi:10.4049/jimmunol.1103556
64. Flores-Villalva S, Suarez-Guemes F, Espitia C, Whelan AO, Vordermeier M, Gutierrez-Pabello JA. Specificity of the tuberculin skin test is modified by use of a protein cocktail containing ESAT-6 and CFP-10 in cattle naturally infected with *Mycobacterium bovis*. *Clin Vaccine Immunol* (2012) **19**:797–803. doi:10.1128/CVI.05668-11
65. Casal C, Bezos J, Diez-Guerrier A, Alvarez J, Romero B, de Juan L, et al. Evaluation of two cocktails containing ESAT-6, CFP-10 and Rv-3615c in the intradermal test and the interferon-gamma assay for diagnosis of bovine tuberculosis. *Prev Vet Med* (2012) **105**:149–54. doi:10.1016/j.prevetmed.2012.02.007
66. Saleem IY, Vordermeier M, Barralet JE, Coombes AG. Improving peptide-based assays to differentiate between vaccination and *Mycobacterium bovis* infection in cattle using nanoparticle carriers for adsorbed antigens. *J Control Release* (2005) **102**:551–61. doi:10.1016/j.jconrel.2004.10.034
67. Ruhwald M, Aabye MG, Ravn P. IP-10 release assays in the diagnosis of tuberculosis infection: current status and future directions. *Expert Rev Mol Diagn* (2012) **12**:175–87. doi:10.1586/erm.11.97
68. Aabye MG, Latorre I, Diaz J, Maldonado J, Mialdea I, Eugen-Olsen J, et al. Dried plasma spots in the diagnosis of tuberculosis: IP-10 release assay on filter paper. *Eur Respir J* (2013) **42**:495–503. doi:10.1183/09031936.00129412
69. Uplekar S, Heym B, Friocourt V, Rougemont J, Cole ST. Comparative genomics of Esx genes from clinical isolates of *Mycobacterium tuberculosis* provides evidence for gene conversion and epitope variation. *Infect Immun* (2011) **79**:4042–9. doi:10.1128/IAI.05344-11
70. Jones GJ, Gordon SV, Hewinson RG, Vordermeier HM. Screening of predicted secreted antigens from *Mycobacterium bovis* reveals the immunodominance of the ESAT-6 protein family. *Infect Immun* (2010) **78**:1326–32. doi:10.1128/IAI.01246-09
71. Chegou NN, Heyckendorf J, Walzl G, Lange C, Ruhwald M. Beyond the IFN-gamma horizon: biomarkers for immunodiagnosis of infection with *M. tuberculosis*. *Eur Respir J* (2013). doi:10.1183/09031936.00151413
72. Wilkinson RJ, Vordermeier HM, Wilkinson KA, Sjolund A, Moreno C, Pasvol G, et al. Peptide-specific T cell response to *Mycobacterium tuberculosis*: clinical spectrum, compartmentalization, and effect of chemotherapy. *J Infect Dis* (1998) **178**:760–8. doi:10.1086/515336
73. Dieli F, Friscia G, Di Sano C, Ivanyi J, Singh M, Spallek R, et al. Sequestration of T lymphocytes to body fluids in tuberculosis: reversal of anergy following chemotherapy. *J Infect Dis* (1999) **180**:225–8. doi:10.1086/314852
74. Caccamo N, Dieli F. Are polyfunctional cells protective in *M. tuberculosis* infection? In: Cardona P-J, editor. *Understanding Tuberculosis-Analyzing the Origin of Mycobacterium tuberculosis Pathogenicity*. Rieka: InTech (2012). p. 313–42.
75. Goletti D, Carrara S, Mayanja-Kizza H, Baseke J, Mugerwa MA, Girardi E, et al. Response to *M. tuberculosis* selected RD1 peptides in Ugandan HIV-infected patients with smear positive pulmonary tuberculosis: a pilot study. *BMC Infect Dis* (2008) **8**:11. doi:10.1186/1471-2334-8-11
76. Geldmacher C, Ngwenyama N, Schuetz A, Petrovas C, Reither K, Heeregrave EJ, et al. Preferential infection and depletion of *Mycobacterium tuberculosis*-specific CD4 T cells after HIV-1 infection. *J Exp Med* (2010) **207**:2869–81. doi:10.1084/jem.20100090
77. Singh SP, Mehra NK, Dingley HB, Pande JN, Vaidya MC. Human leukocyte antigen (HLA)-linked control of susceptibility to pulmonary tuberculosis and association with HLA-DR types. *J Infect Dis* (1983) **148**:676–81. doi:10.1093/infdis/148.4.676
78. Brahmajothi V, Pitchappan RM, Kakkanaiah VN, Sashidhar M, Rajaram K, Ramu S, et al. Association of pulmonary tuberculosis and HLA in south India. *Tubercle* (1991) **72**:123–32. doi:10.1016/0041-3879(91)90039-U
79. Bothamley GH, Beck JS, Schreuder GM, D'Amaro J, de Vries RR, Kardjito T, et al. Association of tuberculosis and *M. tuberculosis*-specific antibody levels with HLA. *J Infect Dis* (1989) **159**:549–55. doi:10.1093/infdis/159.3.549
80. Ivanyi J, Thole J. Specificity and function of T and B cell recognition in tuberculosis. In: Bloom BR, editor. *Tuberculosis: Pathogenesis, Protection and Control*. Washington, DC: ASM Press (1994). p. 437–58.

81. Rossi-Bergmann B, Muller I, Godinho EB. TH1 and TH2 T-cell subsets are differentially activated by macrophages and B cells in murine leishmaniasis. *Infect Immun* (1993) **61**:2266–9.
82. Pitchappan RM, Agrewala JN, Dheenadhayalan V, Ivanyi J. Major histocompatibility complex restriction in tuberculosis susceptibility. *J Biosci* (1997) **22**:47–57. doi:10.1007/BF02703617
83. Brett S, Orrell JM, Swanson Beck J, Ivanyi J. Influence of H-2 genes on growth of *Mycobacterium tuberculosis* in the lungs of chronically infected mice. *Immunology* (1992) **76**:129–32.
84. Ivanyi J, Sharp K. Control by H-2 genes of murine antibody responses to protein antigens of *Mycobacterium tuberculosis*. *Immunology* (1986) **59**:329–32.
85. Barcenas-Morales G, Merkenschlager M, Wahid F, Doffinger R, Ivanyi J. Recessive expression of the H2A-controlled immune response phenotype depends critically on antigen dose. *Immunology* (2000) **99**:221–8. doi:10.1046/j.1365-2567.2000.00956.x
86. Brett SJ, Ivanyi J. Genetic influences on the immune repertoire following tuberculous infection in mice. *Immunology* (1990) **71**:113–9.
87. Orrell JM, Brett SJ, Ivanyi J, Coghill G, Grant A, Beck JS. Morphometric analysis of *Mycobacterium tuberculosis* infection in mice suggests a genetic influence on the generation of the granulomatous inflammatory response. *J Pathol* (1992) **166**:77–82. doi:10.1002/path.1711660112
88. Verbon A, Hartskeerl RA, Moreno C, Kolk AH. Characterization of B cell epitopes on the 16K antigen of *Mycobacterium tuberculosis*. *Clin Exp Immunol* (1992) **89**:395–401. doi:10.1111/j.1365-2249.1992.tb06969.x
89. Vordermeier HM, Harris DP, Moreno C, Singh M, Ivanyi J. The nature of the immunogen determines the specificity of antibodies and T cells to selected peptides of the 38 kDa mycobacterial antigen. *Int Immunol* (1995) **7**:559–66. doi:10.1093/intimm/7.4.559
90. Harris DP, Hill M, Vordermeier HM, Jones M, Hewinson G, Thangaraj H, et al. Mutagenesis of an immunodominant T cell epitope can affect recognition of different T and B determinants within the same antigen. *Mol Immunol* (1997) **34**:315–22. doi:10.1016/S0161-5890(97)00041-2
91. Zhu X, Stauss HJ, Ivanyi J, Vordermeier HM. Specificity of CD8+ T cells from subunit-vaccinated and infected H-2b mice recognizing the 38 kDa antigen of *Mycobacterium tuberculosis*. *Int Immunol* (1997) **9**:1669–76. doi:10.1093/intimm/9.11.1669
92. Zhu X, Venkataprasad N, Thangaraj HS, Hill M, Singh M, Ivanyi J, et al. Functions and specificity of T cells following nucleic acid vaccination of mice against *Mycobacterium tuberculosis* infection. *J Immunol* (1997) **158**:5921–6.
93. Bennekov T, Dietrich J, Rosenkrands I, Stryhn A, Doherty TM, Andersen P. Alteration of epitope recognition pattern in Ag85B and ESAT-6 has a profound influence on vaccine-induced protection against *Mycobacterium tuberculosis*. *Eur J Immunol* (2006) **36**:3346–55. doi:10.1002/eji.200636128
94. Tchilian E, Ahuja D, Hey A, Jiang S, Beverley P. Immunization with different formulations of *Mycobacterium tuberculosis* antigen 85A induces immune responses with different specificity and protective efficacy. *Vaccine* (2013) **31**:4624–31. doi:10.1016/j.vaccine.2013.07.040
95. Suzuki D, Nagata T, Eweda G, Matsumoto S, Matsumoto M, Tsujimura K, et al. Characterization of murine T-cell epitopes on mycobacterial DNA-binding protein 1 (MDP1) using DNA vaccination. *Vaccine* (2010) **28**:2020–5. doi:10.1016/j.vaccine.2009.10.062
96. Wang LX, Nagata T, Tsujimura K, Uchijima M, Seto S, Koide Y. Identification of HLA-DR4-restricted T-cell epitope on MPT51 protein, a major secreted protein derived from *Mycobacterium tuberculosis* using MPT51 overlapping peptides screening and DNA vaccination. *Vaccine* (2010) **28**:2026–31. doi:10.1016/j.vaccine.2009.10.063
97. Uto T, Tsujimura K, Uchijima M, Seto S, Nagata T, Suda T, et al. A novel vaccine strategy to induce mycobacterial antigen-specific Th1 responses by utilizing the C-terminal domain of heat shock protein 70. *FEMS Immunol Med Microbiol* (2011) **61**:189–96. doi:10.1111/j.1574-695X.2010.00762.x
98. Cox JH, Ivanyi J, Young DB, Lamb JR, Syred AD, Francis MJ. Orientation of epitopes influences the immunogenicity of synthetic peptide dimers. *Eur J Immunol* (1988) **18**:2015–9. doi:10.1002/eji.1830181222
99. De Smet KA, Vordermeier HM, Ivanyi J. A versatile system for the production of recombinant chimeric peptides. *J Immunol Methods* (1994) **177**:243–50. doi:10.1016/0022-1759(94)90162-7
100. Wilkinson KA, Vordermeier MH, Kajtar J, Jurcevic S, Wilkinson R, Ivanyi J, et al. Modulation of peptide specific T cell responses by non-native flanking regions. *Mol Immunol* (1997) **34**:1237–46. doi:10.1016/S0161-5890(98)00009-1
101. Wilkinson KA, Hudecz F, Vordermeier HM, Ivanyi J, Wilkinson RJ. Enhancement of the T cell response to a mycobacterial peptide by conjugation to synthetic branched polypeptide. *Eur J Immunol* (1999) **29**:2788–96. doi:10.1002/(SICI)1521-4141(199909)29:09<2788::AID-IMMU2788>3.0.CO;2-4
102. Gowthaman U, Singh V, Zeng W, Jain S, Siddiqui KF, Chodisetti SB, et al. Promiscuous peptide of 16 kDa antigen linked to Pam2Cys protects against *Mycobacterium tuberculosis* by evoking enduring memory T-cell response. *J Infect Dis* (2011) **204**:1328–38. doi:10.1093/infdis/jir548
103. Hewitt J, Coates AR, Mitchison DA, Ivanyi J. The use of murine monoclonal antibodies without purification of antigen in the serodiagnosis of tuberculosis. *J Immunol Methods* (1982) **55**:205–11. doi:10.1016/0022-1759(82)90032-1
104. Sinha S, Sengupta U, Ramu G, Ivanyi J. Serological survey of leprosy and control subjects by a monoclonal antibody-based immunoassay. *Int J Lepr Other Mycobact Dis* (1985) **53**:33–8.
105. Bothamley GH, Rudd R, Festenstein F, Ivanyi J. Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis. *Thorax* (1992) **47**:270–5. doi:10.1136/thx.47.4.270
106. Wilkins EG, Ivanyi J. Potential value of serology for diagnosis of extrapulmonary tuberculosis. *Lancet* (1990) **336**:641–4. doi:10.1016/0140-6736(90)92144-7
107. Bothamley G, Batra H, Ramesh V, Chandramui A, Ivanyi J. Serodiagnostic value of the 19 kilodalton antigen of *Mycobacterium tuberculosis* in Indian patients. *Eur J Clin Microbiol Infect Dis* (1992) **11**:912–5. doi:10.1007/BF01962372
108. Ivanyi J. Serodiagnosis of tuberculosis: due to shift track. *Tuberculosis (Edinb)* (1989) **92**:31–7. doi:10.1016/j.tube.2011.09.001
109. Chandramuki A, Bothamley GH, Brennan PJ, Ivanyi J. Levels of antibody to defined antigens of *Mycobacterium tuberculosis* in tuberculous meningitis. *J Clin Microbiol* (1989) **27**:821–5.
110. Ivanyi J. Could active case finding reduce the transmission of tuberculosis? *The Lancet* (2014) **383**:1035–6. doi:10.1016/S0140-6736(14)60510-9
111. Gaseitsiwe S, Valentini D, Mahdavifar S, Magalhaes I, Hoft DF, Zerweck J, et al. Pattern recognition in pulmonary tuberculosis defined by high content peptide microarray chip analysis representing 61 proteins from *M. tuberculosis*. *PLoS One* (2008) **3**:e3840. doi:10.1371/journal.pone.0003840
112. Balu S, Reljic R, Lewis MJ, Pleass RJ, McIntosh R, van Kooten C, et al. A novel human IgA monoclonal antibody protects against tuberculosis. *J Immunol* (2011) **186**:3113–9. doi:10.4049/jimmunol.1003189
113. Buccheri S, Reljic R, Caccamo N, Meraviglia S, Ivanyi J, Salerno A, et al. Prevention of the post-chemotherapy relapse of tuberculous infection by combined immunotherapy. *Tuberculosis (Edinb)* (2009) **89**:91–4. doi:10.1016/j.tube.2008.09.001
114. Harris DP, Vordermeier HM, Arya A, Bogdan K, Moreno C, Ivanyi J. Immunogenicity of peptides for B cells is not impaired by overlapping T-cell epitope topology. *Immunology* (1996) **88**:348–54. doi:10.1046/j.1365-2567.1996.d01-673.x
115. Engberg J, Krogsgaard M, Fugger L. Recombinant antibodies with the antigen-specific, MHC restricted specificity of T cells: novel reagents for basic and clinical investigations and immunotherapy. *Immunotechnology* (1999) **4**:273–8.
116. Engberg J, Yenidunya AF, Clausen R, Jensen LB, Sorensen P, Kops P, et al. Human recombinant Fab antibodies with T-cell receptor-like specificities generated from phage display libraries. *Methods Mol Biol* (2003) **207**:161–77.
117. Cohen CJ, Denkberg G, Lev A, Epel M, Reiter Y. Recombinant antibodies with MHC-restricted, peptide-specific, T-cell receptor-like specificity: new tools to study antigen presentation and TCR-peptide-MHC interactions. *J Mol Recognit* (2003) **16**:324–32. doi:10.1002/jmr.640
118. Denkberg G, Reiter Y. Recombinant antibodies with T-cell receptor-like specificity: novel tools to study MHC class I presentation. *Autoimmun Rev* (2006) **5**:252–7. doi:10.1016/j.autrev.2005.07.004
119. Liddy N, Molloy PE, Bennett AD, Boulter JM, Jakobsen BK, Li Y. Production of a soluble disulfide bond-linked TCR in the cytoplasm of Escherichia coli trxB gor mutants. *Mol Biotechnol* (2010) **45**:140–9. doi:10.1007/s12033-010-9250-0
120. Michaeli Y, Denkberg G, Sinik K, Lantzy L, Chih-Sheng C, Beauverd C, et al. Expression hierarchy of T cell epitopes from melanoma differentiation antigens: unexpected high level presentation of tyrosinase-HLA-A2 Complexes revealed by peptide-specific, MHC-restricted, TCR-like antibodies. *J Immunol* (2009) **182**:6328–41. doi:10.4049/jimmunol.0801898
121. Epel M, Carmi I, Soueid-Baumgarten S, Oh SK, Bera T, Pastan I, et al. Targeting TARP, a novel breast and prostate tumor-associated antigen, with

T cell receptor-like human recombinant antibodies. *Eur J Immunol* (2008) **38**:1706–20. doi:10.1002/eji.200737524
122. Klechevsky E, Gallegos M, Denkberg G, Palucka K, Banchereau J, Cohen C, et al. Antitumor activity of immunotoxins with T-cell receptor-like specificity against human melanoma xenografts. *Cancer Res* (2008) **68**:6360–7. doi:10.1158/0008-5472.CAN-08-0928
123. Caccamo N, Milano S, Di Sano C, Cigna D, Ivanyi J, Krensky AM, et al. Identification of epitopes of *Mycobacterium tuberculosis* 16-kDa protein recognized by human leukocyte antigen-A*0201 CD8(+) T lymphocytes. *J Infect Dis* (2002) **186**:991–8. doi:10.1086/344174
124. Geluk A, van Meijgaarden KE, Franken KL, Drijfhout JW, D'Souza S, Necker A, et al. Identification of major epitopes of *Mycobacterium tuberculosis* AG85B that are recognized by HLA-A*0201-restricted CD8+ T cells in HLA-transgenic mice and humans. *J Immunol* (2000) **165**:6463–71.
125. Chang ST, Linderman JJ, Kirschner DE. Multiple mechanisms allow *Mycobacterium tuberculosis* to continuously inhibit MHC class II-mediated antigen presentation by macrophages. *Proc Natl Acad Sci U S A* (2005) **102**:4530–5. doi:10.1073/pnas.0500362102
126. Dadaglio G, Nelson CA, Deck MB, Petzold SJ, Unanue ER. Characterization and quantitation of peptide-MHC complexes produced from hen egg lysozyme using a monoclonal antibody. *Immunity* (1997) **6**:727–38. doi:10.1016/S1074-7613(00)80448-3
127. Croft NP, Smith SA, Wong YC, Tan CT, Dudek NL I, Flesch E, et al. Kinetics of antigen expression and epitope presentation during virus infection. *PLoS Pathog* (2013) **9**:e1003129. doi:10.1371/journal.ppat.1003129
128. Molloy A, Laochumroonvorapong P, Kaplan G. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. *J Exp Med* (1994) **180**:1499–509. doi:10.1084/jem.180.4.1499
129. Fratazzi C, Arbeit RD, Carini C, Balcewicz-Sablinska MK, Keane J, Kornfeld H, et al. Macrophage apoptosis in mycobacterial infections. *J Leukoc Biol* (1999) **66**:763–4.
130. Matthey B, Engert A, Klimka A, Diehl V, Barth S. A new series of pET-derived vectors for high efficiency expression of *Pseudomonas* exotoxin-based fusion proteins. *Gene* (1999) **229**:145–53. doi:10.1016/S0378-1119(99)00038-4
131. Dalken B, Giesubel U, Knauer SK, Wels WS. Targeted induction of apoptosis by chimeric granzyme B fusion proteins carrying antibody and growth factor domains for cell recognition. *Cell Death Differ* (2006) **13**:576–85. doi:10.1038/sj.cdd.4401773
132. Kurschus FC, Kleinschmidt M, Fellows E, Dornmair K, Rudolph R, Lilie H, et al. Killing of target cells by redirected granzyme B in the absence of perforin. *FEBS Lett* (2004) **562**:87–92. doi:10.1016/S0014-5793(04)00187-5
133. Dharap SS, Qiu B, Williams GC, Sinko P, Stein S, Minko T. Molecular targeting of drug delivery systems to ovarian cancer by BH3 and LHRH peptides. *J Control Release* (2003) **91**:61–73. doi:10.1016/S0168-3659(03)00209-8
134. Moreau C, Cartron PF, Hunt A, Meflah K, Green DR, Evan G, et al. Minimal BH3 peptides promote cell death by antagonizing anti-apoptotic proteins. *J Biol Chem* (2003) **278**:19426–35. doi:10.1074/jbc.M209472200
135. Condos R, Raju B, Canova A, Zhao BY, Weiden M, Rom WN, et al. Recombinant gamma interferon stimulates signal transduction and gene expression in alveolar macrophages in vitro and in tuberculosis patients. *Infect Immun* (2003) **71**:2058–64. doi:10.1128/IAI.71.4.2058-2064.2003
136. Cousens LP, Najafian N, Mingozzi F, Elyaman W, Mazer B, Moise L, et al. In vitro and in vivo studies of IgG-derived Treg epitopes (Tregitopes): a promising new tool for tolerance induction and treatment of autoimmunity. *J Clin Immunol* (2013) **33**(Suppl 1):S43–9. doi:10.1007/s10875-012-9762-4
137. Cousens LP, Su Y, McClaine E, Li X, Terry F, Smith R, et al. Application of IgG-derived natural Treg epitopes (IgG Tregitopes) to antigen-specific tolerance induction in a murine model of type 1 diabetes. *J Diabetes Res* (2013) **2013**:621693. doi:10.1155/2013/621693
138. Cousens LP, Tassone R, Mazer BD, Ramachandiran V, Scott DW, De Groot AS. Tregitope update: mechanism of action parallels IVIg. *Autoimmun Rev* (2013) **12**:436–43. doi:10.1016/j.autrev.2012.08.017
139. Olivares N, Leon A, Lopez Y, Puig A, Cadiz A, Falero G, et al. The effect of the administration of human gamma globulins in a model of BCG infection in mice. *Tuberculosis (Edinb)* (2006) **86**:268–72. doi:10.1016/j.tube.2006.01.006
140. Olivares N, Puig A, Aguilar D, Moya A, Cadiz A, Otero O, et al. Prophylactic effect of administration of human gamma globulins in a mouse model of tuberculosis. *Tuberculosis (Edinb)* (2009) **89**:218–20. doi:10.1016/j.tube.2009.02.003
141. Hougardy JM, Place S, Hildebrand M, Drowart A, Debrie AS, Locht C, et al. Regulatory T cells depress immune responses to protective antigens in active tuberculosis. *Am J Respir Crit Care Med* (2007) **176**:409–16. doi:10.1164/rccm.200701-084OC
142. Boer MC, van Meijgaarden KE, Bastid J, Ottenhoff TH, Joosten SA. CD39 is involved in mediating suppression by *Mycobacterium bovis* BCG-activated human CD8(+) CD39(+) regulatory T cells. *Eur J Immunol* (2013) **43**:1925–32. doi:10.1002/eji.201243286
143. Li L, Lao SH, Wu CY. Increased frequency of CD4(+)CD25(high) Treg cells inhibit BCG-specific induction of IFN-gamma by CD4(+) T cells from TB patients. *Tuberculosis (Edinb)* (2007) **87**:526–34. doi:10.1016/j.tube.2007.07.004
144. Quinn KM, McHugh RS, Rich FJ, Goldsack LM, de Lisle GW, Buddle BM, et al. Inactivation of CD4+ CD25+ regulatory T cells during early mycobacterial infection increases cytokine production but does not affect pathogen load. *Immunol Cell Biol* (2006) **84**:467–74. doi:10.1111/j.1440-1711.2006.01460.x
145. Quinn KM, Rich FJ, Goldsack LM, de Lisle GW, Buddle BM, Delahunt B, et al. Accelerating the secondary immune response by inactivating CD4(+)CD25(+) T regulatory cells prior to BCG vaccination does not enhance protection against tuberculosis. *Eur J Immunol* (2008) **38**:695–705. doi:10.1002/eji.200737888

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 February 2014; paper pending published: 23 February 2014; accepted: 03 March 2014; published online: 24 March 2014.
*Citation: Ivanyi J (2014) Function and potentials of M. tuberculosis epitopes. Front. Immunol. **5**:107. doi: 10.3389/fimmu.2014.00107*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 24 March 2014
doi: 10.3389/fimmu.2014.00124

Definition of CD4 immunosignatures associated with MTB

Cecilia S. Lindestam Arlehamn* and Alessandro Sette

Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA

Edited by:
Tom H. M. Ottenhoff, Leiden University Medical Center, Netherlands

Reviewed by:
Dirk Dittmer, University of North Carolina at Chapel Hill, USA
Heinrich Korner, Menzies Research Institute Tasmania, Australia

****Correspondence:***
Cecilia S. Lindestam Arlehamn, Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037, USA
e-mail: cecilia@liai.org

We have recently described the first true genome-wide screen for $CD4^+$ T-cell reactivity directed against *Mycobacterium tuberculosis* (MTB) in latent TB-infected individuals. The approach relied on predictions of HLA-binding capacity for a panel of DR, DP, and DQ alleles representative of those most commonly expressed in the general population, coupled with high throughput ELISPOT assays. The results identified hundreds of novel epitopes and antigens, and documented the novel observation that T cells in latent MTB infection are confined to the $CXCR3^+CCR6^+$ phenotype and largely directed against three antigenic "islands" within the MTB genome. In parallel, we have made generally available to the scientific community the technical approaches and reagents developed in the process, such as motifs, algorithms, and binding assays for several common HLA class II alleles, and a panel of single allele HLA class II transfected cell lines representative of the most frequent specificities in the general population. Recent efforts have been focused on characterization of epitopes and antigens recognized by patients with active TB and individuals vaccinated with BCG, with the aim of providing the first systematic evaluation of the overlap between latent, active, and BCG cohorts. The definition of a broad range of epitopes restricted by common HLA molecules, will facilitate development of diagnostic reagents, allow a rigorous evaluation of T-cell responses associated with TB infection in humans, and enable the evaluation of the immunogenicity of different vaccine candidates. Furthermore, it might suggest new candidates for vaccine and diagnostic development.

Keywords: tuberculosis, T cells, epitope, HLA, genome-wide

TB AS A WORLDWIDE MEDICAL PROBLEM

Tuberculosis is the second leading cause of death from infectious diseases worldwide (1). The World Health Organization (WHO) estimates that approximately one-third of the world's population (two billion total) is infected with *Mycobacterium tuberculosis* (MTB). MTB is responsible for 1.4 million deaths annually and 9 million new infections are reported each year. The majority of infected individuals control the pathogen by mounting a successful, long-lived, and protective immune response, leading to either resolution or a clinically latent infection. Approximately, 10% of latently infected individuals subsequently develop active TB (2, 3). The risk of developing active tuberculosis is higher in immunocompromised individuals (due to age, corticosteroids, malnutrition, HIV infection, etc.). Treatment is lengthy and expensive, requiring a combination of antibiotics. In many parts of the world, access to these drugs is limited and compliance with the drug regime is often poor, thus precipitating the development of drug-resistant strains. Worldwide, 3.7% of new cases and 20% of previously treated cases are infected with multidrug-resistant TB (MDR-TB), extensively drug-resistant TB (XDR-TB) and recently virtually untreatable totally drug-resistant (TDR) strains (1, 4). The prevalence of these drug-resistant cases, which complicates the schedule and increases cost of treatment, has heightened interest in the development of effective vaccines, and prompted inclusion of MTB in the list of A–C pathogens. The vaccination of children with *Mycobacterium bovis* BCG results in a 60–80% decrease in the incidence of active tuberculosis. However, in most developed countries BCG vaccination is not recommended due to the relatively low incidence of disease and variable effectiveness in preventing pulmonary TB in adults, a large fraction of active disease cases.

CD4 T-CELL RESPONSES IN TB INFECTION

Due to the intracellular lifestyle of MTB, immunity relies on a successful T-cell response against a repertoire of antigenic targets. Defining this is central to understanding the immune response against TB and it has been vigorously pursued. Human T-cell responses to MTB involve CD4, CD8, CD1, and γδ T cells, though protective immunity to MTB is commonly ascribed to a Th1 profile (3, 5–8). $CD4^+$ T cells are central to the defense against MTB, as exemplified by the fact that HIV-infected patients are more susceptible to primary TB infection, reinfection, and reactivation (9–11). Seminal studies in human T-cell responses to MTB showed that memory Th1 cells secreted IFNγ (12). It was further shown that IFNγ has an essential role in the protective immunity to mycobacteria, as individuals with genetic defects in the IFNγ receptor have an increased susceptibility to infection with mycobacteria (13). Furthermore, TNFα is important in host resistance to TB, as evidenced by studies following anti-TNFα therapy for autoimmune disease, where patients with LTBI have been observed to develop active TB (14, 15). Other Th subsets and cytokines have been shown to be involved in the T-cell response to MTB. Several studies indicate that lower IFNγ/IL-4 or IFNγ/IL-5 ratios are found in active TB patients compared to healthy TB controls (16,

17). Furthermore, healthcare workers that have worked in close proximity to TB patients, and subsequently developed TB, showed increased IL-5 levels compared to healthcare workers that did not develop TB (18). Definition of the exact role of Th2 responses still requires more investigation. Furthermore, several studies suggest that the capacity to secrete multiple cytokines can determine pathogen clearance versus persistence (19). Indeed, several studies in TB have suggested that multifunctional T cells are not only a potential correlate of protection, but have also been implicated in pathology (20–23). Recently, MTB-specific T-cell expression of IL-17 has been described (24–27). It has been demonstrated that the BCG vaccine and purified protein derivative (PPD) are able to expand memory $CD4^+IL\text{-}17^+$ cells (24, 26). Furthermore, $IL\text{-}17^+$ T cells have been described in active TB patients, particularly in those infected by MDR MTB strains (27, 28), suggesting a pathogenic role for this cytokine. There is also evidence for IL-10 as a factor in humans with active TB, where IL-10 mediates inhibition of antigen presentation to T cells, and therefore mediates a decreased ability to clear infection contributing to TB pathogenesis (29). IL-10 has been shown to be elevated in serum from active pulmonary TB patients (30). Therefore, the involvement of different Th subsets in TB infection remains to be clarified and definition of human $CD4^+$ T-cell epitopes to specifically track pathogen-specific T-cell subsets remains a high priority.

THE CONCEPT OF HLA PROMISCUITY

T cells recognize specific complexes formed between MHC molecules and particular peptide epitopes. Accordingly, a given epitope will elicit a T-cell response only in individuals expressing MHC molecules having the capacity to bind it with sufficiently high affinity. Both class I and class II MHC molecules are extremely polymorphic, and thousands of different variants are known in humans (31, 32). The most frequent HLA class II alleles with population coverage of almost 90% at each locus are shown in **Table 1**. Much of the polymorphism is concentrated on residues located in the peptide-binding groove, giving each allelic variant a distinct binding specificity. As a result, the prediction, identification, and validation of epitopes restricted by each HLA type represent tasks of such complexity as to be practically unfeasible. Further, different MHC types are expressed at dramatically different frequencies in and across different ethnicities. Thus, without careful consideration, ethnically unbiased population coverage is difficult to obtain.

One mean of circumventing the problem is to focus on the HLA types that are most widely represented in different ethnicities worldwide (**Table 1**), while at the same time selecting epitopes that are capable of binding multiple common HLA types (promiscuous epitopes). In this respect, it has been found that both class I and II HLA molecules can be classified into groups, denominated as supertypes that reflect shared or largely overlapping peptide-binding repertoires and specificities. Indeed, a large body of evidence demonstrates that the repertoire of peptides bound by different HLA class II molecules significantly overlap (32), and peptides with promiscuous binding capacity are also quite common [see, e.g., (34, 35)]. Overlaps in the repertoires of DRB1 and DRB3/4/5 molecules, leading to the definition of a DR supertype, as well as the identity of well-characterized CD4 T-cell epitopes

Table 1 | Genotype and phenotype frequencies of HLA class II alleles.

Locus	Allele	Genotype frequency	Phenotype frequency
DRB1	**DRB1*01:01**	2.8	5.4
	DRB1*03:01	7.1	13.7
	DRB1*03:02	1.1	2.1
	DRB1*04:01	2.3	4.6
	DRB1*04:02	1.1	2.2
	DRB1*04:03	2.3	4.5
	DRB1*04:04	1.9	3.8
	DRB1*04:05	3.1	6.2
	DRB1*04:07	2.4	4.8
	DRB1*04:11	1.6	3.3
	DRB1*07:01	7	13.5
	DRB1*08:02	2.5	4.9
	DRB1*09:01	3.1	6.2
	DRB1*11:01	6.1	11.8
	DRB1*11:02	1.1	2.2
	DRB1*11:03	0.3	0.5
	DRB1*11:04	1.4	2.8
	DRB1*12:01	2	3.9
	DRB1*13:01	3.2	6.3
	DRB1*13:02	3.9	7.7
	DRB1*13:03	1.2	2.4
	DRB1*13:04	0.1	0.2
	DRB1*14:01	3.4	6.7
	DRB1*14:02	2.8	5.6
	DRB1*15:01	6.3	12.2
	DRB1*16:01	1	1.9
DRB1	Total	71.1	91.7
DRB3/4/5	**DRB3*01:01**	14	26.1
	DRB3*02:02	18.9	34.3
	DRB3*03:01	6.7	13
	DRB4*01:01	23.7	41.8
	DRB5*01:01	8.3	16
	DRB5*01:02	5.1	9.8
DRB3/4/5	Total	76.7	94.6
DQA1/DQB1	**DQA1*05:01/DQB1*02:01**	5.8	11.3
	DQA1*02:01/DQB1*02:01	5.7	11.1
	DQA1*05:01/DQB1*03:01	19.5	35.1
	DQA1*03:01/DQB1*03:02	10	19
	DQA1*04:01/DQB1*04:02	6.6	12.8
	DQA1*01:01/DQB1*05:01	7.6	14.6
	DQA1*01:02/DQB1*05:02	3.5	6.9
	DQA1*01:02/DQB1*06:02	7.6	14.6
DQA1/DQB1	Total	66.3	88.7
DPA1/DPB1	**DPA1*02:01/DPB1*01:01**	8.4	16
	DPA1*01:03/DPB1*02:01	9.2	17.5
	DPA1*01:03/DPB1*04:01	20.1	36.2
	DPA1*01:03/DPB1*04:02	23.6	41.6
	DPA1*02:02/DPB1*05:01	11.5	21.7
	DPA1*02:01/DPB1*14:01	3.8	7.4
DPA1/DPB1	Total	76.5	94.5

Average genotype and phenotype frequencies for individual alleles are based on data available at dbMHC, as previously described by McKinney et al. (33). Alleles previously characterized in detail for binding specificity are highlighted in bold (32).

with promiscuous DR-binding capacity, have been known for over a decade (34–36). Other studies have similarly addressed repertoire overlaps and the existence of corresponding supertypes for DP (32, 37–39) and DQ (32, 40, 41).

Following upon earlier computational, structural, and functional approaches to define class II supertypes (42–45), we utilized a large library of HLA DR-, DQ-, and DP-binding data to define seven different class II supertypes (main DR, DR4, DRB3, main DQ, DQ7, main DP, and DP2) (32). The molecules associated with the respective supertypes fell largely along lines defined by MHC locus and reflect, in broad terms, commonalities in reported peptide-binding motifs. Repertoire overlaps between molecules within the same class II supertype were found to be similar in magnitude to what has been observed for HLA class I supertypes. Surprisingly, however, the degree to which repertoires between molecules in the different class II supertypes overlapped was found to be fivefold to tenfold higher than repertoire overlaps typically noted between molecules in different class I supertypes. These results highlight the existence of a high degree of repertoire overlap amongst all HLA class II molecules, regardless of supertype association. Further, in terms of implications for epitope identification studies, these data also validate the idea that broadly reactive HLA class II epitopes can be defined.

HLA PROMISCUITY IMPLICATIONS FOR EPITOPE IDENTIFICATION

Peptides with highly promiscuous binding capacity are frequently recognized by immune individuals (34, 46–49) and epitope immunodominance is highly influenced by promiscuous recognition in the context of multiple HLA class II molecules (50). Furthermore, a dominant fraction of the pathogen or allergen-specific response can be identified by selection of the most promiscuous binding peptides using bioinformatic predictions (51–54). The advantage of this approach is that it would identify the optimal set of peptide candidates for immunogenicity testing, eliminating the necessity of synthesizing a large number of overlapping peptides and, more importantly, circumvent the need to test each one of them for binding to numerous HLA class II molecules *in vitro*.

We have recently described the selection of a panel of HLA DR, DQ, and DP specificities that provide worldwide population (phenotypic) coverage of almost 90% at each locus, and accounts for over 66% of all genes at each locus (32) (**Table 1**). Considering up to eight different class II alleles expressed per individual (i.e., up to two at each of the four class II loci – DRB1, DRB3/4/5, DQ, and DP), this panel afforded coverage of at least four alleles in over 95% of the individuals in four different study populations of diverse ethnicity from the USA and South Africa (33). For each of these allelic variants, single HLA class II allele-transfected cell lines have been generated (33). These transfected cell lines can be used for high throughput determination of HLA restriction, enabling better characterization of T-cell responses, and facilitating the development of tetrameric staining reagents. Also, for the vast majority of these alleles high throughput binding assays have been established, peptide-binding motifs defined, and predictive algorithms developed and made publically available (32, 55), enabling efficient and thorough identification of candidate epitopes, as well as characterization of their HLA-binding capacity and potential population coverage.

Taken together, these data highlight that broadly reactive HLA class II epitopes can be identified, and that these promiscuous epitopes can account for a large fraction of the specific immune response. Further, the bioinformatic tools necessary to identify candidate epitopes, as well as specific cellular and immunochemical reagents to allow detailed characterization of epitope-specific responses are available and have been well-validated in several studies.

SCREEN OF A GENOME-WIDE LIBRARY OF MTB-DERIVED PREDICTED HLA CLASS II EPITOPES IN LTBI DONORS

The MTB genome encodes more than 4,000 different open reading frames (ORFs) (56), generally highly conserved amongst different strains, including drug-resistant ones. Identification of T-cell epitopes from such a large and complex target is a complex task, yet necessary for disease monitoring, vaccine evaluations, and development. A comprehensive genome-wide screen for HLA class II epitopes was recently performed (57). This genome-wide screen analyzed the reactivity of latent TB-infected (LTBI) individuals from the San Diego area. LTBIs were initially chosen as representative of a patient population that is, at least in part, capable of containing TB infection. Several 100 novel CD4-restricted epitopes and many antigens were identified (57). Furthermore, this study documented the novel observation that T cells in latent MTB infection are confined to the recently described $CXCR3^{+}CCR6^{+}$ phenotype (24, 58) and largely directed against three antigenic "islands" within the MTB genome. Still, important gaps in epitope knowledge remain, as also highlighted in the TB research community, in particular after the disappointing results of the MVA85A BCG boost human vaccine trial (59).

To enable a genome-wide screen for epitopes recognized by LTBIs (57), protein sequences from five complete (CDC1551, F11, H37Ra, H37Rv, and KZN 1435) MTB genomes and 16 draft assemblies available in the NCBI Protein database were aligned. To select candidate promiscuous epitopes, the binding capacity of all possible 15-mer peptides was predicted for 22 HLA DR, DP, and DQ class II alleles commonly expressed in the general population and for which validated algorithms were available (32, 52). This approach eliminates the need to test each peptide *in vitro* for HLA class II binding, as well as the necessity of synthesizing overlapping peptides. The resulting synthetic peptide library of 20,610 peptides (2–10 per ORF, average 5), were tested in high throughput *ex vivo* IFNγ ELISPOT using circulating T cells from LTBI donors. Each individual donor tested recognized 24 epitopes on average, revealing striking heterogeneity of responses to MTB. The epitopes identified were ranked on the basis of magnitude of response to assess their relative dominance. Overall, the top 80 epitopes accounted for 75% of the total response and the top 175 epitopes accounted for 90% of the total response. The epitopes were mapped to individual MTB antigens using the H37Rv as a reference genome. A total of 82 antigens were recognized by more than 10% of LTBI donors, accounting for approximately 80% of the total response. Thus, natural immunity to MTB is multiantigenic. Taken together, these results demonstrate the feasibility, novelty, and success of the genome-wide screen for epitopes recognized by LTBIs.

CHARACTERISTICS OF HLA CLASS II RESTRICTED ANTIGENS IDENTIFIED BY THE GENOME-WIDE APPROACH

The protein category and the genomic location of the identified antigens were determined using the TubercuList database (60). This revealed enrichment for responses against cell wall-associated and secreted proteins, however, strong immune responses were induced by both secreted and non-secreted proteins, consistent with earlier antigen discovery efforts (61–63). The localization of antigens recognized by the LTBI donors was visualized by plotting the recognition data on a linear map of the MTB genome. This revealed striking clusters of reactivity within certain regions of the genome. In particular, three significant antigenic islands, which encode 0.55% of the total ORFs, accounted for 42% of the total response. All three islands were shown to contain ESX protein pairs, such as the well-known Rv3875 (Early Secretory Target-6, ESAT-6) and Rv3874 (Culture Filtrate Protein 10, CFP10) (64), and two also contain Type VII secretion systems ESX-1 and ESX-3.

METHODS TO CHARACTERIZE AND VALIDATE IDENTIFIED EPITOPES

Characterization and tracking of pathogen-specific T cells can be achieved once specific T-cell epitopes have been defined. Here, we briefly review our published data relating to the characterization of T cells derived from LTBI donors (57). A variety of approaches were employed in parallel including multiparameter intracellular cytokine staining (ICS) assays, tetramer staining, and T-cell libraries (65). It was found that $CD4^+$ T cells recognizing epitopes derived from different TB antigens were associated with similar multifunctional cytokine expression patterns. The most frequent $CD4^+$ T cells were $IFN\gamma^+TNF\alpha^+IL\text{-}2^+$ or $IFN\gamma^+TNF\alpha^+$, followed by $TNF\alpha^+$ single producing $CD4^+$ T cells. To a lesser extent, $TNF\alpha^+IL\text{-}2^+$, single $IFN\gamma^+$, and single $IL\text{-}2^+$ cells were also detected.

To characterize the responding T cells in depth, HLA-epitope tetramer reagents were prepared for representative epitopes for staining of $CD4^+$ purified cells. To overcome low T-cell frequency, a magnetic bead enrichment technique was preformed (50, 66). This allowed phenotypic characterization of epitope-specific memory subsets (57) as well as Th subset characterization (manuscript in preparation).

An alternative and complementary approach to ICS and tetrameric staining reagents is the screening of T-cell libraries (65). This high throughput method allows determination of frequency and distribution of pathogen/antigen/epitope-specific T cells (67).

THE REPERTOIRE OF T-CELL EPITOPES IN THE DIFFERENT CLINICAL MANIFESTATIONS OF MTB IS NOT FULLY DEFINED

The work described above developed reagents and approaches to broadly characterize human T-cell epitopes in the general human population (32, 33, 39, 41, 68), and characterized in detail the T-cell epitopes recognized in a panel of model TB antigens (50). Most importantly, using LTBI donor PBMCs, we performed the first truly genome-wide screen of *ex vivo* human $CD4^+$ MTB T-cell reactivity. Since latently infected individuals are able to control infection, they provided a logical relevant "first step" population to study protective responses. Mapping T-cell responses from these individuals identified immunodominant $CD4^+$ T-cell antigens associated with potentially protective responses, and thus relevant to vaccine design.

According to most classifications, three primary and different outcomes can follow MTB exposure. The first, active TB infection is usually associated with evidence of bacterial replication. The second, LTBI, is usually associated with no disease symptoms and an effective immune response. And thirdly, reactivation of tuberculosis is often triggered by immunosuppression (69). MTB is believed to express different proteins in different stages of infection that may give rise to stage-specific immune responses and recognition of different antigens. Evidence of infection and stage-specific antigens in humans has indeed been reported (70, 71). In granulomas, MTB is believed to be in a dormant state, triggered by a range of stress factors including hypoxia, low pH, NO, nutrient deprivation, and host immune pressure (72). Under these conditions, genes encoded by the DosR regulon are upregulated (73, 74) and several antigens encoded by this regulon have been described as preferentially recognized by individuals with LTBI (71, 75–77). In addition, some proteins have been described and referred to as "resuscitation antigens" (78, 79). These are small bacterial proteins that promote proliferation of dormant mycobacteria, and are therefore believed to be involved in the reactivation of MTB (80). However, these antigens have not been described as being preferentially associated with a certain stage of infection. The availability of prediction methods and high throughput assays makes it possible to investigate genome-wide disease stage-specific TB reactivity and they can be applied to other pathogen systems.

VACCINATION AGAINST TB: NEED FOR REAPPRAISAL OF BCG

One of the long-term strategies essential for control of the global TB epidemic is effective vaccination. The only available licensed TB vaccine to date, BCG, protects against disseminated tuberculosis in young children but offers very variable protection against pulmonary tuberculosis (the contagious transmittable form of the disease) in children and adults (81–83).

Several candidate TB vaccines are in clinical trial and many of them are designed to boost the BCG response. One candidate is MVA85A (modified Vaccinia Ankara virus expressing antigen 85A; Rv3804c), which was developed as a heterologous boost for BCG (84, 85). Rv3804c is highly conserved amongst mycobacterial species and it is present in all strains of BCG (86). MVA85A was shown to boost pre-existing antimycobacterial immune responses induced by either environmental mycobacteria or BCG vaccination (84). A recent phase 2b study demonstrated the feasibility of a large efficacy trial of a new TB vaccine in a high-burden setting (59). This landmark trial was the first infant efficacy trial in over 50 years and its protocols and design will provide a reference for future trials and vaccination strategies. Unfortunately, however, the trial failed to show any efficacy against MTB infection in infants. Various hypotheses have been proposed to explain the lack of efficacy. Amongst them is the hypothesis that boosting with an antigen conserved in all mycobacterial species does not provide any additional benefit over the immunity already induced by exposure to environmental mycobacteria or BCG.

BCG was developed 90 years ago, but there is still a fundamental need for more knowledge regarding the actual mechanism

of BCG immunogenicity, immunodominance, and crossreactivity with TB. Several studies have investigated mycobacterial antigen-specific human T-cell responses primed by vaccination with BCG (62, 87, 88). However, a genome-wide screen for epitopes has not previously been performed and would provide important immunological answers. First, it is imperative to clearly define the TB antigens that are primed following BCG vaccination. Identification of the antigens that are dominantly recognized following BCG vaccination and also recognized in natural TB infection would be important information for the design of BCG prime-boosting vaccines. Conversely, identification of antigens that are primed by BCG but are either weakly or not recognized in natural TB infection could provide new vaccination strategies, as elimination of such antigens may improve BCG efficacy. Second, the characterization of the T-cell phenotypes associated with recognition of various antigens in the context of BCG vaccination and natural infection may provide clues to the type of immune response that might be most desirable, and additionally indicate how the vaccine response should be modulated (i.e., by use of specific adjuvants). Finally, the identification and characterization of epitopes and antigens recognized following BCG vaccination would provide important tools to monitor and evaluate different vaccine candidates, or different vaccination strategies examining dose, route, and the use of different adjuvants. In conclusion, as it is likely that a large proportion of future vaccine recipients will be BCG immunized at birth, and because several new vaccine candidates aim at either replacing or augmenting the efficacy of the BCG vaccine (89–91), a thorough understanding of the immune response following BCG vaccination and in different disease stages of TB infection will provide much needed information toward future TB vaccine design and more efficacious predictive diagnostic tests.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health contract N01-A1-900044C.

REFERENCES

1. WHO. *Global Tuberculosis Report 2012*. Geneva: World Health Organization (2012).
2. Comstock GW. Epidemiology of tuberculosis. *Am Rev Respir Dis* (1982) **125**:8–15.
3. Kaufmann SH. Is the development of a new tuberculosis vaccine possible? *Nat Med* (2000) **6**:955–60. doi:10.1038/79631
4. Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, Ziazarifi AH, et al. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest* (2009) **136**:420–5. doi:10.1378/chest.08-2427
5. Boom WH. The role of T-cell subsets in *Mycobacterium tuberculosis* infection. *Infect Agents Dis* (1996) **5**:73–81.
6. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol* (2001) **19**:93–129. doi:10.1146/annurev.immunol.19.1.93
7. Kaufmann SHE. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol* (2001) **1**:20–30. doi:10.1038/35095558
8. Jung Y-J, Ryan L, Lacourse R, North RJ. Properties and protective value of the secondary versus primary T helper type 1 response to airborne *Mycobacterium tuberculosis* infection in mice. *J Exp Med* (2005) **201**:1915–24. doi:10.1084/jem.20050265
9. Barnes PF, Bloch AB, Davidson PT, Snider DE Jr. Tuberculosis in patients with human immunodeficiency virus infection. *N Engl J Med* (1991) **324**:1644–50. doi:10.1056/NEJM199106063242307
10. Hopewell PC. Impact of human immunodeficiency virus infection on the epidemiology, clinical features, management, and control of tuberculosis. *Clin Infect Dis* (1992) **15**:540–7. doi:10.1093/clind/15.3.540
11. Raviglione MC, Snider DE Jr, Kochi A. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. *JAMA* (1995) **273**:220–6. doi:10.1001/jama.1995.03520270054031
12. Del Prete GF, De Carli M, Mastromauro C, Biagiotti R, Macchia D, Falagiani P, et al. Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J Clin Invest* (1991) **88**:346–50. doi:10.1172/JCI115300
13. Newport MJ, Huxley CM, Huston S, Hawrylowicz CM, Oostra BA, Williamson R, et al. A Mutation in the interferon-γ-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med* (1996) **335**:1941–9. doi:10.1056/NEJM199612263352602
14. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor α-neutralizing agent. *N Engl J Med* (2001) **345**:1098–104. doi:10.1056/NEJMoa011110
15. Galloway JB, Hyrich KL, Mercer LK, Dixon WG, Fu B, Ustianowski AP, et al. Anti-TNF therapy is associated with an increased risk of serious infections in patients with rheumatoid arthritis especially in the first 6 months of treatment: updated results from the British Society for Rheumatology Biologics Register with special emphasis on risks in the elderly. *Rheumatology* (2011) **50**:124–31. doi:10.1093/rheumatology/keq242
16. van Crevel R, Karyadi E, Preyers F, Leenders M, Kullberg BJ, Nelwan H, et al. Increased production of interleukin 4 by CD4+ and CD8+ T cells from patients with tuberculosis is related to the presence of pulmonary cavities. *J Infect Dis* (2000) **181**:1194–7. doi:10.1086/315325
17. Morosini M, Meloni F, Marone Bianco A, Paschetto E, Uccelli M, Pozzi E, et al. The assessment of IFN-gamma and its regulatory cytokines in the plasma and bronchoalveolar lavage fluid of patients with active pulmonary tuberculosis. *Int J Tuberc Lung Dis* (2003) **7**:994–1000.
18. Ordway DJ, Costa L, Martins M, Silveira H, Amaral L, Arroz MJ, et al. Increased interleukin-4 production by CD8 and gammadelta T cells in health-care workers is associated with the subsequent development of active tuberculosis. *J Infect Dis* (2004) **190**:756–66. doi:10.1086/422532
19. Harari A, Vallelian F, Meylan PR, Pantaleo G. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J Immunol* (2005) **174**:1037–45.
20. Beveridge NE, Price DA, Casazza JP, Pathan AA, Sander CR, Asher TE, et al. Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional *Mycobacterium tuberculosis*-specific CD4+ memory T lymphocyte populations. *Eur J Immunol* (2007) **37**:3089–100. doi:10.1002/eji.200737504
21. Day CL, Mkhwanazi N, Reddy S, Mncube Z, van der Stok M, Klenerman P, et al. Detection of polyfunctional *Mycobacterium tuberculosis*-specific T cells and association with viral load in HIV-1-infected persons. *J Infect Dis* (2008) **197**:990–9. doi:10.1086/529048
22. Sutherland JS, Adetifa IM, Hill PC, Adegbola RA, Ota MOC. Pattern and diversity of cytokine production differentiates between *Mycobacterium tuberculosis* infection and disease. *Eur J Immunol* (2009) **39**:723–9. doi:10.1002/eji.200838693
23. Scriba TJ, Tameris M, Mansoor N, Smit E, Van Der Merwe L, Isaacs F, et al. Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. *Eur J Immunol* (2010) **40**:279–90. doi:10.1002/eji.200939754
24. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* (2007) **8**:639–46. doi:10.1038/ni1467
25. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat Immunol* (2007) **8**:369–77. doi:10.1038/ni1449
26. Scriba TJ, Kalsdorf B, Abrahams D-A, Isaacs F, Hofmeister J, Black G, et al. Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J Immunol* (2008) **180**:1962–70.
27. Basile JI, Geffner LJ, Romero MM, Balboa L, Sabio YGC, Ritacco V, et al. Outbreaks of *Mycobacterium tuberculosis* MDR strains induce high IL-17 T-cell

response in patients with MDR tuberculosis that is closely associated with high antigen load. *J Infect Dis* (2011) **204**:1054–64. doi:10.1093/infdis/jir460
28. Jurado JO, Pasquinelli V, Alvarez IB, Peña D, Rovetta AI, Tateosian NL, et al. IL-17 and IFN-γ expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease. *J Leukoc Biol* (2012) **91**:991–1002. doi:10.1189/jlb.1211619
29. Redford PS, Murray PJ, O'Garra A. The role of IL-10 in immune regulation during *M. tuberculosis* infection. *Mucosal Immunol* (2011) **4**:261–70. doi:10.1038/mi.2011.7
30. Verbon A, Juffermans N, Van Deventer SJ, Speelman P, Van Deutekom H, Van Der Poll T. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clin Exp Immunol* (1999) **115**:110–3. doi:10.1046/j.1365-2249.1999.00783.x
31. Sette A, Sidney J. Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* (1999) **50**:201–12. doi:10.1007/s002510050594
32. Greenbaum J, Sidney J, Chung J, Brander C, Peters B, Sette A. Functional classification of class II human leukocyte antigen (HLA) molecules reveals seven different supertypes and a surprising degree of repertoire sharing across supertypes. *Immunogenetics* (2011) **63**:325–35. doi:10.1007/s00251-011-0513-0
33. McKinney DM, Southwood S, Hinz D, Oseroff C, Arlehamn CS, Schulten V, et al. A strategy to determine HLA class II restriction broadly covering the DR, DP, and DQ allelic variants most commonly expressed in the general population. *Immunogenetics* (2013) **65**:357–70. doi:10.1007/s00251-013-0684-y
34. Alexander J, Sidney J, Southwood S, Ruppert J, Oseroff C, Maewal A, et al. Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. *Immunity* (1994) **1**:751–61. doi:10.1016/S1074-7613(94)80017-0
35. Southwood S, Sidney J, Kondo A, Del Guercio M-F, Appella E, Hoffman S, et al. Several common HLA-DR types share largely overlapping peptide binding repertoires. *J Immunol* (1998) **160**:3363–73.
36. O'Sullivan D, Arrhenius T, Sidney J, Del Guercio MF, Albertson M, Wall M, et al. On the interaction of promiscuous antigenic peptides with different DR alleles. Identification of common structural motifs. *J Immunol* (1991) **147**:2663–9.
37. Castelli FA, Buhot C, Sanson A, Zarour H, Pouvelle-Moratille S, Nonn C, et al. HLA-DP4, the most frequent HLA II molecule, defines a new supertype of peptide-binding specificity. *J Immunol* (2002) **169**:6928–34.
38. Berretta F, Butler RH, Diaz G, Sanarico N, Arroyo J, Fraziano M, et al. Detailed analysis of the effects of Glu/Lys beta69 human leukocyte antigen-DP polymorphism on peptide-binding specificity. *Tissue Antigens* (2003) **62**:459–71. doi:10.1046/j.1399-0039.2003.00131.x
39. Sidney J, Steen A, Moore C, Ngo S, Chung J, Peters B, et al. Five HLA-DP molecules frequently expressed in the worldwide human population share a common HLA supertypic binding specificity. *J Immunol* (2010) **184**:2492–503. doi:10.4049/jimmunol.0903655
40. Sidney J, Del Guercio MF, Southwood S, Sette A. The HLA molecules DQA1*0501/B1*0201 and DQA1*0301/B1*0302 share an extensive overlap in peptide binding specificity. *J Immunol* (2002) **169**:5098–108.
41. Sidney J, Steen A, Moore C, Ngo S, Chung J, Peters B, et al. Divergent motifs but overlapping binding repertoires of six HLA-DQ molecules frequently expressed in the worldwide human population. *J Immunol* (2010) **185**:4189–98. doi:10.4049/jimmunol.1001006
42. Chelvanayagam G. A roadmap for HLA-DR peptide binding specificities. *Hum Immunol* (1997) **58**:61–9.
43. Ou D, Mitchell LA, Tingle AJ. A new categorization of HLA DR alleles on a functional basis. *Hum Immunol* (1998) **59**:665–76. doi:10.1016/S0198-8859(98)00067-6
44. Doytchinova IA, Flower DR. In silico identification of supertypes for class II MHCs. *J Immunol* (2005) **174**:7085–95.
45. Nielsen M, Lund O, Buus S, Lundegaard C. MHC class II epitope predictive algorithms. *Immunology* (2010) **130**:319–28. doi:10.1111/j.1365-2567.2010.03268.x
46. Lamonaca V, Missale G, Urbani S, Pilli M, Boni C, Mori C, et al. Conserved hepatitis C virus sequences are highly immunogenic for CD4(+) T cells: implications for vaccine development. *Hepatology* (1999) **30**:1088–98. doi:10.1002/hep.510300435
47. Doolan DL, Southwood S, Chesnut R, Appella E, Gomez E, Richards A, et al. HLA-DR-promiscuous T cell epitopes from *Plasmodium falciparum* pre-erythrocytic-stage antigens restricted by multiple HLA class II alleles. *J Immunol* (2000) **165**:1123–37.
48. Wilson CC, Palmer B, Southwood S, Sidney J, Higashimoto Y, Appella E, et al. Identification and antigenicity of broadly cross-reactive and conserved human immunodeficiency virus type 1-derived helper T-lymphocyte epitopes. *J Virol* (2001) **75**:4195–207. doi:10.1128/JVI.75.9.4195-4207.2001
49. Tangri S, Mothe BR, Eisenbraun J, Sidney J, Southwood S, Briggs K, et al. Rationally engineered therapeutic proteins with reduced immunogenicity. *J Immunol* (2005) **174**:3187–96.
50. Lindestam Arlehamn CS, Sidney J, Henderson R, Greenbaum JA, James EA, Moutaftsi M, et al. Dissecting mechanisms of immunodominance to the common tuberculosis antigens ESAT-6, CFP10, Rv2031c (hspX), Rv2654c (TB7.7), and Rv1038c (EsxJ). *J Immunol* (2012) **188**:5020–31. doi:10.4049/jimmunol.1103556
51. Assarsson E, Bui H-H, Sidney J, Zhang Q, Glenn J, Oseroff C, et al. Immunomic analysis of the repertoire of T-cell specificities for influenza A virus in humans. *J Virol* (2008) **82**:12241–51. doi:10.1128/JVI.01563-08
52. Oseroff C, Sidney J, Kotturi MF, Kolla R, Alam R, Broide DH, et al. Molecular determinants of T cell epitope recognition to the common Timothy grass allergen. *J Immunol* (2010) **185**:943–55. doi:10.4049/jimmunol.1000405
53. Oseroff C, Sidney J, Tripple V, Grey H, Wood R, Broide DH, et al. Analysis of T cell responses to the major allergens from German cockroach: epitope specificity and relationship to IgE production. *J Immunol* (2012) **189**:679–88. doi:10.4049/jimmunol.1200694
54. Oseroff C, Sidney J, Vita R, Tripple V, Mckinney DM, Southwood S, et al. T cell responses to known allergen proteins are differently polarized and account for a variable fraction of total response to allergen extracts. *J Immunol* (2012) **189**:1800–11. doi:10.4049/jimmunol.1200850
55. Wang P, Sidney J, Kim Y, Sette A, Lund O, Nielsen M, et al. Peptide binding predictions for HLA DR, DP and DQ molecules. *BMC Bioinformatics* (2010) **11**:568. doi:10.1186/1471-2105-11-568
56. Cole S, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* (1998) **393**:537–544. doi:10.1038/31159
57. Lindestam Arlehamn CS, Gerasimova A, Mele F, Henderson R, Swann J, Greenbaum JA, et al. Memory T cells in latent *Mycobacterium tuberculosis* infection are directed against three antigenic islands and largely contained in a CXCR3+CCR6+ Th1 subset. *PLoS Pathog* (2013) **9**:e1003130. doi:10.1371/journal.ppat.1003130
58. Gosselin A, Monteiro P, Chomont N, Diaz-Griffero F, Said EA, Fonseca S, et al. Peripheral blood CCR4+CCR6+ and CXCR3+CCR6+CD4+ T cells are highly permissive to HIV-1 infection. *J Immunol* (2010) **184**:1604–16. doi:10.4049/jimmunol.0903058
59. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* (2013) **381**:1021–8. doi:10.1016/S0140-6736(13)60177-4
60. Lew JM, Kapopoulou A, Jones LM, Cole ST. TubercuList – 10 years after. *Tuberculosis* (2011) **91**:1–7. doi:10.1016/j.tube.2010.09.008
61. Andersen P. The T cell response to secreted antigens of *Mycobacterium tuberculosis*. *Immunobiology* (1994) **191**:537–47. doi:10.1016/S0171-2985(11)80460-2
62. Boesen H, Jensen B, Wilcke T, Andersen P. Human T-cell responses to secreted antigen fractions of *Mycobacterium tuberculosis*. *Infect Immun* (1995) **63**:1491–7.
63. Blythe M, Zhang Q, Vaughan K, De Castro R, Salimi N, Bui H-H, et al. An analysis of the epitope knowledge related to mycobacteria. *Immunome Res* (2007) **3**:10. doi:10.1186/1745-7580-3-10
64. Gey Van Pittius N, Gamieldien J, Hide W, Brown G, Siezen R, Beyers A. The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. *Genome Biol* (2001) **2**:research0044.0041–research0044.0018. doi:10.1186/gb-2001-2-10-research0044
65. Geiger R, Duhen T, Lanzavecchia A, Sallusto F. Human naive and memory CD4+ T cell repertoires specific for naturally processed antigens analyzed using libraries of amplified T cells. *J Exp Med* (2009) **206**:1525–34. doi:10.1084/jem.20090504
66. Barnes E, Ward SM, Kasprowicz VO, Dusheiko G, Klenerman P, Lucas M. Ultrasensitive class I tetramer analysis reveals previously undetectable populations of antiviral CD8+ T cells. *Eur J Immunol* (2004) **34**:1570–7. doi:10.1002/eji.200424898
67. Zielinski CE, Corti D, Mele F, Pinto D, Lanzavecchia A, Sallusto F. Dissecting the human immunologic memory for pathogens. *Immunol Rev* (2011) **240**:40–51. doi:10.1111/j.1600-065X.2010.01000.x

68. Sidney J, Southwood S, Moore C, Oseroff C, Pinilla C, Grey HM, et al. Measurement of MHC/peptide interactions by gel filtration or monoclonal antibody capture. *Curr Protoc Immunol* (2013) **Chapter 18**:Unit18.13. doi:10.1002/0471142735.im1803s100
69. Young DB, Gideon HP, Wilkinson RJ. Eliminating latent tuberculosis. *Trends Microbiol* (2009) **17**:183–8. doi:10.1016/j.tim.2009.02.005
70. Wilkinson RJ, De Smet KA, Haslov K, Pasvol G, Singh M, Svarcova I, et al. Human T- and B-cell reactivity to the 16kDa alpha-crystallin protein of *Mycobacterium tuberculosis*. *Scand J Immunol* (1998) **48**:403–9. doi:10.1046/j.1365-3083.1998.00420.x
71. Leyten EMS, Lin MY, Franken KLMC, Friggen AH, Prins C, Van Meijgaarden KE, et al. Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*. *Microbes Infect* (2006) **8**:2052–60. doi:10.1016/j.micinf.2006.03.018
72. Wayne LG, Sohaskey CD. Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annu Rev Microbiol* (2001) **55**:139–63. doi:10.1146/annurev.micro.55.1.139
73. Sherman DR, Voskuil M, Schnappinger D, Liao R, Harrell MI, Schoolnik GK. Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding alpha-crystallin. *Proc Natl Acad Sci U S A* (2001) **98**:7534–9. doi:10.1073/pnas.121172498
74. Roberts DM, Liao RP, Wisedchaisri G, Hol WG, Sherman DR. Two sensor kinases contribute to the hypoxic response of *Mycobacterium tuberculosis*. *J Biol Chem* (2004) **279**:23082–7. doi:10.1074/jbc.M401230200
75. Demissie A, Leyten EMS, Abebe M, Wassie L, Aseffa A, Abate G, et al. Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with *Mycobacterium tuberculosis*. *Clin Vaccine Immunol* (2006) **13**:179–86. doi:10.1128/CVI.13.2.179-186.2006
76. Geluk A, Lin MY, Van Meijgaarden KE, Leyten EMS, Franken KLMC, Ottenhoff THM, et al. T-cell recognition of the HspX protein of *Mycobacterium tuberculosis* correlates with latent *M. tuberculosis* infection but not with *M. bovis* BCG vaccination. *Infect Immun* (2007) **75**:2914–21. doi:10.1128/IAI.01990-06
77. Roupie V, Romano M, Zhang L, Korf H, Lin MY, Franken KLMC, et al. Immunogenicity of eight dormancy regulon-encoded proteins of *Mycobacterium tuberculosis* in DNA-vaccinated and tuberculosis-infected mice. *Infect Immun* (2007) **75**:941–9. doi:10.1128/IAI.01137-06
78. Schuck SD, Mueller H, Kunitz F, Neher A, Hoffmann H, Franken KLCM, et al. Identification of T-cell antigens specific for latent *Mycobacterium Tuberculosis* infection. *PLoS One* (2009) **4**:e5590. doi:10.1371/journal.pone.0005590
79. Commandeur S, Van Meijgaarden KE, Lin MY, Franken KLMC, Friggen AH, Drijfhout JW, et al. Identification of human T-cell responses to *Mycobacterium tuberculosis* resuscitation promoting factors in long-term latently infected individuals. *Clin Vaccine Immunol* (2011) **18**:676–83. doi:10.1128/CVI.00492-10
80. Kell DB, Young M. Bacterial dormancy and culturability: the role of autocrine growth factors. *Curr Opin Microbiol* (2000) **3**:238–43. doi:10.1016/S1369-5274(00)00082-5
81. Colditz GA, Berkey CS, Mosteller F, Brewer TF, Wilson ME, Burdick E, et al. The efficacy of bacillus Calmette-Guerin vaccination of newborns and infants in the prevention of tuberculosis: meta-analyses of the published literature. *Pediatrics* (1995) **96**:29–35.
82. Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* (1995) **346**:1339–45. doi:10.1016/S0140-6736(95)92348-9
83. Trunz BB, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* (2006) **367**:1173–80. doi:10.1016/S0140-6736(06)68507-3
84. McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat Med* (2004) **10**:1240–4. doi:10.1038/nm1128
85. Scriba TJ, Tameris M, Mansoor N, Smit E, Van Der Merwe L, Mauff K, et al. Dose-finding study of the novel tuberculosis vaccine, MVA85A, in healthy BCG-vaccinated infants. *J Infect Dis* (2011) **203**:1832–43. doi:10.1093/infdis/jir195
86. D'Souza S, Rosseels V, Romano M, Tanghe A, Denis O, Jurion F, et al. Mapping of murine Th1 helper T-cell epitopes of mycolyl transferases Ag85A, Ag85B, and Ag85C from *Mycobacterium tuberculosis*. *Infect Immun* (2003) **71**:483–93. doi:10.1128/IAI.71.1.483-493.2003
87. Ravn P, Boesen H, Pedersen BK, Andersen P. Human T cell responses induced by vaccination with *Mycobacterium bovis* bacillus Calmette-Guerin. *J Immunol* (1997) **158**:1949–55.
88. Kagina BM, Abel B, Scriba TJ, Hughes EJ, Keyser A, Soares A, et al. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns. *Am J Respir Crit Care Med* (2010) **182**:1073–9. doi:10.1164/rccm.201003-0334OC
89. Hanekom WA. The immune response to BCG vaccination of newborns. *Ann N Y Acad Sci* (2005) **1062**:69–78. doi:10.1196/annals.1358.010
90. Orme IM. The Achilles heel of BCG. *Tuberculosis* (2010) **90**:329–32. doi:10.1016/j.tube.2010.06.002
91. Abebe F. Is interferon-gamma the right marker for bacille Calmette-Guérin-induced immune protection? The missing link in our understanding of tuberculosis immunology. *Clin Exp Immunol* (2012) **169**:213–9. doi:10.1111/j.1365-2249.2012.04614.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 January 2014; paper pending published: 24 February 2014; accepted: 11 March 2014; published online: 24 March 2014.
*Citation: Lindestam Arlehamn CS and Sette A (2014) Definition of CD4 immunosignatures associated with MTB. Front. Immunol. **5**:124. doi: 10.3389/fimmu.2014.00124 This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 25 June 2014
doi: 10.3389/fimmu.2014.00256

Innovative strategies to identify *M. tuberculosis* antigens and epitopes using genome-wide analyses

Annemieke Geluk*, Krista E. van Meijgaarden, Simone A. Joosten, Susanna Commandeur and Tom H. M. Ottenhoff

Department of Infectious Diseases, Leiden University Medical Center, Leiden, Netherlands

Edited by:
Juraj Ivanyi, King's College London, UK

Reviewed by:
Alexis DeHaven Dunkle, Lawrence Livermore National Laboratory, USA
Rajko Reljic, St George's University of London, UK

****Correspondence:***
Annemieke Geluk, Department of Infectious Diseases, Leiden University Medical Center, PO Box 9600, Leiden 2300 RC, Netherlands
e-mail: a.geluk@lumc.nl

In view of the fact that only a small part of the Mtb expressome has been explored for identification of antigens capable of activating human T-cell responses, which is critically required for the design of better TB vaccination strategies, more emphasis should be placed on innovative ways to discover new Mtb antigens and explore their function at the several stages of infection. Better protective antigens for TB-vaccines are urgently needed, also in view of the disappointing results of the MVA85 vaccine, which failed to induce additional protection in BCG-vaccinated infants (1). Moreover, immune responses to relevant antigens may be useful to identify TB-specific biomarker signatures. Here, we describe the potency of novel tools and strategies to reveal such Mtb antigens. Using proteins specific for different Mtb infection phases, many new antigens of the latency-associated Mtb DosR-regulon as well as resuscitation promoting factor proteins, associated with resuscitating TB, were discovered that were recognized by $CD4^+$ and $CD8^+$ T-cells. Furthermore, by employing MHC binding algorithms and bioinformatics combined with high-throughput human T-cell screens and tetramers, HLA-class Ia restricted polyfunctional $CD8^+$ T-cells were identified in TB patients. Comparable methods, led to the identification of HLA-E-restricted Mtb epitopes recognized by $CD8^+$ T-cells. A genome-wide unbiased antigen discovery approach was applied to analyze the in vivo Mtb gene expression profiles in the lungs of mice, resulting in the identification of IVE-TB antigens, which are expressed during infection in the lung, the main target organ of Mtb. IVE-TB antigens induce strong T-cell responses in long-term latently Mtb infected individuals, and represent an interesting new group of TB antigens for vaccination. In summary, new tools have helped expand our view on the Mtb antigenome involved in human cellular immunity and provided new candidates for TB vaccination.

Keywords: cellular immunity, CD4, CD8, *Mycobacterium tuberculosis*, TB, T-cell epitopes, vaccines

INTRODUCTION

M. TUBERCULOSIS ANTIGEN DISCOVERY: TRADITIONAL APPROACHES

Antigen discovery efforts have been a core component of mycobacterial research for over several decades, and have been markedly facilitated since the availability of the *Mycobacterium tuberculosis* (*Mtb*) genome sequence (2). A lot of different antigen discovery approaches have been described, among which are biochemical-, genetic-, expression library-, and peptide T-cell epitope-based approaches. Using animal models, antigens with protective potential against *Mtb* infection have been discovered, some of which have moved into human clinical trials (phase 1/2a) (3). Nevertheless, T-cell epitopes have been identified in only 7% of all predicted 4000 open reading frames (ORFs) of *Mtb*, and the top 30 most frequently studied protein antigens contain 65% of the known epitopes (4). This leaves the *Mtb* "antigenome" incompletely identified, which may be especially relevant in relation to *Mtb's* phase-dependent variation in gene expression (see below) in response to varying environmental factors (5, 6).

Selection of vaccine candidates has been based largely on empirical observations in the mouse, guinea pig, and non-human primate *Mtb* infection models and only a few antigens (mostly those secreted during active replication of *Mtb*) have been exploited as human vaccine candidates (7). Since *Mtb* alters its gene expression profile significantly during intracellular stress inside host macrophages, its antigen repertoire which is expressed and exposed to the immune system varies considerably during different stages of infection under the pressure of various human host defense mechanisms. A more profound understanding of the actual *Mtb* antigenome expressed during different phases of *Mtb* infection, particularly in the lung, the main target organ of *Mtb* and the identification of the major T-cell epitopes involved, is key to the design of better TB-vaccines and TB correlates of immunity.

Almost all TB-vaccine antigen discovery approaches have implicitly relied on the assumption that the *Mtb* antigens studied are expressed and presented by infected cells, where they are supposedly recognized by T-cells that execute an appropriate effector response. The latter either assist phagocytes in controlling or eliminating live bacteria through various intracellular pathways (phagosomal maturation and phagolysosomal fusion; oxidative/nitrate intermediates; oxygen/nutrient deprivation; the activity of defensins and other anti-microbial peptides and enzymes;

autophagy; apoptosis) (8), or – alternatively direct killing of infected cells. Despite the significant advances made recently, relatively little is known about the *Mtb* antigen repertoire, which is truly expressed by the tubercle bacillus during its infection cycle in human cells. Although abundantly expressed proteins of *Mtb* are often the primary targets of research, less prominently expressed antigens may have equally good or even superior vaccine potential. Better insight into the antigen repertoire available for immune recognition on infected cells, its dynamic changes as well as the quantitative relationship between the various antigens expressed, should provide new directions for antigen discovery and vaccine testing, with the potential to complement or change current strategies used in TB-vaccine-design.

CLASSICAL *Mtb* ANTIGEN DISCOVERY METHODS AND THE RISK OF TUNNEL VISION

Indirect discovery approaches have mostly been the basis for currently available evidence supporting the recognition of *Mtb* antigens, including those on infected cells. Whereas the protective potential of *Mtb* antigens is typically demonstrated using effective vaccine platforms in animal models, the selection of the antigens to be tested in such platforms is often biased and limited by the antigen discovery procedures used. For example, as outlined above, many studies have concentrated on antigens that are highly expressed by bacteria under *in vitro* culture conditions in liquid growth media. However, it is improbable that the same bacterial transcriptomic or proteomic profiles expressed under optimal laboratory growth conditions are the same as those expressed during *in vivo* host infection.

A further important bias is that most *Mtb* antigens recognized by human cells have been identified using IFN-γ assays as read outs. As mentioned above, a considerable number of antigens eliciting $CD4^+$ IFN-γ Th1-cell responses has been identified (9), but this represents almost certainly only a fraction of the potential *Mtb* "antigenome." The number of IFN-γ-releasing antigen-specific T-cells and the amount of total IFN-γ released have remained widely used surrogate markers for the pro-inflammatory immune response against *Mtb.* Thus, the antigens activating other immune cells, including $CD4^+$ T-cells that produce other cytokines than IFN-γ such as Th2 cells, Tregs, Th17/22, cells and cytolytic cells, as well as non-classically restricted (MHC-Ib, see below) human T-cell subsets, remain largely incomplete or even unknown. However, the lack of (application of) well-developed methods to identify *Mtb*-responsive T-cells other than classical Th1 or proliferative responses have contributed to this bias in *Mtb* antigens identified. Thus, it is vital to develop better and more diverse assays that can be applied to detect *Mtb*-induced responses across all relevant human T-cell compartments.

SCOPE OF THIS REVIEW

Since our knowledge of the human *Mtb* antigenome is far from complete as most currently known *Mtb* antigens were identified using IFN-γ (Th1) production as read-out, and because there is limited knowledge about the vaccine potential (protective efficacy) of most antigens, we have followed several alternative strategies to discover new *Mtb* antigens. Below, we will review the five approaches we have pursued in recent years (**Figure 1**). It is likely that many unexplored antigens for classical or non-classical T-cells exist in the *Mtb* antigenome that may possess vaccine potential, but appropriate tools and technologies are required to reveal these. Improved and rational selection is needed to identify candidate antigens with vaccine potential, based on comprehensive knowledge of their patterns of expression, broad immunogenicity, suitability for processing, HLA-binding and induction of protective immunity in relevant model systems.

NEW *Mtb* ANTIGEN DISCOVERY APPROACHES

INFECTION STAGE-SPECIFIC *Mtb* GENE EXPRESSION ANALYSES

Mtb DosR-regulon encoded proteins as latency antigens

As mentioned above, we have hypothesized that the design of improved vaccination strategies requires a better understanding of the *Mtb* proteins that are expressed during the different phases of the *Mtb* intracellular life cycle, and their recognition by human T-cell subsets.

During *in vivo Mtb* infection, environmental and host immune factors induce bacterial dormancy, in the course of which *Mtb* enters a state of non-or slowly replicating persistence, decreases its metabolic activity and alters its gene expression pattern (5, 10). This adjustment by the bacterium is thought to enhance its resistance to environmental and host immune stress. Under *in vitro* conditions that are considered to imitate part of the environment encountered by tubercle bacilli *in vivo* in the immunocompetent host (11) such as hypoxia, low dose nitric oxide, CO exposure or in IFN-γ-activated macrophages, *Mtb* induces the expression of the 48 gene encoding DosR (Rv3133c) regulon (12). In view of this infection phase-specific upregulation, we first decided to

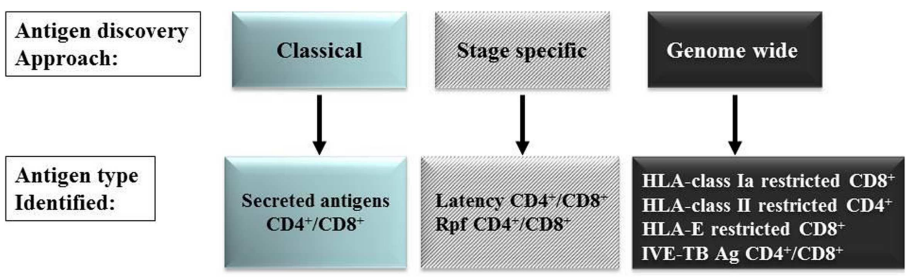


FIGURE 1 | *Mtb* antigen discovery approaches.

study T-cell responses to these 48 DosR-regulon encoded antigens (so-called TB latency antigens) in various ethnically and geographically distinct cohorts in Europe (Italy, Germany, The Netherlands) and Africa (The Gambia, Ethiopia, Uganda, and South Africa) (13–19). In all cohorts we observed T-cell recognition of *Mtb* DosR-regulon encoded latency antigens, particularly in TST^+ individuals [designated latent TB (LTBI)] compared to (ex-) TB patients. Thus, T-cell proliferation and IFN-γ production to *Mtb* DosR-regulon encoded latency antigens are characteristically seen in LTBI in diverse genetic and geographic populations.

An unexpected but important finding was that *Mtb* DosR-regulon encoded antigens were very poorly if at all recognized in previously BCG-vaccinated individuals (20). Additionally, BCG-vaccinated HLA-DR3 and HLA-A2 transgenic mice (21) failed to respond to these antigens. The explanation for this lack of induction of immune responses was not due to BCG's ability to express the DosR-regulon because transcriptional profiling of 14 different BCG strains, cultured under hypoxia, and nitric oxide exposure *in vitro,* showed that genes of the DosR-regulon (except Rv3133) were expressed to similar extents as observed in *Mtb.* Moreover, comparison of the amino acid sequences of *M. bovis* BCG and *Mtb* showed at least 97% homology with 85% of the genes being completely identical (20). The explanation for this phenomenon thus remains unknown, although it may relate to an inability of BCG to enter a state of latency following intradermal immunization. In agreement with the findings in human LTBI, the *Mtb* DosR-regulon encoded latency antigens Rv1733c, Rv2031c, and Rv2626c, were more prominently recognized in chronic infected mice than in acute phase infection, whereas the secreted protein Ag85B (Rv3804c) showed the opposite phenotype (18), again highlighting the preferential recognition of these antigens in latent or long term as opposed to acute, early phase infection.

An important group of individuals, the study of which shed more light into this matter was composed of individuals who had been *Mtb* infected for over 30 years ago, as witnessed by their positive Mantoux skin tests, yet never developed disease despite not having received any therapeutic treatment. Detailed immune profiling experiments with cells from these long-term infected individuals demonstrated strong *Mtb* DosR-regulon encoded antigen-specific T-cell responses. Various epitopes were identified that induced mono- and polyfunctional $CD4^+$ and $CD8^+$ T-cell responses, in particular IFN-γ^+TNF-α^+ $CD8^+$ effector memory- or effector T-cells (22).

Considering the inability of BCG to induce immune responses to latency antigens, TB vaccination strategies started to consider incorporating DosR-regulon encoded antigens as a strategy to complement and improve the current BCG vaccine. This was demonstrated in TB mouse models using rBCG_ureC:hly expressing defined latency-associated antigens and test this construct for long-term protection against an isolate of the *Mtb* Beijing/W lineage (23). Expression of Rv2659c, Rv3407, and Rv1733c by rBCG_ureC:hly showed long-term protection superior to BCG in both lung and spleen compared to rBCG not expressing latency antigens at day 200 post-infection after intradermal vaccination of mice. Even a latency antigen hsp16-derived peptide linked to PAM2Cys adjuvant induced more efficient protection than BCG (24).

Another type of latency antigens is represented by the so-called "starvation" antigens, which are expressed under conditions of nutrient deprivation. A hybrid protein consisting of two secreted, acute phase-specific antigens, Ag85B and ESAT-6, fused to the nutrient stress-induced antigen Rv2660c, was constructed. In pre-exposure mouse models this hybrid protein formulated in CAF01, promoted hybrid-specific T-cells, in particular polyfunctional $CD4^+$ T-cells and more efficient containment of late-stage infection than the Ag85B-ESAT-6 vaccine and BCG (25). Moreover, in latent TB mouse models, post-exposure immunization with this hybrid controlled TB reactivation and significantly lowered the number of bacteria in the lung compared to adjuvant control mice.

Immunization of cynomolgus macaques with the multistage Ag85B-ESAT-6-Rv2660c protein in IC31 adjuvant as a boost to vaccination with BCG delayed and reduced clinical disease after challenge with *M. tuberculosis* and also prevented reactivation of latent infection (26). This boosting regimen resulted in efficient control of *M. tuberculosis* infection and reduced rates of clinical disease. Importantly, it improved survival of the NHP compared to BCG alone and the vaccinated monkeys did not reactivate latent infection after treatment with anti-TNF antibody.

Considering routine BCG vaccination practice in most TB endemic countries, improved, rationally designed *Mtb* subunit vaccines could be employed by simultaneous vaccination with BCG, or booster vaccination on top of BCG, or by constructing improved recombinant BCG strains expressing such antigens.

Resuscitation promoting factors as antigens

Although the factors that trigger bacterial reactivation and resumption of intracellular growth remain largely known, *Mtb* resuscitation promoting factors (Rpfs), which are secreted proteins with high homology to the hormone-like protein secreted by *Micrococcus luteus* (*M. luteus*) are believed to play an important role in this respect (27, 28). The *Mtb* genome encodes five such *rpf* genes [*Rv0867c (rpfA)*, *Rv1009 (rpfB)*, *Rv1884c (rpfC)*, *Rv2389c (rpfD)*, and *Rv2450c (rpfE)*] that are able to stimulate the growth of dormant mycobacteria and expression of the five Rpf proteins is observed *in vitro* in actively replicating *Mtb*, in BCG as well as in *Mtb*-infected human tissue (29–31). Addition of Rpf proteins to sputa from TB patients improves the sensitivity of culture based detection of live *Mtb* in certain conditions (32, 33). There is differential *rpf* expression in cultures grown under hypoxia, nutrient starvation, acidic conditions, stationary, non-cultivable, and resuscitation phase-like conditions, suggesting that the role of *Mtb* Rpfs may be infection stage dependent but likely is not identical for all Rpfs (34).

In view of the role of *Mtb* Rpf proteins in the resuscitation of mycobacteria, immunity against these proteins may reflect the ability to detect the presence of actively replicating *Mtb* organisms at an early stage. Thus, immunity against Rpf proteins may play a role in host control bacterial reactivation. In line with the immunogenicity found in mice for Rv0867c, Rv1009, Rv2389c, and Rv2450c (35), we identified the first human *Mtb* Rpf-specific T-cell responses against *Mtb* Rpfs (19), showing

IFN-γ production in TST^+ individuals in response to Rv1009, Rv1884c, and Rv2450c and to a lesser extent Rv0867c, whereas hardly any IFN-γ was detected in individuals without a positive Mantoux. More detailed analyses of the immune responses to *Mtb* Rpf proteins in *Mtb*-exposed individuals, including long-term LTBI non-progressors showed frequent and significant T-cell responses against both Rv0867c and Rv2389c and identified novel *Mtb* Rpf epitopes, including a single highly dominant peptide epitope in Rv2389c. Of note, *Mtb* Rpf-specific polyfunctional memory $CD4^+$ and particularly $CD8^+$ T-cell memory responses were observed in response to Rv0867c and Rv2389c Rpf proteins. The polyfunctional phenotype of these cells was both single and double cytokine producing $CD4^+$ and $CD8^+$ T-cells, supporting the concept that $CD8^+$ T-cells may be important in long-term control of *Mtb* infection. Based on these collective studies, we envisage that a combination of multiple phase-specific antigens may significantly improve the protective potential of new TB-vaccines (25, 36). Thus, subunit vaccines based on a combination of latency and Rpf antigens could be designed that induce responses able to eliminate *Mtb* bacilli in their dormant state, as well as inhibit *Mtb* bacilli that are trying to recommence active replication.

UNBIASED *Mtb* "GENOME WIDE" ANTIGEN DISCOVERY APPROACHES

HLA-class Ia presented human $CD8^+$ T-cell epitopes

Despite the fact that $CD4^+$ T-cells play a vital role in immunity against *Mtb*, it is becoming evident that also $CD8^+$ T-cells contribute to host defense against *Mtb* by virtue of their ability to produce pro-inflammatory cytokines (9, 37, 38), lyse infected host cells (39), and kill mycobacteria (40). Despite the fact that the antigens and epitopes activating human *Mtb*-specific $CD8^+$ T-cell responses have been less well defined than those for $CD4^+$ T-cells, some groups have been able to isolate such $CD8^+$ T-cells from humans (41–44). Unexpectedly, it was reported that *ex vivo* frequencies of $CD8^+$ T-cells recognizing epitopes from six different *Mtb* proteins in patients with active TB were lower as evaluated by specific tetramers, but normalized following therapy to frequencies comparable to subjects with LTBI. Additionally, $CD8^+$ T-cells with an IL-2^+/IFN-γ^+ phenotype were particularly reduced or found absent in active TB patients (45). Nevertheless, it has remained challenging to identify the role and function of $CD8^+$ T-cells in TB, urging for new strategies and appropriate tools to decipher their specificities. Using classic approaches it would not be feasible to screen the approximately 1 million possible 9-mers in the *Mtb* proteome for $CD8^+$ T-cell responses.

A recently described approach addressed this issue exploiting an integrated computational and proteomic approach to screen 10% of the *Mtb* proteome for antigens that are recognized by $CD8^+$ T-cells: using a synthetic *Mtb* peptide library consisting of 15-mers (11 aa overlap) with high probability of containing $CD8^+$ T-cell epitopes, IFN-γ release by *Mtb*-specific, HLA-class I-restricted $CD8^+$ T-cell clones was measured by ELISPOT assay (46). This study identified the EsxJ family, PE9, and PE_PGRS42 as three novel CD8 antigens and validated the use of peptide library-based approaches a new tool for identification of *Mtb* epitopes recognized by $CD8^+$ T-cells.

An alternative method of combined bioinformatics- and functional immunological screening strategies, called "reverse antigen discovery," was applied by our group to identify HLA-class Ia restricted, $CD8^+$ T-cell antigens. Through peptide-binding prediction algorithms, potential peptide epitopes of *Mtb* antigens were identified restricted by three major HLA-class Ia supertypes (HLA-A2, -A3, and -B7) which together cover more than 80% of the population from different ethnic groups (47). Over 400 synthetic *Mtb* peptides with predicted binding affinities for HLA-A*0201, HLA-A*0301, and HLA-B*0702 (representing the above supertypes) were tested for HLA-binding and induction of proliferation of $CD8^+$ T-cells. This study led to the identification of >60 new *Mtb* epitopes. Further validation of the most interesting epitopes was executed using HLA-class I-tetramers and assessment of peptide-induced intracellular cytokine staining to measure multifunctional $CD8^+$ T-cell responses in cured TB patients and healthy control individuals. In depth analysis of 18 prominently recognized HLA-A*0201-binding, *Mtb* peptides using $CD8^+$ T-cells of cured TB patients, showed IFN-γ, IL-2, and TNF-α, mono-, dual-, and triple-positive $CD8^+$ T-cells. Interestingly, in *Mtb*-non-infected individuals these polyfunctional $CD8^+$ T-cells were absent, which argues for their priming during *in vivo Mtb* infection. Thus, this study is consistent with the notion that there is a much broader repertoire of $CD8^+$ T-cells, which can be identified with specific bioinformatic approaches combined with functional immune assays.

An additional alternative strategy, not yet applied in the identification of new *Mtb* antigens, could be the use of HLA-conditional ligands for high-throughput tetramer generation (48). This technique allows production of HLA ligands that form stable complexes with HLA molecules but can be cleaved upon UV irradiation. The resulting empty, peptide-receptive HLA molecules can be loaded under native conditions with selected epitopes in a high-throughput and HLA-epitope tetramers can be generated and subsequently used for T-cell detection.

Summarized innovative detection methods for $CD8^+$ T-cells can and will allow identification of protective- and pathogenic immunity, which can be used to monitor vaccine- and treatment efficacy.

HLA-class II presented$^+$ human T-cell epitopes

A similar genome-wide approach was recently followed by Sette et al. to identify $CD4^+$ T-cell epitopes: the *Mtb* genome was mined for potential peptide epitopes presented by HLA-class II molecules to $CD4^+$ T-cells (49). The approach relied on predictions of HLA-binding capacity for a panel of DR, DP, and DQ alleles representative of those most commonly expressed in the general population, coupled with high-throughput ELISPOT assays. They found that secreted antigens as well as proteins involved in the active secretion process were dominant targets of the $CD4^+$ T-cell response in the latently infected individuals tested in San Diego who successfully contain *Mtb* infection.

Responses were highly focused on three broadly immunodominant antigenic islands, all related to bacterial secretion systems and composed by several distinct ORFs. These data suggest that vaccination with one or few defined antigens will fail to replicate the response associated with natural immunity. Importantly, they also

found that the $CD4^+$ T-cells responding were largely restricted to the $CXCR3^+CCR6^+$ memory subset. The identification of this immunodominant population of memory T-cells characterized suggests that the response is shaped uniquely by *Mtb*-associated factors.

In analogy to the above results for $CD8^+$ T-cells, these results again underline the power of an unbiased, genome-wide, *Mtb* antigen discovery approaches.

HLA-class Ib presented CD8$^+$ human T-cell epitopes

The identification of *Mtb* antigens for human $CD8^+$ T-cells focus has been focused almost entirely on classical HLA-class Ia and to a lesser extent, on CD1 a, b, c restricted $CD8^+$ T-cell responses (50). In this respect, the polymorphism of the classical HLA-class Ia molecules (HLA-A, -B, -C) which include more than 500, 850, or 270 unique alleles, respectively, is an important factor to be considered for vaccine development due to the considerable variations in peptides that can bind to each HLA-class Ia molecule. In contrast, the HLA-class Ib genes HLA-E, -F, and -G exhibit restricted polymorphism with only 3, 4, and 10 alleles, respectively (51). This might argue for distinct roles for MHC-class Ia and class Ib molecules in host defense to infectious diseases. Non-classical HLA-class I molecules can present antigens of both self and foreign (microbial) origin to $CD8^+$ T-cells (52–54). The fact that their allelic variation is limited provides an opportunity for vaccine-design. Since the two most important variants of HLA-E, HLA-E^R (E*0101), and HLA-E^G (E*0103), occur in equal frequencies amongst different populations (55) and can present antigens derived from pathogens including *Mtb, Mtb* antigen(s) recognized by HLA-E restricted $CD8^+$ T-cell clones represent interesting targets for vaccine development. Moreover, HLA-E is not downregulated by HIV in contrast to HLA-A and -B offering possibilities for vaccination of HIV-infected individuals as well (56). However, the nature of the epitopes recognized by HLA-E restricted $CD8^+$ T-cells remained unknown until recently (57).

Although HLA-E molecules can clearly interact with CD94 molecular complexes expressed predominantly by NK cells, HLA-E is also known to trigger microbial-specific cytotoxic $CD8^+$ T-cells (58, 59). In mice, $CD8^+$ T-cells restricted by the murine HLA-E equivalent, Qa-1 were found to have the ability to induce immune-suppression (60), revealing yet another function of non-classically restricted $CD8^+$ T-cells.

To identify HLA-E-restricted *Mtb* antigens which could be exploited for TB vaccination, we applied bioinformatics, HLA-E peptide-binding assays, and immunological screening, in analogy to the approach used for identification of classical MHC-Ia restricted CD8 T-cell epitopes (61). With this method we identified 69 *Mtb* peptides, derived from a large variety of *Mtb* antigens, which were presented by HLA-E molecules to human $CD8^+$ T-cells. We could demonstrate that $CD8^+$ T-cells from both mycobacterium responsive adults and BCG-vaccinated infants, but not negative controls, proliferated in response to the identified *Mtb* peptides. These $CD8^+$ T-cells displayed cytotoxic activity against target-cells expressing HLA-E loaded with specific peptides, in the absence of any class Ia molecules, and were able to lyse *M. bovis* BCG infected human macrophages demonstrating that HLA-E restricted antigens are naturally processed during infection. Besides, several HLA-E-restricted, *Mtb*-specific $CD8^+$ T-cells were able to suppress proliferation of bystander $CD4^+$ T-cells. This suppression depended on cell–cell contact and was mediated, at least in part, by membrane bound TGFβ1. These data underscore that human $CD8^+$ T-cells are highly multifunctional, and can combine diverse functions such as suppressive and cytotoxic functions. If BCG vaccination would be able to prime HLA-E restricted T-cell responses, HLA-E peptide-based vaccines might be able to boost such BCG-primed responses. The dual character of the response induced in the context of HLA-E may lead to induction of protection as well as balanced regulation of inflammation, which, might be exploited to limit inflammatory pathology in TB.

In vivo expressed Mtb (IVE-TB) antigen-derived human T-cell epitopes

Since the lung represents the principal organ where TB disease is manifested, vaccine-induced immune responses need to target *Mtb* during pulmonary infection. In this respect, it is important to note the significant impact of the alteration of *Mtb*'s gene expression profile during intracellular stress inside host alveolar macrophages, on the *Mtb* antigen repertoire that is presented to the immune system. Thus, we hypothesized that a more profound understanding of the real time *Mtb* "antigenome" expressed during infection in the lung and its recognition by the human immune system, including non-classical T- and B-cells, is vital to develop better vaccination strategies. To identify novel antigens with vaccine potential, we used an unbiased genome-wide approach based on comprehensive gene expression data from *Mtb* during infection of four genetically related but distinct mouse strains (62). These strains represent key features of human TB with a spectrum of TB susceptibility, including the development of necrotic lesions and granuloma formation, which are regulated by the *super-susceptibility to tuberculosis 1* (*sst1*) locus as well as modifier background genes (62). We investigated the *in vivo* expression of 2170 *Mtb* genes, most of which represent the first gene of each predicted *Mtb* operon, during infection in the lungs. To select candidate antigens, stringent selection approaches were then applied to identify a list of *in vivo* expressed *Mtb* (designated IVE-TB) genes. The resulting 16 most consistently expressed *Mtb* genes were produced as recombinant proteins and their immunogenicity was analyzed in PBMC of TST^+ healthy, TB affected individuals, TB patients as well as long-term LTBI. Seven of the identified IVE-TB antigens were strongly immunogenic in TST^+, ESAT-6/CFP10-responsive individuals, but not in E/C negative TST^+ individuals and healthy mycobacterial naïve individuals, indicating that these antigens are presented during natural *Mtb* infection. Importantly, IVE-TB antigen-specific responses could be detected in long-term LTBI, who had been exposed to *Mtb* many years ago yet never developed TB symptoms despite not having had preventive treatment. The most pronounced T-cell subsets recognizing IVE-TB antigens were identified as IFN-γ^+/TNF-α^+ $CD8^+$ T-cells and TNF-α^+/IL-2^+ $CD154^+CD4^+$ T-cells of which the former were major contributors to IFN-γ production. Since IFN-γ^+/TNF-α^+ $CD8^+$ T-cells were also the most prominent subset in the response to Rpf and DosR proteins, this suggests that the development of

specific differential T-cell subsets may be unrelated to the nature of the specific protein antigen involved.

Thus, the analysis of *in vivo* expression patterns during pulmonary *Mtb* infection to identify IVE-TB antigens, combined with detailed immune profiling in humans, led to the identification of IVE-TB as a new class of TB antigens, with the potential for TB vaccination. Our most recent results indeed reveal their protective efficacy in various animal models (63). Importantly, IVE-TB antigen discovery strategies can be applied also to other infectious diseases caused by complex pathogens and represent a novel approach for antigen discovery in general.

ACKNOWLEDGMENTS

We gratefully acknowledge the support of the following organizations: the European Commission (FP7 projects NEWTBVAC, IDEA, ADITEC, EURIPRED, and VACTRAIN); the Bill and Melinda Gates Foundation Grand Challenges in Global Health (grants GC6#74); EDCTP through a project entitled *AE-TBC* under Grant Agreement No. IP_09_32040, the Netherlands Leprosy Relief Foundation (NLR) together with the Turing Foundation (ILEP#: 701.02.49), the Heiser Program for Research in Leprosy in The New York Community Trust (P13-000392), the Order of Malta-Grants-for-Leprosy-Research (MaLTALEP), and the Q. M. Gastmann Wichers Foundation. We thank all members of the laboratory for their great support. We would also like to express our gratitude to all other colleagues which have contributed greatly to parts of this research, in particular: May Young Lin, Francesco Dieli, Nadia Caccamo, Ole Lund, Sheila Tan, Marta Romano, Kris Huygen, Gregory Dolganov, Gary Schoolnik and Fredrik Oftung, and members of the BMGF supported Biomarkers for TB consortium.

REFERENCES

1. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* (2013) **381**(9871):1021–8. doi:10.1016/S0140-6736(13)60177-4
2. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* (1998) **393**:537–44. doi:10.1038/31159
3. Ottenhoff TH, Doherty TM, van Dissel JT, Bang P, Lingnau K, Kromann I, et al. First in humans: a new molecularly defined vaccine shows excellent safety and strong induction of long-lived *Mycobacterium tuberculosis*-specific Th1-cell like responses. *Hum Vaccin* (2010) **6**:1007–15. doi:10.4161/hv.6.12.13143
4. Blythe MJ, Zhang Q, Vaughan K, de Castro R Jr, Salimi N, Bui HH, et al. An analysis of the epitope knowledge related to mycobacteria. *Immunome Res* (2007) **3**:10. doi:10.1186/1745-7580-3-10
5. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol Microbiol* (2002) **43**:717–31. doi:10.1046/j.1365-2958.2002.02779.x
6. Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, et al. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med* (2003) **198**:705–13. doi:10.1084/jem.20030205
7. Ottenhoff TH, Kaufmann SH. Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathog* (2012) **8**:e1002607. doi:10.1371/journal.ppat.1002607
8. Ottenhoff TH. New pathways of protective and pathological host defense to mycobacteria. *Trends Microbiol* (2012) **20**:419–28. doi:10.1016/j.tim.2012.06.002
9. Ottenhoff TH, Lewinsohn DA, Lewinsohn DM. Human CD4 and CD8 T cell responses to *Mycobacterium tuberculosis*: antigen specificity, function, implications and applications. In: Kaufmann SH, Britton WJ, editors. *Handbook of Tuberculosis*. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA (2008). p. 119–156.
10. Wayne LG, Hayes LG. An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun* (1996) **64**:2062–9.
11. Shi L, Jung YJ, Tyagi S, Gennaro ML, North RJ. Expression of Th1-mediated immunity in mouse lungs induces a *Mycobacterium tuberculosis* transcription pattern characteristic of nonreplicating persistence. *Proc Natl Acad Sci U S A* (2003) **100**:241–6. doi:10.1073/pnas.0136863100
12. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, et al. Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J Exp Med* (2003) **198**:693–704. doi:10.1084/jem.20030846
13. Black GF, Thiel BA, Ota MO, Parida SK, Adegbola R, Boom WH, et al. Immunogenicity of novel DosR regulon-encoded candidate antigens of *Mycobacterium tuberculosis* in three high-burden populations in Africa. *Clin Vaccine Immunol* (2009) **16**:1203–12. doi:10.1128/CVI.00111-09
14. Goletti D, Butera O, Vanini V, Lauria FN, Lange C, Franken KL, et al. Response to Rv2628 latency antigen associates with cured tuberculosis and remote infection. *Eur Respir J* (2010) **36**:135–42. doi:10.1183/09031936.00140009
15. Leyten EM, Lin MY, Franken KL, Friggen AH, Prins C, van Meijgaarden KE, et al. Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*. *Microbes Infect* (2006) **8**:2052–60. doi:10.1016/j.micinf.2006.03.018
16. Lin MY, Ottenhoff TH. Host-pathogen interactions in latent *Mycobacterium tuberculosis* infection: identification of new targets for tuberculosis intervention. *Endocr Metab Immune Disord Drug Targets* (2008) **8**:15–29. doi:10.2174/187153008783928398
17. Lin MY, Ottenhoff TH. Not to wake a sleeping giant: new insights into host-pathogen interactions identify new targets for vaccination against latent *Mycobacterium tuberculosis* infection. *Biol Chem* (2008) **389**:497–511. doi:10.1515/BC.2008.057
18. Roupie V, Romano M, Zhang L, Korf H, Lin MY, Franken KL, et al. Immunogenicity of eight dormancy regulon-encoded proteins of *Mycobacterium tuberculosis* in DNA-vaccinated and tuberculosis-infected mice. *Infect Immun* (2007) **75**:941–9. doi:10.1128/IAI.01137-06
19. Schuck SD, Mueller H, Kunitz F, Neher A, Hoffmann H, Franken KL, et al. Identification of T-cell antigens specific for latent *Mycobacterium tuberculosis* infection. *PLoS One* (2009) **4**:e5590. doi:10.1371/journal.pone.0005590
20. Lin MY, Geluk A, Smith SG, Stewart AL, Friggen AH, Franken KL, et al. Lack of immune responses to *Mycobacterium tuberculosis* DosR regulon proteins following *Mycobacterium bovis* BCG vaccination. *Infect Immun* (2007) **75**:3523–30. doi:10.1128/IAI.01999-06
21. Geluk A, Lin MY, van Meijgaarden KE, Leyten EM, Franken KL, Ottenhoff TH, et al. T-cell recognition of the HspX protein of *Mycobacterium tuberculosis* correlates with latent *M. tuberculosis* infection but not with *M. bovis* BCG vaccination. *Infect Immun* (2007) **75**:2914–21. doi:10.1128/IAI.01990-06
22. Commandeur S, Lin MY, van Meijgaarden KE, Friggen AH, Franken KL, Drijfhout JW, et al. Double- and monofunctional CD4 and CD8 T-cell responses to *Mycobacterium tuberculosis* DosR antigens and peptides in long-term latently infected individuals. *Eur J Immunol* (2011) **41**:2925–36. doi:10.1002/eji.201141602
23. Reece ST, Nasser-Eddine A, Dietrich J, Stein M, Zedler U, Schommer-Leitner S, et al. Improved long-term protection against *Mycobacterium tuberculosis* Beijing/W in mice after intra-dermal inoculation of recombinant BCG expressing latency associated antigens. *Vaccine* (2011) **29**:8740–4. doi:10.1016/j.vaccine.2011.07.144
24. Gowthaman U, Singh V, Zeng W, Jain S, Siddiqui KF, Chodisetti SB, et al. Promiscuous peptide of 16 kDa antigen linked to Pam2Cys protects against *Mycobacterium tuberculosis* by evoking enduring memory T-cell response. *J Infect Dis* (2011) **204**:1328–38. doi:10.1093/infdis/jir548
25. Aagaard C, Hoang T, Dietrich J, Cardona PJ, Izzo A, Dolganov G, et al. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nat Med* (2011) **17**:189–94. doi:10.1038/nm.2285
26. Lin PL, Dietrich J, Tan E, Abalos RM, Burgos J, Bigbee C, et al. The multistage vaccine H56 boosts the effects of BCG to protect cynomolgus macaques against

active tuberculosis and reactivation of latent *Mycobacterium tuberculosis* infection. *J Clin Invest* (2012) **122**:303–14. doi:10.1172/JCI46252
27. Biketov S, Mukamolova GV, Potapov V, Gilenkov E, Vostroknutova G, Kell DB, et al. Culturability of *Mycobacterium tuberculosis* cells isolated from murine macrophages: a bacterial growth factor promotes recovery. *FEMS Immunol Med Microbiol* (2000) **29**:233–40. doi:10.1111/j.1574-695X.2000.tb01528.x
28. Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB. A bacterial cytokine. *Proc Natl Acad Sci U S A* (1998) **95**:8916–21. doi:10.1073/pnas.95.15.8916
29. Davies AP, Dhillon AP, Young M, Henderson B, McHugh TD, Gillespie SH. Resuscitation-promoting factors are expressed in *Mycobacterium tuberculosis*-infected human tissue. *Tuberculosis (Edinb)* (2008) **88**:462–8. doi:10.1016/j.tube.2008.01.007
30. Mukamolova GV, Turapov OA, Young DI, Kaprelyants AS, Kell DB, Young M. A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Mol Microbiol* (2002) **46**:623–35. doi:10.1046/j.1365-2958.2002.03184.x
31. Rachman H, Strong M, Ulrichs T, Grode L, Schuchhardt J, Mollenkopf H, et al. Unique transcriptome signature of *Mycobacterium tuberculosis* in pulmonary tuberculosis. *Infect Immun* (2006) **74**:1233–42. doi:10.1128/IAI.74.2.1233-1242.2006
32. Mukamolova GV, Turapov O, Malkin J, Woltmann G, Barer MR. Resuscitation-promoting factors reveal an occult population of tubercle bacilli in sputum. *Am J Respir Crit Care Med* (2010) **181**:174–80. doi:10.1164/rccm.200905-0661OC
33. Huang W, Qi Y, Diao Y, Yang F, Zha X, Ren C, et al. Use of resuscitation-promoting factors proteins improves the sensitivity of culture-based tuberculosis testing in special samples. *Am J Respir Crit Care Med* (2014) **189**(5):612–4. doi:10.1164/rccm.201310-1899LE
34. Gupta RK, Srivastava BS, Srivastava R. Comparative expression analysis of rpf-like genes of *Mycobacterium tuberculosis* H37Rv under different physiological stress and growth conditions. *Microbiology* (2010) **156**:2714–22. doi:10.1099/mic.0.037622-0
35. Yeremeev VV, Kondratieva TK, Rubakova EI, Petrovskaya SN, Kazarian KA, Telkov MV, et al. Proteins of the Rpf family: immune cell reactivity and vaccination efficacy against tuberculosis in mice. *Infect Immun* (2003) **71**:4789–94. doi:10.1128/IAI.71.8.4789-4794.2003
36. Geluk A, van den Eeden SJ, van Meijgaarden KE, Dijkman K, Franken KL, Ottenhoff TH. A multistage-polyepitope vaccine protects against *Mycobacterium tuberculosis* infection in HLA-DR3 transgenic mice. *Vaccine* (2012) **30**(52):7513–21. doi:10.1016/j.vaccine.2012.10.045
37. Cho S, Mehra V, Thoma-Uszynski S, Stenger S, Serbina N, Mazzaccaro RJ, et al. Antimicrobial activity of MHC class I-restricted CD8+ T cells in human tuberculosis. *Proc Natl Acad Sci U S A* (2000) **97**:12210–15. doi:10.1073/pnas.210391497
38. Flynn JL, Chan J. Tuberculosis: latency and reactivation. *Infect Immun* (2001) **69**:4195–201. doi:10.1128/IAI.69.7.4195-4201.2001
39. Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Andersen P, et al. Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* (1998) **95**:270–5. doi:10.1073/pnas.95.1.270
40. Stenger S, Mazzaccaro RJ, Uyemura K, Cho S, Barnes PF, Rosat JP, et al. Differential effects of cytolytic T cell subsets on intracellular infection. *Science* (1997) **276**:1684–7. doi:10.1126/science.276.5319.1684
41. Ab BK, Kiessling R, Van Embden JD, Thole JE, Kumararatne DS, Pisa P, et al. Induction of antigen-specific CD4+ HLA-DR-restricted cytotoxic T lymphocytes as well as nonspecific nonrestricted killer cells by the recombinant mycobacterial 65-kDa heat-shock protein. *Eur J Immunol* (1990) **20**:369–77. doi:10.1002/eji.1830200221
42. Lewinsohn DA, Winata E, Swarbrick GM, Tanner KE, Cook MS, Null MD, et al. Immunodominant tuberculosis CD8 antigens preferentially restricted by HLA-B. *PLoS Pathog* (2007) **3**:1240–9. doi:10.1371/journal.ppat.0030127
43. Smith SM, Brookes R, Klein MR, Malin AS, Lukey PT, King AS, et al. Human CD8+ CTL specific for the mycobacterial major secreted antigen 85A. *J Immunol* (2000) **165**:7088–95. doi:10.4049/jimmunol.165.12.7088
44. Smith SM, Klein MR, Malin AS, Sillah J, McAdam KP, Dockrell HM. Decreased IFN-gamma and increased IL-4 production by human CD8(+) T cells in response to *Mycobacterium tuberculosis* in tuberculosis patients. *Tuberculosis (Edinb)* (2002) **82**:7–13. doi:10.1054/tube.2001.0317
45. Caccamo N, Guggino G, Meraviglia S, Gelsomino G, Di CP, Titone L, et al. Analysis of *Mycobacterium tuberculosis*-specific CD8 T-cells in patients with active tuberculosis and in individuals with latent infection. *PLoS One* (2009) **4**:e5528. doi:10.1371/journal.pone.0005528
46. Lewinsohn DM, Swarbrick GM, Cansler ME, Null MD, Rajaraman V, Frieder MM, et al. Human CD8 T Cell antigens/epitopes identified by a proteomic peptide library. *PLoS One* (2013) **8**:e67016. doi:10.1371/journal.pone.0067016
47. Tang ST, van Meijgaarden KE, Caccamo N, Guggino G, Klein MR, van Weeren P, et al. Genome-based in silico identification of new *Mycobacterium tuberculosis* antigens activating polyfunctional CD8+ T cells in human tuberculosis. *J Immunol* (2011) **186**:1068–80. doi:10.4049/jimmunol.1002212
48. Rodenko B, Toebes M, Hadrup SR, van Esch WJ, Molenaar AM, Schumacher TN, et al. Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. *Nat Protoc* (2006) **1**:1120–32. doi:10.1038/nprot.2006.121
49. Lindestam Arlehamn CS, Gerasimova A, Mele F, Henderson R, Swann J, Greenbaum JA, et al. Memory T cells in latent *Mycobacterium tuberculosis* infection are directed against three antigenic islands and largely contained in a CXCR3+CCR6+ Th1 subset. *PLoS Pathog* (2013) **9**:e1003130. doi:10.1371/journal.ppat.1003130
50. Kaufmann SH. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol* (2001) **1**:20–30. doi:10.1038/35095558
51. Available from: http://hla.alleles.org/alleles/class1.html
52. Mazzarino P, Pietra G, Vacca P, Falco M, Colau D, Coulie P, et al. Identification of effector-memory CMV-specific T lymphocytes that kill CMV-infected target cells in an HLA-E-restricted fashion. *Eur J Immunol* (2005) **35**:3240–7. doi:10.1002/eji.200535343
53. Pietra G, Romagnani C, Mazzarino P, Falco M, Millo E, Moretta A, et al. HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8+ cytolytic T lymphocytes. *Proc Natl Acad Sci U S A* (2003) **100**:10896–901. doi:10.1073/pnas.1834449100
54. Salerno-Goncalves R, Fernandez-Vina M, Lewinsohn DM, Sztein MB. Identification of a human HLA-E-restricted CD8+ T cell subset in volunteers immunized with *Salmonella enterica* serovar typhi strain Ty21a typhoid vaccine. *J Immunol* (2004) **173**:5852–62. doi:10.4049/jimmunol.173.9.5852
55. Strong RK, Holmes MA, Li P, Braun L, Lee N, Geraghty DE. HLA-E allelic variants. Correlating differential expression, peptide affinities, crystal structures, and thermal stabilities. *J Biol Chem* (2003) **278**:5082–90. doi:10.1074/jbc.M208268200
56. Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL, et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* (1999) **10**:661–71. doi:10.1016/S1074-7613(00)80065-5
57. Heinzel AS, Grotzke JE, Lines RA, Lewinsohn DA, McNabb AL, Streblow DN, et al. HLA-E-dependent presentation of Mtb-derived antigen to human CD8+ T cells. *J Exp Med* (2002) **196**:1473–81. doi:10.1084/jem.20020609
58. Garcia P, Llano M, de Heredia AB, Willberg CB, Caparros E, Aparicio P, et al. Human T cell receptor-mediated recognition of HLA-E. *Eur J Immunol* (2002) **32**:936–44. doi:10.1002/1521-4141(200204)32:4<936::AID-IMMU936>3.3.CO;2-D
59. Pietra G, Romagnani C, Falco M, Vitale M, Castriconi R, Pende D, et al. The analysis of the natural killer-like activity of human cytolytic T lymphocytes revealed HLA-E as a novel target for TCR alpha/beta-mediated recognition. *Eur J Immunol* (2001) **31**:3687–93. doi:10.1002/1521-4141(200112)31:12<3687::AID-IMMU3687>3.0.CO;2-C
60. Sarantopoulos S, Lu L, Cantor H. Qa-1 restriction of CD8+ suppressor T cells. *J Clin Invest* (2004) **114**:1218–21. doi:10.1172/JCI200423152
61. Joosten SA, van Meijgaarden KE, van Weeren PC, Kazi F, Geluk A, Savage ND, et al. *Mycobacterium tuberculosis* peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS Pathog* (2010) **6**:e1000782. doi:10.1371/journal.ppat.1000782
62. Commandeur S, van Meijgaarden KE, Prins C, Pichugin AV, Dijkman K, van den Eeden SJ, et al. An unbiased genome-wide *Mycobacterium tuberculosis* gene expression approach to discover antigens targeted by human T cells expressed during pulmonary infection. *J Immunol* (2013) **190**:1659–71. doi:10.4049/jimmunol.1201593

63. Commandeur S, van den Eeden SJF, Dijkman K, Clark SO, van Meijgaarden KE, Wilson L, et al. The *in vivo* expressed *Mycobacterium tuberculosis* (IVE-TB) antigen Rv2034 induces CD4+ T-cells that protect against pulmonary infection in HLA-DR transgenic mice and guinea pigs. *Vaccine* (2014) **32**:3580–8. doi:10.1016/j.vaccine.2014.05.005

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 March 2014; paper pending published: 02 May 2014; accepted: 16 May 2014; published online: 25 June 2014.

*Citation: Geluk A, van Meijgaarden KE, Joosten SA, Commandeur S and Ottenhoff THM (2014) Innovative strategies to identify M. tuberculosis antigens and epitopes using genome-wide analyses. Front. Immunol. **5**:256. doi: 10.3389/fimmu.2014.00256 This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 22 April 2014
doi: 10.3389/fimmu.2014.00180

Functional signatures of human CD4 and CD8 T cell responses to *Mycobacterium tuberculosis*

Teresa Prezzemolo, Giuliana Guggino, Marco Pio La Manna, Diana Di Liberto, Francesco Dieli and Nadia Caccamo*

Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi and Central Laboratory of Advanced Diagnosis and Biomedical Research, University of Palermo, Palermo, Italy

Edited by:
Tom H. M. Ottenhoff, Leiden University Medical Center, Netherlands

Reviewed by:
Tom H. M. Ottenhoff, Leiden University Medical Center, Netherlands
Simone Joosten, Leiden University Medical Center, Netherlands

****Correspondence:***
Nadia Caccamo, Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi, University of Palermo, Corso Tukory n. 211, 90134 Palermo, Italy
e-mail: nadia.caccamo@unipa.it

With 1.4 million deaths and 8.7 million new cases in 2011, tuberculosis (TB) remains a global health care problem and together with HIV and Malaria represents one of the three infectious diseases world-wide. Control of the global TB epidemic has been impaired by the lack of an effective vaccine, by the emergence of drug-resistant forms of *Mycobacterium tuberculosis* (Mtb) and by the lack of sensitive and rapid diagnostics. It is estimated, by epidemiological reports, that one third of the world's population is latently infected with Mtb, but the majority of infected individuals develop long-lived protective immunity, which controls and contains Mtb in a T cell-dependent manner. Development of TB disease results from interactions among the environment, the host, and the pathogen, and known risk factors include HIV co-infection, immunodeficiency, diabetes mellitus, overcrowding, malnutrition, and general poverty; therefore, an effective T cell response determines whether the infection resolves or develops into clinically evident disease. Consequently, there is great interest in determining which T cells subsets mediate anti-mycobacterial immunity, delineating their effector functions. On the other hand, many aspects remain unsolved in understanding why some individuals are protected from Mtb infection while others go on to develop disease. Several studies have demonstrated that $CD4^+$ T cells are involved in protection against Mtb, as supported by the evidence that $CD4^+$ T cell depletion is responsible for Mtb reactivation in HIV-infected individuals. There are many subsets of $CD4^+$ T cells, such as T-helper 1 (Th1), Th2, Th17, and regulatory T cells (Tregs), and all these subsets co-operate or interfere with each other to control infection; the dominant subset may differ between active and latent Mtb infection cases. Mtb-specific-$CD4^+$ Th1 cell response is considered to have a protective role for the ability to produce cytokines such as IFN-γ or TNF-α that contribute to the recruitment and activation of innate immune cells, like monocytes and granulocytes. Thus, while other antigen (Ag)-specific T cells such as $CD8^+$ T cells, natural killer (NK) cells, γδ T cells, and CD1-restricted T cells can also produce IFN-γ during Mtb infection, they cannot compensate for the lack of $CD4^+$ T cells. The detection of Ag-specific cytokine production by intracellular cytokine staining (ICS) and the use of flow cytometry techniques are a common routine that supports the studies aimed at focusing the role of the immune system in infectious diseases. Flow cytometry permits to evaluate simultaneously the presence of different cytokines that can delineate different subsets of cells as having "multifunctional/polyfunctional" profile. It has been proposed that polyfunctional T cells, are associated with protective immunity toward Mtb, in particular it has been highlighted that the number of Mtb-specific T cells producing a combination of IFN-γ, IL-2, and/or TNF-α may be correlated with the mycobacterial load, while other studies have associated the presence of this particular functional profile as marker of TB disease activity. Although the role of CD8 T cells in TB is less clear than CD4 T cells, they are generally considered to contribute to optimal immunity and protection. CD8 T cells possess a number of anti-microbial effector mechanisms that are less prominent or absent in CD4 Th1 and Th17 T cells. The interest in studying CD8 T cells that are either MHC-class Ia or MHC-class Ib-restricted, has gained more attention. These studies include the role of HLA-E-restricted cells, lung mucosal-associated invariant T-cells (MAIT), and CD1-restricted cells. Nevertheless, the knowledge about the role of $CD8^+$ T cells in Mtb infection is relatively new and recent studies have delineated that CD8 T cells, which display a functional profile termed "multifunctional," can be a better marker of protection in TB than $CD4^+$ T cells. Their effector mechanisms could contribute to control Mtb infection, as upon activation, CD8 T cells release cytokines or cytotoxic molecules, which cause apoptosis of target cells. Taken together, the balance of the immune response in the control of infection and possibly bacterial eradication is important in understanding whether the host immune response will be appropriate in contrasting the infection or not, and, consequently, the inability of the immune response, will determine the dissemination and the transmission of bacilli to new subjects. In conclusion, the recent highlights on the role of different functional signatures of T cell subsets in the immune response toward Mtb infection will be discerned in this review, in order to summarize what is known about the immune response in human TB. In particular, we will discuss the role of CD4 and CD8 T cells in contrasting the advance of the intracellular pathogen in already infected people or the progression to active disease in subjects with latent infection. All the information will be aimed at increasing the knowledge of this complex disease in order to improve diagnosis, prognosis, drug treatment, and vaccination.

Keywords: *M. tuberculosis*, cytokines, human memory T cells, disease, infection

INTRODUCTION

Tuberculosis, with approximately 9 million cases annually, determines a world-wide mortality and morbidity, especially in low-income countries (1–3). *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, is transmitted via aerosol droplets that are suspended in the air for prolonged periods of time (4), determining a risk of infection to people who inhalate these droplets. However, infection does not necessarily lead to TB disease; in fact, as reported in several studies, only 3–10% of immunocompetent individuals that are infected will develop the disease during their life-time (5), while more than 90% of infected subjects contain infection in a subclinical stage known as latent TB infection (LTBI), in which the pathogen remains in a quiescent state (4). One of the important aspects that can contribute to reactivation depends on the immune system of each individual that can be perturbed by several factors during life-time, such as chronic diseases: diabetes, alcoholic liver disease, HIV co-infection, and in some circumstances, the use of steroids or other immunosuppressive drugs. Another occurrence of active disease in later life is attributable to reactivation of latent Mtb bacilli or to a new infection with another Mtb strain. However, this huge reservoir contributes to fuel the high numbers of new active TB disease (3, 6); therefore, in order to diminish the risk of new active TB disease, it is important to treat LTBI cases by chemoprophylaxis, successfully eradicating the infection in the majority of cases. LTBI subjects, due to the increasing use of biological drugs, such as tumor necrosis factor-α (TNF-α)/Interleukin (IL)-12/IL-23 blockers for the treatment of inflammatory diseases like rheumatoid arthritis, Crohn's disease, and psoriasis, have major risk to progress toward active disease more than other subjects (3, 7). Diagnosis of LTBI remains a priority for TB control within high income, low TB prevalence countries (8, 9), where a high proportion of TB cases occurs in immigrants from countries with high TB incidence (10, 11).

The study of subjects that are able to control Mtb infection in the long-term may be particularly informative in this respect. Despite two decades of intensified research, the mechanisms involved in the protective immune response against Mtb are not well understood. So, the comprehension of the pathways involved in protection in the host could represent biomarkers useful as correlates of protection, while the inhibition of the pathways involved in the surviving of host pathogens, could represent a biological target to contrast the bacilli growth and replication (12, 13).

Mycobacterium tuberculosis involves several conventional and unconventional T cell subsets that are characterized by distinct effector functions and surface phenotype markers (14). Th1 CD4 T cells activate effector functions in macrophages that control intracellular Mtb, and their role has been correlated with protection (14). Moreover, several studies have reported that Th17 cells, which are able to produce IL-17, are involved in immune protection against Mtb, primarily due to the effect of this cytokine in attracting and activating neutrophils (14, 15). Th17 cells have been involved in protection against TB at early stages (15, 16), for their capacity to recruit monocytes and Th1 lymphocytes to the site of granuloma formation (14, 15, 17). On the contrary, several studies have demonstrated that unrestricted Th17 stimulation determines an exaggerated inflammation mediated by neutrophils and inflammatory monocytes that rush to the site of disease causing tissue damage (14, 18–20).

CD4 T cells recognize antigenic peptides derived from the phagosomal compartment in the context of MHC-class II molecules (21). Mtb preferentially resides in the phagosome, where mycobacterial Ags can be processed and assembled to MHC-class II molecules (14, 22, 23). Another conventional lymphocytes subset, CD8 T cells, contributes to immune protection against TB (24): upon specific Ag recognition, CD8 T cells differentiate into effector cells, which produce cytolytic molecules and cytokines that kill both host cells and the intracellular Mtb (14, 25).

CD8 T lymphocytes recognize antigenic peptides, which are generally loaded in the cytosolic compartment in the context of MHC-class I molecules (21). MHC-class I loading can occur because of the intracellular pathogen or Mtb proteins diversification from the phagosome to the cytosol (14, 26). Moreover, apoptotic vesicles coming from infected macrophages and dendritic cells (DCs) can be uptaken by DCs (27, 28), which, in turn, will process and shuttled peptides into the canonical MHC-class I presentation pathway, a process termed cross-presentation (29).

Other cells play a role in the control or in the suppression of immune responses during Mtb infection such as Th2 cells, which counter-regulate Th1 cells and likely impair protective immunity against TB (30, 31), and regulatory T (Treg) cells (32, 33), which also contribute to the down modulation of the immune response to the pathogen (14) and to TB reactivation (14, 32–34).

The so-called unconventional T cells are activated during TB; these cells are able to recognize lipids that are abundant in the mycobacterial cell wall, in the context of non-polymorphic CD1 molecules (35). Very recently, mucosal-associated invariant T cells (MAIT) have been found to recognize protein Mtb (Ags) presented by the non-classical molecule MR1 (36). γδ T cells, recognize "phosphoAgs" of host or bacterial origin and may also contribute to the immune response to Mtb as well (14, 37). **Figure 1** shows the different cell populations involved in the immunopathology of TB.

In the last years, the potential role of distinct T cell subsets as biomarkers of active TB and/or LTBI has been studied. Functional CD4 and CD8 T cell subsets have been defined on the bases of cytokine production as single, double, or triple producer cells. These different cytokine signatures have been differently associated with disease stage, mycobacterial load or treatment, and several studies, mostly derived from vaccination in animals, have highlighted that polyfunctional CD4 T cells are associated with protective immunity. In contrast, more recent studies have suggested that these cells may be not correlated with protection, but rather with TB disease activity (38, 39).

In this review, we will analyze the complexity of the immune response of conventional CD4 and CD8 T cells widely described by recent studies in patients with pulmonary and extra-pulmonary disease and in subjects with LTBI, in order to better define the potential of different functional signatures of T cells as potential biomarkers.

POPULATIONS OF HUMAN MEMORY T CELLS

Individuals that have encountered a pathogen, develop an adaptive immune response with the induction of memory cells that

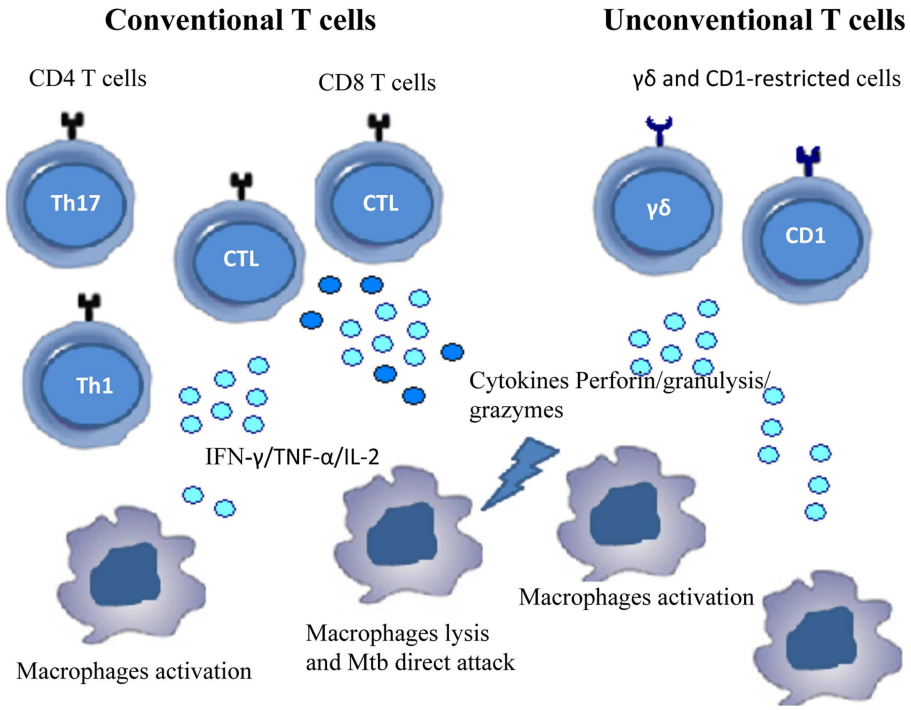


FIGURE 1 | Cells involved in immune response during Mtb infection. The figure shows conventional and unconventional T cell subsets that contribute to the immune response against Mtb.

will recognize the same Ag, upon the second encounter, dictating the type of immune response. Several studies have delineated that the quality of the memory response is important to dissect the real difference between protection and immunopathology, and to design strategies for vaccination (40).

Generally, the generation of memory T cells is characterized by different phases (41). The first encounter with an Ag, defined priming, determines a massive proliferation and clonal expansion of Ag-specific T cells followed by a phase of contraction, where the majority of these cells, named effector cells, are eliminated by apoptosis (42, 43). During this primary response, memory T cells develop and are maintained for extended periods due to several mechanisms such as the retention of Ag, stimulation/boosters, or homeostatic proliferation, that will insure the maintenance of a pool of cells that can rapidly respond to subsequent encounters with the pathogen.

The induction of memory T cells by vaccination against intracellular pathogens has definitively led a major challenge for the development of new subunit vaccines (40).

In humans, the functional properties of memory T and B cells can be defined, at least for those cells circulating in the blood, using techniques that detect typical surface markers (44). The combinatorial expression of surface markers such as adhesion molecules, chemokine receptors, and memory markers, allows for tissue specific homing of memory and effector lymphocytes and thus provides full characterization of that particular subsets of memory T cells, in terms of preferential residence inside tissues (40, 45, 46).

At least dozens of subsets can be identified and enumerated on the basis of distinct cellular functions that express unique combinations of surface and intracellular markers (47).

Memory T cells could be divided into $CD62L^+$ and $CD62L^-$ subsets; moreover some surface markers are specific for T cells homing to mucosa and skin that are confined to the $CD62L^-$ subset (48, 49). The development of techniques that allow to measure cytokines production at the single-cell level and the analysis of several surface markers has permitted to correlate the functional properties of T cells with their phenotype (50). $CCR7^+$ memory cells are named central memory (T_{CM}) cells: they are able to home to secondary lymphoid tissues, produce high amounts of IL-2 but low levels of other effector cytokines (41), while their $CCR7^-$ counter parts, named effector memory (T_{EM}) cells, are able to produce high levels of cytokines, exert rapid effector functions and home to peripheral tissues (41). It has been established a relationship between T_{CM} and T_{EM} cells suggested by the analysis of the telomeres that are longer in T_{CM} than T_{EM} cells and T_{CM} cells are capable of generating T_{EM} cells *in vitro*, but not *vice versa* (41). Studies performed in humans and rhesus macaques both *in vitro* and *in vivo* have led to the identification of T cells with multiple stem cell-like properties, termed memory T stem cells (T_{SCM}). These cells constitute a relatively rare memory population having a largely T naive (T_N) phenotype, while overexpressing CD95 (51, 52), which is usually expressed at high levels by all memory cells (53, 54). T_{SCM} cells, precede T_{CM} cells in differentiation. These type of cells are capable of generating all memory subsets, including T_{CM} cells (51, 52); no

other memory subset thus far has been found to regenerate T_{SCM} cells (44).

Another subset of "transitional" memory T cells (T_{TM}) has been defined, mostly of which were isolated in the peripheral blood of healthy individuals (55, 56). These T_{TM} cells are more differentiated than T_{CM} cells but not as fully differentiated as T_{EM} cells in terms of phenotype (55, 56) and ability to expand in response to IL-15 *in vivo* (57, 58).

Very recently, Mahnke et al. propose that the phenotypic, functional, and gene expression properties of human memory T cell differentiation follow a linear progression along a continuum of major clusters (T_N, T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells) (44). According to this linear progression, memory T cells, progressively acquire or lose their specific functions (**Figure 2**). Other molecules that mediate lymphocyte functions, including markers of migration, co-stimulation, and cytotoxic molecules and adhesion markers can better define these different T cell subsets (**Table 1**).

Seder et al. have proposed that T cells progressively acquire their functions with further differentiation, until they reach the phase that is adequate for their effector function (such as the production of cytokines or cytotoxic activity) (44, 59). The authors have demonstrated that the continued antigenic stimulation led to progressive loss of memory potential as well as the ability to produce cytokines, until the last step of the differentiation pathway represented by effector cells that are able to produce only IFN-γ and are short-lived, named terminally differentiated effector cells (T_{EMRA}) (59). Another aspect that can optimize this linear differentiation process will depend on the amount of initial Ag exposure or the different conditions that are present in the microenvironment, which will dictate the extent of differentiation (44, 59).

Hierarchical expression of cytolytic molecules and surface markers, such as CD27, CD28, and CD57, has been delineated for CD8 T cell subsets. Granzyme (Gr)A is the first cytotoxic molecule detected in memory cells, followed by GrB and subsequently by perforin (60–62). GrB is always expressed in the presence of GrA, while, perforin$^+$ cells are primarily positive for GrA and GrB, making it a choice indicator for cytolytic cells (62). Usually, perforin is present in cells that are CD27$^-$ and CD28$^-$ (63), while this molecule is always associated with the expression of the senescence marker CD57, which can be used as marker for T cells with high cytolytic potential (44, 62). Finally, the identification of the different subsets of human memory T cells, through the analysis of the expression of exclusive markers in that particular population could have a potential implications in T cell-based immunotherapy for infectious disease or other immune pathological conditions. Several studies have evaluated the different distribution of Ag-specific memory T cells subsets as good model of correlate of protection; for example, in response to chronic infectious agents such as HIV-1, hepatitis C virus (HCV), and Mtb, the increase of the frequency of Ag-specific T_{CM} cells, which produce high levels of IL-2, is associated with individuals' ability to control the viral load (64–68).

Moreover, the response to cytokines used to differentiate or to maintain the different human memory T cells has been characterized (69). It has been shown that T_{EM} cells can proliferate in response to IL-7 and IL-15 *in vitro* but do not expand because of spontaneous apoptosis; conversely, T_{CM} proliferate and differentiate to T_{EM} cells, in the absence of these cytokines (70, 71).

Therefore, the quality of T cell responses can be modulated by several factors, and it is crucial for establishing the disease outcome in the context of various infections or pathologies.

In summary, the definition of the different subsets of memory T cells can be used to delineate the quality of a given T

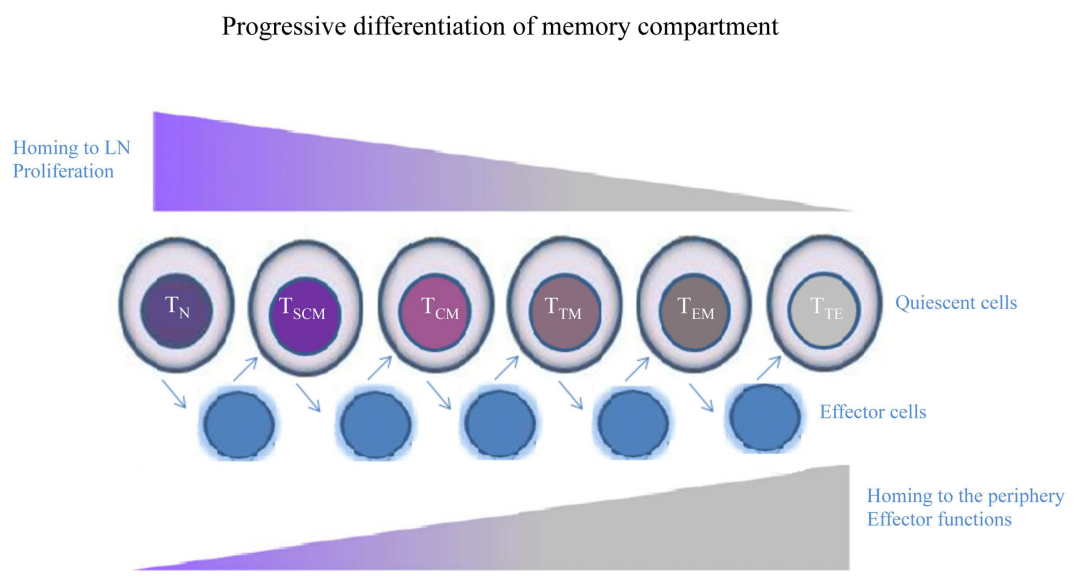


FIGURE 2 | Human memory T cell subsets. Following encounter with Ag, quiescent T cells develop into effectors, whose phenotype is highly dynamic and largely unpredictable. When the Ag is cleared, effector T cells that survive return to a quiescent memory state. Cells differentiate from T_N to T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and culminating in T_{TE} cells. Memory T cells progressively lose or acquire specific functions, such as the ability to migrate to peripheral tissues or to proliferate or produce effector molecules.

Table 1 | Expression of functional molecules by circulating T cell subsets.

Subsets	T_N	T_{SCM}	T_{CM}	T_{TM}	T_{EM}	T_{TE}	Category	Ag	Function
	+	++	++	++	−	−	Co-stimulation/survival	CD28	Co-stimulation
	++	+	+	+	±	−		CD27	Co-stimulation
	++	+++	+++	++	±	−		CD127	IL-7 signaling
	−	±	+	++	+	+		PD-1	Inhibition of effector function
	−	+	++	+++	+++	+++		CD122	IL-2/IL-15 signaling
	+	+	+	+	+	+		CD132	γc cytokine signaling
	−	ND	±	+	++	+++		KLRG-1	Inhibition of effector function
	+	++	++	+++	+++	+++	Adhesion	CD11a	Adhesion to APC/endothelium
	−	+	++	+++	+++	+++		CD58	Adhesion to APC
	±	+	++	++	++	++		CD99	Transendothelial migration
	+	+	+	−	−	−	Migration	CD62L	Secondary lymphoid tissues homing
	−	−	−	−	+	−		CD103	Gut homing
	±	+	++	+++	+++	±		CCR4	Chemokine response/Th2 associated
	−	−	+	++	+++	++		CCR5	Homing to inflamed tissues
	−	−	++	+++	+++	−		CCR6	Chemokine response/Th17 associated
CD4	−	ND	+	−	−	−		CCR9	Gut homing
CD8	−	ND	+	++	++	−			
	−	−	+	ND	++	−		CCR10	Skin homing
CD4	−	±	+	++	+++	+++		CXCR3	Homing to inflamed tissues
CD8	++	+++	+++	++	+	+			
	+	++	+++	+++	++	++		CXCR4	Homing to Bone Marrow
	−	ND	+	ND	++	ND		CLA	Skin homing
CD4	−	−	−	−	±	+	Cytolitic molecules	Granzyme A	Cleavage of cellular proteins
CD8	−	−	±	++	+++	+++			
CD4	−	−	−	−	±	±		Granzyme B	Cleavage of cellular proteins
CD8	−	−	−	+	++	+++			
CD4	−	−	−	−	±	±		Perforin	Pore forming
CD8	−	−	±	+	++	+++			

Combination of + and – indicates the expression level respect to TN cells. ND = not determined.

cell response, and this can be achieved by the combination of cell-surface phenotype, functional properties, and the capacity to traffic to lymphoid and non-lymphoid tissues: such a complex analysis should confer more intuition if an immune response will be protective or not.

SUBSETS OF MEMORY CD4 T CELLS IN TB

Mycobacterium tuberculosis-specific-$CD4^+$ T cell protective response is typically due to Th1 cells and is mediated by IFN-γ and TNF-α that recruit monocytes and granulocytes and promote their anti-microbial activities (72–74).

Recent studies have shown that polyfunctional T cells (i.e., T cells equipped with multiple effector functions) (44, 75), could exert immune protection toward viral infections such as HIV (76, 77), models of TB vaccine (78–81), or in murine models of leishmania (36). However, the role of polyfunctional T cells during Mtb infection is controversial and different from that observed in chronic viral infections (36, 40, 81).

The definition of polyfunctional T cells was attributed to their ability to proliferate and to secrete multiple cytokines and these cells were found to play a protective role in antiviral immunity in chronic infections (when Ag load is low). Conversely, single IFN-γ-secreting CD4 and CD8 T cells typically predominate in acute infections (when Ag load is high), and in chronic infection characterized by the failure of immune control: in the case of HIV-1 infection, in fact, the response is dominated by HIV-1-specific-CD4 and -CD8 T cells that are able to produce only IFN-γ in both the primary and chronic phases of infection. On the other hand, the distinct cytokines profile during intracellular pathogens infection, comprises a very wide spectrum of T cell subpopulations (75).

Several authors have recently shown that polyfunctional T cells release multiple cytokines simultaneously in a relatively short period. The analysis of different aspects that could contribute to the release of cytokines, such as the methodologies used to stimulate the cells, peptides, or proteins used, the different cohort groups

included in the study, should be taken into account, considering that very often the results obtained are controversial (75, 82).

Earlier studies in human TB have investigated on the role of polyfunctional T cells able to produce IFN-γ in combination with IL-2 (75, 83–86), and later on, a subset of cells able to simultaneously produce IFN-γ, TNF-α, and/or IL-2 was detected in patient with active TB disease compared to latently infected individuals (87–90), whose frequency decreased after anti-TB treatment. In another study, high frequencies of CD4 T cells expressing three cytokines simultaneously (IFN-γ, TNF-α, and IL-2) was found in adults with active TB disease, as compared to the frequency found in LTBI subjects, in which IFN-γ single and IFN-γ/IL-2 dual secreting CD4 T cells dominated the anti-mycobacterial response. Therefore, the presence of multifunctional CD4 T cells in TB patients was associated with the bacterial loads, as suggested by their decrease after completion of anti-TB chemotherapy (82, 91). This implies that multifunctional CD4 T cells are indicative of active TB rather than assuming a protective role. However, during these years, several contrasting findings have been reported, which do not allow a clear-cut conclusion on the role of polyfunctional CD4 T cells (40). In fact, some authors have found a reduced frequency of polyfunctional T cells in patients with active TB disease compared to latently infected individuals, which is recovered with the anti-TB therapy (75, 92, 93). Similar recovery of dual IFN-γ/IL-2-producing cells with the anti-TB therapy was also previously reported (82, 94).

Finally, a higher proportion of Ag-specific effector memory T_{EM} cells and a decreased frequency of T_{CM} $CD4^+$ T cells has been found in patients with active TB (95, 96), as compared to the distribution found in LTBI individuals (75).

Since it is not possible to associate any specific cytokine profile with protection against active TB, recent studies have tried to find a correlation between functional signatures of CD4 or CD8 T cells and the state of infection/disease.

Marin et al. have analyzed the Th1 and Th17 responses through the counts of IFN-γ and IL-17 producing T cells by elispot assay, the frequencies of polyfunctional T cells producing IFN-γ, TNF-α, IL-2, and IL-17 by ICS, and the amounts of the above cited cytokines released after 1 day (short term) and 6 days (long-term) of *in vitro* stimulation using different Ags (CFP-10, PPD, or Mtb) (75) by ELISA. The evaluation of different T cell subsets after short- and long-term *in vitro* stimulation with different Ags has permitted to find a significant increase in single and double producer $CD4^+$cells in long-term *in vitro* stimulation compared to short term *in vitro* stimulation in LTBI subjects and a significant increase of the frequency of single producer cells in patients with active disease (75). Mtb stimulation determined an increase in the frequency of single and triple producer T cells in LTBI subjects in 6 days compared to the frequency found in 1 day *in vitro* stimulated cells, with a significant value found for the frequency of double producer T cells in patients with active disease (75). These results suggest that the use of different mycobacterial Ags could induce distinct T cell functional signatures in LTBI subjects and in patients with active disease, highlighting that it is possible to define "functional signatures" of CD4 T cells correlated with the state of infection and that could be used as indicators of the clinical activity of the disease (82).

Very recently, Petruccioli et al. have correlated bifunctional "RD1-proteins"-specific-CD4 T cells with effector memory phenotype with active TB disease, while "RD1-proteins"-specific-CD4 T cells with a central memory phenotype were associated with cured TB and LTBI subjects (82). According to this study, the EM phenotype should be associated with inactive TB due to the presence of live and replicating bacteria, whereas the contraction of this phenotype and the further differentiation toward CM T cells in LTBI and cured TB subjects could indicate Mtb control, suggesting that the different expression of the memory/effector status may be used to monitor treatment efficacy, as previously suggested in patients with active TB with HIV co-infection (82, 97, 98).

A more detailed study on the role of Ag-specific T cell phenotype and function has been carried out by Lalvani et al. who delineated the association of TB disease stage with Mtb-specific cellular immunity. The authors have found the same trend of functional signature demonstrated by Petruccioli, but in response to different antigenic stimulation, namely PPD and RD1-peptides: in fact, Ag-specific-CD4 T cells were principally of the CM phenotype in subjects with latent infection compared to EM cells predominantly found in patients with active disease. Combined measurement of both functional profile and differentiation phenotype, in this study, reflects a discriminatory immunological status in the different cohort groups studied (patients with active disease vs. LTBI) (99). Moreover, HIV infection did not influence the number of Mtb-specific-CD4 effector cells, which instead was influenced by TB disease stage. This last aspect could be intriguing for the fact that assessment of cellular changes could be used also for immune compromised patients; in fact, it is known that HIV and active TB both impact Mtb-specific T cell immunity, such as skin test anergy, and therefore, dissection of distinct subsets as biomarkers could have an impact also in HIV co-infection.

Altogether, the above studies highlight the concept that the protective immune response against mycobacterial infection seems to depend more on the quality of CD4 T cell response assessed as the capacity to exert multiple functions, than on their magnitude, which is due to their Ag-specific frequency (44, 75). Finally, several methodologies used for the evaluation of the profiles of Mtb-specific-CD4 T cells in the reported studies led to different results: these include Ag specificity and type, *in vitro* stimulation conditions (short- or long-term *in vitro* stimulation), variability of the study cohort characteristics and at least, the monoclonal antibodies used to distinguish the subsets of CD4 T cells or intracellular cytokines content (40).

Thus, further studies are necessary to define particular phenotypes of Mtb-specific-CD4 T cells, assessing several functional properties such as activation, memory, migratory and inhibitory receptors, and ligands.

SUBSETS OF MEMORY CD8 T CELLS IN TB

$CD8^+$ T cells contribute to protective response against TB (100, 101). $CD8^+$T cells recognize Ags derived from an intracellular environment and could serve as sensors of bacterial burden. In fact, human $CD8^+$T cells preferentially recognize cells heavily infected with Mtb (102) and in animal models, the magnitude of the CD8 response correlates with bacterial load (103–105).

The mechanisms involved in $CD8^+$ T cell activation during Mtb infection are incompletely defined. DCs possess several pathways to load MHC-class I molecules, such as classical cytosolic processing, or alternative processing of phagosome located pathogens and endosome-located Ags. The recent evidences that virulent mycobacteria can escape from the phagosome into the cytoplasm and the possibility to direct access MHC-class I processing/presentation pathway provide a new mechanism (27). DCs also can take up vesicles derived from apoptotic Mtb-infected cells, after which the Ags are cross-presented through MHC-class I and class II molecules (28, 29). Finally, autophagy, which has a prominent role in cellular homeostasis and bacterial sequestration into vacuolar organelles, is involved in Ag presentation and cross-priming of T cells in response to intracellular pathogens, including Mtb (106, 107).

It has been demonstrated that several pathways are used in order to activate $CD8^+$ T cells by phagosomal Ags, and, very recently, MHC-class Ib-restricted $CD8^+$ T cells have received attention, including a role for HLA-E, which presents peptides from a wide range of mycobacterial Ags (34, 108). CD1-restricted CD8T cells recognize lipids such as mycolic acids and lipoarabinomannan from the bacterial cell wall (34) and lung MAIT recognize Mtb Ags in the context of the non-classical MR1 molecule (109).

Thus, $CD8^+$ T cell immunity offers evidences of their clear synergy of action and complementarities in association with $CD4^+$ T cell immunity, for the fact that $CD8^+$ T cells display other direct effector functions such as the secretion of granules that contain cytotoxic molecules as perforin, granzymes, and granulysin. These molecules can lyse host cells, or can have a direct killing toward Mtb and other bacteria. Moreover, $CD8^+$ T cells can induce apoptosis of infected target cells through molecules such as Fas or TNF-R family-related cell-death receptors. Finally, $CD8^+$ T cells release, upon activation, cytokines such as IFN-γ, TNF-α, and in many cases also IL-2. These functions are also used by MHC-class Ib-restricted $CD8^+$ T cells, suggesting a role for classical as well as non-classical $CD8^+$ T cells in TB protection.

From the functional point of view, different studies conducted in mice and non-human models have delineated a role for Mtb-specific $CD8^+$T cells in the control of Mtb infection (102–104). In these studies, it has been demonstrated that IFN-γ and perforin released by Mtb-specific $CD8^+$ T cells were necessary to induce protection in Mtb-infected mice (102, 105). The role of these molecules has been efforted in humans' studies that have reported the same conclusions (21, 110).

Hence, other *in vitro* studies have indicated that perforin- and/or granulysin-containing Mtb-specific $CD8^+$ T cell lines were able to kill Mtb-infected macrophages or even free bacteria (25, 111, 112), other studies have found the complete absence of these molecules released by Mtb-specific $CD8^+$ T cells from lung-associated tissues (113, 114).

Though it is not still possible to attribute a role to polyfunctional T cells as marker of protective immunity or of disease activity, multi-, or polyfunctionality of CD8 T cells is referred to the simultaneous production of several cytokines (IFN-γ, IL-2, TNF-α) and/or the expression of multiple effector functions (perforin, granulysin, cytolysis, etc.). However, contrary to initial expectations, these cells do not appear to correlate with BCG-induced protection in infants (115) and adults (116). Moreover, they are also present in active TB, although they may nevertheless be part of the protective host response attempting to limit infection rather than contributing to active disease.

Previously, we have correlated the frequency of Mtb-Ag85A-specific $CD8^+$ T cells with the efficacy of anti-mycobacterial therapy in children. In particular, we found that Ag85A epitope-specific $CD8^+$ T cells in children with active disease were able to produce low levels of IFN-γ and perforin, which recovered after successful therapy (117). In a later study, the analysis of the *ex vivo* frequencies, cytokine production, and memory phenotype of circulating CD8 T cells specific for different non-amers of Mtb proteins was performed in adult HLA-A*0201 different cohorts (87).

We found a lower percentage of circulating tetramer specific CD8 T cells in TB patients before therapy respect to LTBI subjects, but values increased after 4 months of anti-mycobacterial therapy to those found in subjects with LTBI. In this study, we also found high percentages of IL-2^+/IFN-γ^+ and single IFN-γ^+ in subjects with LTBI, and a reduction of IL-2^+/IFN-γ^+ population in TB patients, suggesting a restricted functional profile of Mtb-specific CD8 T cells during active disease (87).

Many studies have focused on the response to different Mtb Ags expressed in the early phase of infection such as ESAT6, CFP-10, and Ag85B proteins but further studies should also incorporate those Ags expressed at different phases of infection (40).

Another study, using defined cohorts of individuals with smear-positive and smear-negative TB and LTBI subjects, evaluated Mtb-specific responses in correlation to mycobacterial load (93). The authors found, in individuals with high mycobacterial load smear-positive TB, a decrease of polyfunctional and IL-2-producing cells, and an increase of TNF-α^+ Mtb-specific-CD4 T cells and CD8 T cells, both of which had an impaired proliferative capacity (40). These patients were followed during the anti-mycobacterial therapy and it was shown that the percentage of triple positive CD8 T cells (producing IFN-γ, IL-2, and TNF-α) increased over time in 7 out of 13 patients and this increase was paralleled by decrease of the frequency of IFN-γ^+ T cells, providing another evidence that the cytokine production capacity of Mtb-specific CD8 T cells is associated with mycobacterial load.

In children or immunocompromised individuals, where it is very difficult to distinguish Mtb infection from disease, and in people that are at high risk to develop active disease, the increase of polyfuntional CD8 T cells and the reduction of single IFN-γ or TNF-α producing cells may be used to correlate these CD8 T cell subsets with TB disease progression, highlighting a new possible role as indicator of successful response to treatment.

Mycobacterium tuberculosis DosR-regulon encoded Ags (118) expressed by Mtb during *in vitro* conditions, represent rational targets for TB vaccination because they mimic intracellular infection. It has been shown that LTBI individuals are able to recognize Mtb DosR-regulon encoded Ags belonging to different ethnically and geographically distinct populations (40, 111, 118, 119). Moreover, Mtb DosR Ag-specific-$CD4^+$ and -$CD8^+$ polyfunctional T cells were found in LTBI subjects. In detail, a hierarchy of response, in terms of the ability of Ag-specific CD8 T cells to produce one or more cytokines, was found. The highest response was observed

among single cytokine producing $CD4^+$ and $CD8^+$ T cell subsets, followed by double producing $CD4^+$ and particularly $CD8^+$ T cells. In particular, the most frequent multiple-cytokine producing T cells were IFN-γ^+TNF-α^+ $CD8^+$ T cells. These cells were effector memory ($CCR7^-$ and $CD45RA^-$) or terminally differentiated effector memory ($CCR7^-$ and $CD45RA^+$) T cells, both phenotypes associated with the protective role of $CD8^+$ T cells in Mtb infection (40, 111, 120). Another important observation was the number of epitopes identified, in accordance with their immunogenicity and recognition by a wide variety of HLA backgrounds (121, 122).

Therefore, the role of Mtb DosR-regulon encoded peptide Ag-specific single and double functional $CD4^+$ and $CD8^+$ T cell responses in LTBI, significantly improves the understanding of the immune response to Mtb phase-dependent Ags in the control of infection, and suggests a possible role for using MtbDosR-Ag and/or peptide based diagnostic tests or vaccination approaches to TB.

Several studies have tried to correlate the frequency, the phenotype, and the effector functions of CD8 T cells in patients with disease and subjects with latent infection. Here, we report other additional recent studies aimed at identify biological indicators useful to discriminate between patients with active disease, subjects with latent infection and patients that recovery after successful therapy.

Niendak et al. have observed that specific $CD8^+$ T cell response decreased by 58.4% at 24 weeks, with the majority of the decrease (38.7%) noted at 8 weeks in subjects receiving successful anti-TB treatment (123); decrease of the $CD8^+$ T cell response was relatively unaffected by malnutrition, supporting the hypothesis that the frequency of Mtb-specific $CD8^+$ T cells declines with anti-tuberculosis therapy potentially as consequence of decreasing intracellular mycobacterial Ags, and may prove to be a surrogate marker of response to therapy (34, 124). The authors postulate that each individual has a CD8 "set point," which reflects the complex interplay of antigenic exposure, in conjunction with host factors such as the HLA background. Nonetheless, these findings are concordant with the observation that removal of Ag results in decreasing T cell frequencies, and help to explain the observed reduction in $CD8^+$ T cell frequency following anti-tuberculosis therapy.

Another recent study of Harari et al. (92) highlighted phenotypic and functional properties of Mtb-specific CD8 T cell responses in 326 TB patients and LTBI subjects in order to correlate their presence with different clinical form of Mtb infection (74). Authors found a higher frequency of Mtb-specific CD8 T cell responses in TB patients, which was correlated with the presence of higher Ag load (74, 92). These results were confirmed by two different studies, the first performed in children with active disease, where Mtb-specific CD8 T cells were detected in active TB disease but not in healthy children recently exposed to Mtb (92), and the second that demonstrated the presence of higher number of granulomas in TB patients as compared with those in LTBI subjects (74). Moreover, major phenotypic and functional differences were observed between TB and LTBI subjects, as Mtb-specific $CD8^+$ T cells were mostly represented by terminally differentiated effector memory cells (T_{EMRA}) in LTBI and of T_{EM} cells in TB patients. These results also suggests that T_{EMRA} and T_{EM} cell subsets, are involved in the control of Mtb infection, as already demonstrated in chronic controlled and uncontrolled virus infection, respectively (74, 125).

The authors did not find any statistically significant difference in the cytokines profile of Mtb-specific $CD8^+$ T cell responses between LTBI subjects and TB patients, while they found that Mtb-specific $CD8^+$ T cells were more polyfunctional (i.e., IFN-γ^+TNF-α^+IL-2^+) in LTBI subjects, according to the role that these cells play in anti-viral immunity (74, 125). Instead, it was found that Mtb-specific $CD8^+$ T cells have a higher frequency as single TNF-α-producer cells in TB patients, as occurred for $CD4^+$ T cells (125). Further analysis of the functional properties of these Mtb-specific $CD8^+$ T cells, permitted to detect significant high levels of GrB and GrA, but low level of perforin, suggesting a mechanism of action of Mtb-specific $CD8^+$ T cells that is independent on the expression of perforin (74).

Another intriguing aspect of that study was the finding of a higher prevalence of Mtb-specific $CD8^+$ T cell responses in pulmonary TB patients compared with extra-pulmonary TB patients and the higher magnitude of these responses in smear-positive versus smear-negative pulmonary TB patients (74). Moreover, Mtb-specific $CD8^+$ T cells from pulmonary TB patients were not able to proliferate compared to CD8 T cells from extra-pulmonary TB patients (74). These functional differences of the CD8 T cell responses, in term of cytokines release or proliferation, most likely depend on antigenic stimulation that occur at different anatomic sites, that could be correlated with high Ag burden (88, 126, 127), attributing to tropism of responding T cells (74).

In conclusion, Mtb-specific CD8 T cell response, as defined by the qualitative and the quantitative aspects above cited, could have significance in understand how the immune system fails to control the progression of TB, or how the quality of the response could facilitate early diagnosis in order to reduce TB associated morbidity and mortality and to individuate subjects that are at high risk to develop active disease (40).

ROLE OF T CELLS IN TB-HIV CO-INFECTION

HIV infection has led to an increase in the incidence of TB, and TB-HIV co-infection has determined not easy decisions in both the diagnosis and treatment. The treatment of co-infected patients requires anti-tuberculosis and antiretroviral drugs to be administered together. The therapeutic treatment leads to different results, according to patient compliance, drug toxic effects, and, finally to a syndrome that appears following the initiation of antiretroviral therapy (ART) named immune reconstitution inflammatory syndrome (IRIS).

Several studies have provided to clarify the relationship that exists between HIV and Mtb pathogens and how they interact both *in vitro* and *in vivo*, highlighting how HIV infection could increase the risk of TB and how Mtb infection may accelerate the evolution of HIV infection. Flynn et al., very recently, have summarized the results obtained from different studies, discerning the several hypotheses on the role of the immune system in the co-infection (128).

It is well known that TB-HIV co-infection is destructive (129–131), but nowadays the mechanisms involved in the impairment

of the immune system, guiding to the morbidity and mortality of co-infected subjects, remain to be elucidated (132). In countries with low rates of TB and, of course, with high-burden TB, the identification of LTBI within individuals co-infected with HIV is important due to the high risk to develop active TB. One of the control strategy adopted by the WHO is the use of preventive therapy of LTBI with isoniazid (INH) treatment (133). HIV-infected individuals are at high risk to develop active TB for the progressive CD4 depletion in the first few years after infection, even if the number of peripheral CD4 T cells is still high at the beginning (134–136). Although, the ART could restore absolute CD4 T cell numbers, it does not reduce the risk of TB progression in HIV patients (137). Conversely, TB infection has a negative impact on clinical progression of HIV infection (138).

Studies of human disease have characterized functional defects in CD4 T cells in TB-HIV co-infection by the analysis of cytokine production (e.g., IFN-γ) by CD4 cells in response to Mtb Ags (139–142) and by the analysis of phenotype distribution of CD4 T cells in lymphoid tissue, peripheral blood, and at the sites of disease (139, 143, 144). The correlation of different phenotypes of Ag-specific-CD4 T cells, and their role on the protection or susceptibility to infection, has been clearly demonstrated by the emerging characterization of polyfunctional CD4 T cells in TB-HIV co-infection. In the peripheral blood of TB-HIV-infected people, CD4 T cells are less able to secrete more than one cytokine when the viral load is high (145). Kalsdorf et al. have demonstrated that polyfunctional T cells specific for mycobacterial Ags are reduced in BAL from latent TB-HIV-infected subjects with no symptoms of active TB. The impairment of mycobacterial specific T cells could contribute to develop active TB, suggesting that HIV infection affects the frequency of Ag-specific polyfunctional T cells in the BAL of people with latent TB-HIV (140). Therefore, several studies have tried to correlate the presence of these cells in blood or in fluids recovered at the site of infection, highlighting how their presence can be reduced or increased, in term of absolute number. In fact, some authors have found a reduction of polyfunctional CD4 T cells in the peripheral blood of HIV-infected infants, in response to restimulation with BCG, compared with HIV-uninfected infants, or in BAL samples from HIV-infected subjects compared with HIV-uninfected healthy subjects, and finally, an increase in pericardial fluid of TB-HIV patients, with a terminally effector phenotype (143). Matthews et al. have found a lower proportions of Ag-specific polyfunctional T cells, with the less mature phenotype of CD4 T memory, at the site of disease of both HIV-infected and uninfected TB patients, supporting the hypothesis that their presence could correlate with Ag load and disease status, instead than with protection (143). Finally, understanding how the immune system contributes to TB-HIV co-infection could provide the basis for the discovery and development of new drugs and vaccines that can prevent or cure TB in co-infected people. At the moment, an early ART treatment still represents the gold standard in the control of TB-HIV co-infection.

CONCLUDING REMARKS

Tuberculosis research in the field of vaccine and diagnostic tests development suffers from lack of rigorous correlates of protection in order to better understand the basic mechanisms underlying pathophysiology. Therefore, the identification of biosignatures that predict risk of disease, but also vaccine efficacy would be important.

Studies of human T cell responses, using different protocols of *in vitro* stimulation, have made possible to delineate some functional signatures indicative of the immunological status of each studied individual (40).

From the above cited studies, it has clearly emerged that, for TB diagnosis it is necessary to investigate on several biomarkers. The different expression levels of several cytokines, evaluated *ex vivo* in cells obtained from blood samples, comparing uninfected subjects, LTBI individuals, and patients with active disease, led to not unique results. This issue, therefore, requires further investigation by different analytical platforms. In particular, we believe that TB biomarkers research may continue to generate signatures with clinical applicability and additionally provides novel hypotheses related to disease pathophysiology (146).

Finally, the identification of such functional T cell signatures could help to better make diagnosis of different stages of TB, including also the cases of risk of reactivation and/or progression to active disease such as occurs in HIV patients (146).

ACKNOWLEDGMENTS

This work was supported by grants from the European Commission within the 7th Framework Program, NEWTBVAC contract no. HEALTH-F3-2009-241745. The text represents the authors' views and does not necessarily represent a position of the Commission who will not be liable for the use made of such information.

REFERENCES

1. WHO. *Global Tuberculosis Report 2011*. Geneva: WHO Press (2011). Available from: http://whqlibdoc.who.int/publications/2011/9789241564380_eng.pdf
2. Raviglione M, Marais B, Floyd K, Lönnroth K, Getahun H, Migliori GB, et al. Scaling up interventions to achieve global tuberculosis control: progress and new developments. *Lancet* (2012) **379**:1902–13. doi:10.1016/S0140-6736(12)60727-2
3. Whitworth HS, Scott M, Connell DW, Dongés B, Lalvani A. IGRAs – the gateway to T cell based TB diagnosis. *Methods* (2013) **15**:52–62. doi:10.1016/j.ymeth.2012.12.012
4. McNerney R, Maeurer M, Abubakar I, Marais B, McHugh TD, Ford N, et al. Tuberculosis diagnostics and biomarkers: needs, challenges, recent advances, and opportunities. *J Infect Dis* (2012) **15**:S147–58. doi:10.1093/infdis/jir860
5. Zumla A, Atun R, Maeurer M, Mwaba P, Ma Z, O'Grady J, et al. Viewpoint: scientific dogmas, paradoxes and mysteries of latent *Mycobacterium tuberculosis* infection. *Trop Med Int Health* (2011) **16**:79–83. doi:10.1111/j.1365-3156.2010.02665.x
6. Lillebaek T, Andersen AB, Dirksen A, Smith E, Skovgaard LT, Kok-Jensen A. Persistent high incidence of tuberculosis in immigrants in a low-incidence country. *Emerg Infect Dis* (2002) **8**:679–84. doi:10.3201/eid0807.010482
7. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* (2001) **345**:1098–104. doi:10.1056/NEJMoa011110
8. National Collaborating Centre for Chronic Conditions (UK), Centre for Clinical Practice at NICE (UK). *Tuberculosis: Clinical Diagnosis and Management of Tuberculosis, and Measures for Its Prevention and Control*. London: National Institute for Health and Clinical Excellence (2011). Available from: http://www.nice.org.uk/nicemedia/live/13422/53642/53642.pdf
9. Broekmans JF, Migliori GB, Rieder HL, Lees J, Ruutu P, Loddenkemper R, et al. European framework for tuberculosis control and elimination in countries with a low incidence. Recommendations of the World Health Organization (WHO), International Union Against Tuberculosis and Lung Disease

(IUATLD) and Royal Netherlands Tuberculosis Association (KNCV) Working Group. *Eur Respir J* (2002) **19**:765–75.
10. Pareek M, Abubakar I, White PJ, Garnett GP, Lalvani A. Tuberculosis screening of migrants to low-burden nations: insights from evaluation of UK practice. *Eur Respir J* (2011) **37**:1175–82. doi:10.1183/09031936.00105810
11. Abubakar I, Lipman M, Anderson C, Davies P, Zumla A. Tuberculosis in the UK-time to regain control. *BMJ* (2011) **343**:d4281. doi:10.1136/bmj.d4281
12. Parida SK, Kaufmann SH. The quest for biomarkers in tuberculosis. *Drug Discov Today* (2010) **15**:148–57. doi:10.1016/j.drudis.2009.10.005
13. Mittrücker HW, Steinhoff U, Köhler A, Krause M, Lazar D, Mex P, et al. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proc Natl Acad Sci U S A* (2007) **104**:12434–9. doi:10.1073/pnas.0703510104
14. Kaufmann SH. Tuberculosis vaccines: time to think about the next generation. *Semin Immunol* (2013) **25**:172–81. doi:10.1016/j.smim.2013.04.006
15. Perreau M, Rozot V, Welles HC, Belluti-Enders F, Vigano S, Maillard M, et al. Lack of *Mycobacterium tuberculosis*-specific interleukin-17A-producing CD4+ T cells inactive disease. *Eur J Immunol* (2013) **43**:939–48. doi:10.1002/eji.201243090
16. Ottenhoff TH. New pathways of protective and pathological host defense to mycobacteria. *Trends Microbiol* (2012) **20**:419–28. doi:10.1016/j.tim.2012.06.002
17. Jurado JO, Pasquinelli V, Alvarez IB, Peña D, Rovetta AI, Tateosian NL, et al. IL-17 and IFN-γ expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease. *J Leukoc Biol* (2012) **91**:991–1002. doi:10.1189/jlb.1211619
18. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* (2009) **27**:669–92. doi:10.1146/annurev.immunol.021908.132557
19. Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol* (2008) **26**:389–420. doi:10.1146/annurev.immunol.26.021607.090404
20. Nathan C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* (2006) **6**:173–82. doi:10.1038/nri1785
21. Dorhoi A, Reece ST, Kaufmann SHE. Fundamental immunology. 7th ed. In: Paul WE editor. *Immunity to Intracellular Bacteria*. Philadelphia: Wolters KluwerHealth, Lippincott Williams & Wilkins (2012). p. 973–1000.
22. Harding CV, Boom WH. Regulation of antigen presentation by *Mycobacterium tuberculosis*: a role for Toll-like receptors. *Nat Rev Microbiol* (2010) **8**:296–307. doi:10.1038/nrmicro2321
23. Kaufmann SHE. Fundamental immunology. 5th ed. In: Paul WE editor. *Immunityto Intracellular Bacteria*. Philadelphia, NY: Lippincott-Raven (2003). p. 1229–61.
24. Cooper AM. Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol* (2009) **27**:393–422. doi:10.1146/annurev.immunol.021908.132703
25. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* (1998) **282**:121–5. doi:10.1126/science.282.5386.121
26. Van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, et al. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* (2007) **129**:1287–98. doi:10.1016/j.cell.2007.05.059
27. Schaible UE, Winau F, Sieling PA, Fischer K, Collins HL, Hagens K, et al. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat Med* (2003) **9**:1039–46. doi:10.1038/nm906
28. Winau F, Weber S, Sad S, de Diego J, Hoops SL, Breiden B, et al. Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity* (2006) **24**:105–17. doi:10.1016/j.immuni.2005.12.001
29. Lin MY, Ottenhoff TH. Host-pathogen interactions in latent *Mycobacterium tuberculosis* infection: identification of new targets for tuberculosis intervention. *Endocr Metab Immune Disord Drug Targets* (2008) **8**:15–29. doi:10.2174/187153008783928398
30. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* (1989) **7**:145–73. doi:10.1146/annurev.iy.07.040189.001045
31. Romagnani S. "Immunology. 10th ed. In: Kaufmann SHE, Steward MW editors. *Cytokines*. London: Hodder Arnold/ASM Press (2005). p. 273–99.
32. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* (2008) **26**:677–704. doi:10.1146/annurev.immunol.26.021607.090331
33. De Libero G, Mori L. Recognition of lipid antigens by T cells. *Nat Rev Immunol* (2005) **5**:485–96. doi:10.1038/nri1631
34. Gold MC, Cerri S, Smyk-Pearson S, Cansler ME, Vogt TM, Delepine J, et al. Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol* (2010) **8**:e1000407. doi:10.1371/journal.pbio.1000407
35. Scotet E, Nedellec S, Devilder MC, Allain S, Bonneville M. Bridging innate and adaptive immunity through gammadelta T-dendritic cell crosstalk. *Front Biosci* (2008) **13**:6872–85. doi:10.2741/3195
36. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* (2007) **13**:843–50. doi:10.1038/nm1592
37. Derrick SC, Yabe IM, Yang A, Morris SL. Vaccine-induced anti-tuberculosis protective immunity in mice correlates with the magnitude and quality of multifunctional CD4 T cells. *Vaccine* (2011) **29**:2902–9. doi:10.1016/j.vaccine.2011.02.010
38. Joosten SA, Ottenhoff TH. Human CD4 and CD8 regulatory T cells in infectious diseases and vaccination. *Hum Immunol* (2008) **69**:760–70. doi:10.1016/j.humimm.2008.07.017
39. Urdahl KB, Shafiani S, Ernst JD. Initiation and regulation of T-cell responses in tuberculosis. *Mucosal Immunol* (2011) **4**:288–93. doi:10.1038/mi.2011.10
40. Caccamo N, Dieli F. *Are Polyfunctional Cells Protective in M. tuberculosis Infection? Understanding Tuberculosis – Analyzing the Origin of Mycobacterium tuberculosis Pathogenicity*. Available from: http://www.intechopen.com/books/understanding-tuberculosis-analyzing-the-origin-of-mycobacterium-tuberculosis-pathogenicity/are-polyfunctional-cells-protective-in-m-tuberculosis-infection-
41. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* (1999) **401**:708–12. doi:10.1038/44385
42. Lanzavecchia A, Sallusto F. Understanding the generation and function of memory T cell subsets. *Curr Opin Immunol* (2005) **17**:326–32. doi:10.1016/j.coi.2005.04.010
43. Zanetti M, Franchini G. T cell memory and protective immunity by vaccination: is more better? *Trends Immunol* (2006) **27**:511–7. doi:10.1016/j.it.2006.09.004
44. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: human memory T-cell subsets. *Eur J Immunol* (2013) **43**:2797–809. doi:10.1002/eji.201343751
45. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science* (1996) **272**:60–6. doi:10.1126/science.272.5258.60
46. Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol* (2000) **18**:593–620. doi:10.1146/annurev.immunol.18.1.593
47. Appay V, van Lier RA, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* (2008) **73**:975–83. doi:10.1002/cyto.a.20643
48. Picker LJ, Treer JR, Ferguson-Darnell B, Collins PA, Buck D, Terstappen LW. Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selectin on T cells during the virgin to memory cell transition. *J Immunol* (1993) **150**:1105–21.
49. Picker LJ, Treer JR, Ferguson-Darnell B, Collins PA, Bergstresser PR, Terstappen LW. Control of lymphocyte recirculation in man. II. Differential regulation of the cutaneous lymphocyte-associated antigen, a tissue-selective homing receptor for skin-homing T cells. *J Immunol* (1993) **150**:1122–36.
50. Picker LJ, Singh MK, Zdraveski Z, Treer JR, Waldrop SL, Bergstresser PR, et al. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* (1995) **86**:1408–19.
51. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A human memory T cell subset with stem cell-like properties. *Nat Med* (2011) **17**:1290–7. doi:10.1038/nm.2446
52. Lugli E, Dominguez MH, Gattinoni L, Chattopadhyay PK, Bolton DL, Song K, et al. Superior T memory stem cell persistence supports long-lived T cell memory. *J Clin Invest* (2013) **123**:594–9. doi:10.1172/JCI66327

53. Fagnoni FF, Vescovini R, Passeri G, Bologna G, Pedrazzoni M, Lavagetto G, et al. Shortage of circulating naive CD8(+) T cells provides new insights on immunodeficiency in aging. *Blood* (2000) **95**:2860–8.
54. Lugli E, Pinti M, Nasi M, Troiano L, Ferraresi R, Mussi C, et al. Subject classification obtained by cluster analysis and principal component analysis applied to flow cytometric data. *Cytometry A* (2007) **71**:334–44. doi:10.1002/cyto.a.20387
55. Fritsch RD, Shen X, Sims GP, Hathcock KS, Hodes RJ, Lipsky PE. Stepwise differentiation of CD4 memory T cells defined by expression of CCR7 and CD27. *J Immunol* (2005) **175**:6489–97.
56. Okada R, Kondo T, Matsuki F, Takata H, Takiguchi M. Phenotypic classification of human $CD4^+$ T cell subsets and their differentiation. *Int Immunol* (2008) **20**:1189–99. doi:10.1093/intimm/dxn075
57. Picker LJ, Reed-Inderbitzin EF, Hagen SI, Edgar JB, Hansen SG, Legasse A, et al. IL-15 induces CD4 effector memory T cell production and tissue emigration in nonhuman primates. *J Clin Invest* (2006) **116**:1514–24. doi:10.1172/JCI27564
58. Lugli E, Goldman CK, Perera LP, Smedley J, Pung R, Yovandich JL, et al. Transient and persistent effects of IL-15 on lymphocyte homeostasis in nonhuman primates. *Blood* (2010) **116**:3238–48. doi:10.1182/blood-2010-03-275438
59. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* (2008) **8**:247–58. doi:10.1038/nri2274
60. Bird JJ, Brown DR, Mullen AC, Moskowitz NH, Mahowald MA, Sider JR, et al. Helper T cell differentiation is controlled by the cell cycle. *Immunity* (1998) **9**:229–37. doi:10.1016/S1074-7613(00)80605-6
61. Romero P, Zippelius A, Kurth I, Pittet MJ, Touvrey C, Iancu EM, et al. Four functionally distinct populations of human effector-memory $CD8^+$ T lymphocytes. *J Immunol* (2007) **178**:4112–9.
62. Takata H, Takiguchi M. Three memory subsets of human $CD8^+$ T cells differently expressing three cytolytic effector molecules. *J Immunol* (2006) **177**:4330–40.
63. Chattopadhyay PK, Betts MR, Price DA, Gostick E, Horton H, Roederer M, et al. The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. *J Leukoc Biol* (2009) **85**:88–97. doi:10.1189/jlb.0208107
64. Tomiyama H, Takata H, Matsuda T, Takiguchi M. Phenotypic classification of human $CD8^+$ T cells reflecting their function: inverse correlation between quantitative expression of CD27 and cytotoxic effector function. *Eur J Immunol* (2004) **34**:999–1010. doi:10.1002/eji.200324478
65. Harari A, Petitpierre S, Vallelian F, Pantaleo G. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood* (2004) **103**:966–72. doi:10.1182/blood-2003-04-1203
66. Younes SA, Yassine-Diab B, Dumont AR, Boulassel MR, Grossman Z, Routy JP, et al. HIV-1 Viremia prevents the establishment of interleukin 2-producing HIV-specific memory $CD4^+$ T cells endowed with proliferative capacity. *J Exp Med* (2003) **198**:1909–22. doi:10.1084/jem.20031598
67. Semmo N, Day CL, Ward SM, Lucas M, Harcourt G, Loughry A, et al. Preferential loss of IL-2-secreting $CD4^+$ T helper cells in chronic HCV infection. *Hepatology* (2005) **41**:1019–28. doi:10.1002/hep.20669
68. Zielinski CE, Corti D, Mele F, Pinto D, Lanzavecchia A, Sallusto F. Dissecting the human immunologic memory for pathogens. *Immunol Rev* (2011) **240**:40–51. doi:10.1111/j.1600-065X.2010.01000.x
69. Millington KA, Innes JA, Hackforth S, Hinks TS, Deeks JJ, Dosanjh DP, et al. Dynamic relationship between IFN-gamma and IL-2 profile of *Mycobacterium tuberculosis*-specific T cells and antigen load. *J Immunol* (2007) **178**:5217–26.
70. Unutmaz D, Pileri P, Abrignani S. Antigen-independent activation of naive and memory resting T cells by a cytokine combination. *J Exp Med* (1994) **180**:1159–64. doi:10.1084/jem.180.3.1159
71. Geginat J, Lanzavecchia A, Sallusto F. Proliferation and differentiation potential of human $CD8^+$ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* (2003) **101**:4260–6. doi:10.1182/blood-2002-11-3577
72. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med* (2001) **194**:1711–9. doi:10.1084/jem.194.12.1711
73. Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. *Nat Rev Immunol* (2011) **11**:343–54. doi:10.1038/nri2960
74. Rozot V, Vigano S, Mazza-Stalder J, Idrizi E, Day CL, Perreau M, et al. *Mycobacterium tuberculosis*-specific CD8+ T cells are functionally and phenotypically different between latent infection and active disease. *Eur J Immunol* (2013) **43**:1568–77. doi:10.1002/eji.201243262
75. Marín ND, París SC, Rojas M, García LF. Functional profile of CD4+ and CD8+ T cells in latently infected individuals and patients with active TB. *Tuberculosis (Edinb)* (2013) **93**:155–66. doi:10.1016/j.tube.2012.12.002
76. Flynn JL, Chan J. Tuberculosis: latency and reactivation. *Infect Immun* (2001) **69**:4195–201. doi:10.1128/IAI.69.7.4195-4201.2001
77. Almeida JR, Price DA, Papagno L, Arkoub ZA, Sauce D, Bornstein E, et al. Superior control of HIV-1 replication by $CD8^+$ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* (2007) **204**:2473–85. doi:10.1084/jem.20070784
78. Scriba TJ, Tameris M, Mansoor N, Smit E, van der Merwe L, Isaacs F, et al. Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional $CD4^+$ T cells. *Eur J Immunol* (2010) **40**:279–90. doi:10.1002/eji.200939754
79. Abel B, Tameris M, Mansoor N, Gelderbloem S, Hughes J, Abrahams D, et al. The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional $CD4^+$ and $CD8^+$ T cells in adults. *Am J Respir Crit Care Med* (2010) **181**:1407–17. doi:10.1164/rccm.200910-1484OC
80. Soares AP, Scriba TJ, Joseph S, Harbacheuski R, Murray RA, Gelderbloem SJ, et al. Bacillus Calmette-Guérin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. *J Immunol* (2008) **180**:3569–77.
81. Beveridge NE, Price DA, Casazza JP, Pathan AA, Sander CR, Asher TE, et al. Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional *Mycobacterium tuberculosis*-specific CD4+ memory T lymphocyte populations. *Eur J Immunol* (2007) **37**:3089–100. doi:10.1002/eji.200737504
82. Petruccioli E, Petrone L, Vanini V, Sampaolesi A, Gualano G, Girardi E, et al. γ/TNFα specific-cells and effector memory phenotype associate with active tuberculosis. *J Infect* (2013) **66**:475–86. doi:10.1016/j.jinf.2013.02.004
83. Ciuffreda D, Comte D, Cavassini M, Giostra E, Bühler L, Perruchoud M, et al. Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication. *Eur J Immunol* (2008) **38**:2665–77. doi:10.1002/eji.200838336
84. Chiacchio T, Petruccioli E, Vanini V, Butera O, Cuzzi G, Petrone L, et al. Higher frequency of T-cell response to *M. tuberculosis* latency antigen Rv2628 at the site of active tuberculosis disease than in peripheral blood. *PLoS One* (2011) **6**:e27539. doi:10.1371/journal.pone.0027539
85. El Fenniri L, Toossi Z, Aung H, El Iraki G, Bourkkadi J, Benamor J, et al. Polyfunctional *Mycobacterium tuberculosis*-specific effector memory $CD4^+$ T cells at sites of pleural TB. *Tuberculosis (Edinb)* (2011) **91**:224–30. doi:10.1016/j.tube.2010.12.005
86. Sargentini V, Mariotti S, Carrara S, Gagliardi MC, Teloni R, Goletti D, et al. Cytometric detection of antigen-specific IFN-gamma/IL-2 secreting cells in the diagnosis of tuberculosis. *BMC Infect Dis* (2009) **9**:99. doi:10.1186/1471-2334-9-99
87. Caccamo N, Guggino G, Meraviglia S, Gelsomino G, Di Carlo P, Titone L, et al. Analysis of *Mycobacterium tuberculosis*-specific CD8 T-cells in patients with active tuberculosis and in individuals with latent infection. *PLoS One* (2009) **4**:e5528. doi:10.1371/journal.pone.0005528
88. Sester U, Fousse M, Dirks J, Mack U, Prasse A, Singh M, et al. Whole-blood flow-cytometric analysis of antigen-specific CD4 T-cell cytokine profiles distinguishes active tuberculosis from non-active states. *PLoS One* (2011) **6**:e17813. doi:10.1371/journal.pone.0017813
89. Young JM, Adetifa IM, Ota MO, Sutherland JS. Expanded polyfunctional T cell response to mycobacterial antigens in TB disease and contraction post-treatment. *PLoS One* (2010) **5**:e11237. doi:10.1371/journal.pone.0011237
90. Sutherland JS, Adetifa IM, Hill PC, Adegbola RA, Ota MO. Pattern and diversity of cytokine production differentiates between *Mycobacterium tuberculosis* infection and disease. *Eur J Immunol* (2009) **39**:723–9. doi:10.1002/eji.200838693
91. Caccamo N, Guggino G, Joosten SA, Gelsomino G, Di Carlo P, Titone L, et al. Multifunctional CD4(+) T cells correlate with active *Mycobacterium tuberculosis* infection. *Eur J Immunol* (2010) **40**:2211–20. doi:10.1002/eji.201040455

92. Harari A, Rozot V, Enders FB, Perreau M, Stalder JM, Nicod LP, et al. Dominant TNF-α^+ *Mycobacterium tuberculosis*-specific $CD4^+$ T cell responses discriminate between latent infection and active disease. *Nat Med* (2011) **17**:372–6. doi:10.1038/nm.2299
93. Day CL, Abrahams DA, Lerumo L, Janse van Rensburg E, Stone L, O'Rie T, et al. Functional capacity of *Mycobacterium tuberculosis*-specific T cell responses in humans is associated with mycobacterial load. *J Immunol* (2011) **187**:2222–32. doi:10.4049/jimmunol.1101122
94. Mueller H, Detjen AK, Schuck SD, Gutschmidt A, Wahn U, Magdorf K, et al. *Mycobacterium tuberculosis*-specific $CD4^+$, IFNgamma$^+$, and TNFalpha$^+$ multifunctional memory T cells coexpress GM-CSF. *Cytokine* (2008) **43**:143–8. doi:10.1016/j.cyto.2008.05.002
95. Casey R, Blumenkrantz D, Millington K, Montamat-Sicotte D, Kon OM, Wickremasinghe M, et al. Enumeration of functional T-cell subsets by fluorescence-immunospot defines signatures of pathogen burden in tuberculosis. *PLoS One* (2010) **5**:e15619. doi:10.1371/journal.pone.0015619
96. Wang X, Cao Z, Jiang J, Niu H, Dong M, Tong A, et al. Association of mycobacterial antigen-specific CD4($^+$) memory T cell subsets with outcome of pulmonary tuberculosis. *J Infect* (2010) **60**:133–9. doi:10.1016/j.jinf.2009.10.048
97. Schuetz A, Haule A, Reither K, Ngwenyama N, Rachow A, Meyerhans A, et al. Monitoring CD27 expression to evaluate *Mycobacterium tuberculosis* activity in HIV-1 infected individuals in vivo. *PLoS One* (2011) **6**:e27284. doi:10.1371/journal.pone.0027284
98. Pollock KM, Whitworth HS, Montamat-Sicotte DJ, Grass L, Cooke GS, Kapembwa MS, et al. T-cell immunophenotyping distinguishes active from latent tuberculosis. *J Infect Dis* (2013) **208**:952–68. doi:10.1093/infdis/jit265
99. Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Andersen P, et al. Human cytolytic and interferon gamma-secreting $CD8^+$ T lymphocytes specific for *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* (1998) **95**:270–5. doi:10.1073/pnas.95.1.270
100. Ladel CH, Daugelat S, Kaufmann SH. Immune response to *Mycobacterium bovis* bacille Calmette Guérin infection in major histocompatibility complex class I- and II-deficient knock-out mice: contribution of CD4 and CD8 T cells to acquired resistance. *Eur J Immunol* (1995) **25**:377–84. doi:10.1002/eji.1830250211
101. Ottenhoff TH, Lewinsohn DA, Lewinsohn DM. Human CD4 and CD8 T cell responses to *Mycobacterium tuberculosis*: antigen specificity, function, implications and applications. In: *Handbook of Tuberculosis: Immunology and Cell Biology*. Weinheim: Wiley-VCH (2008). p. 119–56.
102. Brighenti S, Andersson J. Induction and regulation of $CD8^+$ cytolytic T cells in human tuberculosis and HIV infection. *Biochem Biophys Res Commun* (2010) **396**:50–7. doi:10.1016/j.bbrc.2010.02.141
103. Chen CY, Huang D, Wang RC, Shen L, Zeng G, Yao S, et al. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS Pathog* (2009) **5**:e1000392. doi:10.1371/journal.ppat.1000392
104. Mazzaccaro RJ, Stenger S, Rock KL, Porcelli SA, Brenner MB, Modlin RL, et al. Cytotoxic T lymphocytes in resistance to tuberculosis. *Adv Exp Med Biol* (1998) **452**:85–101. doi:10.1007/978-1-4615-5355-7_11
105. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature* (2011) **469**:323–35. doi:10.1038/nature09782
106. Deretic V. Autophagy in infection. *Curr Opin Cell Biol* (2010) **22**:252–62. doi:10.1016/j.ceb.2009.12.009
107. Joosten SA, van Meijgaarden KE, van Weeren PC, Kazi F, Geluk A, Savage ND, et al. *Mycobacterium tuberculosis* peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS Pathog* (2010) **6**:e1000782. doi:10.1371/journal.ppat.1000782
108. Heinzel AS, Grotzke JE, Lines RA, Lewinsohn DA, McNabb AL, Streblow DN, et al. HLA-E-dependent presentation of Mtb-derived antigen to human $CD8^+$ T cells. *J Exp Med* (2002) **196**:1473–81. doi:10.1084/jem.20020609
109. Cohen NR, Garg S, Brenner MB. Antigen presentation by CD1 lipids, T cells, and NKT cells in microbial immunity. *Adv Immunol* (2009) **102**:1–94. doi:10.1016/S0065-2776(09)01201-2
110. Bruns H, Meinken C, Schauenberg P, Härter G, Kern P, Modlin RL, et al. Anti-TNF immunotherapy reduces $CD8^+$ T cell-mediated antimicrobial activity against *Mycobacterium tuberculosis* in humans. *J Clin Invest* (2009) **119**:1167–77. doi:10.1172/JCI38482
111. Stenger S, Mazzaccaro RJ, Uyemura K, Cho S, Barnes PF, Rosat JP, et al. Differential effects of cytolytic T cell subsets on intracellular infection. *Science* (1997) **276**:1684–7. doi:10.1126/science.276.5319.1684
112. Semple PL, Watkins M, Davids V, Krensky AM, Hanekom WA, Kaplan G, et al. Induction of granulysin and perforin cytolytic mediator expression in 10-week-old infants vaccinated with BCG at birth. *Clin Dev Immunol* (2011) **2011**:438463. doi:10.1155/2011/438463
113. Rahman S, Gudetta B, Fink J, Granath A, Ashenafi S, Aseffa A, et al. Compartmentalization of immune responses in human tuberculosis: few $CD8^+$ effector T cells but elevated levels of FoxP3$^+$ regulatory t cells in the granulomatous lesions. *Am J Pathol* (2009) **174**:2211–24. doi:10.2353/ajpath.2009.080941
114. Andersson J, Samarina A, Fink J, Rahman S, Grundström S. Impaired expression of perforin and granulysin in $CD8^+$ T cells at the site of infection in human chronic pulmonary tuberculosis. *Infect Immun* (2007) **75**:5210–22. doi:10.1128/IAI.00624-07
115. Kagina BM, Abel B, Scriba TJ, Hughes EJ, Keyser A, Soares A, et al. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after Bacillus Calmette-Guérin vaccination of newborns. *Am J Respir Crit Care Med* (2010) **182**:1073–9. doi:10.1164/rccm.201003-0334OC
116. Smith SG, Lalor MK, Gorak-Stolinska P, Blitz R, Beveridge NE, Worth A, et al. *Mycobacterium tuberculosis* PPD-induced immune biomarkers measurable in vitro following BCG vaccination of UK adolescents by multiplex bead array and intracellular cytokine staining. *BMC Immunol* (2010) **11**:35. doi:10.1186/1471-2172-11-35
117. Caccamo N, Meraviglia S, La Mendola C, Guggino G, Dieli F, Salerno A. Phenotypical and functional analysis of memory and effector human CD8 T cells specific for mycobacterial antigens. *J Immunol* (2006) **177**:1780–5.
118. Leyten EM, Lin MY, Franken KL, Friggen AH, Prins C, van Meijgaarden KE, et al. Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*. *Microbes Infect* (2006) **8**:2052–60. doi:10.1016/j.micinf.2006.03.018
119. Roupie V, Romano M, Zhang L, Korf H, Lin MY, Franken KL, et al. Immunogenicity of eight dormancy regulon-encoded proteins of *Mycobacterium tuberculosis* in DNA-vaccinated and tuberculosis-infected mice. *Infect Immun* (2007) **75**:941–9. doi:10.1128/IAI.01137-06
120. Schuck SD, Mueller H, Kunitz F, Neher A, Hoffmann H, Franken KL, et al. Identification of T-cell antigens specific for latent *mycobacterium tuberculosis* infection. *PLoS One* (2009) **4**:e5590. doi:10.1371/journal.pone.0005590
121. Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proc Natl Acad Sci U S A* (1992) **89**:12013–7. doi:10.1073/pnas.89.24.12013
122. Commandeur S, Lin MY, van Meijgaarden KE, Friggen AH, Franken KL, Drijfhout JW, et al. Double-and monofunctional CD4ss$^+$ and $CD8^+$ T-cell responses to *Mycobacterium tuberculosis* DosR antigens and peptides in long-term latently infected individuals. *Eur J Immunol* (2011) **41**:2925–36. doi:10.1002/eji.201141602
123. Nyendak MR, Park B, Null MD, Baseke J, Swarbrick G, Mayanja-Kizza H, et al. Tuberculosis research unit and the tuberculosis trials consortium. *Mycobacterium tuberculosis* specific CD8($^+$) T cells rapidly decline with antituberculosis treatment. *PLoS One* (2013) **8**:e81564. doi:10.1371/journal.pone.0081564
124. Kunnath-Velayudhan S, Davidow AL, Wang HY, Molina DM, Huynh VT, Salamon H, et al. Proteome-scale antibody responses and outcome of *Mycobacterium tuberculosis* infection in nonhuman primates and in tuberculosis patients. *J Infect Dis* (2012) **206**:697–705. doi:10.1093/infdis/jis421
125. Harari A, Dutoit V, Cellerai C, Bart PA, Du Pasquier RA, Pantaleo G. Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol Rev* (2006) **211**:236–54. doi:10.1111/j.0105-2896.2006.00395.x
126. Lewinsohn DA, Heinzel AS, Gardner JM, Zhu L, Alderson MR, Lewinsohn DM. *Mycobacterium tuberculosis*-specific $CD8^+$ T cells preferentially recognize heavily infected cells. *Am J Respir Crit Care Med* (2003) **168**:1346–52. doi:10.1164/rccm.200306-837OC
127. Lancioni C, Nyendak M, Kiguli S, Zalwango S, Mori T, Mayanja-Kizza H, et al. Tuberculosis research unit. $CD8^+$ T cells provide an immunologic signature of tuberculosis in young children. *Am J Respir Crit Care Med* (2012) **185**:206–12. doi:10.1164/rccm.201107-1355OC

128. Flynn Diedrich CR, Flynn JL. HIV-1/*Mycobacterium tuberculosis* coinfection immunology: how does HIV-1 exacerbate tuberculosis? *Infect Immun* (2011) **79**:1407–17. doi:10.1128/IAI.01126-10
129. Nambuya A, Sewankambo N, Mugerwa J, Goodgame R, Lucas S. *Tuberculous lymphadenitis* associated with human immunodeficiency virus (HIV) in Uganda. *J Clin Pathol* (1988) **41**:93–6. doi:10.1136/jcp.41.1.93
130. Pitchenik AE, Burr J, Suarez M, Fertel D, Gonzalez G, Moas C. Human T-cell lymphotropic virus-III (HTLV-III) seropositivity and related disease among 71 consecutive patients in whom tuberculosis was diagnosed. A prospective study. *Am Rev Respir Dis* (1987) **135**:875–9.
131. Selwyn PA, Sckell BM, Alcabes P, Friedland GH, Klein RS, Schoenbaum EE. High risk of active tuberculosis in HIV-infected drug users with cutaneous anergy. *JAMA* (1992) **268**:504–9. doi:10.1001/jama.1992.03490040080029
132. Lawn SD, Butera ST, Shinnick TM. Tuberculosis unleashed: the impact of human immunodeficiency virus infection on the host granulomatous response to *Mycobacterium tuberculosis*. *Microbes Infect* (2002) **4**:635–46. doi:10.1016/S1286-4579(02)01582-4
133. *WHO Publications, Guidelines, Reports on HIV/AIDS* Available from: http://www.who.int/publications/en/
134. Geldmacher C, Zumla A, Hoelscher M. Interaction between HIV and *Mycobacterium tuberculosis*: HIV-1-induced CD4 T-cell depletion and the development of active tuberculosis. *Curr Opin HIV AIDS* (2012) **7**:268–75. doi:10.1097/COH.0b013e3283524e32
135. Sonnenberg P, Glynn JR, Fielding K, Murray J, Godfrey-Faussett P, Shearer S. How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African gold miners. *J Infect Dis* (2005) **191**:150–8. doi:10.1086/426827
136. Sonnenberg P, Murray J, Glynn JR, Shearer S, Kambashi B, Godfrey-Faussett P. HIV-1 and recurrence, relapse, and reinfection of tuberculosis after cure: a cohort study in South African mineworkers. *Lancet* (2001) **358**:1687–93. doi:10.1016/S0140-6736(01)06712-5
137. Lawn SD, Myer L, Edwards D, Bekker LG, Wood R. Short-term and long-term risk of tuberculosis associated with CD4 cell recovery during antiretroviral therapy in South Africa. *AIDS* (2009) **23**:1717–25. doi:10.1097/QAD.0b013e32832d3b6d
138. Kizza HM, Rodriguez B, Quinones-Mateu M, Mirza M, Aung H, Yen-Lieberman B, et al. Persistent replication of human immunodeficiency virus type 1 despite treatment of pulmonary tuberculosis in dually infected subjects. *Clin Diagn Lab Immunol* (2005) **12**:1298–304.
139. Geldmacher C, Schuetz A, Ngwenyama N, Casazza JP, Sanga E, Saathoff E, et al. Early depletion of *Mycobacterium tuberculosis* specific T helper 1 cell responses after HIV-1 infection. *J Infect Dis* (2008) **198**:1590–8. doi:10.1086/593017
140. Kalsdorf B, Scriba TJ, Wood K, Day CL, Dheda K, Dawson R, et al. HIV-1 infection impairs the bronchoalveolar T-cell response to mycobacteria. *Am J Respir Crit Care Med* (2009) **180**:1262–70. doi:10.1164/rccm.200907-1011OC
141. Mansoor N, Scriba TJ, de Kock M, Tameris M, Abel B, Keyser A, et al. HIV-1 infection in infants severely impairs the immune response induced by Bacille Calmette–Guerin vaccine. *J Infect Dis* (2009) **199**:982–90. doi:10.1086/597304
142. Rangaka MX, Diwakar L, Seldon R, van Cutsem G, Meintjes GA, Morroni C, et al. Clinical, immunological, and epidemiological importance of antituberculosis T cell responses in HIV-infected Africans. *Clin Infect Dis* (2007) **44**:1639–46. doi:10.1086/518234
143. Matthews K, Ntsekhe M, Syed F, Scriba T, Russell J, Tibazarwa K, et al. HIV-1 infection alters CD4+ memory T-cell phenotype at the site of disease in extrapulmonary tuberculosis. *Eur J Immunol* (2012) **42**:147–57. doi:10.1002/eji.201141927
144. Wilkinson KA, Seldon R, Meintjes G, Rangaka MX, Hanekom WA, Maartens G, et al. Dissection of regenerating T-cell responses against tuberculosis in HIV-infected adults sensitized by *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med* (2009) **180**:674–83. doi:10.1164/rccm.200904-0568OC
145. Day CL, Mkhwanazi N, Reddy S, Mncube Z, van der Stok M, Klenerman P, et al. Detection of polyfunctional *Mycobacterium tuberculosis*-specific T cells and association with viral load in HIV-1-infected persons. *J Infect Dis* (2008) **197**:990–9. doi:10.1086/529048
146. Weiner J, Maertzdorf J, Kaufmann SH. The dual role of biomarkers for understanding basic principles and devising novel intervention strategies in tuberculosis. *Ann N Y Acad Sci* (2013) **1283**:22–9. doi:10.1111/j.1749-6632.2012.06802.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 January 2014; accepted: 07 April 2014; published online: 22 April 2014.
*Citation: Prezzemolo T, Guggino G, La Manna MP, Di Liberto D, Dieli F and Caccamo N (2014) Functional signatures of human CD4 and CD8 T cell responses to Mycobacterium tuberculosis. Front. Immunol. **5**:180. doi: 10.3389/fimmu.2014.00180*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 02 June 2014
doi: 10.3389/fimmu.2014.00243

Epitope-specific antibody levels in tuberculosis: biomarkers of protection, disease, and response to treatment

Graham H. Bothamley*

Department of Respiratory Medicine, Homerton University Hospital, London, UK

Edited by:
Juraj Ivanyi, Kings College London, UK

Reviewed by:
Armando Acosta, Universiti Sains Malaysia, Malaysia
Utpal Sengupta, The Leprosy Mission, India

****Correspondence:***
Graham H. Bothamley, Department of Respiratory Medicine, Homerton University Hospital, Homerton Row, London E9 6SR, UK
e-mail: graham.bothamley@homerton.nhs.uk

Monoclonal antibodies restricted to *Mycobacterium tuberculosis* can measure epitope-specific antibody levels in a competition assay. Immunodominant epitopes were defined from clinical samples and related to the clinical spectrum of disease. Antibody to the immunodominant epitopes was associated with HLA-DR15. Occupational exposure showed a different response and was consistent with recognition of dormancy-related proteins and protection despite exposure to tuberculosis (TB). Studies in leprosy revealed the importance of immune deviation and the relationships between T and B cell epitopes. During treatment, antibody levels increased, epitope spreading occurred, but the affinity constants remained the same after further antigen exposure, suggesting constraints on the process of epitope selection. Epitope-specific antibody levels have a potential role as biomarkers for new vaccines which might prevent the progression of latent to active TB and as tools to measure treatment effects on subpopulations of tubercle bacilli.

Keywords: tuberculosis, epitopes, B-lymphocyte, biomarkers, antibodies, monoclonal, antibody specificity

INTRODUCTION

There are many unanswered questions in tuberculosis (TB) for which an understanding of both clinical aspects and the adaptive immune response is critical. Most research has concentrated on the processes of infection and the initial, innate immune response to *Mycobacterium tuberculosis* (Mtb). It has long been clear that BCG vaccination is excellent at preventing primary forms of TB and that the immunodeficiencies caused by HIV infection or by increasing age give rise to these same forms of TB. On the other hand, BCG does not prevent post-primary disease, particularly sputum smear-positive pulmonary tuberculosis (S + PTB). Many animal models which attempt to elucidate the nature of reactivation of latent infection merely recapitulate the same pattern of immunodeficiency found in primary disease.

Post-primary TB is characterized by an immune response to both cross-reactive antigens, as in the tuberculin response, and species-restricted antigens, such as those found in the RD1 sequence, namely esat-6 and cfp-10. Destructive caseation is an essential feature of post-primary disease and much has been made of the difference between apoptotic and necrotic cell death as the pathogenetic mechanism (1). HIV infection has shown that CD4+ T cells are essential in this process, as lung cavities become rarer as the CD4 count falls (2). Sette et al. observed that antigen concentration was important in predicting T helper responses and that antibody responses reflected both the CD8 T cell response to early antigens and the CD4+ T cell response to late and structural antigens (2).

This paper will describe the data available on antibody responses to species-restricted B cell epitopes according to clinical parameters. It will explore whether these immunological markers can discriminate among the clinical states of TB infection and disease. Underlying this discussion remain the problems of why some B cell epitopes are immunodominant, how antibody diversity becomes fixed, whether conformational epitopes are more important than linear epitopes, and the relationship between T and B cell epitopes.

THE MEASUREMENT OF EPITOPE-SPECIFIC ANTIBODY

A soluble extract from irradiated Mtb, prepared by crushing with glass beads or ultrasonic degradation, gave a better range of antigens than tuberculin (4). Mouse monoclonal antibodies (Mabs) were created by inoculation with either Mtb or its soluble extract and tested for specificity to Mtb (4). Competition with human sera was tested using labeled Mabs (5), or by exploiting the difference between mouse and human heavy chains in an ELISA (6).

LIPOPROTEIN ANTIGENS OF *MYCOBACTERIUM TUBERCULOSIS*

The importance of lipoproteins to the immune response has been demonstrated by deleting the prolipoprotein signal peptidase (IspA) (7). S + PTB is characterized by higher levels of antibody to mycobacterial antigens than are other forms of TB with smaller bacterial loads. More than 80% of patients with this form of disease recognize epitopes of the 38-kDa lipoprotein antigen (Rv0934, Antigen 5, Antigen 78, PstS1, PhoS) and epitope-specific antibody correlates well with antibody levels to the purified antigen (8–12). The extent of pulmonary disease has shown a positive association with IgG antibody to the 38-kDa antigen, levels of which were also higher in the few who died from TB (13). In S + PTB, there is a clear association with HLA-DR15, which is also associated with higher anti-38-kDa antibody levels (noting that the control population in this study was from a high incidence area and the

majority were nurses on a TB ward who were regularly exposed to Mtb but who did not develop disease) (14). One suggestion is that TB-associated HLA phenotypes have more focused epitope recognition of TB antigens than those associated with healthy controls (15). Another possibility is that this antigen preferentially provokes a pro-inflammatory (Th17) immune response (16). In this context, it should be noted that the same HLA association has been found with S + PTB in other studies in different populations (17, 18), but more importantly that this association was not found in those with smear-negative pulmonary disease (19).

Epitope-specific (TB23 Mab) and antibody levels to the purified 19-kDa antigen (Rv3763, LpqH) contributed most to improving the serological sensitivity after the 38-kDa antigen in a study of six Mabs and human sera (12). Antibody levels to the purified antigen were more important than its TB23 epitope in the diagnosis of extra-pulmonary TB (20). Analysis of human T cell epitopes showed that p61–80 peptide was important, especially in patients with lymph node TB (21, 22).

HIV–TB co-infection has demonstrated three facts about the role of CD4+ T cells in TB (2). Firstly, they prevent the reactivation of latent TB. Secondly, they prevent disseminated disease and their loss is associated with primary TB. Thirdly, they have a pathogenetic role in cavitation, as cavities and significant pulmonary infiltration become rarer as the CD4 count falls. The majority of proteins secreted by actively dividing bacteria and recognized by polyclonal sera are fibronectin-binding proteins, such as the antigen 85 complex, but this group includes the 38-kDa antigen (23). Thus, these antigens are likely good serodiagnostic agents for infectious TB but poor candidates for vaccines as their recognition occurs at the same time as cavitary lung disease.

Children with TB and those with extra-pulmonary (EP) TB did not have antibody to the 38-kDa antigen (5). Patients with TB meningitis, of whom 90% have no pulmonary focus, also did not have anti-38-kDa antibody (24), nor did other patients with primary TB (20). Studies of contacts of TB did indeed show that antibody level to one of the 38-kDa epitopes (defined by TB72) could be found at low but measurable levels in those with a statistically high probability of being infected (25). Perhaps early recognition of the 38-kDa antigen indicates a subject more likely to develop infectious S + PTB.

DORMANCY-RELATED ANTIGENS

One of the surprising findings in measuring epitope-specific antibody levels was the presence of antibody to the TB68 epitope of the 16-kDa antigen (Rv2031, 14 kDa, hsp16, hspX, Acr) in nurses on a TB ward with frequent exposure to infectious TB (26). The bacterial load was estimated to be small in such healthy individuals and, according to the Th1/Th2 hypothesis, the response in such individuals should have been of cellular rather than humoral immunity. Although this epitope was species-restricted, it was not the immunodominant epitope of the purified antigen. Antibody to the whole antigen was associated with a better prognosis, self-healed disease, and more limited pulmonary disease (13). T cell responses to the 16-kDa p21–40 and p111–130 peptides were also more likely in those with BCG vaccination or EPTB compared to S + PTB (27).

EPITOPE-SPECIFIC ANTIBODY IN LEPROSY

A series of Mabs which reacted to lipoarabinomannan (LAM) were able to define two groups, one which reacted equally with LAM derived from both Mtb and *Mycobacterium leprae*, and one series which bound predominantly to *M. leprae* (28). The structural basis for these epitopes was determined by noting the predominance of mannose capping of LAM in Mtb (29) and using knockout mice for *embA*, *embB*, and *embC* together with competitive binding to synthetic carbohydrates (20, 30, 31). Sera from TB patients showed no binding to the leprosy-specific epitope (unpublished data, using the Mabs ML02 and ML34).

However, for protein antigens, antibody to the Mtb-specific epitopes could be detected in sera from patients with leprosy, although no antibody to the leprosy-specific ML04 epitope (35-kDa antigen) was found in TB patients (32). Two explanations exist for this finding. Firstly, shared T cell epitopes between homologous proteins in the two mycobacterial species might "help" B cells, which had originally been stimulated in response to previous TB infection. Secondly, the antibody epitopes on homologous proteins of *M. leprae* might overlap the binding site of the Mtb-specific Mabs sufficiently to inhibit binding, there being no homolog of the *M. leprae* 35-kDa antigen in Mtb. Bystander stimulation of B cells seems less likely although the probability of exposure to leprosy would have been less than TB (33). Conformational B cell epitopes are flat, oblong ovals with hydrophobic amino acids at the center surrounded by a halo of charged residues (34). Thus, antibody epitope cross-reactivity is unlikely. The effect of trapping of antigen by surface immunoglobulin influences the T cell repertoire (35) and cryptic T cell epitopes may be revealed (36).

EPITOPE-SPECIFIC ANTIBODY DURING TB TREATMENT

Antibody levels are proportional to antigen levels and strong T follicular helper cell responses can often initiate bystander B cell activation hypergammaglobulinemia (3, 33). Patients with TB characteristically have hypergammaglobulinemia. An early finding in the quest for a serodiagnostic test for TB was that antibody levels rose during treatment. This meant that evaluation of tests required pre-treatment sera. In a detailed study of sera from 40 TB patients during treatment (37), antibody to LAM showed a single rise and fall in antibody titer, whereas anti-protein antibody had an early rise within the first 2 weeks of treatment followed by a fall and a second rise during the continuation phase of treatment. This would be consistent with killing of different populations of tubercle bacilli, as suggested from chemotherapy trials by Mitchison (38). The rapidly dividing population is sensitive to isoniazid and standard treatment kills 99% of bacilli in the first 2 weeks. In this study, those with isoniazid-resistant strains of Mtb failed to show the first rise in antibody titers (37).

In an acute inflammation, the immune response tends to be focused on a few immunodominant antigens and a phase of chronic inflammation is associated with epitope spreading (39). Antigen processing by B cells is thought to be important in the process of epitope spreading (36). In cancer immunotherapy, such epitope spreading is associated with a good response to treatment (40). Using vaccinia as a model of complex immune responses, Sette et al. observed that the concentration of antigen was important in predicting T helper responses and that antibody responses

reflected both the CD8 T cell response to early antigens and the CD4+ T cell response to late and structural antigens (3). The number of epitopes recognized by TB patients increased with treatment and was especially marked for epitopes other than the immunodominant determinants of the 38-kDa antigen (29, 37).

Following epitope-specific antibody levels during treatment, despite changes in antibody levels, the affinity constant for the antibody or antibodies to an individual epitope did not change (37). In HIV and influenza responses, there appears to be a convergence of epitope recognition, but "deep" sequencing has suggested that this is accompanied by a divergence in the aminoacid sequences forming the antibody binding site (41). This would suggest that affinity should continue to improve and the absence of such a change during TB treatment is therefore surprising and requires further investigation.

Can the changes in epitope-specific antibody levels predict cure or relapse? Preliminary evidence suggests that antibody to the dormancy antigen α-crystallin (TB68 epitope of the 16-kDa antigen) might be helpful in predicting relapse during treatment, but as antibody levels persisted beyond successful treatment, a biomarker of cure still eludes us (37).

CONCLUSION

Antigen recognition varies across the TB spectrum. Antigen concentration is likely responsible for the immunodominance of epitopes of secreted proteins in S + PTB. There may be a role for measuring antibody/T cell responses to dormancy antigens and some lipoproteins as predictors of disease and biomarkers of protection and response to treatment.

ACKNOWLEDGMENTS

The studies referenced in this review could not have been undertaken without the monoclonal antibodies created by Juraj Ivanyi's group at Wellcome Ltd. and donated to the UK Medical Research Council.

REFERENCES

1. Davis JM, Ramakrishnan L. The role of granulomas in expansion and dissemination or early tuberculous infection. *Cell* (2009) **136**:37–49. doi:10.1016/j.cell.2008.11.014
2. Jones BE, Young SMM, Antonisus D, Davidson PT, Kramer F, Barnes PF. Relationship of the manifestations of tuberculosis to CD4 counts in patients with human immunodeficiency virus infection. *Am Rev Respir Dis* (1993) **148**:1292–7. doi:10.1164/ajrccm/148.5.1292
3. Sette A, Grey H, Oseroff C, Peters B, Moutaftsi M, Crotty S, et al. Definition of epitopes and antigens recognized by vaccinia specific immune responses: their conservation in variola virus sequences, and use as a model system to study complex pathogens. *Vaccine* (2009) **27**(Suppl 6):G21–6. doi:10.1016/j.vaccine.2009.10.011
4. Ivanyi J, Morris JA, Keen M. Studies with monoclonal antibodies. In: Macario AJL, Conway de Macario E, editors. *Monoclonal Antibodies Against Bacteria*. (Vol. 1). New York: Acad Press Inc. (1985). p. 59–90.
5. Bothamley G, Udani P, Rudd R, Festenstein F, Ivanyi J. Humoral response to defined epitopes of tubercle bacilli in adult pulmonary and child tuberculosis. *Eur J Clin Microbiol Infect Dis* (1988) **7**:639–45. doi:10.1007/BF01964242
6. Wilkins E, Bothamley G, Jackett P. A rapid simple enzyme immunoassay for detection of antibody to individual epitopes in the serodiagnosis of tuberculosis. *Eur J Clin Microbiol Infect Dis* (1991) **10**:559–63. doi:10.1007/BF01967273
7. Seshradi C, Turner MT, Lewisohn DM, Moody DB, van Rhijn I. Lipoproteins are major targets of the polyclonal human T cell response to *Mycobacterium tuberculosis*. *J Immunol* (2013) **190**(1):278–84. doi:10.4049/jimmunol.1201667
8. Benjamin RG, Daniel TM. Serodiagnosis of tuberculosis using the enzyme-linked immunosorbent assay (ELISA) of antibody to *Mycobacterium tuberculosis* antigen 5. *Am Rev Respir Dis* (1982) **126**:1013–6.
9. Uma Devi KR, Ramalingam B, Brennan PJ, Narayanan PR, Raja A. Specific and early detection of IgG, IgA and IgM antibodies to *Mycobacterium tuberculosis* 38 kDa antigen in pulmonary tuberculosis. *Tuberculosis* (2001) **81**:249–53. doi:10.1054/tube.2001.0293
10. Kadival GV, Chaparas SD, Hussong D. Characterization of serologic and cell-mediated reactivity of a 38-kDa antigen isolated from *Mycobacterium tuberculosis*. *J Immunol* (1987) **139**:2447–51.
11. Espitia C, Cervera I, Gonzalez R, Manchilla R. A 38-kD *Mycobacterium tuberculosis* antigen associated with infection. Its isolation and serologic evaluation. *Clin Exp Immunol* (1989) **77**:373–7.
12. Jackett PS, Bothamley GH, Batra HV, Mistry A, Young DB, Ivanyi J. Specificity of antibodies to immunodominant mycobacterial antigens in tuberculosis. *J Clin Microbiol* (1988) **26**:2313–8.
13. Bothamley GH, Rudd R, Festenstein F, Ivanyi J. Clinical value of the measurement of *Mycobacterium tuberculosis*-specific antibody in pulmonary tuberculosis. *Thorax* (1992) **47**:270–5. doi:10.1136/thx.47.4.270
14. Bothamley GH, Beck JS, Schreuder GMTH, D'Amaro J, de Vries RRP, Kardjito T, et al. Association of tuberculosis and *M. tuberculosis*-specific antibody levels with HLA. *J Infect Dis* (1989) **159**:549–55. doi:10.1093/infdis/159.3.549
15. Contini S, Pallante M, Vejbaesya S, Park MH, Chierakul N, Kim HS, et al. A model of phenotypic susceptibility to tuberculosis: deficient in silico selection of *Mycobacterium tuberculosis* epitopes by HLA alleles. *Sarcoidosis Vasc Diffuse Lung Dis* (2008) **25**:21–8.
16. Palma C, Spallek R, Piccaro G, Pardini M, Jonas F, Oehlmann W, et al. The *M. tuberculosis* phosphate-binding lipoproteins PstS1 and PstS3 induce Th1 and Th17 responses that are not associated with protection against *M. tuberculosis* infection. *Clin Dev Immunol* (2011) **2011**:690238. doi:10.1155/2011/690328
17. Khomenko AG, Litivinov VI, Chukanova VP, Pospelov LE. Tuberculosis in patients with various HLA phenotypes. *Tubercle* (1990) **71**:187–92. doi:10.1016/0041-3879(90)90074-I
18. Meyer CG, May J, Stark K. Human leukocyte antigens in tuberculosis and leprosy. *Trends Microbiol* (1998) **6**:148–54. doi:10.1016/S0966-842X(98)01240-2
19. Brahmajothi V, Pitchappan RM, Kakkanaiah VN, Sashidhar M, Rajaram K, Ramu S, et al. Association of pulmonary tuberculosis and HLA in south India. *Tubercle* (1991) **72**:123–32. doi:10.1016/0041-3879(91)90039-U
20. Bothamley G, Batra J, Ramesh V, Chandramuki A, Ivanyi J. Serodiagnostic value of the 19 kilodalton antigen of *Mycobacterium tuberculosis* in Indian patients. *Eur J Clin Microbiol Infect Dis* (1992) **11**:912–5. doi:10.1007/BF01962372
21. Faith A, Moreno C, Lathigra R, Roman E, Fernandez M, Brett S, et al. Analysis of human T-cell epitopes in the 19,000 MW antigen of *Mycobacterium tuberculosis*: influence of HLA-DR. *Immunology* (1991) **74**:1–7.
22. Harris DP, Vordermeier HM, Friscia G, Román E, Surcel HM, Pasvol G, et al. Genetically permissive recognition of adjacent epitopes from the 19-kDa antigen of *Mycobacterium tuberculosis* by human and murine T cells. *J Immunol* (1993) **150**:5041–50.
23. Wiker HG, Harboe M, Nagai S. A localization index for distinction between extracellular and intracellular antigens of *Mycobacterium tuberculosis*. *J Gen Microbiol* (1991) **137**:875–84. doi:10.1099/00221287-137-4-875
24. Chandramuki A, Bothamley GH, Brennan PJ, Ivanyi J. Levels of antibody to defined antigens of *Mycobacterium tuberculosis* in tuberculous meningitis. *J Clin Microbiol* (1989) **27**:821–5.
25. Bothamley GH, Rudd RM. Clinical evaluation of a serological assay using a monoclonal antibody (TB72) to the 38 kDa antigen of *Mycobacterium tuberculosis*. *Eur Respir J* (1994) **7**:240–6. doi:10.1183/09031936.94.07020240
26. Bothamley GH, Beck JS, Potts RC, Grange JM, Kardjito T, Ivanyi J. Specificity of antibodies and tuberculin response after occupational exposure to tuberculosis. *J Infect Dis* (1992) **166**:182–6. doi:10.1093/infdis/166.1.182
27. Wilkinson RJ, Vordermeier HM, Wilkinson KA, Sjolund A, Moreno C, Pasvol G, et al. Peptide-specific T cell responses to *Mycobacterium tuberculosis*: clinical spectrum, compartmentalization and effect of chemotherapy. *J Infect Dis* (1998) **178**:760–8. doi:10.1086/515336
28. Gaylord H, Brennan PJ, Young DB, Buchanan TM. Most *Mycobacterium leprae* carbohydrate-reactive monoclonal antibodies are directed to lipoarabinomannan. *Infect Immun* (1987) **55**(11):2860–3.

29. Chatterjee D, Lowell K, Rivoire B, McNeil MR, Brennan PJ. Lipoarabinomannan of *Mycobacterium tuberculosis*. *J Biol Chem* (1992) **267**:6234–9.
30. Kaur D, Lowary TL, Vissa VD, Crick DC, Brennan PJ. Characterization of the epitope of lipoarabinomannan antibodies as the hexa aribonfuransyl motif of mycobacterial arabinans. *Microbiology* (2002) **148**:3049–57.
31. Rademacher C, Shoemaker GK, Kim HS, Zheng RB, Taha H, Liu C, et al. Ligand specificity of CS-35, a monoclonal antibody that recognizes mycobacterial lipoarabinomannan: a model system for oligofuranoside-protein recognition. *J Am Chem Soc* (2007) **129**(34):10489–502. doi:10.1021/ja0723380
32. Bothamley G, Beck JS, Britton W, Elsaghier A, Ivanyi J. Antibodies to *Mycobacterium tuberculosis*-specific epitopes in lepromatous leprosy. *Clin Exp Immunol* (1991) **86**:426–32. doi:10.1111/j.1365-2249.1991.tb02948.x
33. Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity* (2013) **38**(3):596–605. doi:10.1016/j.immuni.2012.11.020
34. Kringelum JV, Nielsen M, Padkjaer SN, Lund O. Structural analysis of B-cell epitopes in antibody:protein complexes. *Mol Immunol* (2013) **53**:24–34. doi:10.1016/j.molimm.2012.06.001
35. Yuseff MI, Pierobon P, Reversat A, Lennon-Duménil AM. How B cells capture, process and present antigens: a crucial role for cell polarity. *Nat Rev Immunol* (2013) **13**(7):475–86. doi:10.1038/nri3469
36. Dai YD, Caravanniotis G, Sercarz E. Antigen processing by autoreactive B cells promotes determinant spreading. *Cell Mol Immunol* (2005) **2**:169–75.
37. Bothamley GH. Epitope-specific antibody levels demonstrate recognition of new epitopes and changes in titer but not affinity during treatment of tuberculosis. *J Clin Lab Immunol* (2004) **11**:942–51. doi:10.1128/CDLI.11.5.942-951.2004
38. Mitchison DA. Basic mechanism of chemotherapy. *Chest* (1979) **76**:771–81. doi:10.1378/chest.76.6_Supplement.771
39. Sercarz EE. Driver clones and determinant spreading. *J Autoimmun* (2000) **14**:275–7. doi:10.1006/jaut.2000.0380
40. Disis ML. Immunologic biomarkers as correlates of clinical response to cancer immunotherapy. *Cancer Immunol Immunother* (2011) **60**:433–42. doi:10.1007/s00262-010-0960-8
41. Mathonet P, Ullman CG. The application of next generation sequencing to understanding the antibody repertoire. *Front Immunol* (2013) **4**:265. doi:10.3389/fimmu.2013.00265

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 February 2014; accepted: 09 May 2014; published online: 02 June 2014.
*Citation: Bothamley GH (2014) Epitope-specific antibody levels in tuberculosis: biomarkers of protection, disease, and response to treatment. Front. Immunol. **5**:243. doi: 10.3389/fimmu.2014.00243*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 09 July 2014
doi: 10.3389/fimmu.2014.00321

The immunodominant T-cell epitopes of the mycolyl-transferases of the antigen 85 complex of *M. tuberculosis*

Kris Huygen*

Service Immunology, O.D. Communicable and Infectious Diseases, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium

Edited by:
Juraj Ivanyi, Kings College London, UK

Reviewed by:
Katalin Wilkinson, University of Cape Town, South Africa
Martin Vordermeier, Animal Health and Veterinary Laboratories Agency, UK

****Correspondence:***
Kris Huygen, Scientific Institute of Public Health (WIV-ISP, Site Ukkel), 642 Engelandstraat, B1180 Brussels, Belgium
e-mail: kris.huygen@wiv-isp.be

The Ag85 complex is a 30–32 kDa family of three proteins (Ag85A, Ag85B, and Ag85C), which all three possess enzymatic mycolyl-transferase activity involved in the coupling of mycolic acids to the arabinogalactan of the cell wall and in the biogenesis of cord factor. By virtue of their strong potential to induce Th1-type immune responses, important for the control of intracellular infections, members of the Ag85 family rank among the most promising TB vaccine candidate antigens. Ag85A and Ag85B, initially purified from *Mycobacterium bovis* bacillus Calmette–Guérin (BCG)/*Mycobacterium tuberculosis* culture filtrate respectively, induce strong T-cell proliferation and IFN-γ production in most healthy individuals latently infected with *M. tuberculosis* and in BCG-vaccinated mice and humans but not in tuberculosis patients. Members of the Ag85 complex are highly conserved in other mycobacterial species. Mice and humans infected with *Mycobacterium ulcerans* or cattle infected with *M. bovis* or *Mycobacterium avium* subsp. *paratuberculosis* also show strong T-cell responses to this protein family. Using synthetic overlapping peptides, bio-informatic prediction programs and tetramer-binding studies, a number of immunodominant $CD4^+$ and $CD8^+$ T-cell epitopes have been identified in experimental animal models as well as in humans, using proliferation and Th1 cytokine secretion as main read-outs. The results from these studies are summarized in this review.

Keywords: antigen 85, mycolyl transferase, Th1 helper T-cell, immunodominance, promiscuous epitopes

INTRODUCTION

The Ag85 complex is actually a 30–32 kDa family of three proteins (Ag85A, Ag85B, and Ag85C), which each possess enzymatic mycolyl-transferase activity involved in the coupling of mycolic acids to the arabinogalactan of the cell wall and in the biogenesis of cord factor (1). These proteins are also known for their capacity to bind to the extracellular matrix proteins fibronectin and elastin (2, 3). In literature members of the Ag85 complex are known under different names: ***Mtb*Ag85A**: Rv3804c, P32, FbpA; ***Mtb* 85B**: Rv1886c, 30 kDa antigen, α-antigen, FbpB; ***Mtb*85C**: Rv0129c, FbpC2; ***M. bovis*85A**: Mb3834c, MPB44; ***M. bovis*** *Ag85B*:MPB59; ***M. bovis*** bacillus Calmette–Guérin **(BCG) 85A**: BCG_3866c; ***M. ulcerans* 85A**: MUL4987; ***Map*85A**: MAP0216; ***Map*85B**: MAP 1609c.

Members of the Ag85 family are found in all mycobacteria, and sequence comparisons indicate that the Ag85 gene family arose by duplication of an ancestral gene, before the emergence of the actually known mycobacterial species (4). The genes encoding these proteins are not physically linked, but located at distinct sites on the mycobacterial genome. The genes of Ag85 encode for a characteristic leader sequence of about 40 aa, which is cleaved off during export and release of the mature proteins into mycobacterial culture filtrate (CF). The Ag85A and Ag85B components are detected essentially as secreted proteins, whereas the Ag85C component is more tightly associated with the bacterial cell wall envelope. The calculated secretion index of the three proteins reflects this difference in localization (5). The mycolyl-transferase activity of these proteins generates trehalose dimycolate (TDM), an envelope lipid essential for *Mtb* virulence, and cell wall arabinogalactan-linked mycolic acids. A novel inhibitor of Ag85C, 2-amino-6-propyl-4,5,6,7-tetrahydro-1-benzothiophene-3-carbonitrile (I3-AG85) inhibits *Mtb* survival in infected primary macrophages and quantification of mycolic acid-linked lipids of the *Mtb* envelope showed a specific blockade of TDM synthesis (6). Members of the Ag85 complex are highly conserved in other mycobacterial species and mice infected with *Mycobacterium ulcerans* or with some non-tuberculous mycobacteria belonging to the MAIS-group (*Mycobacterium avium*, *M. intracellulaire*, and *M. scrofulaceum*) show cross-reactive Th1-type immune responses to Ag85 components purified from BCG CF or produced as recombinant *E. coli* derived proteins (5, 7). By virtue of their strong Th1-type cytokine inducing potential, members of the tuberculosis Ag85 complex (particularly the Ag85A and Ag85B component) are among the most promising tuberculosis vaccine candidates today. Many of the new TB vaccines tested in preclinical and clinical trials, are composed of Ag85 components, expressed as recombinant fusion proteins or encoded by recombinant viral vectors (8, 9).

AMINO-ACID SEQUENCE ALIGNMENTS OF Ag85A, Ag85B, Ag85C OF *Mtb*, AND OF Ag85A OF *M. ULCERANS*, *M. AVIUM* SUBSP. *PARATUBERCULOSIS (Map)*, AND *M. LEPRAE*

As shown in **Figure 1**, amino-acid sequences (aa) of the mature Ag85A homologs (without their leader sequence) are highly conserved between mycobacterial species. The three aa essential for the mycolyl-transferase activity, i.e., Serine in position 125, Glutamic acid in position 230 and Histidine in position 262 are conserved in all sequences (highlighted in red). Although some aa stretches are 100% conserved between the different species, there are small variations (indicated in bold as compared to the *Mtb* Ag85A sequence). The aa sequence of Ag85A of *Mycobacterium bovis* and *M. bovis* BCG (1173P2 strain) is identical to the aa sequence of the Ag85A component of *Mtb* (H37Rv) and is therefore not shown. The Ag85B sequence of *M. bovis* differs in one aa from the Ag85B sequence of *Mtb*: Phe100Leu. On the other hand, expression levels of these proteins may differ and whereas Ag85A is the major component in CF of surface-pellicle grown BCG, Ag85B is the major component in CF from *Mtb* and *Map*. The Ag85C component is found in lesser concentrations in the CF, as it is localized more internally in the cell wall. In 2000, M. Horwitz reported that recombinant BCG vaccines expressing the *Mtb* 30-kDa (Ag85B) major secretory protein induced greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model, i.e., the guinea pig (10). The rationale for the construction of this recombinant BCG, was

```
     1

85A:FSRPGLPVEY LQVPSPSMGR DIKVQFQSGG ANSPALYLLD GLRAQDDFSG WDINTPAFEW
85B:FSRPGLPVEY LQVPSPSMGR DIKVQFQSGG NNSPAVYLLD GLRAQDDYNG WDINTPAFEW
85C:FSRPGLPVEY LQVPSASMGR DIKVQFQGGG PH..AVYLLD GLRAQDDYNG WDINTPAFEE

Mul:FSRPGLPVEY LQVPSVAMGR NIKVQFQSGG ANSPALYLLD GMRAQDDFSG WDINTPAFEW
MAP:FSRPGLPVEY LQVPSAAMGR DIKVQFQSGG ANSPALYLLD GMRAQDDFNG WDINTPAFEW
Mle:FSRPGLPVEY LQVPSPSMGR DIKVQFQNGG ANSPALYLLD GLRAQDDFSG WDINTTAFEW


    61
85A:YDQSGLSVVM PVGGQSSFYS DWYQPACGKA GCQTYKWETF LTSELPGWLQ ANRHVKPTGS
85B:YYQSGLSIVM PVGGQSSFYS DWYSPACGKA GCQTYKWETF LTSELPQWLS ANRAVKPTGS
85C:YYQSGLSVIM PVGGQSSFYT DWYQPSQSNG QNYTYKWETF LTREMPAWLQ ANKGVSPTGN

Mul:YYQSGISVAM PVGGQSSFYS DWYNPACGKA GCTTYKWETF LTSELPQYLS ANKGVKPTGS
Map:YNQSGISVAM PVGGQSSFYS DWYKPACGKA GCTTYKWETF LTSELPQYLS AQKQVKPTGS
Mle:YYQSGISVVM PVGGQSSFYS DWYSPACGKA GCQTYKWETF LTSELPQYLQ SNKQIKPTGS


    121
85A:AVVGLSMAAS SALTLAIYHP QQFVYAGAMS GLLDPSQAMG PTLIGLAMGD AGGYKASDMW
85B:AAIGLSMAGS SAMILAAYHP QQFIYAGSLS ALLDPSQGMG PSLIGLAMGD AGGYKAADMW
85C:AAVGLSMSGG SALILAAYYP QQFPYAASLS GFLNPSEGWW PTLIGLAMND SGGYNANSMW

Mul:GVVGLSMAGS SALILAAYHP DQFVYSGSLS ALLDPSQGIG PSLIGLAMGD AGGYKASDMW
Map:GVVGLSMAGS SALILAAYHP DQFVYAGSLS ALLDSSQGMG PSLIGLAMGD AGGYKAADMW
Mle:AAVGLSMAGL SALTLAIYHP DQFIYVGSMS GLLDPSNAMG PSLIGLAMGD AGGYKAADMW

    181
85A:GPKEDPAWQR NDPLLNVGKL IANNTRVWVY CGNGKPSDLG GNNLPAKFLE GFVRTSNIKF
85B:GPSSDPAWER NDPTQQIPKL VANNTRLWVY CGNGTPNELG GANIPAEFLE NFVRSSNLKF
85C:GPSSDPAWKR NDPMVQIPRL VANNTRIWVY CGNGTPSDLG GDNIPAKFLE GLTLRTNQTF

Mul:GPKDDPAWAR NDPMLQVGKL VANNTRIWVY CGNGKPSDLG GDNLPAKFLE GFVRTSNMKF
Map:GPKEDPAWAR NDPSLQVGKL VANNTRIWVY CGNGKPSDLG GDNLPAKFLE GFVRTSNLKF
Mle:GPSTDPAWKR NDPTVNVGTL IANNTRIWMY CGNGKPTELG GNNLPAKLLE GLVRTSNIKF


    241
85A:QDAYNAGGGH NGVFDFPDSG THSWEYWGAQ LNAMKPDLQR AL.GATPNTG PAPQGA
85B:QDAYNAAGGH NAVFNFPPNG THSWEYWGAQ LNAMKGDLQS SL.GAG
85C:RDTYAADGGR NGVFNFPPNG THSWPYWNEQ LVAMKADIQH VLNGATPPAA PAAPAA

Mul:FQAAYNAAGG HNAVWNFDDG THSWEYWGAQ LNAMRPDLQH TL.GATPNTG DTQGA
Map:QDAYNGAGGH NAVWNFDANG THDWPYWGAQ LQAMKPDLQS VL.GATPGAG PATAAATNAGN GGGT
Mle:QDGYNAGGGH NAVFNFPDSG THSWEYWGEQ LNDMKPDLQQ YL.GATPGA
```

FIGURE 1 | Amino-acid sequence alignment of *Mtb*Ag85A, *Mtb*Ag85B, *Mtb*Ag85C, and Ag85A sequence of *M. ulcerans*, *M. avium* subsp. *paratuberculosis*, and *M. leprae*. Aa differences with the *Mtb*Ag85A sequence are underlined and bold. The three aa essential for the mycolyl-transferase activity are indicated in red.

among others the sequence difference of Ag85B of *Mtb* and that of the *M. bovis* BCG Tokyo strain (three aa differences Phe100Leu, Asn245Lys, and Ala246Pro) (11). As already mentioned, Ag85B of other BCG strains and *M. bovis* only differ in position 100 from the sequence of Ag85B of *Mtb.*

T-CELL EPITOPES OF Ag85 OF *M. TUBERCULOSIS*/*M. BOVIS* AND *M. BOVIS* BCG (TABLE 1)

Murine studies

M. bovis BCG. The first T-cell epitope mapping of Ag85A was performed 20 years ago in seven different mouse strains vaccinated with live *M. bovis* BCG (12). Twenty-eight overlapping 20-mer peptides covering the complete mature 295-amino-acid (AA) protein were synthesized. Significant interleukin-2 (IL-2) and gamma interferon (IFN-γ) secretion was measured following *in vitro* stimulation of spleen cells with these peptides. H-2^d haplotype mice (BALB/c and DBA/2) reacted preferentially against the amino-terminal half of the protein, i.e., against peptide 5 (aa 41–60) and especially against peptide 11 (aa **101–120**), which contains a predicted I-E^d binding motif. H-2^b haplotype mice, on the other hand, reacted against peptides from both amino- and carboxy-terminal halves of the protein, peptide 25 (aa **241–260**) and peptide 27 (aa **261–280**) being the most potent stimulators of IL-2 and IFN-γ production. Finally, CBA/J (H-2^k) and major histocompatibility complex class II mutant B6.C.bml2 mice, carrying a mutant I-A^{bml2} allele on an H-2^b background, reacted only very weakly to the 85A peptides. (12).

M. tuberculosis H37Rv. BALB/c and C57BL/6 mice were infected intravenously with *Mycobacterium tuberculosis* H37Rv and Th1-type spleen cell cytokine secretion was analyzed in response to purified Ag85A, Ag85B, and Ag85C components and synthetic overlapping peptides covering the three mature sequences (13). Tuberculosis-infected C57BL/6 mice reacted strongly to some peptides from Ag85A and Ag85B but not from Ag85C and more specifically strong responses were detected against peptide 25 (aa 241–260) of Ag85A and against the same sequence of Ag85B (13). This latter peptide region was also identified by Yanagisawa et al. (14) and TCR-transgenic mice with MHC class II A^b-restricted $CD4^+$ T-cells expressing TCR α and β chains for the mycobacterial **$Ag85B_{240–254}$** have been generated (15). Tuberculosis-infected BALB/c mice reacted only to peptides from Ag85A: p11 (aa 101–120), p16 (aa 151–160), and p20 (aa 191–210) (13).

Plasmid DNA vaccines/viral vectors encoding Ag85A, Ag85B, and Ag85C of Mtb. Plasmid DNA vaccination is a powerful tool to identify protective antigens of tuberculosis and to identify immunodominant $CD4^+$ and particularly $CD8^+$ T-cell epitopes (16). BALB/c and C57BL/6 mice were vaccinated intramuscularly with plasmid DNA encoding the three components of the Ag85 complex. Ag85A and Ag85B encoding plasmids induced a robust Th1 like response to native Ag85 purified from BCG CF, characterized by elevated levels of IL-2, IFN-γ, and TNF-α. Levels of IL-4, IL-6, and IL-10 were low or undetectable. Plasmid encoding Ag85C was only weakly immunogenic. Whereas BALB/c mice reacted preferentially to the Ag85A component, C57BL/6 mice reacted to both Ag85A and Ag85B with more or less the same magnitude (17). Furthermore, vaccination with plasmid DNA encoding Ag85A or Ag85B but not Ag85C conferred significant protection against mycobacterial replication in lungs from C57BL/6 mice (17). T-cell epitopes could be identified in BALB/c and C57BL/6 mice vaccinated with plasmid DNA encoding Ag85A, Ag85B, and Ag85C DNA using synthetic peptides spanning the three Ag85 proteins, and the epitope repertoire was found to be broader than in infected mice (13). Despite pronounced sequence homology, a number of immunodominant regions contained component specific epitopes. Thus, BALB/c mice vaccinated against all three Ag85 antigens reacted against the same amino-acid region, **101–120** (already identified in BCG vaccinated and TB infected mice) but responses were completely component specific. In C57BL/6 mice, a cross-reactive T-cell response was detected against two carboxy-terminal peptides spanning amino acids 241–260 and 261–280 of Ag85A and Ag85B. These regions were not recognized at all in C57BL/6 mice vaccinated with Ag85C DNA.

T-cell repertoire of BALB/c mice vaccinated with plasmid DNA encoding Ag85A was broader that of *Mtb* infected mice (13, 18). Besides peptide regions spanning aa **11–30** and **191–210** inducing both IL-2 and IFN-γ responses, three peptides induced strong IFN-γ but weak to no IL-2 responses. More detailed analysis of the Ag85A sequence for predicted MHC class I binding motifs using "human leukocyte antigen (HLA) peptide motif" (http://www-bimas.cit.nih.gov/molbio/hla_bind/) showed that these three peptides spanned four predicted $CD8^+$ T-cell epitopes (18). The following half-time dissociation scores (reflecting affinity for the respective MHC class I molecules) were found: aa **61–68** YDQSGLSV: half-time dissociation score 600, predicted K^d; aa **71–78** PVGGQSSF: half-time dissociation score 390, predicted L^d; aa **145–152** YAGAMSGL: half-time dissociation score 2000, predicted K^d; aa **161–168** PTLIGLAM: half-time dissociation score 150, predicted L^d. CTL activity against these peptides was demonstrated using a ^{51}Cr release assay (**Figure 2**) and showed cross-reactive responses against Ag85B for both K^d restricted peptides (18). A particularly interesting region was identified in peptide 15 spanning aa 141–160, which besides the K^d restricted epitope 145–152 also contains a $CD4^+$ epitope with a predicted Rothbard and Taylor motif spanning aa 147–154 and an amphipathic stretch spanning aa 149–157 (according to T sites program) (19).

Immunization with DNA followed by modified vaccinia virus Ankara strain, both expressing the antigen 85A, induced both $CD4^+$- and $CD8^+$-T-cell responses in BALB/c mice, directed against the K^d restricted epitope WYDQSGLSV (aa 60–67) and the I-E^d restricted epitope TFLTSELPGWLQANRHVKPT (aa 99–119), respectively (20). DNA priming, followed by a MVA85A boost induced both $CD4^+$ and $CD8^+$ responses, whereas priming with MVA followed by a DNA boost only induced CD4 responses. Following immunization with dendritic cells pulsed with the antigen 85A $CD4^+$- or $CD8^+$-restricted epitope, alone or in combination, copresentation of both epitopes on the same dendritic cell was required for protection, demonstrating that induced $CD8^+$ T-cells can play a protective role against tuberculosis (20).

A single intranasal, but not i.m., immunization with a recombinant replication-deficient adenoviral-based vaccine expressing Ag85A (AdAg85A) provided potent protection against airway *M.*

Table 1 | Summary of immunodominant Ag85 T-cell epitopes of *M. tuberculosis*.

Infection/vaccination	Position	Sequence	Restriction	Host	Reference
***M. tuberculosis* INFECTION**					
Rv3804c	241–260	QDAYNAGGGH NGVFDFPDSG	**I-A^b**	**Mouse**	(18)
Rv1886c	240–254	**F**QDAYNA**A**GGHN**A**VF	**I-A^b**	**Mouse**	(14)
Rv3804c	101–120	LTSELPGWLQANRHVKPTGS	**I-E^d**	**Mouse**	(18)
Rv3804c	151–170	GLLDPSQAMG PTLIGLAMGD	**H-2^d**	**Mouse**	(13)
Rv3804c	191–210	NDPLLNVGKL IANNTRVWVY	**H-2^d**	**Mouse**	(13)
Rv3804c	51–70	WDINTPAFEWYDQSGLSVVM	**Promiscuous**	**LTBI**	(23)
Rv3804c	141–160	QQFVYAGAMSGLLDPSQAMG	**Promiscuous**	**LTBI**	(23)
Rv1886c	100–117	**F**LTSELP**Q**WL**S**ANR**A**VKP	**Promiscuous**	**LTBI**	(24, 25)
Rv1886c	91–115	GCQTYKWETFLTSEL	**Promiscuous**	**LTBI**	(26)
Rv1886c	193–214	P**TQQIP**KL**V**ANNTR**L**WVYCGNG	**Promiscuous**	**LTBI**	(26)
Rv0129c	70–79	MPVGGQSSFY	**HLA-B*35**	**Human/*in silico***	(29)
Rv0129c	160–168	WPTLIGLAM	**HLA-B*35**	**Human/*in silico***	(29)
Rv1886c	224–232	IPA**E**FL**E**NF	**HLA-B*35**	**Human/*in silico***	(29)
Rv3804c/Rv1886c	90–104	**A**GCQTYKWETFLTSE	**DPB1*04:01**	**LTBI**	(28)
***M. bovis* BCG VACCINATION**					
Rv3804c	101–120	LTSELPGWLQANRHVKPTGS	**I-E^d**	**Mouse**	(12)
Rv3804c	241–260	QDAYNAGGGH NGVFDFPDSG	**I-A^b**	**Mouse**	(12)
Rv3804c	261–280	T**H**SWEYWGAQ LNAMKPDLQR	**I-A^b**	**Mouse**	(12)
MPB59	51–70	WDINTPAFEW Y**Y**QSGLS**I**VM	**Promiscuous**	**Human**	(32)
MPB59	11–30	LQVPSPSMGR DIKVQFQSGG	**Promiscuous**	**Human**	(32)
***Mtb* PLASMID DNA VACCINATION**					
Rv3804c	101–120	LTSELPGWLQANRHVKPTGS	**I-E^d**	**Mouse**	(13, 18)
Rv1886c	100–117	**F**LTSELP**Q**WL**S**ANR**A**VKP	**I-A^d**	**Mouse**	(13)
Rv0129c	101–120	LT**R**E**M**P**A**WLQAN**KG**V**S**PTG**N**	**H-2^d**	**Mouse**	(13)
Rv3804c/Rv1886c	141–160	QQFV**Y**AGAMSG**L**LDPSQAMG	**H-2^d**	**Mouse**	(18)
Rv3804c	191–120	NDPLLNVGKLIANNTRVWVY	**H-2^d**	**Mouse**	(13)
Rv0129c	191–210	NDP**MVQIPR**L**V**ANNTR**I**WVY	**H-2^d**	**Mouse**	(13)
Rv3804c	241–260	QDAYNAGGGH NGVFDFPDSG	**I-A^b**	**Mouse**	(13)
Rv3804c	261–280	T**H**SWEYWGAQ LNAMKPDLQR	**I-A^b**	**Mouse**	(13)
Rv3804c	61–68	YDQSGLSV	**K^d**	**Mouse**	(18)
Rv3804c/Rv1886c	71–78	PVGGQSSF	**L^d**	**Mouse**	(18)
Rv3804c/Rv1886c	145–152	YAGAMSGL	**K^d**	**Mouse**	(18)
Rv3804c	161–168	PTLIGLAM	**L^d**	**Mouse**	(18)
Rv1886c	145–152	FIYAG**SL**S	**HLA-A*0201**	**HLA-tg**	(27)
Rv1886c	199–207	KL**V**ANNTR**L**	**HLA-A*0201**	**HLA-tg**	(27)
PROTEIN VACCINATION (*Mtb* Ag85 COMPLEX)					
Rv1886c	11–30	LQVPSPSMGRDIKVQFQSGG	**HLA-DRA/B1*0302**	**HLA-tg**	(27)
Rv3804c	121–145	AVVGL**S**MAASSALTL	**Epimer**	**Guinea pigs**	(11)
Rv3804c	196–215	NVGKLIANNTRVWVYCGNGK	**Epimer**	**Guinea pigs**	(11)
Rv1886c	101–122	LTSELP**Q**WL**S**ANR**A**VKPTGSAA	**Epimer**	**Guinea pigs**	(11)
Rv1886c	126–140	SMAGSSA**M**ILA**A**YHP	**Epimer**	**Guinea pigs**	(11)
Rv1886c	261–275	T**H**SWEYWGAQLNAMK	**Epimer**	**Guinea pigs**	(11)
***M. leprae* INFECTION**					
Rv3804c	11–30	LQVPSPSMGR DIKVQFQSGG	**Promiscuous**	**Lepromin+**	(23)
***M. ulcerans* PLASMID VACCINATION**					
MUL4987	21–40	**N**IKVQFQSGG ANSPALYLLD	**H-2^b**	**Mouse**	(39)
MUL4987	61–80	Y**Y**QSG**I**SV**A**MPVGGQSSFYS	**H-2^b**	**Mouse**	(39)
MUL4987	81–100	DWY**N**PACGKAGC**T**TYKWETF	**H-2^b**	**Mouse**	(39)
MUL4987	240–259	**FQ**A**AYNAA**G**GHNAVWNF**DD	**H-2^b**	**Mouse**	(39)
MUL4987	261–280	T**H**SWEYWGAQ LNAMRPDLQ**H**	**H-2^b**	**Mouse**	(39)

(Continued)

Table 1 | Continued

Infection/vaccination	Position	Sequence	Restriction	Host	Reference
***Map* ATCC 19698 INFECTION**					
Rv3804c	241–260	QDAYNAGGGHNGVFDFPDSG	**I-A^b**	**B6 $^{bg/bg}$**	(41)
Rv1886c	241–260	QDAYNA**A**GGHN**A**VF**N**FPP**N**G	**I-A^b**	**B6 $^{bg/bg}$**	(41)
Rv3804c	261–280	T**H**SWEYWGAQLNAMKPDLQR	**I-A^b**	**B6 $^{bg/bg}$**	(41)
Rv1886c	262–279	**H**SWEYWGAQ LNAMK**G**D**L**Q	**I-A^b**	**B6 $^{bg/bg}$**	(41)
Rv0129c	261–280	T**H**SW**P**YW**NE**Q L**V**AMK**A**D**I**Q**H**	**I-A^b**	**B6 $^{bg/bg}$**	(41)
Rv0129c	21–40	DIKVQFQ**G**GG **PH**.A**V**YLLD	**I-A^b**	**B6 $^{bg/bg}$**	(41)
Rv1886c	145–162	YAG**SL**S**A**LLDPSQ**G**MGPS	**Promiscuous**	***Bos taurus***	(41)
***Map* PLASMID DNA VACCINATION**					
Rv3804c	241–260	QDAYNAGGGH NGVFDFPDSG	**I-A^b**	**B6**	(42)
Rv1886c	240–260	**F**QDAYNA**A**GGHN**A**VF**N**FPP**N**G	**I-A^b**	**B6**	(42)
Rv3804c	91–110	GCQTYKWETF LTSELPGWLQ	**I-A^b**	**B6**	(42)
Rv1886c	145–162	YAG**SL**S**A**LLDPSQ**G**MGP**S**	**I-A^b**	**B6**	(42)

Immunodominant T-cell epitopes of Ag85, as defined in experimental vaccination models and infection (LTBI, latent TB infection).
Amino acids different from aa sequence of MtbAg85A (Rv3804c) are indicated in bold/underlined.
The three aa involved in mycolyl-transferase activity are highlighted in red.

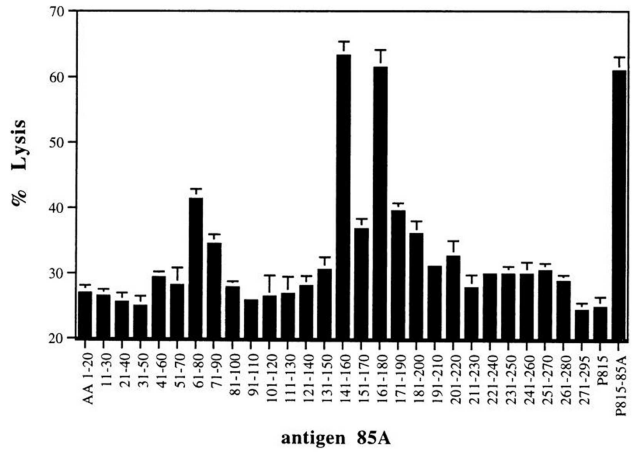


FIGURE 2 | CTL activity against P815 target cells loaded with synthetic 20-mer peptides (overlapping by 10 aa, covering the complete Ag85A sequence) of spleen cells from BALB/c mice vaccinated with mature Ag85A DNA. Reproduced from Ref. (18).

tuberculosis challenge at an improved level over that by cutaneous BCG vaccination. Systemic priming with an Ag85A DNA vaccine and mucosal boosting with AdAg85A conferred a further enhanced immune protection, which was remarkably better than BCG vaccination. Such superior protection triggered by AdAg85 mucosal immunization was correlated with much greater retention of Ag-specific T-cells, particularly CD4 T-cells, in the lung and was shown to be mediated by both CD4+ (LTSELPGWLQANRHVKPTGS, aa 101–120) and CD8$^+$ (MPVGGQSSF, aa 70–78) T-cells (21).

For H-2^b haplotype mice, no MHC class I restricted epitopes have been identified so far on Ag85A or Ag85B to our knowledge, neither in BCG or plasmid DNA vaccinated nor in TB infected mice.

Human studies

Tuberculosis. In our first paper on Ag85A (called P32 at that time), we reported that healthy Mantoux positive volunteers showed a much stronger lymphoproliferative and IFN-γ response to this antigen than tuberculosis patients (22). This was the initial indication that T-cell responses against this protein could confer protection against *Mtb*. Subsequently, we reported on T-cell epitope mapping of Ag85A from *Mtb* using peripheral blood mononuclear cell (PBMC) cultures from healthy tuberculin-positive volunteers and from patients with tuberculosis, using the same synthetic 20-mer peptides of the murine study. Peptide recognition was largely promiscuous, with a variety of HLA haplotypes reacting to the same peptides. PBMC from all tuberculin-positive subjects reacted to Ag85A, and the majority proliferated in response to peptide 6 (amino acids 51–70), peptides 13, 14, and 15 (amino acids 121–160), or peptides 20 and 21 (amino acids 191–220). PBMC from tuberculosis patients demonstrated a variable reactivity to Ag85 and its peptides, and the strongest proliferation was observed against peptide 7 (amino acids 61–80) (23). Nine out of ten of the tuberculin-positive volunteers in this study reacted to aa **141–160**, precisely the peptide characterized by the presence of both a CD4$^+$ and a CD8$^+$ epitope in BALB/c mice. In contrast, the most immunogenic CD4$^+$ peptide of Ag85A for BALB/c mice, i.e., p11 was not recognized by PBMCs from healthy PPD-positive humans. However, two reports published in 2000 and 2001 showed that aa **100–117** of **Ag85B** is recognized in a similar promiscuous manner by T-cells from a majority of PPD-positive human volunteers (24, 25). Ag85A differs from Ag85B in three aa in this region Gly107Gln, Gln110Ser, and His114Ala. Ag85A sequences from *M. ulcerans*, *M. leprae*, *Map*, and *Maa* also show strong differences as compared to *Mtb*Ag85A sequence in this region (see **Figure 1**). These aa shifts probably explain the difference in the human responses to Ag85A and Ag85B, as also in DNA vaccinated BALB/c mice, IL-2 and IFN-γ responses to region 100–120 are specific for both Ag85 components (13).

In 1995, Silver et al. had already assessed the T-cell epitopes of *Mtb* Ag85B using blastogenic responses of PBMC from 12 healthy purified protein derivative-positive subjects to a set of synthetic 15-mer peptides based on the full 325-amino-acid sequence (leader sequence included) (26). Seven immunodominant regions were identified and each subject responded to at least one of the two most dominant epitopes, which corresponded to aa **91–115** and aa **193–217**. Peptides of these two epitopes induced production of IFN-γ by sorted $CD4^+$ T-cells.

Human leukocyte antigen-transgenic mice can be a powerful tool to identify human T-cell epitopes in an experimental mouse model. In 1998, A. Geluk reported on the identification of an HLA-class II restricted epitope of Ag85. HLA-DRA/B1*0302 (DR3) transgenic mice were vaccinated with Ag85 protein, purified from *Mtb* CF in Incomplete Freund's adjuvant (27). Using 20-mer peptides, covering the entire *Mtb* 85B sequence, they identified one single peptide epitope in the NH_2-terminal region, spanning aa **11–30** (aa 51–70 in the numbering including the signal peptide): LQVPSPSMGRDIKVQFSGG. This sequence is identical in Ag85A and Ag85B, but differs in two positions in Ag85C: Pro16Ala and Ser28Gly. Also in *M. ulcerans*, *Map*, and *Maa*, position 16 has the shift to Alanine. In *M. leprae* there is also one aa shift: Ser28Asn.

Whereas most of these studies on human T-cell epitope mapping were performed during the mid-nineties, one more recent paper of Lindestam Arlehamn et al. reported on the memory phenotype of *Mtb*-specific $CD4^+$ T-cells, using HLA-class II tetramers for a peptide shared between Ag85A and Ag85B, i.e., aa **90–104** AGCQTYKWETFLTSE in healthy PPD-positive donors, latently infected with *Mtb* (28). Tetramer positive T-cells predominantly consisted of $CD45RA^-CCR7^+$ central memory T-cells in all donors tested, followed by effector memory ($CD45^-CCR7^-$) T-cells. Only a minor fraction appeared to be naïve or effector T-cells. Interesting to note that this sequence is shared with Ag85A of *M. leprae* that there is only Q93T shift in *M. ulcerans*, *Map*, and *Maa* but that the sequence of *Mtb* Ag85C differs in five positions.

Less is known on human MHC class I restricted epitopes of Ag85. Klein et al. reported on a HLA-B*35 restricted $CD8^+$ T-cell epitope of Ag85C (29). Using reverse immunogenetics, they tested 23 motif-bearing peptides of the Ag85 complex for binding to HLA-B*35, one of the most common HLA-B types in West Africa. Three 9-mer peptides bound with high affinity to HLA-B*3501. Peptide MPVGGQSSFY (spanning aa 70–79 of the mature protein), a highly conserved region shared by all three members of the Ag85 complex of *Mtb* and also identical in *M. ulcerans*, *M. avium*, and *M. leprae*. This peptide encompasses an L^d predicted epitope, recognized by BALB/c mice vaccinated with pAg85A DNA (see Plasmid DNA vaccines/viral vectors encoding Ag85A, Ag85B, and Ag85C of *Mtb*) and also an IL-2/IFN-γ inducing region for C57BL/6 mice vaccinated with pAg85C (13). Peptide WPTLIGLAM of Ag85C (spanning aa 160–168) with a W160G change as compared to all other sequences, and a T162S change in Ag85B, and the three other non-tuberculous mycobacteria. Finally peptide IPAEFLENF of Ag85B (spanning aa 224–232), with an isoleucine in position 224 shared with Ag85C, and a leucine in Ag85A of *Mtb* and the four non-tuberculous mycobacteria. WPTLIGLAM stimulated effector cells were able to kill *Mtb* or BCG infected macrophages and produced IFN-γ and TNF-α (30). Interestingly, an L^d restricted epitope spanning the same aa 161–168 (PTLIGLAM) was identified in Ag85A DNA vaccinated BALB/c mice, which did not cross-react with the corresponding Ag85B peptide (because of the Thr162Ser shift).

A comprehensive epitope mapping to HLA-A*0101, A*0201, A*1101, A*2402, B*0702, B*0801, and B*1501 of Ag85B was published in 2007 (31). Affinity and half-life ($t_{1/2}$ off-rate) analysis for individual peptide species on HLA-A and HLA-B molecules revealed binding ranges between 10^{-3} and 10^{-7} M. After selection of the best matches, major histocompatibility complex class I/peptide tetramer complexes were constructed to measure the $CD8^+$ T-cell responses directly *ex vivo* in PBMC derived from 57 patients with acute pulmonary tuberculosis. Three patterns of (allele-) specific $CD8^+$ recognition were identified: (a) Focus on one dominant epitope, (b) Co-dominant recognition of two distinct groups of peptides, and (c) Diverse and broad recognition of peptides (presented by HLA-A*0201). Peptides that bound with slow off-rates to class I alleles, that is HLA-A*0201, were associated with low frequency of $CD8^+$ T-cells in PBMCs from patients with tuberculosis. HLA-B alleles showed fast off-rates in peptide binding and restricted high numbers (up to 6%) of antigen-specific $CD8^+$ T-cells in patients with pulmonary tuberculosis (31). Functional analysis (*in vitro* IL-2 and IFN-γ production) revealed that tetramer-binding T-cells in PBMCs from these patients were little or not responsive to the nominal peptide epitope, confirming the notion of a deficient Ag85 specific T-cell response in TB patients. The study focused on TB patients and not on latently infected subjects, which could have been more relevant in the context of TB vaccine development.

***M. bovis* BCG.** In 1994, Roche et al. reported on the T-cell determinants of Ag85B of *M. bovis* (MPB 59) in BCG vaccinees and TB patients. The mature 85B protein of *M. bovis* (MPB59) has a high degree of amino-acid identity with the *M. bovis* 85A protein (76%) and the *Mtb* 85B (99%) and *Mtb*85A (76%) proteins. Proliferative assays with recombinant MPB59 demonstrated that PBMC from 95% of BCG vaccinees and 52% of tuberculosis patients responded to the whole mature protein. Using a set of synthetic 20-mer peptides, five peptides were found to be recognized in more than half of the MPB59 responders. The T-cell-reactive regions were essentially identical in the *M. bovis* and *Mtb* 85B proteins. Subjects with a variety of HLA-DR phenotypes responded to a number of these peptides and there was no difference in the pattern of responses between BCG vaccinees and TB patients. A promiscuous recognition pattern was observed in response to peptides spanning aa 51–70 (recognized by 87% of the responsive BCG vaccinees and 93% of the responding TB patients) and aa 11–30 (recognized by 73% of the responders). Peptides in the C-terminal region (aa 131–150 and aa 191–210) were more frequently recognized by patients than by BCG vaccinees (32). More recently, Finan et al. reported on 236 healthy Gambian babies vaccinated at birth with *M. bovis* BCG (33). Using a whole blood assay 2 months after vaccination, cytokine analysis showed that 89% of the babies produced Ag85 complex specific IFN-γ responses, albeit that response varied up

to 10 log-fold within this population and 25 and 31% of the babies also produced detectable levels of the Th2 cytokines IL-5 and IL-13, respectively. Unfortunately, T-cell epitopes were not mapped in this study.

A. Geluk et al. reported on the identification of two HLA-A*0201 restricted $CD8^+$ T-cell epitopes of Ag85B using pDNA vaccination encoding Ag85B of HLA-A2/K^b transgenic mice. (34). HLA-A*0201 is one of the most prevalent class I alleles, with a frequency of over 30% in most populations. The two peptides spanned aa 145–152 FIYAGSLS and aa 199–207 KLVANNTRL. As already mentioned, the first region is also recognized by K^d restricted $CD8^+$ T-cells of Ag85A/B DNA vaccinated mice. The second peptide differs from that of Ag85A only in position 201, with a leucine in the Ag85A and a valine in the Ag85B sequence (both with a non-polar side chain), change that does not affect the binding affinity for HLA-A*0201 (34). As precursor frequencies of these cells were low in the periphery of human BCG vaccinees, restimulation with *M. bovis* BCG was necessary to visualize the cells by tetramer staining. Stable human $CD8^+$ T-cell lines were generated against the two peptides, using CD4 depletion and peptide-pulsed autologous Dcs derived, from HLA-A*0201+ BCG-responsive donors. These T-cell lines were able to lyse HLA-A*0201+ peptide-pulsed targets and produced the pro-inflammatory cytokines IFN-γ and TNF-α. The group of H. Dockrell also demonstrated Ag85A specific CTL responses using BCG-specific cell lines generated from PBMC of BCG-vaccinated donors stimulated for 2 weeks with live *M. bovis* BCG in the presence of IL-2 and IL-7 (35). In this study, two HLA-A*0201 restricted epitopes were identified, one spanning aa 5–13 GLPVEYLQV and the other spanning aa 199–207 KLIANNTRV. This second peptide spans exactly the same region of Ag85B identified by Geluk et al., using HLA-A2/K^b transgenic mice, suggesting the existence of a cross-reactive CTL epitope in this region for Ag85A and Ag85B.

Leprosy. In 1994, we also reported on T-cell epitope mapping of *Mtb*Ag85A using PBMCs from healthy lepromin-positive volunteers and from patients with leprosy. As for tuberculosis, peptide recognition was largely promiscuous, with a variety of HLA haplotypes reacting to the same peptides. However, despite a 90% homology between the 85A proteins of *M. leprae* and *Mtb*, the peptides recognized were different. PBMC from lepromin-positive healthy contacts reacted against peptide 2 (aa 11–30), peptide 5 (aa 41–60), and peptides 25 and 26 (aa 241–270). PBMC from paucibacillary patients reacted preferentially against peptide 1 (amino acids 1–20) and peptide 5. Multibacillary patients were not reactive to Ag85 or the Ag85A peptides (23). It is interesting to note that responses to aa 11–30 were also identified in BCG vaccinees (27, 32). It is well known that BCG vaccination exerts some degree of protection against leprosy and that a second BCG immunization can increase this protection (36).

Guinea pigs

Lee and Horwitz reported on T-cell epitope mapping of Ag85A and Ag85B in outbred Hartley strain guinea pigs, immunized with the purified *Mtb* proteins and tested for splenocyte proliferation in response to a series of overlapping 15-mer peptides spanning the mature proteins (11). Three of the nine immunoreactive regions of Ag85B identified in the guinea pigs (aa 101–122, 126–140, and 261–275) overlapped with epitopes predicted by the EpiMer computer program (37). Two immunodominant T-cell epitopes were identified in Ag85A immunized guinea pigs, spanning aa 121–145 and 196–215, and these regions were also predicted by the EpiMer program (37).

T-CELL EPITOPES OF Ag85 OF *M. ULCERANS*

The gene encoding Ag85A from *M. ulcerans* 5150 (MUL4987) shares 84.1% amino-acid sequence identity and 91% conserved residues with the gene encoding Ag85A from *Mtb* (38) (see also sequence alignment). We characterized the H-2^b restricted immunodominant T-cell epitopes, using synthetic 20-mer peptides spanning the entire mature sequence of Ag85A from *M. ulcerans* and from *Mtb* (39). *M. ulcerans* DNA vaccinated mice reacted against *M. ulcerans* peptides both from the NH_2-terminal and COOH-terminal part of the protein, whereas *Mtb* DNA vaccinated mice reacted almost exclusively against *M. ulcerans* peptide spanning aa 240–259, albeit that its sequence is quite different from that of *Mtb*. *M. ulcerans* DNA vaccinated mice also recognized this peptide very effectively. Responses against the NH_2-terminal peptides spanning aa 61–80 and 81–100 of *M. ulcerans* were only observed in *M. ulcerans* DNA vaccinated mice, indicating that this NH_2-terminal region was responsible for a partial species-specificity.

T-CELL EPITOPES OF Ag85 OF *M. AVIUM* SUBSP. *PARATUBERCULOSIS (Map)*

The genes encoding the three Ag85 components from *M. avium* subsp. *paratuberculosis* (*Map)* have been sequenced, and at the protein level, a 99% sequence identity with *M. avium* subsp. *avium (Maa)* was found, with a single amino-acid residue difference for each protein: Ag85A: Ser155Pro, Ag85B: Ser120Asn, and Ag85C: Ileu284Thr for *Map* vs. *Maa.* Compared to the mature protein sequences of *M. bovis*, *Map*85A (Map 0216) shares 82%, *Map*85B (Map 1609c) shares 86%, and the *Map*85C (Map 3531c) shares 87% identity (40). *Map* ATCC 19698 was adapted to grow as a surface pellicle on synthetic, protein-free Sauton medium supplemented with mycobactin J. Comparison of the 4-week-old *Map* CF with the protein profile of a 2-week-old *Mtb* H37Rv CF by SDS-PAGE indicated that in the region of the Ag85 complex, only one protein of approximately 30 kDa (presumably Map1609c) was strongly expressed in *Map* CF (41).

Cross-reactive $CD4^+$ epitopes of Ag85A, Ag85B, and Ag85C of *Mtb* were identified in H-2^b mice intravenously infected with *Map* ATCC 19698 (**Figure 3**) (41). Spleen cells from susceptible *Map* infected $B6^{bg/bg}$ mice reacted against peptides of Ag85A and Ag85B from *Mtb*. These epitopes were the same as those we have previously identified in B6 mice infected with *Mtb* or vaccinated with DNA encoding the *Mtb* Ag85 components (13). Peptides from Ag85C were more weakly recognized by spleen cells from *Map* infected mice.

Cross-reactive $CD4^+$ epitopes of Ag85A, Ag85B, and Ag85C were also identified in C57BL/6 mice vaccinated with plasmid DNA encoding the *Map* antigens (42). Plasmid DNA encoding the *Map*Ag85A component induced the strongest IFN-γ response,

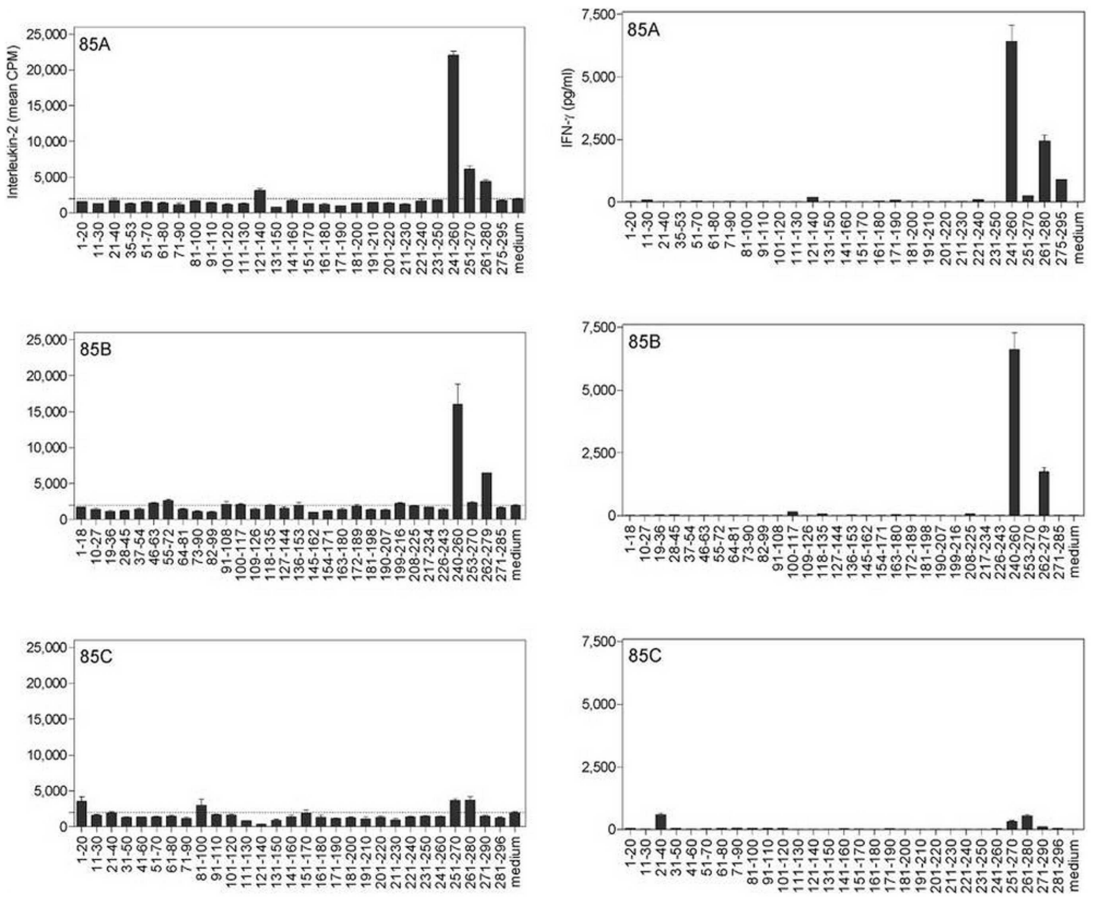


FIGURE 3 | Characterization of cross-reactive T-cell epitopes of Ag85A, Ag85B and Ag85C in *M. avium* subsp. *paratuberculosis*-infected B6$^{bg/bg}$ mice, using synthetic peptides spanning the mature sequences of Ag85A, Ag85B, and Ag85C from *Mtb*. IL-2 (left) and IFN-γ (right) production was analyzed in 24 and 72-h culture supernatants, respectively, of spleen cells from a pool of five animals infected intravenously with 3×10^5 CFU of *Map* and stimulated with overlapping synthetic peptides (10 μg/ml). Reproduced from Ref. (41).

whereas DNA encoding *Map*Ag85B and *Map*Ag85C induced about 10-fold lower titers. More or less the same epitopes were recognized by pDNA vaccinated as by *Map* infected mice. However, and in contrast to infection, peptide 27 of Ag85A or peptide 27 from Ag85C were not recognized in mice vaccinated with the respective DNAs. On the other hand, three new epitopes were identified in pDNA vaccinated mice that were not recognized in infected animals: peptide 10 (aa 91–110) on Ag85A, peptide 17 (aa 145–162) on Ag85B, and the corresponding region of Ag85C covered by peptides 15–16 (aa 141–170). The strongest Th1 epitope identified in this study spanned region 241–260 of the Ag85A and Ag85B sequence. These sequences are very similar to each other, but – as for *M. tuberculosis* – the Ag85C sequence is completely different.

Ag85 specific T-cell epitopes were also mapped in experimentally infected cattle, the target species of Johne's disease. Five 2- to 3-week-old calves were infected by the oral route with 10 mg (10^8 CFU) of *Map* (ATCC 19698) cells per day for 10 consecutive days. (41). Strong proliferative and *ex vivo* IFN-γ responses against Ag85, purified from *M. bovis* BCG CF, could be detected in cattle as early as 10 weeks after oral *Map* infection. Synthetic peptides from the Ag85A and Ag85B components of this complex were strongly recognized, whereas T-cell responses were weaker against peptides from the Ag85C protein. A promiscuous T-cell epitope spanning amino acids 145–162 of Ag85B (identical sequence in *M. bovis* and *Map*) was identified in experimentally infected cattle.

CONCLUDING REMARKS

Members of the Ag85 complex are immunodominant mycobacterial antigens, which induce strong Th1-type immune responses in situations of controlled mycobacterial infection. By virtue of their role in cell wall integrity and synthesis of cord factor, these abundantly expressed proteins have long been considered as virulence factors. On the other hand, because of the strong T-cell responses they induce, these proteins can also be regarded as a means of both host and pathogen to reach a state of equilibrium, advantageous for both parties. An estimated one third of the world population is infected with *Mtb*, an immense reservoir for this successful microbe. However, most humans latently infected with *Mtb* will never develop the active disease, precisely

because of the strong elicited T-cell response. A similar scenario holds true for infections caused by other mycobacteria such as *M. leprae* and non-tuberculous mycobacteria from the environment such as *M. ulcerans* and various *M. avium* subspecies. The MHC class I and MHC class II restricted epitopes of *Mtb*/*M. bovis* Ag85A and Ag85B have been identified in experimental animal models and in healthy Mantoux positive subjects. In humans, a small number of dominant T-cell epitopes were found to be promiscuously recognized by subjects with many different HLA haplotypes. Also in experimental mouse models (particularly of H-2^d haplotype), the same epitopes have been identified. More in particular, regions spanning aa 10–30, 60–80, 100–120, 140–160, and 199–207 of the mature Ag85A and Ag85B span these IFN-γ/IL-2 inducing Th1/CTL epitopes. The Ag85C component differs in its sequence from the two other components and overall T-cell responses against this third component are lower. The Ag85C molecules are buried more in the cell wall and hence may be less accessible for rapid antigenic processing and presentation to T-cells. It is also possible that because of this less exposed localization, there has been less evolutionary pressure on the gene of Ag85C to encode for such immunodominant, promiscuous T-cell epitopes. Members of the Ag85 complex are highly conserved in other mycobacterial species and cross-reactive T-cell responses against *Mtb* antigens can be found in *M. ulcerans*, *Map*, and *M. leprae* infection. Sequence comparisons indicate that the Ag85A sequences of these three non-tuberculous mycobacteria are more similar to the sequence of *Mtb*Ag85B than of *Mtb*Ag85A, particularly in aa stretches spanning the immunodominant epitope regions. Moreover, expression of the two components by different mycobacterial species seems to be differentially regulated, with a preference for the Ag85B orthologs in NTM.

Recently, a randomized, placebo-controlled phase 2b trial in a rural region near Cape Town, South Africa, analyzing safety and efficacy of MVA85A in infants previously vaccinated with BCG, showed that the MVA85A boost was well tolerated but induced only modest cell-mediated immune responses (lower that responses observed in previous studies in adult BCG vaccinees in United Kingdom) and furthermore did not augment protective efficacy of BCG (43). The reasons for this vaccine trial failure are not clear but conclusions as to the protective nature of Ag85A should not be taken too hastily. It is important to stress that the rationale for all BCG boosting strategies is based on the assumption that BCG-induced protection is waning in time through gradual attrition of BCG-induced T-cells. One could argue that in the Tameris study, the time between neonatal BCG vaccination and MVA85A boost was too short to measure effects of waning immunity. On the other hand, there may be other factors than just waning that are responsible for the variable efficacy of BCG and besides magnitude, the *quality* of the memory response induced by the BCG vaccine may be insufficient (44). More specifically, the BCG vaccine is a very poor inducer of $CD8^+$ T-cells, which are especially important for the control of a latent TB infection. As BCG vaccination primes almost exclusively for MHC class II restricted responses, it is obvious that boosting strategies with proteins and even with recombinant viral vectors will augment preferentially the $CD4^+$ T-cell population. Priming with plasmid DNA encoding Ag85A can increase the protective efficacy of BCG in mice as measured in a long term survival study and this increased efficacy is accompanied by increased Ag85A specific $CD8^+$ responses (45). More recently, we have shown in an experimental mouse model and also in a large mammalian species (*Sus scrofa*) that the vaccine potential of live BCG can be augmented by coadministration with plasmid DNA encoding PPE44 and Ag85A respectively, as measured by Th1-type cytokine secretion, specific IgG antibodies, as well as specific IFN-γ producing $CD8^+$ T-cells (46) (Bruffaerts et al. submitted for publication). These results have provided a proof of concept for a new TB vaccine, based on BCG-plasmid DNA combination, approach that now needs to be tested in non-human primates, the only animal species in which reactivation of latent *Mtb* infection can be monitored properly (47).

ACKNOWLEDGMENTS

I want to express my sincere gratitude to all my colleagues and collaborators with whom I had the opportunity to work during the last 25 years. A special word of thanks to Kamiel Palfliet, Fabienne Jurion, Jacqueline De Bruyn, Pascal Launois, Jean Content, Margaret Liu, Evelyne Lozes, Audrey Tanghe, Valerie Rosseels, Virginie Roupie, Sushila D'Souza, Olivier Denis, and Marta Romano.

REFERENCES

1. Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, Besra GS. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* (1997) **276**:1420–2. doi:10.1126/science.276.5317.1420
2. Naito M, Ohara N, Matsumoto S, Yamada T. The novel fibronectin-binding motif and key residues of mycobacteria. *J Biol Chem* (1998) **273**:2905–9. doi:10.1074/jbc.273.5.2905
3. Kuo CJ, Ptak CP, Hsieh CL, Akey BL, Chang YF. Elastin, a novel extracellular matrix protein adhering to mycobacterial antigen 85 complex. *J Biol Chem* (2013) **288**:3886–96. doi:10.1074/jbc.M112.415679
4. Content J, de La Cuvellerie A, De Wit L, Vincent-Levy-Frebault V, Ooms J, De Bruyn J. The genes coding for the antigen 85 complexes of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG are members of a gene family: cloning, sequence determination, and genomic organization of the gene coding for Antigen 85-C of *M. tuberculosis*. *Infect Immun* (1991) **59**:3205–12.
5. Wiker HG, Harboe M. The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. *Microbiol Rev* (1992) **56**:648–61.
6. Warrier T, Tropis M, Werngren J, Diehl A, Gengenbacher M, Schlegel B, et al. Antigen 85C inhibition restricts *Mycobacterium tuberculosis* growth through disruption of cord factor biosynthesis. *Antimicrob Agents Chemother* (2012) **56**:1735–43. doi:10.1128/AAC.05742-11
7. Lozes E, Denis O, Drowart A, Jurion F, Palfliet K, Vanonckelen A, et al. Cross-reactive immune responses against *Mycobacterium bovis* BCG in mice infected with non-tuberculous mycobacteria belonging to the MAIS-group. *Scand J Immunol* (1997) **46**:16–26. doi:10.1046/j.1365-3083.1997.d01-99.x
8. Brennan MJ, Clagett B, Fitzgerald H, Chen V, Williams A, Izzo A, et al. Preclinical evidence for implementing a prime-boost vaccine strategy for tuberculosis. *Tuberculosis (Edinb)* (2012) **30**:2811–23. doi:10.1016/j.vaccine.2012.02.036
9. Brennan MJ, Thole J. Tuberculosis vaccines: a strategic blueprint for the next decade. *Tuberculosis (Edinb)* (2013) **92**:S6–13. doi:10.1016/S1472-9792(12)70005-7
10. Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic S. Recombinant bacillus Calmette-Guérin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30 kDa major secretory protein induce greater protective immunity against tuberculosis than conventional vaccines in a highly susceptible animal model. *Proc Natl Acad Sci U S A* (2000) **97**:13853–8. doi:10.1073/pnas.250480397
11. Lee BY, Horwitz MA. T cell epitope mapping of the three most abundant extracellular proteins of *Mycobacterium tuberculosis* in outbred guinea pigs. *Infect Immun* (1999) **67**:2665–70.

12. Huygen K, Lozes E, Gilles B, Drowart A, Palfliet K, Jurion F, et al. Mapping of Th1 helper T-cell epitopes on major secreted mycobacterial antigen 85A in mice infected with live *Mycobacterium bovis* BCG. *Infect Immun* (1994) **62**:363–70.
13. D'Souza S, Rosseels V, Romano M, Tanghe A, Denis O, Jurion F, et al. Mapping of murine Th1 helper T-cell epitopes of mycolyl transferases Ag85A, Ag85B and Ag85C from *M. tuberculosis*. *Infect Immun* (2003) **71**:483–93. doi:10.1128/IAI.71.1.483-493.2003
14. Yanagisawa S, Koike M, Kariyone A, Nagai S, Takatsu K. Mapping of V beta 11+ helper T cell epitopes on mycobacterial antigen in mouse primed with *Mycobacterium tuberculosis*. *Int Immunol* (1997) **9**:227–37. doi:10.1093/intimm/9.2.227
15. Tamura T, Ariga H, Kinashi T, Uehara S, Kikuchi T, Nakada M, et al. The role of antigenic peptide in CD4+ T helper phenotype development in a T cell receptor transgenic model. *Int Immunol* (2004) **16**:1691–9. doi:10.1093/intimm/dxh170
16. Huygen K, Content J, Denis O, Montgomery DL, Yawman AM, Deck RR, et al. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nat Med* (1996) **2**:893–8. doi:10.1038/nm0896-893
17. Lozes E, Huygen K, Content J, Denis O, Montgomery DL, Yawman AM, et al. Immunogenicity and efficacy of tuberculosis DNA vaccine encoding the components of the secreted antigen 85 complex. *Vaccine* (1997) **15**:830–3. doi:10.1016/S0264-410X(96)00274-5
18. Denis O, Tanghe A, Palfliet K, Jurion F, Van den Berg TP, Vanonckelen A, et al. Vaccination with plasmid DNA encoding mycobacterial antigen 85A stimulates a CD4+ and CD8+ T-cell epitopic repertoire broader than that stimulated by *Mycobacterium tuberculosis* H37Rv infection. *Infect Immun* (1998) **66**:1527–33.
19. Feller DC, de La Cruz VF. Identifying antigenic T-cell sites. *Nature* (1991) **349**:720–1. doi:10.1038/349720a0
20. McShane H, Behboudi S, Goonetilleke N, Brookes R, Hill AVS. Protective immunity against *M. tuberculosis* induced by dendritic cells pulsed with both CD8+ and CD4+ T cell epitopes from Antigen 85A. *Infect Immun* (2002) **70**:1623–6. doi:10.1128/IAI.70.3.1623-1626.2002
21. Wang J, Thorson L, Stokes RW, Santosuosso M, Huygen K, Zganiacz A, et al. Single mucosal, but not parenteral immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. *J Immunol* (2004) **173**:6357–65. doi:10.4049/jimmunol.173.10.6357
22. Huygen K, Van Vooren JP, Turneer M, Bosmans R, Dierckx P, De Bruyn J. Specific lymphoproliferation, gamma interferon production, and serum immunoglobulin G directed against a purified 32 kDa mycobacterial protein antigen (P32) in patients with active tuberculosis. *Scand J Immunol* (1988) **27**:187–94. doi:10.1111/j.1365-3083.1988.tb02338.x
23. Launois P, Deleys R, N'Diaye Niang M, Drowart A, Andrien M, Dierckx P, et al. T cell epitope mapping of the major secreted mycobacterial antigen Ag85A in tuberculosis and leprosy. *Infect Immun* (1994) **62**:3679–87.
24. Mustafa AS, Shaban FA, Abal AT, Al-Attiyah R, Wiker HG, Lundin K, et al. Identification and HLA restriction of naturally derived Th1-cell epitopes from the secreted *Mycobacterium tuberculosis* antigen 85B recognized by antigen-specific human CD4+ T-cell lines. *Infect Immun* (2000) **68**:3933–40. doi:10.1128/IAI.68.7.3933-3940.2000
25. Valle MT, Megiovanni AM, Merlo A, Li Pira G, Bottone L, Angelini G, et al. Epitope focus, clonal composition and Th1 phenotype of the human CD4 response to the secretory mycobacterial antigen Ag85. *Clin Exp Immunol* (2001) **123**:226–32. doi:10.1046/j.1365-2249.2001.01450.x
26. Silver RF, Wallis RS, Ellner JJ. Mapping of T cell epitopes of the 30-kDa a antigen of *Mycobacterium bovis* strain Bacillus Calmette-Guérin in purified protein derivative (PPD)-positive individuals. *J Immunol* (1995) **154**:4665–74.
27. Geluk A, Taneja V, van Meijgaarden KE, Zanelli E, Abou-Zeid C, Thole JE, et al. Identification of HLA class II-restricted determinants of *Mycobacterium tuberculosis*-derived proteins by using HLA-transgenic, class II-deficient mice. *Proc Natl Acad Sci U S A* (1998) **95**:10797–802. doi:10.1073/pnas.95.18.10797
28. Lindestam Arlehamn CS, Gerasimova A, Mele F, Henderson R, Swann J, Greenbaum JA, et al. Memory T cells in latent *Mycobacterium tuberculosis* infection are directed against three antigenic islands and largely contained in a $CXCR3^+CCR6^+$ Th1 subset. *PLoS Pathog* (2013) **9**:e1003130. doi:10.1371/journal.ppat.1003130
29. Klein MR, Smith SM, Hammond AS, Ogg GS, King AS, Vekemans J, et al. HLA-B*35-restricted CD8 T cell epitopes in the antigen 85 complex of *Mycobacterium tuberculosis*. *J Infect Dis* (2001) **183**:928–34. doi:10.1086/319267
30. Bartek IL, Rutherford R, Gruppo V, Morton RA, Morris RP, Klein MR, et al. The DosR regulon of *M. tuberculosis* and antibacterial tolerance. *Tuberculosis (Edinb)* (2009) **89**:310–6. doi:10.1016/j.tube.2009.06.001
31. Weichold FF, Mueller S, Kortsik C, Hitzler WE, Wulf MJ, Hone DM, et al. Impact of MHC class I alleles on the *M. tuberculosis* antigen-specific CD8+ T-cell response in patients with pulmonary tuberculosis. *Genes Immun* (2007) **8**:334–43. doi:10.1038/sj.gene.6364392
32. Roche PW, Peake PW, Billman-Jacobe H, Doran T, Britton WJ. T cell determinants and antibody binding sites on the major mycobacterial secretory protein MPB59 of *Mycobacterium bovis*. *Infect Immun* (1994) **62**:5319–26.
33. Finan C, Ota MO, Marchant A, Newport MJ. Natural variation in immune responses to neonatal *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) vaccination in a cohort of gambian infants. *PLoS One* (2008) **3**:e3485. doi:10.1371/journal.pone.0003485
34. Geluk A, van Meijgaarden KE, Franken KLMC, Drijfhout JW, D'Souza S, Necker A, et al. Identification of major epitopes of *Mycobacterium tuberculosis* Ag85B that are recognized by HLA-A*0201 restricted CD8+ T cells in HLA-transgenic mice and humans. *J Immunol* (2000) **165**:6463–71. doi:10.4049/jimmunol.165.11.6463
35. Smith SM, Brooks R, Klein MR, Malin AS, Lukey PT, King AS, et al. Human CD8+ CTL specific for the mycobacterial major secreted antigen 85A. *J Immunol* (2000) **165**:7088–95. doi:10.4049/jimmunol.165.12.7088
36. Pönnighaus JM, Fine PEM, Sterne JAC, Wilson RJ, Msosa E, Gruer PJK, et al. Efficacy of BCG vaccine against leprosy and tuberculosis in northern Malawi. *Lancet* (1992) **339**:636–9. doi:10.1016/0140-6736(92)90794-4
37. Meister GE, Roberts CGP, Berzofsky JA, DeGroot AS. Two novel T cell epitope predicted algorithm based on MHC-binding motifs: comparison of predicted and published epitopes from *Mycobacterium tuberculosis* and HIV protein sequences. *Vaccine* (1995) **13**:581–91. doi:10.1016/0264-410X(94)00014-E
38. Tanghe A, Content J, Van Vooren JP, Portaels F, Huygen K. Protective efficacy of a DNA vaccine encoding Ag85A from *M. bovis* BCG against Buruli ulcer. *Infect Immun* (2001) **69**:5403–11. doi:10.1128/IAI.69.9.5403-5411.2001
39. Tanghe A, Danzy JP, Pluschke G, Huygen K. Improved protective efficacy of a species-specific DNA vaccine encoding mycolyl-transferase Ag85A from *Mycobacterium ulcerans* by homologous protein boosting. *PLoS Negl Trop Dis* (2008) **2**:e199. doi:10.1371/journal.pntd.0000199
40. Dheenadhayalan V, Shin KS, Chang CF, Chang CD, Wang SJ, McDonough P, et al. Cloning and characterization of the genes coding for Antigen 85A, 85B and 85C of *Mycobacterium avium* subsp. paratuberculosis. *DNA Seq* (2002) **13**:287–94. doi:10.1080/1042517021000019269
41. Rosseels V, Marché S, Roupie V, Govaerts M, Godfroid J, Walravens K, et al. Members of the 30- to 32-kDa mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. paratuberculosis are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. *Infect Immun* (2006) **74**:202–12. doi:10.1128/IAI.74.1.202-212.2006
42. Rosseels V, Scanlan V, Vanonckelen A, Jurion F, Palfliet K, Marché S, et al. Development of a plasmid DNA based *M. paratuberculosis* vaccine encoding immunodominant T cell antigens identified in mycobacterial culture filtrate. Seventh International Colloquium on Paratuberculosis International Association for Paratuberculosis, Madison, WI (2003). p. 108–13.
43. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomized, placebo-controlled phase 2b trial. *Lancet* (2013) **381**:1021–8. doi:10.1016/S0140-6736(13)60177-4
44. Romano M, Huygen K. An update on vaccines for tuberculosis-there is more to it than just waning of BCG efficacy with time. *Expert Opinion Biol Ther* (2012) **12**:1601–10. doi:10.1517/14712598.2012.721768
45. Romano M, D'Souza S, Adnet PY, Laali R, Jurion F, Palfliet K, et al. Priming but not boosting with plasmid DNA encoding mycolyl-transferase Ag85A from *M. tuberculosis* increases the survival time of *M. bovis* BCG vaccinated mice against low dose intravenous challenge with *M. tuberculosis* H37Rv. *Vaccine* (2006) **24**:3353–64. doi:10.1016/j.vaccine.2005.12.066
46. Bruffaerts N, Romano M, Denis O, Jurion F, Huygen K. Increasing the vaccine potential of live *M. bovis* BCG by coadministration with plasmid DNA encoding a tuberculosis prototype antigen. *Vaccine* (2014) **2**:181–95. doi:10.3390/vaccines2010181

47. Lin PL, Rodgers M, Smith LR, Bigbee M, Myers A, Bigbee C, et al. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infect Immun* (2009) **77**:4631–42. doi:10.1128/IAI.00592-09

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 March 2014; accepted: 25 June 2014; published online: 09 July 2014.

*Citation: Huygen K (2014) The immunodominant T-cell epitopes of the mycolyl-transferases of the antigen 85 complex of M. tuberculosis. Front. Immunol. **5**:321. doi: 10.3389/fimmu.2014.00321*

This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 23 April 2014
doi: 10.3389/fimmu.2014.00188

Role of fused *Mycobacterium tuberculosis* immunogens and adjuvants in modern tuberculosis vaccines

Ana Paula Junqueira-Kipnis*, Lázaro Moreira Marques Neto and André Kipnis

Department of Microbiology, Immunology, Pathology and Parasitology, Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, Brazil

Edited by:
Tom H. M. Ottenhoff, Leiden University Medical Center, Netherlands

Reviewed by:
Christian Bogdan, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany; Universitätsklinikum Erlangen, Germany
George W. Anderson, Lawrence Livermore National Laboratory, USA

****Correspondence:***
Ana Paula Junqueira-Kipnis, Laboratory of Immunopathology of Infectious Diseases, Department of Microbiology, Immunology, Pathology and Parasitology, Institute of Tropical Pathology and Public Health, Federal University of Goiás, Rua 235 esquina com Primeira Avenida, Setor Universitário, Goiânia, Goiás 74605-050, Brazil
e-mail: apkipnis@gmail.com

Several approaches have been developed to improve or replace the only available vaccine for tuberculosis (TB), BCG (Bacille Calmette Guerin). The development of subunit protein vaccines is a promising strategy because it combines specificity and safety. In addition, subunit protein vaccines can be designed to have selected immune epitopes associated with immunomodulating components to drive the appropriate immune response. However, the limited antigens present in subunit vaccines reduce their capacity to stimulate a complete immune response compared with vaccines composed of live attenuated or killed microorganisms. This deficiency can be compensated by the incorporation of adjuvants in the vaccine formulation. The fusion of adjuvants with *Mycobacterium tuberculosis* (*Mtb*) proteins or immune epitopes has the potential to become the new frontier in the TB vaccine development field. Researchers have addressed this approach by fusing the immune epitopes of their vaccines with molecules such as interleukins, lipids, lipoproteins, and immune stimulatory peptides, which have the potential to enhance the immune response. The fused molecules are being tested as subunit vaccines alone or within live attenuated vector contexts. Therefore, the objectives of this review are to discuss the association of *Mtb* fusion proteins with adjuvants; *Mtb* immunogens fused with adjuvants; and cytokine fusion with *Mtb* proteins and live recombinant vectors expressing cytokines. The incorporation of adjuvant molecules in a vaccine can be complex, and developing a stable fusion with proteins is a challenging task. Overall, the fusion of adjuvants with *Mtb* epitopes, despite the limited number of studies, is a promising field in vaccine development.

Keywords: fusion, adjuvant, peptides, protection

INTRODUCTION

It is undeniable that vaccination is the best strategy available to efficiently control infectious diseases. For instance, the eradication of several infectious diseases concomitant to the lowering of morbidity and mortality rate of others can currently only be achieved by vaccination strategies. However, in the case of tuberculosis (TB), the development of a vaccine (*Mycobacterium bovis* BCG) did not have the capacity to eradicate the illness, and it persists as the second leading cause of deaths by infectious diseases, behind only AIDS (1, 2). Consequently, efforts have been made to develop vaccines that will improve or replace BCG, with the capacity to avoid infection and prevent the development of any of its disease forms and that is safe among immunocompromised individuals and capable of eliciting a protective immune response by several cellular populations (3).

Among the most promising strategies are the protein subunit vaccines that present desirable qualities for a vaccine, which are specificity, safety, and easy production (4). Protein subunit vaccines have been shown to induce a Th1 immune response, which is classically the response primarily associated with protection against TB. Such a response is characterized by the production of cytokines such as gamma interferon (IFN-γ), which is responsible for macrophage activation; tumor necrosis factor alpha (TNF-α), which is important for granuloma development and maintenance; and interleukin 2 (IL-2), which is responsible for the clonal expansion of T lymphocytes and is thus involved in immune response maintenance (5, 6). Due to these characteristics, several protein subunit vaccines are currently in advanced clinical trials (3, 7).

The selection of protein subunit vaccine components is based on the knowledge of which of the microorganism's molecules are capable of eliciting a protective immune response (8). Therefore, due to the high level of complexity involved in the interaction between *Mycobacterium tuberculosis* (*Mtb*) and its host, the understanding of the bacteria's immunogenic repertoire is of utmost importance for the development of an efficient vaccine. Antigens that are recognized by the host cells during active TB, when the *bacilli* are replicating, or during latent infection as well as those involved in the immunologic evasion mechanisms or the elicitation of CD4+ and CD8+ specific T cells are potential targets for immunologically controlling infections (3, 7). As the selection of potential proteins is not easy due to the vast number of MHC polymorphisms, it is necessary to select or design proteins that present promiscuous epitopes (9). Thus, the capacity of a single protein to induce an efficient immune response is inferior to other vaccine strategies (e.g., attenuated and viral vector vaccines), and their utilization is strictly associated with the use of adjuvants and immunomodulators (4, 10, 11).

Adjuvants, in the context of vaccines, are defined as components capable of enhancing and/or shaping antigen-specific immune responses (12). They can be divided into two classes: vehicles, which present vaccine antigens to the immune system in a more efficient way and control the release and storage of antigens to increase the specific immune response; immunostimulants, which affect the immune system and increase the immune responses to antigens (13). An adjuvant to be used in a vaccine against TB must have the capacity to support the generation of a robust and lasting Th1 type response. Few adjuvants are licensed to use in human vaccines, and the majority of them are poor inducers of Th1 type responses (squalene-based emulsions and aluminum-based salts). Currently, several investigations have been conducted with the objective of developing new adjuvants, many of which have searched for adjuvants that are capable of eliciting a Th1 immune response. One of these approaches is the incorporation of molecules that are capable of interaction with the pattern recognition receptors (PRRs) used by the innate immune system to recognize pathogen-associated molecular patterns (PAMPs), which are molecules or motifs that are conserved and present exclusively among pathogens (14).

Given the distinct biochemical properties of PAMPs (peptidoglycan, flagellin, lipopolysaccharide, teichoic and lipoteichoic acids, mannose residues, CpG DNA, and single-stranded RNA, among others), several types of receptors have been described [toll-like receptors (TLRs), RIG-1, NOD, and scavengers for example]. The TLR family is the most abundant and diversified and present on antigen-presenting cells (APCs) and many other cell types not related to the immune system. Signaling through TLRs can result in two possible cascades: the first is dependent on the molecule MYD88 (for myeloid differentiation factor 88) and related to TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9; the second is dependent on TIF (TIR-domain-containing adaptor-inducing interferon-β) and is associated with TLR3 and TLR4 (15). The recognition of PAMPs by TLRs can result in the expression of co-stimulatory molecules such as CD40, CD80, and CD86 as well as the expression of pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, TNF-α, COX-2, and type 1 interferons) that collectively are related to the development of an adaptive immune response by both B and T lymphocytes (16) (**Figure 1**).

To improve the number of epitopes associated with a specific vaccine, fusions of different proteins are being developed. Vaccines based on fusion proteins have specific bacterial protein antigens comprised of proteins with better immunogenic capacities than vaccines composed of a single protein. However, this approach is not sufficient to induce a desired immune response, and several molecules with potential immunogenic properties are needed in association with fusion proteins. In this regard, the fusion of *Mtb* antigens to PAMPs (17–20), damage-associated molecular patterns (DAMPs) (21), and/or to interleukins [**Table 1**; Ref. (22–26)] enhances the innate immune system response, increases the capacity of the antigen to stimulate higher production of cytokines and chemokines, and elicits distinct cell populations that will aid in the defense against the bacteria (**Table 1**). This review covers publications that used different strategies to fuse *Mtb* antigens with adjuvants or with molecules with the capacity to interact and stimulate the immune system, addressing their immunogenicity and protection outcomes in vaccine models.

ASSOCIATION OF *Mtb* FUSION PROTEINS WITH ADJUVANTS

Some protein subunit vaccines against TB that are currently in clinical trials also use adjuvant molecules that are TLR agonists. The fusion Mtb72 [a protein fusion containing the antigens Mtb32 (Rv1196) and Mtb39 (Rv0125)] uses the adjuvants AS01B™(a liposomal formulation) and AS02A™(an oil-in-water emulsion) was developed by GlaxoSmithKline (GSK). The abovementioned adjuvants are composed of MPL (3-deacylated monophosphoryl lipid A) and the detergent QS-21. MPL is a detoxified derivative of lipid A from the Gram-negative bacteria *Salmonella minnesota* R595 LPS, while QS-21 (fraction 1) is a substance purified and fractionated from the bark of the South American tree *Quillaja saponaria*. The known action of MPL is through TLR4, whereas QS-21 has no related TLR agonistic action (27). In animal studies (mice and guinea pigs), vaccination with Mtb72F and the adjuvant AS01B proved to be protective, with strong induction of antibodies (IgG1 and IgG2) and enhanced production of IFN-γ by CD4+ T cells and cytotoxic activity by CD8+ T cells. Although the vaccine formulation using AS02A induced weaker immune responses, they were able to diminish the *Mtb* bacillary load in mice. In clinical trials that compared M72 (Mtb72f with three point mutations aiming to improve antigen processing and enhance protein expression) in combination with AS01B or AS02A, both vaccine formulations were shown to be safe and immunogenic, with memory cell generation (persistency was followed for 3 years) and the production of cytokines protective against *Mtb* by several cell populations, generating similar immune responses (28–30).

Two other fusion proteins (Hybrid 4 and Hybrid 56), which are currently in clinical trials, are combined with the adjuvant IC31™. This adjuvant is made of two components, a TLR9 agonist (the oligodeoxynucleotide ODN1a) and an artificial antimicrobial cationic peptide (KLKL5KLK), which serves as a vehicle. Its mechanism of action is related to TLR activation within endosomes, and as such, IC31 is a good adjuvant for use in vaccines against intracellular microorganisms. This adjuvant was shown in several animal models to aid the skewing of the immune response toward Th1 and Th17, which is most likely associated with its adjuvant effect on dendritic cells, enhancing the expression of co-stimulatory molecules (CD80, CD86, and CD40) and the expression of IL-12p40 (31, 32).

The vaccine ID93, created by the fusion of epitopes from Rv3619, Rv1813, Rv3620, and Rv2608 and proposed to improve TB prophylaxis, includes GLA-SE [a glucopyranosyl lipid (TLR4 agonist) in a stable emulsion] as an adjuvant. Baldwin et al. (33) reported that protection was associated with strong stimulation of Th1 type immune responses, with an increase in polyfunctional cells (producing IL-2, TNF-α, and IFN-γ). This vaccine approach was shown to boost BCG protection and diminish multi-drug resistant *Mtb* infection in mice, guinea pigs, and cynomolgus monkeys (34). A peptide fusion associated with a strong adjuvant that is only mixed with the recombinant protein just before the injection might be a stimulator of the BCG immune response elicited during childhood. To select the best adjuvant to combine with ID-93-GLA, different formulations including this TLR4 agonist

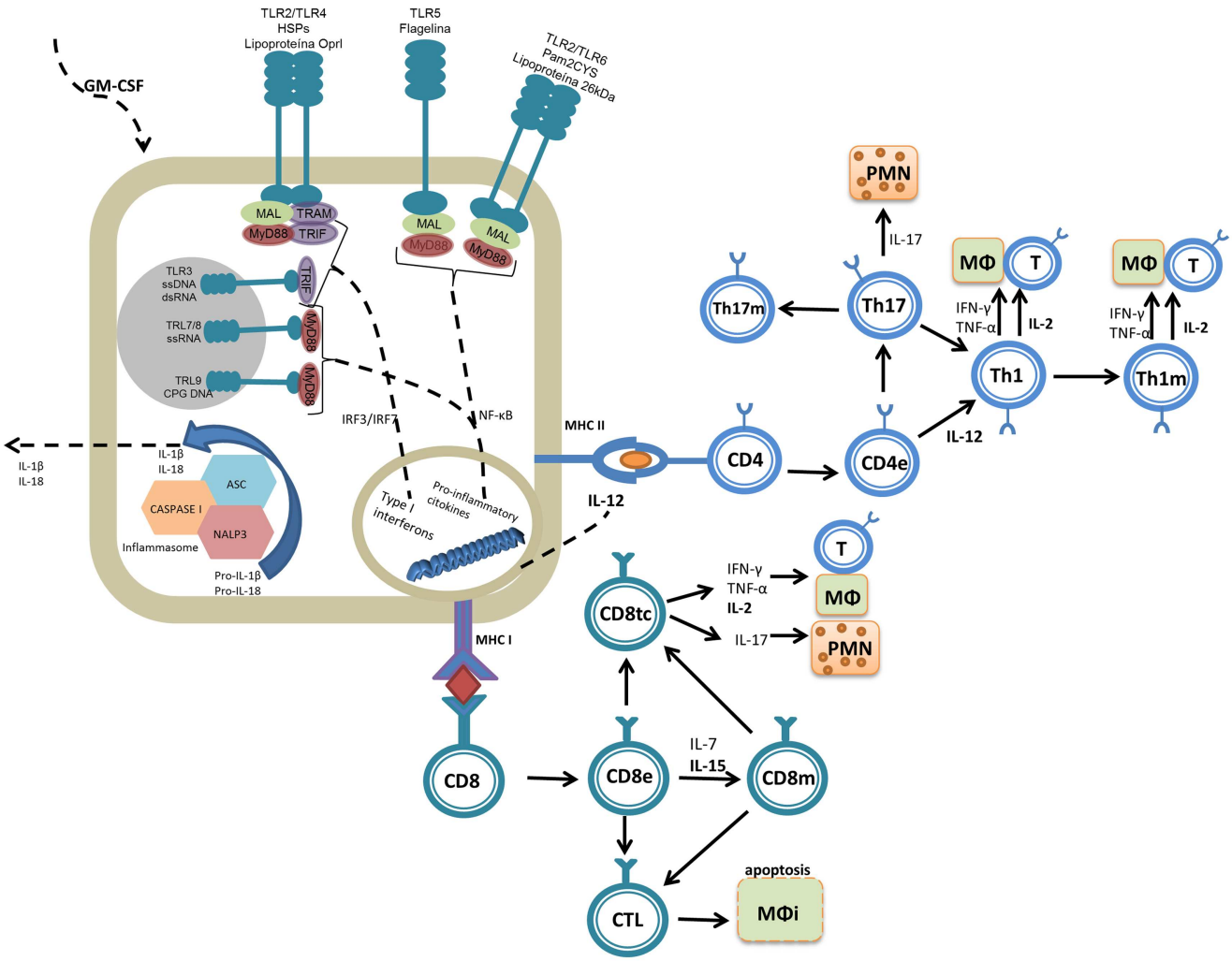


FIGURE 1 | Molecular mechanisms of the adjuvant molecules reviewed. Adjuvants are molecules that promote inflammatory reactions, interacting with the innate immune system and assisting in the generation of adaptive immune responses. PAMPs interact primarily with TLR2 (lipoproteins and Pam2Cys) and TLR5 (flagellin). DAMPs (e.g., Hsp70) can be recognized by many molecules of the immune system, but the innate immune system interacts with Hsp70 primarily through TLR4. Adjuvants lead to the generation of intracellular signaling cascades (dependent on MyD88 and TRIF) that culminate with the production of pro-inflammatory cytokines. The cytokines then act as the third signal, aiding the development of the adaptive immune response in combination with the presentation of the fused antigen (first signal). Effector (e) and memory (m) cells are generated upon antigen presentation. Some cytokines induced by the adjuvants or used as adjuvants, such as IL-2, IL-12, IL-15, and GM-CSF, are important for activation of the protective immune response or to maintain a long-lasting immune response (memory). Among the T cell subsets induced during vaccination, Th1 cells (which produce IL-2, IFN-γ, and TNF-α) can stimulate macrophages (MΦ) and Th17 cells (which produce IL-17) activate primarily polymorphonuclear cells (PMNs), and these have been directly associated with protection against *Mtb*. Some adjuvants also induce antigen presentation via MHC-I and thus activate CD8+ T cells, which differentiate into cytotoxic cells (CD8tc) and act on infected (MΦi) or effector cells (CD8e) to secrete cytokines and differentiate into memory cells (CD8m).

have been tested, including water with aluminum salts, emulsions, and liposomes. The best formulation was prepared in an aqueous nanosuspension containing alum (35). However, further studies should be conducted to best define the adjuvant associated with protective immune responses and the eradication of the *bacilli* from the host.

Although not an adjuvant currently in clinical trials, TDB (trehalose-6,6-dibehenate) has drawn attention because it is a less toxic analog of TDM (trehalose-6,6-dimycolate), a critical component of the cell wall of *Mtb*. TDB arose from changes in TDM, also known as cord factor, which is a potent inducer of Th1 type responses with restricted use in humans because of its toxicity. The effect of TDB was evaluated with the H1 fusion protein (Ag85B-ESAT-6) and shown to be a powerful aid in stimulating the cellular response of Th1 and Th17 populations as well as humoral immune responses (36, 37). The Mincle receptor (a C-type lectin) is responsible for the recognition of TDB (38) and TDM (39). It has been demonstrated that the association of Mincle–Fcrγ–Syk–CARD9 is involved in the response to TDM/TDB to generate Th1 and

Table 1 | Reviewed studies that evaluated fusions of *M. tuberculosis* antigens with adjuvant molecules: molecules used and the generated immune response.

Reference	Antigen	Adjuvant	Elicited response					
			Antibodies	Cytokines	CD4+	CD8+	Memory	Protection[a]
(18)	ESAT-6	26 kDa lipoprotein	N	IL-12p40 ↑	IFNγ ↑	N	N	=
(20)	$HspX_{91–110}$	Pam2Cys	N	IL-6 ↑, IL-12 ↑, IFNγ ↑	CD4+ IFNγ+ ↑	N	$CD44^{hi}$ $CD62^{hi}$ ↑ $CD44^{hi}$ $CD62^{lo}$ ↑	↑
(17)	Ag85A	OprI lipoprotein	IgG ↑, IgG2a ↑	IL-2 ↑, IFNγ ↑, IL-10 ↑	N	N	N	=
(19)	PPE 27 kDa	Flagellin	IgG1 ↑, IgG2a ↑, IgG2b ↑	IFNγ ↑	N	N	N	N
(21)	ESAT-6	C-terminal Hsp70	IgG ↑	IFNγ ↑, IL-4 ↑	N	N	N	N
(24)	Hsp65	IL-2	IgG ↑	IFNγ ↑, IL-2 ↑	N	CTL ↑	N	=
(22)	ESAT-6	IL-2	IgG ↑	IFN-γ ↑	N	CTL ↑	N	N
(25)	ESAT-6	IL-12p70	IgG ↑, IgG1 ↑, IgG2a ↑	IFN-γ ↑	CD4+↑	CD8+↑	N	↓
(23)	Ag85B	IL-15	N	IFN-γ ↑	CD4+ IFNγ+ ↑ CD4+ CD44+↑	CD8+ IFNγ+ ↑ CD8+ CD44+↑	CD4+ CD44+ CD62+↑, CD8+ CD44+ CD62+↑	↑
(26)	ESAT-6	GM-CSF	IgG ↑, IgG1 ↑, IgG2a ↑[1]	IFN-γ ↑, GM-CSF ↑	CD4+ ↑	CD8+ ↑	N	N

[a] *Protection compared to BCG.*

N, not evaluated; =, equal, ↑, higher; ↓, lower.

Th17 type immune responses in addition to activating the Nlrp3 inflammasome, inducing the production of IL-1β (40, 41).

Mtb IMMUNOGENS FUSED WITH ADJUVANTS

The cell wall of *Mtb* is a great source of PAMPs, as TLR2 is responsible for recognizing most of the mycobacterial lipid antigens such as lipoproteins, lipoarabinomannan (LAM), and other glycolipids. In addition to its pro-inflammatory action, TLR2 activation can result in the production of cathelicidin, a peptide with microbicidal function that also acts against intracellular bacteria (42, 43). To effectively recognize these molecules, TLR2 forms heterodimers with TLR1 and TLR6. Normally diacetylated molecules are recognized by the TLR2/TLR6 heterodimer, while triacetylated molecules (the majority are found in Gram-negative bacteria and mycobacteria) and LAM are recognized by the TLR2/TLR1 heterodimer. However, this recognition scheme is not mandatory because the recognition of lipoproteins depends both on their acetylation as well as on the peptide chain (44–46). In addition, the recognition of TLR2 agonists is also affected by accessory molecules, including CD14, CD36, lipopolysaccharide-binding protein, and others (42, 47, 48).

One of the molecules capable of interacting with TLR2 is the 26 kDa lipoprotein (Rv1411) whose recognition is related to increased production of IL-12, which in turn stimulates T lymphocytes and NK cells to produce and secrete IFN-γ, the most important inducer of reactive oxygen and nitrogen species, which are key effector molecules to kill the *bacilli* (49) (**Figure 1**). To take advantage of the Th1 immune response-boosting effects provided by the 26 kDa lipoprotein, this protein was fused to another immunodominant *Mtb* antigen, ESAT-6, to form the fusion protein CSU-F36. This recombinant fusion protein was produced in *Mycobacterium smegmatis*, a non-pathogenic mycobacterial species that is capable of glycosylation and acylation, allowing the molecule to be recognized as a PAMP and to correctly interact with TLR2. This protein fusion was tested in C57BL/6 mice and proved to be capable of inducing strong IL-12 p40 expression, stimulating CD4+ IFN-γ+ lymphocytes and inducing protection similar to BCG in a murine model of infection [**Table 1**; Ref. (18)].

Another TLR2 agonist, S-[2,3-bis(palmitoyloxy)propyl]-cysteine (Pam2Cys), has also presented favorable characteristics for a fusion vaccine against TB. Pam2Cys is a potent dendritic cell stimulator and has the ability to induce antigen cross-presentation. This synthetic molecule was derived from mycoplasma MALP-2 (macrophage-activating lipopeptide) (50). When a promiscuous peptide (capable of binding to several MHC clefts) from the 16 kDa heat shock protein (HSP) (amino acids 91–110) was fused to

Pam2Cys to generate L91, it was observed that this fusion was able to induce dendritic cell maturation by enhancing the expression of the co-stimulatory molecules CD40, CD80, and CD86 and by stimulating the production of IL-6 and IL-12. The immunization of mice with L91 was capable of inducing a strong Th1 response, as evidenced by the increase of IFN-γ-producing CD4+ T cells as well as memory $CD44^{hi}$ $CD62L^{hi}$ (central memory) and $CD44^{hi}$ $CD62L^{lo}$ (effector memory) CD4+ T cells. Last, L91 was more capable than BCG of protecting vaccinated guinea pigs from *Mtb* challenge by reducing the bacterial load and the pathology [**Table 1**; Ref. (20)].

The outer membrane lipoprotein (OprI) of *Pseudomonas aeruginosa* can bind to TLR3/TLR4, and it has been demonstrated to be capable of supporting a strong Th1 response with the production of IFN-γ and TNF-α as well as the induction of IgG2a in a *Leishmania major* model of infection (51). *P. aeruginosa* OprI was fused to antigen 85A (Ag85A) from *Mtb* and introduced in *Escherichia coli*, which has the machinery necessary to N-acylate OprI. This fusion was used in a prime-boost strategy following vaccination with Ag85A DNA or BCG. The intranasal administration of the fusion with the DNA vaccine enhanced the immune response by potentiating the production of antibodies against Ag85A and enhancing IL-2- and IFN-γ-producing cells, but it was not able to improve its protective capacity. Similarly, the OprI-Ag85A fusion improved the Th1 response induced by BCG, but it was unable to increase its protective efficacy [**Table 1**; Ref. (17)].

Flagellin is another PAMP with potential to be used in fusions with *Mtb* antigens. It is recognized by TLR5, a PRR that when stimulated, induces pro-inflammatory responses as well as the maturation of APCs, boosting their capacity to activate naïve T cells to which the cells will present their antigen, thus potentiating the generation of an efficient adaptive response (52). In addition, flagellin may also be recognized by other types of PRRs, such as the Nod-like receptor family CARD 4 (NLRC4) and NIP5 (neuronal apoptosis inhibitory protein 5) (53). The protein p27, a PPE family protein from *Mtb*, was fused to the flagellin of *E. coli*. The recombinant *E. coli* expressing p27 in its flagellum was tested in BALB/c mice and compared with other vaccine strategies, including a DNA vaccine, vaccination with purified p27 and Freund's adjuvant, and vaccination using a purified protein and CpG DNA. Immunization with recombinant bacteria induced strong splenocyte proliferation as well as higher induction of IFN-γ [**Table 1**; Ref. (19)].

Evidently, the fusion of *Mtb* antigens with adjuvant molecules that are agonists of TLR2 and TLR5 can boost the Th1 response. However, with the exception of L91, the fusions with TLR2 agonists were incapable of generating a protective response superior to that provided by BCG. In this regard, as described by McBride et al. (43), mice deficient for TLR2 were not more susceptible to *Mtb* when compared with wild-type mice. Another important aspect of the immune response induced by a vaccine is long-lasting protection that is provided by the generation of memory cells. McBride et al. (43) also evaluated the ability of mice that lacked TLR2 to produce memory cells against TB, and despite the large number of bacterial antigens that are agonists of this particular TLR, its absence did not prevent or hinder the generation of this cell population. However, the fusion of *Mtb* proteins with PAMPs may result in conformational changes that diminish the association with its cognate PRR, reducing the expected immunological response. Furthermore, the use of PAMPs must be considered carefully, as the immune response induced against the adjuvant fused to *Mtb* immune epitopes may result in an enhanced and deleterious immune response upon infection with *Mtb* or other pathogens expressing those PAMPs.

In the case of flagellin use in recombinant live organisms, the strategy requires that the antigen inserted in the flagellin gene does not interfere with the final molecule structure, its ability to transport flagellin, or the final flagellum assembly, which would compromise the exposure of the molecule on the cellular surface and the desired goal (52). Live vaccines, such as those including Gram-negative bacteria, have other PAMPs (LPS that interacts with TLR4, peptidoglycan recognized by TLR2, and CpG DNA recognized by TLR9) and may induce synergistic actions among PRRs such as TLR5 activation, and overstimulation can occur, inducing a deleterious inflammatory response.

Heat shock proteins are expressed both constitutively and under stress conditions in all cells and are essential for several intracellular processes such as protein transport, protection against denaturation and aggregation, and protein folding. Microbial HSPs (mHSPs) have been described as DAMPs that are highly conserved between species and potentially immunogenic, with the ability to induce the production of cytokines and chemokines, increase the expression of co-stimulatory molecules and activate APCs (particularly dendritic cells). They have also been shown to be stimulators of both T cell-mediated and humoral immune responses (54, 55).

Hsp70 from *Mtb* consists of a 44 kDa ATPase, an 18 kDa domain that binds to substrate, and a 10 kDa C-terminal fragment (56). Studies with THP-1 cells indicate that its recognition is mediated through the interaction of the C-terminal portion and the innate system, primarily the heterodimer TLR2/TLR4 and CD14 as well as CD40 and CCR5 (57). This interaction induces the production of IL-12, TNF-α, CCL5, and reactive oxygen and nitrogen species. Hsp70 is also recognized by CD8+ T lymphocytes through CD40, inducing the production of CCL3, CCL4, and CCL5 (56, 58). Based on these properties, a fusion composed of the C-terminal portion of Hsp70 (amino acids 359–610) and the immunodominant antigen ESAT-6 was made and tested in BALB/c mice in a subcutaneous immunization protocol. This fusion vaccine induced an increase in total IgG specific for ESAT-6 as well as IFN-γ production and splenocyte proliferation [**Table 1**; Ref. (21)].

The use of HSPs as vaccines against TB is controversial, as those proteins are able to induce pro-inflammatory and modulatory responses. For instance, Hsp70 from *Mtb* has been reported to inhibit the maturation of mouse dendritic cells *in vitro* and to modulate effects capable of inducing immune tolerance to cutaneous allografts through the induction of regulatory T lymphocytes (CD4+ CD25+ Foxp3+) (59). In addition, Hsp70 has also been shown to stimulate the production of IL-10 by peripheral blood mononuclear cells from patients with arthritis (60). Consequently, despite the capacity to induce IFN-γ production, the fusion has not been tested for its capacity to generate protection against *Mtb* infection, and the production of that cytokine alone is not sufficient to correlate with protection (61).

CYTOKINE FUSION WITH *Mtb* PROTEINS AND LIVE RECOMBINANT VECTORS EXPRESSING CYTOKINES

Cytokines are molecules with biological activities that are produced by immune system cells and are responsible for cell–cell communication and the generation of a response following antigen presentation, acting as co-stimulators. Several cytokines are related to protection against *Mtb* infection, including TNF-α, IL-2, IL-6, IL-8, IL-10, and IL-17 among others, thus providing a different approach for fusion development (3, 62). The fusion of *Mtb* antigens to cytokines is aimed at not only modulating the magnitude of the response but also guiding the development of the protective immune response. However, cytokine action at the injection site is part of a complex network of signaling, and the effects of the administration of a single cytokine may not be the same as those exercised by the endogenous molecule, which acts in combination with several other molecules. Additionally, the use of recombinant cytokines can cause an imbalance of the response, and in some cases, the systemic administration of cytokines (IL-2, IL-12, TNF-α, and IFN-γ) has been associated with toxic effects to the organism. However, the short half-life of several cytokines in the circulation is also related to the inadequate adjuvant properties of some of the reported strategies (63).

IL-2 is related to the suppression of *Mtb* replication through its participation in T cell maintenance and proliferation as well as in the activation of NK and γδ T cells to produce IFN-γ. A fusion protein containing the 65 kDa HSP (Hsp65) and IL-2 was used in a vaccination scheme (DDA and MPL adjuvants) and compared to the unfused Hsp65 protein (with DDA and MPL adjuvants) or BCG. The fusion protein was shown to have a superior ability to induce the production of IFN-γ and IL-2. Vaccinated mouse splenocytes were also capable of generating superior cytotoxic activity over the P815 cell line (expressing Hsp65-hIL-12). Last, the protection conferred by the recombinant fusion protein was similar to that induced by BCG [**Table 1**; Ref. (24)]. Another fusion protein, comprising ESAT-6 and IL-2, was inserted in BCG. BALB/c mice vaccinated with the recombinant bacteria showed significantly greater induction of *ex vivo* splenocyte proliferation and IFN-γ production when stimulated with ESAT-6 and CFPs (culture filtrate proteins), greater production of total IgG against ESAT-6 and greater lymphocyte cytotoxic activity. However, despite the potential immunogenicity, the protection of the recombinant vaccine was not evaluated (22).

IL-12 has diverse biological functions, as it acts on several immune system cells. IL-12 plays roles in both the innate and adaptive immune responses through JAK-STAT signaling, leading to effector Th1 cell differentiation and IFN-γ production by CD4+, NK, and NKT cells in the initial stages of infection (**Figure 1**) (64, 65). The influence of IL-12 on CD8+ T cell differentiation has also been demonstrated through its action as the third signal (66). In addition, this cytokine acts on B cells, favoring IgG2a class switching and inhibiting IgE and IgG1 (67). A recombinant BCG containing a fusion of the IL-12p70 and ESAT-6 genes was shown to induce higher IFN-γ production than the other BCG constructs analyzed (BCG, rBCG-ESAT-6, and rBCG-IL-12), as well as higher total IgG, IgG1, and IgG2a levels. However, none of the recombinant constructs surpassed the protection induced by wild-type BCG [**Table 1**; Ref. (25)].

IL-15 is an important cytokine in the immune response of CD8+ T lymphocytes, as it is involved in clonal expansion, memory cell ($CD44^{hi}$) generation, and antigen recognition, and it further acts as a T lymphocyte chemoattractant (**Figure 1**) (68, 69). A fusion protein was designed to explore the poor CD8+ T cell stimulation capacity of BCG (70); the fusion protein comprised Ag85B and IL-15 and was expressed in BCG [**Table 1**; Ref. (23)]. The recombinant BCG was shown to enhance the control of infection, inducing total memory (CD44+) CD4+ and CD8+ T cells, and to potentiate the production of IFN-γ by both CD4 and CD8 T cells. Finally, the presence of IL-15 was capable of enhancing the protection against *Mtb* when compared with rBCG-Ag85B, with lower bacillary loads in the lungs and milder pathology (23).

Granulocyte macrophage colony-stimulating factor (GM-CSF) has several described biological effects, but from the vaccination perspective, its primary effects are enhancement of the maturation, migration, and immunostimulatory properties of Langerhans, dendritic, and NK cells; increasing MHC class II expression on APCs, which plays a fundamental role in antigen presentation to CD4 T helper cells; increasing the expression of CD80, a co-stimulatory molecule that participates in T lymphocyte activation, on Langerhans giant cells *in vitro*; and inducing local inflammation at the injection site, resulting in the accumulation of neutrophils and mononuclear cells (**Figure 1**). An important role of GM-CSF has also been demonstrated in mice lacking GM-CSF expression, which were more susceptible to *Mtb* infection (71). In one study, GM-CSF was fused to ESAT-6, and the recombinant fusion gene was inserted into BCG, creating rBCG:GE. This rBCG was tested and compared to BCG expressing either ESAT-6 (rBCG:E) or GM-CSF (rBCG:G). rBCG:GE induced higher levels of total IgG, IgG1, and IgG2a. Mice immunized with rBCG:GE also had higher levels of specific CD4+ and CD8+ T cells 8 weeks after immunization when compared with the other recombinant BCG vaccines. Last, rBCG:GE also showed the greatest capacity to stimulate IFN-γ production by the splenocytes of immunized mice. However, no protection assay was described by the authors [**Table 1**; Ref. (26)].

When using cytokines as immune stimulation components in vaccines, one expects that they will enhance the immune response due to their participation in the regulation of both innate and adaptive immune responses. In the published literature, IL-2, IL-12, IL-15, and GM-CSF have been reported as potential immune response stimulators. However, only the Hsp65 antigen fusion with IL-2 was used without being in the context of the BCG vector, and in that case, it was necessary to use a dimethyl dioctadecyl ammonium bromide (DDA) and MPL emulsion as an adjuvant to generate an efficient immune response, demonstrating the inability of a single cytokine to support an antigen-elicited response. Although fusions of *Mtb* antigens with cytokines have been shown to be immunogenic and to improve the response to BCG, only one of the studies we reviewed, using IL-15/Ag85B, induced levels of protection against bacteria that were better than those induced by the current vaccine. Additionally, two studies did not evaluate the protection against *Mtb*, which prevents a more thorough assessment of the real potential of the examined fusion vaccines because there is no consensus in the scientific community of a biomarker for protection.

Among the major obstacles faced in the search for adjuvants capable of stimulating Th1 type responses is the immunotoxicity generated by some molecules. Therefore, an adjuvant should be effective in assisting the generation of protective immune responses while inducing few side effects (72). However, the balance between effectiveness and toxicity in a vaccine for TB is complex because the protective immune response against the agent (Th1 and Th17) is highly inflammatory. Side effects related to the use of adjuvants can be divided into two major groups: local and systemic effects. The most common effects are local injection site tenderness and swelling, while the more severe reactions involve the formation of abscesses and painful nodules. With regard to systemic effects, the most common reaction is a non-specific acute phase reaction, characterized by changes in plasma proteins, fever, fatigue, and anorexia, whereas severe effects may include the generation of autoimmune diseases or worsening thereof and the appearance of neurological disorders (73). Systemic side effects related to the administration of adjuvants typically prevent their use in human vaccines, and these effects usually occur due to the hyperactivation of the immune system as a result of constant exposure to adjuvant, which causes an intense production of pro-inflammatory cytokines (IL-1, IL-6, TNF-α, IFN-γ, and others). Importantly, these effects may occur after the administration of a cytokine adjuvant or after the use of a molecule and subsequent infection by a microorganism that has the same molecule in its constitution, and therefore, such effects must be considered when choosing an adjuvant for a vaccine formulation (73, 74).

CONCLUSION

Several fusions of *Mtb* proteins or immunodominant epitopes have been evaluated as subunit vaccines for TB. Some adjuvants have been incorporated in the vaccine formulation without a physical association with the recombinant fusion proteins, while others have been incorporated in the backbone of the subunit vaccines. Other studies have evaluated recombinant BCG vaccines expressing both *Mtb* proteins and adjuvant molecules. The majority of the vaccine formulations were able to induce higher levels of Th1 and IgG2a responses, although not all of the vaccines discussed here presented better protection against *Mtb* than BCG.

The fusion of *Mtb* antigens with adjuvants can interfere with the induction of specific immune responses, but in most of the reviewed articles, the vaccine formulations did not offer improvements over the protection conferred by BCG. Thus, further studies are needed to develop an effective adjuvant with low toxicity to be used in vaccine formulations to control TB.

AUTHOR CONTRIBUTIONS

Ana Paula Junqueira-Kipnis designed the review and critically wrote and edited the manuscript. Lázaro Moreira Marques Neto critically wrote the draft. André Kipnis critically wrote the manuscript and edited the manuscript. All authors read and approved the final version of the manuscript.

REFERENCES

1. von Reyn CF. New studies of BCG: implications for tuberculosis vaccines. *Lancet Infect Dis* (2012) **12**:259–60. doi:10.1016/S1473-3099(11)70317-6
2. World Health Organization. *Global Tuberculosis Report 2012*. Geneva: WHO (2012). Available from: http://www.who.int/tb/publications/global_report/gtbr12_main.pdf
3. Orme IM. Vaccine development for tuberculosis: current progress. *Drugs* (2013) **73**:1015–24. doi:10.1007/s40265-013-0081-8
4. Moyle PM, Toth I. Modern subunit vaccines: development, components, and research opportunities. *ChemMedChem* (2013) **8**:360–76. doi:10.1002/cmdc.201200487
5. Ottenhoff TH. New pathways of protective and pathological host defense to mycobacteria. *Trends Microbiol* (2012) **20**:419–28. doi:10.1016/j.tim.2012.06.002
6. O'Garra A, Redford PS, Mcnab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. *Annu Rev Immunol* (2013) **31**:475–527. doi:10.1146/annurev-immunol-032712-095939
7. Kaufmann SH. Tuberculosis vaccines: time to think about the next generation. *Semin Immunol* (2013) **25**:172–81. doi:10.1016/j.smim.2013.04.006
8. Pulendran B, Ahmed R. Immunological mechanisms of vaccination. *Nat Immunol* (2011) **12**:509–17. doi:10.1038/ni.2039
9. Alexander J, Fikes J, Hoffman S, Franke E, Sacci J, Appella E, et al. The optimization of helper T lymphocyte (HTL) function in vaccine development. *Immunol Res* (1998) **18**:79–92. doi:10.1007/BF02788751
10. Awate S, Babiuk LA, Mutwiri G. Mechanisms of action of adjuvants. *Front Immunol* (2013) **4**:114. doi:10.3389/fimmu.2013.00114
11. Patronov A, Doytchinova I. T-cell epitope vaccine design by immunoinformatics. *Open Biol* (2013) **3**:120139. doi:10.1098/rsob.120139
12. Reed SG, Orr MT, Fox CB. Key roles of adjuvants in modern vaccines. *Nat Med* (2013) **19**:1597–608. doi:10.1038/nm.3409
13. Montomoli E, Piccirella S, Khadang B, Mennitto E, Camerini R, De Rosa A. Current adjuvants and new perspectives in vaccine formulation. *Expert Rev Vaccines* (2011) **10**:1053–61. doi:10.1586/erv.11.48
14. Alving CR, Peachman KK, Rao M, Reed SG. Adjuvants for human vaccines. *Curr Opin Immunol* (2012) **24**:310–5. doi:10.1016/j.coi.2012.03.008
15. Balamayooran T, Balamayooran G, Jeyaseelan S. Review: toll-like receptors and NOD-like receptors in pulmonary antibacterial immunity. *Innate Immun* (2010) **16**:201–10. doi:10.1177/1753425910366058
16. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* (2011) **34**:637–50. doi:10.1016/j.immuni.2011.05.006
17. Gartner T, Baeten M, Otieno S, Revets H, De Baetselier P, Huygen K. Mucosal prime-boost vaccination for tuberculosis based on TLR triggering OprI lipoprotein from *Pseudomonas aeruginosa* fused to mycolyl-transferase Ag85A. *Immunol Lett* (2007) **111**:26–35. doi:10.1016/j.imlet.2007.04.010
18. Wang B, Henao-Tamayo M, Harton M, Ordway D, Shanley C, Basaraba RJ, et al. A toll-like receptor-2-directed fusion protein vaccine against tuberculosis. *Clin Vaccine Immunol* (2007) **14**:902–6. doi:10.1128/CDLI.00077-07
19. Le Moigne V, Robreau G, Mahana W. Flagellin as a good carrier and potent adjuvant for Th1 response: study of mice immune response to the p27 (Rv2108) *Mycobacterium tuberculosis* antigen. *Mol Immunol* (2008) **45**:2499–507. doi:10.1016/j.molimm.2008.01.005
20. Gowthaman U, Singh V, Zeng W, Jain S, Siddiqui KF, Chodisetti SB, et al. Promiscuous peptide of 16 kDa antigen linked to Pam2Cys protects against *Mycobacterium tuberculosis* by evoking enduring memory T-cell response. *J Infect Dis* (2011) **204**:1328–38. doi:10.1093/infdis/jir548
21. Tebianian M, Hoseini AZ, Ebrahimi SM, Memarnejadian A, Mokarram AR, Mahdavi M, et al. Cloning, expression, and immunogenicity of novel fusion protein of *Mycobacterium tuberculosis* based on ESAT-6 and truncated C-terminal fragment of HSP70. *Biologicals* (2011) **39**:143–8. doi:10.1016/j.biologicals.2011.02.002
22. Fan XL, Yu TH, Gao Q, Yao W. Immunological properties of recombinant *Mycobacterium bovis* bacillus Calmette-Guerin strain expressing fusion protein IL-2-ESAT-6. *Acta Biochim Biophys Sin (Shanghai)* (2006) **38**:683–90. doi:10.1111/j.1745-7270.2006.00217.x
23. Tang C, Yamada H, Shibata K, Maeda N, Yoshida S, Wajjwalku W, et al. Efficacy of recombinant bacille Calmette-Guerin vaccine secreting interleukin-15/antigen 85B fusion protein in providing protection against *Mycobacterium tuberculosis*. *J Infect Dis* (2008) **197**:1263–74. doi:10.1086/586902
24. Shi C, Yuan S, Zhang H, Zhang T, Wang L, Xu Z. Cell-mediated immune responses and protective efficacy against infection with *Mycobacterium tuberculosis* induced by Hsp65 and hIL-2 fusion protein in mice. *Scand J Immunol* (2009) **69**:140–9. doi:10.1111/j.1365-3083.2008.02207.x
25. Deng Y, Bao L, Yang X. Evaluation of immunogenicity and protective efficacy against *Mycobacterium tuberculosis* infection elicited by recombinant

Mycobacterium bovis BCG expressing human Interleukin-12p70 and early secretory antigen target-6 fusion protein. *Microbiol Immunol* (2011) **55**:798–808. doi:10.1111/j.1348-0421.2011.00376.x

26. Yang X, Bao L, Deng Y. A novel recombinant *Mycobacterium bovis* bacillus Calmette-Guerin strain expressing human granulocyte macrophage colony-stimulating factor and *Mycobacterium tuberculosis* early secretory antigenic target 6 complex augments Th1 immunity. *Acta Biochim Biophys Sin (Shanghai)* (2011) **43**:511–8. doi:10.1093/abbs/gmr045
27. Garcon N, Van Mechelen M. Recent clinical experience with vaccines using MPL- and QS-21-containing adjuvant systems. *Expert Rev Vaccines* (2011) **10**:471–86. doi:10.1586/erv.11.29
28. Leroux-Roels I, Leroux-Roels G, Ofori-Anyinam O, Moris P, De Kock E, Clement F, et al. Evaluation of the safety and immunogenicity of two antigen concentrations of the Mtb72F/AS02(A) candidate tuberculosis vaccine in purified protein derivative-negative adults. *Clin Vaccine Immunol* (2010) **17**:1763–71. doi:10.1128/CVI.00133-10
29. Leroux-Roels I, Forgus S, De Boever F, Clement F, Demoitie MA, Mettens P, et al. Improved CD4(+) T cell responses to *Mycobacterium tuberculosis* in PPD-negative adults by M72/AS01 as compared to the M72/AS02 and Mtb72F/AS02 tuberculosis candidate vaccine formulations: a randomized trial. *Vaccine* (2013) **31**:2196–206. doi:10.1016/j.vaccine.2012.05.035
30. Spertini F, Audran R, Lurati F, Ofori-Anyinam O, Zysset F, Vandepapeliere P, et al. The candidate tuberculosis vaccine Mtb72F/AS02 in PPD positive adults: a randomized controlled phase I/II study. *Tuberculosis (Edinb)* (2013) **93**:179–88. doi:10.1016/j.tube.2012.10.011
31. Agger EM, Rosenkrands I, Olsen AW, Hatch G, Williams A, Kritsch C, et al. Protective immunity to tuberculosis with Ag85B-ESAT-6 in a synthetic cationic adjuvant system IC31. *Vaccine* (2006) **24**:5452–60. doi:10.1016/j.vaccine.2006.03.072
32. Kamath AT, Valenti MP, Rochat AF, Agger EM, Lingnau K, Von Gabain A, et al. Protective anti-mycobacterial T cell responses through exquisite in vivo activation of vaccine-targeted dendritic cells. *Eur J Immunol* (2008) **38**:1247–56. doi:10.1002/eji.200737889
33. Baldwin SL, Bertholet S, Reese VA, Ching LK, Reed SG, Coler RN. The importance of adjuvant formulation in the development of a tuberculosis vaccine. *J Immunol* (2012) **188**:2189–97. doi:10.4049/jimmunol.1102696
34. Bertholet S, Ireton GC, Ordway DJ, Windish HP, Pine SO, Kahn M, et al. A defined tuberculosis vaccine candidate boosts BCG and protects against multidrug-resistant *Mycobacterium tuberculosis*. *Sci Transl Med* (2010) **2**:53ra74. doi:10.1126/scitranslmed.3001094
35. Orr MT, Fox CB, Baldwin SL, Sivananthan SJ, Lucas E, Lin S, et al. Adjuvant formulation structure and composition are critical for the development of an effective vaccine against tuberculosis. *J Control Release* (2013) **172**:190–200. doi:10.1016/j.jconrel.2013.07.030
36. Holten-Andersen L, Doherty TM, Korsholm KS, Andersen P. Combination of the cationic surfactant dimethyl dioctadecyl ammonium bromide and synthetic mycobacterial cord factor as an efficient adjuvant for tuberculosis subunit vaccines. *Infect Immun* (2004) **72**:1608–17. doi:10.1128/IAI.72.3.1608-1617.2004
37. Davidsen J, Rosenkrands I, Christensen D, Vangala A, Kirby D, Perrie Y, et al. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6'-dibehenate)-a novel adjuvant inducing both strong CMI and antibody responses. *Biochim Biophys Acta* (2005) **1718**:22–31. doi:10.1016/j.bbamem.2005.10.011
38. Schoenen H, Bodendorfer B, Hitchens K, Manzanero S, Werninghaus K, Nimmerjahn F, et al. Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. *J Immunol* (2010) **184**:2756–60. doi:10.4049/jimmunol.0904013
39. Ishikawa E, Ishikawa T, Morita YS, Toyonaga K, Yamada H, Takeuchi O, et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *J Exp Med* (2009) **206**:2879–88. doi:10.1084/jem.20091750
40. Desel C, Werninghaus K, Ritter M, Jozefowski K, Wenzel J, Russkamp N, et al. The Mincle-activating adjuvant TDB induces MyD88-dependent Th1 and Th17 responses through IL-1R signaling. *PLoS One* (2013) **8**:e53531. doi:10.1371/journal.pone.0053531
41. Schweneker K, Gorka O, Schweneker M, Poeck H, Tschopp J, Peschel C, et al. The mycobacterial cord factor adjuvant analogue trehalose-6,6'-dibehenate (TDB) activates the Nlrp3 inflammasome. *Immunobiology* (2013) **218**:664–73. doi:10.1016/j.imbio.2012.07.029
42. Drage MG, Pecora ND, Hise AG, Febbraio M, Silverstein RL, Golenbock DT, et al. TLR2 and its co-receptors determine responses of macrophages and dendritic cells to lipoproteins of *Mycobacterium tuberculosis*. *Cell Immunol* (2009) **258**:29–37. doi:10.1016/j.cellimm.2009.03.008
43. McBride A, Bhatt K, Salgame P. Development of a secondary immune response to *Mycobacterium tuberculosis* is independent of toll-like receptor 2. *Infect Immun* (2011) **79**:1118–23. doi:10.1128/IAI.01076-10
44. Takeda K, Takeuchi O, Akira S. Recognition of lipopeptides by Toll-like receptors. *J Endotoxin Res* (2002) **8**:459–63. doi:10.1179/096805102125001073
45. Schenk M, Belisle JT, Modlin RL. TLR2 looks at lipoproteins. *Immunity* (2009) **31**:847–9. doi:10.1016/j.immuni.2009.11.008
46. Buwitt-Beckmann U, Heine H, Wiesmuller KH, Jung G, Brock R, Akira S, et al. Toll-like receptor 6-independent signaling by diacylated lipopeptides. *Eur J Immunol* (2005) **35**:282–9. doi:10.1002/eji.200424955
47. Schroder NW, Heine H, Alexander C, Manukyan M, Eckert J, Hamann L, et al. Lipopolysaccharide binding protein binds to triacylated and diacylated lipopeptides and mediates innate immune responses. *J Immunol* (2004) **173**:2683–91.
48. Hoebe K, Georgel P, Rutschmann S, Du X, Mudd S, Crozat K, et al. CD36 is a sensor of diacylglycerides. *Nature* (2005) **433**:523–7. doi:10.1038/nature03253
49. Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, et al. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* (1999) **285**:732–6. doi:10.1126/science.285.5428.732
50. Jackson DC, Lau YF, Le T, Suhrbier A, Deliyannis G, Cheers C, et al. A totally synthetic vaccine of generic structure that targets toll-like receptor 2 on dendritic cells and promotes antibody or cytotoxic T cell responses. *Proc Natl Acad Sci U S A* (2004) **101**:15440–5. doi:10.1073/pnas.0406740101
51. Cote-Sierra J, Bredan A, Toldos CM, Stijlemans B, Brys L, Cornelis P, et al. Bacterial lipoprotein-based vaccines induce tumor necrosis factor-dependent type 1 protective immunity against *Leishmania major*. *Infect Immun* (2002) **70**:240–8. doi:10.1128/IAI.70.1.240-248.2002
52. Cuadros C, Lopez-Hernandez FJ, Dominguez AL, Mcclelland M, Lustgarten J. Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses. *Infect Immun* (2004) **72**:2810–6. doi:10.1128/IAI.72.5.2810-2816.2004
53. Garaude J, Kent A, Van Rooijen N, Blander JM. Simultaneous targeting of toll- and nod-like receptors induces effective tumor-specific immune responses. *Sci Transl Med* (2012) **4**:120ra116. doi:10.1126/scitranslmed.3002868
54. Zugel U, Kaufmann SH. Immune response against heat shock proteins in infectious diseases. *Immunobiology* (1999) **201**:22–35. doi:10.1016/S0171-2985(99)80044-8
55. Calderwood SK, Theriault J, Gray PJ, Gong J. Cell surface receptors for molecular chaperones. *Methods* (2007) **43**:199–206. doi:10.1016/j.ymeth.2007.06.008
56. Lehner T, Wang Y, Whittall T, Mcgowan E, Kelly CG, Singh M. Functional domains of HSP70 stimulate generation of cytokines and chemokines, maturation of dendritic cells and adjuvanticity. *Biochem Soc Trans* (2004) **32**:629–32. doi:10.1042/BST0320629
57. Wang Y, Kelly CG, Karttunen JT, Whittall T, Lehner PJ, Duncan L, et al. CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. *Immunity* (2001) **15**:971–83. doi:10.1016/S1074-7613(01)00242-4
58. Wang Y, Whittall T, Mcgowan E, Younson J, Kelly C, Bergmeier LA, et al. Identification of stimulating and inhibitory epitopes within the heat shock protein 70 molecule that modulate cytokine production and maturation of dendritic cells. *J Immunol* (2005) **174**:3306–16.
59. Borges TJ, Porto BN, Teixeira CA, Rodrigues M, Machado FD, Ornaghi AP, et al. Prolonged survival of allografts induced by mycobacterial Hsp70 is dependent on CD4+CD25+ regulatory T cells. *PLoS One* (2010) **5**:e14264. doi:10.1371/journal.pone.0014264
60. Detanico T, Rodrigues L, Sabritto AC, Keisermann M, Bauer ME, Zwickey H, et al. Mycobacterial heat shock protein 70 induces interleukin-10 production: immunomodulation of synovial cell cytokine profile and dendritic cell maturation. *Clin Exp Immunol* (2004) **135**:336–42. doi:10.1111/j.1365-2249.2004.02351.x
61. Bennekov T, Dietrich J, Rosenkrands I, Stryhn A, Doherty TM, Andersen P. Alteration of epitope recognition pattern in Ag85B and ESAT-6 has a profound influence on vaccine-induced protection against *Mycobacterium tuberculosis*. *Eur J Immunol* (2006) **36**:3346–55. doi:10.1002/eji.200636128
62. Ottenhoff TH. The knowns and unknowns of the immunopathogenesis of tuberculosis. *Int J Tuberc Lung Dis* (2012) **16**:1424–32. doi:10.5588/ijtld.12.0479

63. Tovey MG, Lallemand C. Adjuvant activity of cytokines. *Methods Mol Biol* (2010) **626**:287–309. doi:10.1007/978-1-60761-585-9_19
64. Chan SH, Perussia B, Gupta JW, Kobayashi M, Pospisil M, Young HA, et al. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J Exp Med* (1991) **173**:869–79. doi:10.1084/jem.173.4.869
65. Gerosa F, Paganin C, Peritt D, Paiola F, Scupoli MT, Aste-Amezaga M, et al. Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-gamma and interleukin-10. *J Exp Med* (1996) **183**:2559–69. doi:10.1084/jem.183.6.2559
66. Curtsinger JM, Lins DC, Mescher MF. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J Exp Med* (2003) **197**:1141–51. doi:10.1084/jem.20021910
67. Mendez-Samperio P. Role of interleukin-12 family cytokines in the cellular response to mycobacterial disease. *Int J Infect Dis* (2010) **14**:e366–71. doi:10.1016/j.ijid.2009.06.022
68. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, et al. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* (1998) **9**:669–76. doi:10.1016/S1074-7613(00)80664-0
69. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J Exp Med* (2000) **191**:771–80. doi:10.1084/jem.191.5.771
70. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser Eddine A, et al. Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. *J Clin Invest* (2005) **115**:2472–9. doi:10.1172/JCI24617
71. Gonzalez-Juarrero M, Hattle JM, Izzo A, Junqueira-Kipnis AP, Shim TS, Trapnell BC, et al. Disruption of granulocyte macrophage-colony stimulating factor production in the lungs severely affects the ability of mice to control *Mycobacterium tuberculosis* infection. *J Leukoc Biol* (2005) **77**:914–22. doi:10.1189/jlb.1204723
72. Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK. Adjuvants – a balance between toxicity and adjuvanticity. *Vaccine* (1993) **11**:293–306. doi:10.1016/0264-410X(93)90190-9
73. Batista-Duharte A, Lindblad EB, Oviedo-Orta E. Progress in understanding adjuvant immunotoxicity mechanisms. *Toxicol Lett* (2011) **203**:97–105. doi:10.1016/j.toxlet.2011.03.001
74. Mastelic B, Ahmed S, Egan WM, Del Giudice G, Golding H, Gust I, et al. Mode of action of adjuvants: implications for vaccine safety and design. *Biologicals* (2010) **38**:594–601. doi:10.1016/j.biologicals.2010.06.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 January 2014; accepted: 09 April 2014; published online: 23 April 2014.
*Citation: Junqueira-Kipnis AP, Marques Neto LM and Kipnis A (2014) Role of fused Mycobacterium tuberculosis immunogens and adjuvants in modern tuberculosis vaccines. Front. Immunol. **5**:188. doi: 10.3389/fimmu.2014.00188*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 07 April 2014
doi: 10.3389/fimmu.2014.00152

Recombinant BCG: innovations on an old vaccine. Scope of BCG strains and strategies to improve long-lasting memory

Adeliane Castro da Costa, Sarah Veloso Nogueira, André Kipnis and Ana Paula Junqueira-Kipnis*

Department of Microbiology, Immunology, Parasitology and Pathology, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brazil

Edited by:
Juraj Ivanyi, King's College London, UK

Reviewed by:
Randall Joseph Basaraba, Colorado State University, USA
Helen McShane, University of Oxford, UK

****Correspondence:***
Ana Paula Junqueira-Kipnis, Laboratório de Imunopatologia das Doenças Infecciosas, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Rua 235 Esquina Com Primeira Avenida, Setor Universitário, Goiânia 74605-050, Goiânia, Brazil
e-mail: apkipnis@gmail.com

Bacille Calmette–Guérin (BCG), an attenuated vaccine derived from *Mycobacterium bovis*, is the current vaccine of choice against tuberculosis (TB). Despite its protection against active TB in children, BCG has failed to protect adults against TB infection and active disease development, especially in developing countries where the disease is endemic. Currently, there is a significant effort toward the development of a new TB vaccine. This review article aims to address publications on recombinant BCG (rBCG) published in the last 5 years, to highlight the strategies used to develop rBCG, with a focus on the criteria used to improve immunological memory and protection compared with BCG. The literature review was done in April 2013, using the key words TB, rBCG vaccine, and memory. This review discusses the BCG strains and strategies currently used for the modification of BCG, including: overexpression of *Mycobacterium tuberculosis* (Mtb) immunodominant antigens already present in BCG; gene insertion of immunodominant antigens from Mtb absent in the BCG vaccine; combination of introduction and overexpression of genes that are lost during the attenuation process of BCG; BCG modifications for the induction of CD8+ T-cell immune responses and cytokines expressing rBCG. Among the vaccines discussed, VPM1002, also called rBCGΔureC:hly, is currently in human clinical trials. Much progress has been made in the effort to improve BCG, with some promising candidates, but considerable work is still required to address functional long-lasting memory.

Keywords: rBCG, tuberculosis, vaccine, protection, long-term memory, strain differences

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb), an intracellular pathogen that, after infecting a host, can cause disease or latency. TB continues to kill some 1.3 million people annually and 2 billion people worldwide are infected with Mtb (1, 2). The attenuated *Mycobacterium bovis* strain, known as Bacille Calmette–Guérin (BCG), is currently the only TB vaccine approved for human use, but its protective efficacy remains doubtful (3, 4). BCG was initially obtained from a virulent strain and was developed in France between 1908 and 1921 by Albert Calmette (1863–1933) and Camille Guérin (1872–1961). Although BCG is efficient in some regions of the world, such as in Alaskan American Indians region (5, 6), the protection conferred by BCG varies between 0 and 80% (7–9), although it has efficacy in protecting children from severe forms of TB.

To achieve BCG attenuation, more than 10 years of research with more than 230 serial passages were performed *in vitro* (10). This attenuation promoted genomic deletions, that together with the evolution of *M. bovis*, resulted in 16 genomic regions of differentiation (RD1–RD16, plus nRD18), when compared with the Mtb genome (11, 12). Regarding the region of differentiation lost during attenuation, RD1 is a DNA segment comprising 9.5 kb, which was deleted in all other BCG strains, that encodes T-lymphocyte epitopes such as ESAT-6, CFP-10, Rv3873, and PPE protein among others (13); RD2 is a 10.7 kb DNA segment that encodes many proteins including Mpt64 and CFP-21 (12); RD14 is a 9.1 kb section of DNA encoding proteins of the PE-PGRS and Rv1771 families (gulonolactone dehydrogenase) (14); RD16 is a 7.6 kb DNA section encoding Rv3405 that is responsible for colony morphology characteristics and the formation of cell membrane constituents (15); and nRD18, a 1.5 kb segment containing genes encoding SigI, an alternative RNA polymerase sigma factor, that was only lost in the strains BCG Pasteur, Phipps, Frappier, Connaught, and Tice (12). During the BCG attenuation process and the years that followed, more than 14 sub-strains emerged: BCG Russia (ATCC 35740), BCG Moreau/Rio de Janeiro, BCG Tokyo, BCG Sweden, BCG Birkhaug (ATCC 35731), BCG Denmark 1331 (ATCC 35733), BCG China, BCG Prague, BCG Glaxo (ATCC 35741), BCG Tice (ATCC 35743), BCG Frappier (ATCC 35735), BCG Connaught, BCG Phipps (ATCC 35744), and BCG Pasteur 1173 (16). They are distributed worldwide and have been used for vaccine development to prevent TB. The main concern is that BCG administration does not provide a reliable protection for adults in the developing world, protecting just against the main causes of infant TB, TB meningitis, and miliary TB (2).

To address the evolution of new recombinant BCG (rBCG) vaccines, the immunological status goal for such a vaccine should be defined. This is a controversial issue as there is no consensus as to what is the ideal immune memory phenotype that can confer protection. For instance, in animal models such as mice,

both Mtb infection or BCG vaccine induce increased levels of lung CD4+ effector T cells (phenotype $CD44^{hi}CD62L^{lo}CCR7^{lo}$) as well as memory cells. The current memory cell phenotypes accepted are effector memory T cells (TEM, $CD44^{hi}CD62L^{lo}CCR7^{lo}$) and central memory T cells (TCM, $CD44^{hi}CD62L^{hi}CCR7^{hi}$) (17–19). An important cornerstone for protection against TB is interferon (IFN)-γ production by T cells (20, 21), a cytokine crucial for stimulation of the microbicidal functions of macrophages. More recently, some authors have proposed that the desired protective memory against TB infection should have a central memory characteristic, with polyfunctional ability to produce IFN-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-2 cytokines (22) or a balance between IFN-γ and IL-17 levels to avoid excessive pathology (23).

The ultimate goal of a vaccine is its protective use in humans; consequently, the characterization of memory T cells in humans is also crucial. The major cell surface biomarker expression pattern for human memory T cells with an effector phenotype is $CD45RA^{hi}$, $CD45RO^{neg}$, and $CCR7^{neg}$, while for central memory T-cell populations it is $CD45RA^{hi/low}$, $CD45RO^{neg}$, and $CCR7^{pos}$. A follow-up study conducted among children vaccinated with BCG showed that specific memory T cells were stimulated and present in the peripheral blood of those individuals for at least 52 weeks following vaccination (24). It is interesting to observe that the induced memory cells were polyfunctional (IFN-γ, TNF-α, and IL-2). Although several studies have characterized the memory phenotypes induced by BCG, the direct association of memory T-cell populations with TB protection is still not well-established. Recently, a long-lasting T-cell memory population expressing CD127 was associated with Mtb infection and may correlate with protection shown by some exposed individuals (25). For this review, we consider the memory T-cell population as those that have the following phenotypes: $CD4^{+}$ $CD44^{hi/low}$ $CD62L^{lo}$ or to be antigen-specific CD4+ IFN-γ producing cells.

A significant limitation of TB vaccine development and testing is the lack of an optimal animal model that truly reflects human TB disease and the progress of immune responses. While there are several new vaccines being developed in different laboratories, there is a diversity of animal models (mice, rabbits, guinea pigs, non-human primates) and disease outcomes being used by different laboratories, preventing an adequate comparison between them. In addition, there is no consensus on the protocol to be used for vaccination and challenge, with different routes of immunization/infection, doses, BCG and Mtb strains, and time periods being used. Short period of time between vaccination and challenge does not allow full immunological memory development, thus generating a bias in the correlation between memory T-cell phenotype and protection. The most accepted method for evaluating protection is the determination of the bacterial load following challenge of vaccinated animals compared to non-vaccinated infected controls. Although colony-forming unit (CFU) counting is a widely used method, the organs used to assess the bacterial load vary among researchers, and make it difficult to establish comparisons. Given these many different parameters, in this review protection conferred by the different rBCG vaccines was considered when an overall significant reduction of the bacterial load compared with wild type BCG was achieved.

The factors that determine the induction of immunological memory related to BCG are not well-understood. Some assumptions are directed to the characteristics of the BCG sub-strains, which exhibit genotypic and phenotypic differences after the attenuation process as well as distinct residual virulence levels, the number of epitopes of each BCG strain, or the recombination strategy used for the development of a new vaccine (26, 27). According to the research tools employed in this study, of all sub-strains originated after this process the strains most frequently tested over 5 years were BCG Tokyo (BCG Japan), BCG Tice, BCG Danish (BCG Denmark/BCG SSI 1331), BCG Pasteur, BCG China (BCG Shanghai), and BCG Prague. It is also hypothesized that generation of the immune response and eventually the outcome of vaccination could be influenced by the type of genetic strain background used. However, pre-clinical animal and human data have demonstrated that different strains of BCG confer the same level of protection (28, 29).

The main strategies used to develop new vaccines are based on the formulation of subunit vaccines; the production of non-recombinant viral vector vaccines that can be used as a BCG prime boost, and the construction of rBCG, which could confer similar protection with a better induction of memory than BCG. Methods to construct rBCG include overexpression of promising Mtb immunodominant antigens expressed by BCG, such as α-crystallin: HspX protein and antigen 85 (Ag85) complex proteins (Ag85A, Ag85B, and Ag85C) (30); insertion of Mtb immunodominant antigens absent on BCG, such as those encoded by RD1, RD2, RD3, RD14, RD15, RD16, and nRD18 genes (27); combination of overexpression with reintroduction of genes lost during BCG attenuation; and BCG modification to induce CD8+ T immune response proteins and cytokines (**Tables 1** and **2**).

Therefore, the aim of this review was to analyze which factors associated with rBCG could induce long-lasting memory and promote better protection compared with conventional BCG.

DOES BCG EPITOPE NUMBER INFLUENCE THE INDUCTION OF MEMORY AND PROTECTION OF rBCG VACCINES?

As stated by Zhang et al. (27), the number of epitopes in a particular strain can be important for the development of an enhanced vaccine that could replace BCG. According to this hypothesis, a strain such as BCG Tokyo has the potential to induce a better immune response compared with conventional BCG because it comprises 359 epitopes that can be recognized by lymphocytes (27). To verify if there is sufficient data to support this hypothesis in the last 5 years, we selected criteria as summarized in **Table 1**. The different rBCG vaccines were compared according to their ability to improve protection by reducing the bacterial load relative to wild type BCG and to generate specific memory CD4+ T cells. Three different published studies using rBCG Tokyo, which contains a high number of epitopes, showed better protection than BCG, which was associated with the recombinant generation strategy: overexpression and reintroduction of lost genes, as well as the presence of cytokines (**Table 1**). Nevertheless, the strain that has been most widely used in the last 5 years is BCG Danish (BCG Denmark/BCG SSI 1331), which has approximately 329 epitopes, and provides improved protection and long-lasting memory when associated with the overexpression of Mtb antigens. Two rBCG

Table 1 | BCG sub-strains genetic background used for recombinant BCG vaccines development and ability to induce memory and protection against tuberculosis.

Sub-strains	RDs[a]	Epitopes	No[b]	Protection better than BCG?[c]	Memory?[d]	Reference
Tokyo/Japan	RD1	359	3	Yes	Yes	(31, 32)
				Yes	No	(33)
Pasteur	RD1, RD2, RD14, nRD18,	331	6	Yes	No	(34)
				No	Yes	(35)
				No	No	(36–38)
Danish/Denmark	RD1, RD2	329	11	Yes	Yes	(39)
				No	Yes	(40)
				Yes	No	(41–45)
				No	No	(46–48)
Tice	RD1, RD2, nRD18	328	2	Yes	Yes	(49)
				Yes	No	(50)
China/Shanghai	RD1, RD2	321	6	Yes	No	(51)
				No	No	(52–56)
Prague	RD1, RD2	318	3	Yes	No	(23)
				No	No	(57, 58)

[a] *RD, region of difference.*

[b] *Number of publications in the last 5 years.*

[c] *Protection was evaluated by CFU analyses and considered when the bacterial load of challenged animals were lower than wild type BCG-vaccinated animals.*

[d] *Memory was defined as $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$ or CD4+ IFN-γ producing T cells specific immune responses.*

Table 2 | Description of strains and antigens used in the papers visited for this review.

Reference	Model	Strain	Antigen	Challenge	Protection[a]
(31)	Mice	BCG Tokyo	rBCG1:Ag85B–CFP10(rBCG1)/BCG2:Ag85B–CFP10–IL-12 (rBCG2)	No	> (*Ex vivo*)
(49)	Human	BCG Tice	rBCG30 (Ag 85B)	No	NE
(46)	Guinea pigs	BCG Danish	rBCG–E6 (ESAT-6)	50–100 bacilli of *Mtb*	>
(41, 42)	Guinea pigs	BCG Danish	rBCG*acr*	50–100 bacilli of *Mtb*	>
(39)	Mice	BCG Danish	BCG:HspX/rBCG:85B	10^6 CFU of *Mtb*	>
(53)	Mice	BCG China	rBCG–AE	10^6 CFU of *Mtb*	<
(58)	Mice	BCG Prague	rBCGureC:hly or rBCGureC:hly	10^2 CFU of *Mtb*	=
(45)	Mice	BCG Danish	BCG: rBCG–Ag85B–Mpt64–Mtb8.4	10^6 CFU of *Mtb*	>
(47)	Mice	BCG Danish	rBCG:Ag85B–ESAT-6–Rv2608	No	NE
(33)	Monkey	BCG Tokyo	rBCG–Ag85A	3000 CFU of *Mtb*	>
(48)	Monkey	BCG Danish	rBCG AFRO-1	No	NE
(55)	*In vitro*	BCG China	rBCG:Quimera 85B + ESAT-6	No	NE
(34)	Mice	BCG Pasteur	rBCG:PE–MPT64/rBCG/HSP60MPT64	~200 CFU of *Mtb*	>
(23)	Mice	BCG Prague	rBCGΔureC:hly+	200–400 CFU of *Mtb*	>
(51)	Mice	BCG China	rBCG: Ag85A/rBCG:Ag85B/rBCG:Ag85A–Ag85B	10^6 CFU of *Mtb*	>
(52)	Mice	BCG China	rBCG: Ag85A–ESAT-6/rBCG: Ag85A/rBCG: ESAT-6	No	NE
(40)	Monkey	BCGDanish	AFRO-1, Ag85A, Ag85B e TB10.4.	500 CFU of *Mtb*	>
(37)	Mice	BCG Pasteur	rBCGs:BCG:Ag85c, BCG:INV, BCG:PPE, BCG:FBP e BCG:CFP	20 bacilli of *Mtb*	=
(57)	Mice	BCG Prague	rBCGΔureC:hly+)	No	NE
(38)	Mice	BCG Pasteur	rBCG: pHspX–Ag 85B	100 bacilli of *Mtb/lung*	=
(56)	Mice	BCG China	rBCGs:BCG:GM-CSF/BCG:ESAT-6/BCG:GMCSF–ESAT-6	No	NE
(32)	Mice	BCG Tokyo	rBCG–85B–IL-15/rBCG–85B	2×10^5 CFU of *Mtb*	=
(44)	Guinea pigs	BCG Danish	rBCG–85C	500 bacilli of *Mtb*	>
(59)	Human	BCG Danish	BCG ΔureC:hly Hm^R	No	NE

[a] *Protection was evaluated by CFU analyses and considered when the bacterial load of challenged animals were lower than wild type BCG-vaccinated animals; >, superior than BCG; =, protection similar to BCG; –, less protection than BCG; NE, not evaluated.*

References published and indexed in PubMed from 2008 to April 2013.

Tice (328 epitopes) vaccine constructions also demonstrated good induction of protection but only one induced memory. Based on those publications, it appears that the genetic background of the BCG strains (number of epitopes) does not have a major role in inducing/improving protection and memory.

Contrary to Zhang et al. (27), other studies support the idea that recombinant antigen selection expressed by BCG, and not the BCG strain background, is the significant point to be considered in the construction of an improved vaccine with enhanced induction of memory and protection (32, 34, 46). Moreover, it appears that overexpression of certain antigens in rBCG are critical for enhanced induction of memory and protection compared with BCG.

One limitation of this study is that when analyzing studies only published in the last 5 years, the conclusions might be biased because important work addressing whether the BCG strain background (epitope number) or selected antigen were important for enhanced memory and protection may have been published earlier.

DOES THE QUANTITY OF Mtb ANTIGENS INCORPORATED IN BCG RESULT IN GREATER PROTECTION AND MEMORY DEVELOPMENT?

rBCG VACCINES SUPER EXPRESSING Mtb IMMUNODOMINANT ANTIGENS

An important strategy used for the construction of a new TB vaccine is the development of an rBCG super expressing Mtb immunodominant antigens, such as proteins from the Ag85 Complex, HspX protein, and the association of both proteins in one vaccine construction, which represents a favored approach for TB vaccine construction (44).

Some of the most important antigens used to construct rBCG vaccines are those from the Ag85 Complex that consists of Ag85A (Rv3804c), Ag85B (Rv1886c), and Ag85C (Rv0129c), encoded by fbpA, fbpB, and fbpC2 genes, respectively, and with molecular weights between 30 and 32 kDa (60). Proteins of Ag85 complex have mycolyltransferase activity, thus they play a role in mycolate production and construction of the Mtb cell wall, which is important for maintaining Mtb integrity and pathogenesis (61).

The protein Ag85B, used for construction of the vaccine rBCG:30 (r30–Ag85B), generated protection in guinea pigs after challenge with Mtb (62, 63). A phase I clinical trial of rBCG:30 in human volunteers induced central and effector memory CD4 and CD8 T cells specific for Ag85B (49). Currently, this vaccine is no longer being tested on humans. The same antigen was used by Tullius et al. (50) who developed a mutant rBCG, rBCG(mbt)30, resulting in a strain unable to synthesize mycobactin and exoquelin molecules that are essential for iron acquisition. The vaccine induced greater protection than conventional BCG. Another approach was to design an rBCG pantothenate auxotroph, rBCG(panCD)30. Both vaccines, rBCG(mbt)30 and rBCG(panCD)30, were attenuated to a higher degree than BCG and induced potent protective and cell-mediated immunity in guinea pigs (50). These vaccines may have the potential to provide a safe alternative for HIV positive individuals since BCG is not indicated for use in immunocompromised individuals.

rBCG:30 and other vaccines overexpressing Ag85B were better at conferring protection and memory than BCG. Ag85B (30 kDa) is the most abundant protein of the Ag85 complex, and is the most abundant extracellular protein of Mtb, responsible for nearly one-quarter of the total extracellular protein in broth culture (64). In addition, Ag85B has a high affinity for T-cell recognition, can induce a type Th1 immune response with IFN-γ production, and has a good protective capacity when used in DNA vaccine strategies (65).

Ag85C is also a major secretory protein and immunodominant antigen, being strongly recognized by sera from TB patients. Indeed, it is responsible for almost 40% of the mycolate content of Mtb and its mycolyltransferase activity cannot be substituted by Ag85A or Ag85B (66). For this reason, Jain et al. (44) developed an rBCG expressing Ag85C under the transcriptional control of mycobacteria promoters. Reduced granulomatous infiltration and granuloma formation were observed when compared to a group immunized with BCG and the protection (reduced bacterial load in lungs and spleen compared with ancestor BCG) was associated with reduced levels of IFN-γ, TNF-α, IL-12, and TGF-β mRNA compared to BCG. However, high levels of inducible nitric oxide synthase (iNOS) were observed compared with BCG. Furthermore, previous studies with DNA vaccines using Ag85C demonstrated the reduced production of IL-2 and IFN-γ with insufficient protection when animals were challenged with *M. bovis* BCG (67). Hence, it is important to stress that both the type of antigen and its expression in a suitable vector (BCG itself) is important to confer good protection status. The study by Lozes et al. used the BCG Danish strain, which may have contributed to the disappointing results. Unfortunately, the study did not provide information regarding the ability to generate memory cells.

Ag85A is strongly recognized by T lymphocytes to induce IL-2 and IFN-γ production (67). Immunization of mice and guinea pigs with rBCG:Ag85A promoted the reduction of pulmonary pathology severity and increased protection in lungs and spleen against infection (68). Consequently this vaccine was also tested in *Macaca mulatta*, and, after challenge, the group immunized with rBCG:Ag85A developed light to moderate pneumonia, while the non-vaccinated group developed multilobar pneumonia, lymphadenopathy, and atelectasis. In addition, its protective capacity was previously appraised in a DNA vaccine system with Ag85A (69). It was shown that rBCG–Ag85A induced higher protective efficacy than the parental BCG Tokyo strain (33). In that study, a strategy of over expressing the antigen in addition to the use of a BCG strain containing more natural epitopes was employed (**Table 1**). Hence, this could justify the potential of this vaccine for further studies to measure memory induction.

The construction of rBCG expressing single proteins resulted in promising results. Following those studies, significant progress was made with the construction and testing of recombinant fusion proteins, combining two or more protein coding regions from one or more Mtb proteins, because the combined use of antigens might enhance protective efficacy compared with rBCG expressing only one antigen.

To analyze this hypothesis, Wang et al. developed three vaccine constructions: rBCG:Ag85A (A), rBCG:Ag85B (B), and rBCG:AB, which were used to immunize mice. The vaccine containing fusion

antigens, rBCG:AB, showed better protection after challenge with Mtb, when compared with BCG or rBCG expressing Ag85A or Ag85B alone. Six and 24 weeks after vaccination, splenocytes of mice immunized with rBCG:AB stimulated with specific antigen secreted more IFN-γ than splenocytes from mice immunized with the other rBCG (51). Regrettably, no memory response was evaluated in that study, probably because memory induction was already known for rBCG:Ag85B (49). However, no studies using rBCG expressing Ag85A have assessed memory responses, so it would be meaningful to verify whether Ag85A could contribute to enhanced memory responses. Although an approach using recombinant fusion proteins is valuable for protection against challenge with Mtb, additional studies regarding the induction of functional, long-lasting memory is also required for the development of new vaccines.

Another antigen frequently used for recombinant expression in BCG is the HspX protein (Rv2031c, also known as α-cristalin, molecular weight 16 kDa), a heat shock protein encoded by the *acr* gene (70). This protein is abundantly produced during the latent or persistent Mtb metabolic condition. Shi et al. developed a rBCG over expressing the immunodominant Mtb antigen, HspX (rBCG:X) and demonstrated that rBCG:X provided enhanced and longer lasting protection against Mtb infection than BCG, as evidenced by high levels of IFN-γ production, low bacterial load in tissues, and reduced lung pathology. This was associated with elevated levels of anti-HspX antibodies during week 6 and 24 (168 days) after rBCG:X immunization, indicating that BCG:X might persist longer *in vivo* than BCG (39).

Additionally, results obtained by Shi et al. demonstrated that expression of HspX by BCG could improve its biological effects, which might explain the higher expression of Ag85B in the supernatant and lysate of cells after infection with rBCG:X compared with that by BCG (39). This theory was also corroborated by Kong et al. (38) who constructed an rBCG expressing Mtb Ag85B under the control of a HspX promoter. The expression and immune response to Ag85B was modulated by the HspX promoter. For example, rBCG:PhspX-85B induced intense specific Ag85B T-cell proliferation and IFN-γ production 3 weeks after infection. Increased cell proliferation and IFN-γ production was observed after 12 weeks indicating long-lasting cell-mediated immunity. Despite the intense induction of immune cell responses, the protection in lungs and spleen induced by this vaccine was similar to that by BCG. This indicated that in a model of Ag85B expression under control of a different promoter, there was no improvement in protective efficacy (38). Although Ag85C is responsible for more than 40% of the mycolate present in the mycobacteria cell wall (61), evidence suggests that Ag85B is critical for enhanced BCG induction of memory and protection (49, 50).

The use of fusion proteins has generated great expectations in the scientific community, nonetheless, the use of combined proteins yielded no better memory than BCG, according to the present accepted parameters, generating only better protection. The increased protection observed among the recombinant vaccines cannot be the only improvement desired for the development of a new vaccine, as vaccination of available animal models to study new vaccines to TB does not eliminate all Mtb from the tissues of challenged animals. Therefore, new definitive protection parameters are needed.

ASSOCIATION OF OVEREXPRESSION AND REINTRODUCTION OF ANTIGENS LOST DURING THE ATTENUATION PROCESS

Some virulence regions, such as RD1, were lost during the BCG attenuation process. RD1 is absent in all BCG sub-strains, but present in virulent strains and clinical isolates of *M. bovis* and *M. tuberculosis*. The association of Mtb genes lost in the *M. bovis* attenuation process within rBCG has been used to improve vaccine efficacy (11). The collection of well-defined T-cell antigen epitopes has been a widely used strategy for the construction of new vaccines. This collection is based on the reintroduction of proteins whose gene regions were deleted during the attenuation process and include the 10 kDa culture filtrate protein (CFP-10, Rv3874), ESAT-6, PPE family protein (Rv3873), INV (Rv1474), and MPT64.

When evaluating the induction of immune responses, vaccine constructions containing antigenic epitopes have been the most successful, although most studies did not evaluate the protection or memory induced by these vaccines. Vaccine constructs using antigen epitopes were good inducers of antigen-specific Th1 (IFN-γ) immune responses, IgG2a production, and delayed type hypersensitivity responses compared with BCG. Conversely, some recombinant vaccines (BCG:CFP, BCG:FBP, BCG:PPE, and BCG:INV) showed protection similar to that of BCG (37). Only rBCG vaccines expressing MPT64 antigens fused to a PE antigen (HPE–ΔMPT64–BCG) showed superior protection than those immunized with BCG. This protection was associated with CD4 and CD8 T-cell induction and the emergence of a specific MPT64 T-cell clone (34). Despite use of the same BCG strain in the two studies, the differences in protection observed indicate the importance of antigen choice.

When using proteins of the Ag85 complex, Qie et al. (45) compared the protective efficacy of rBCG–AMM (BCG expressing Ag85B–MPT64190–198–Mtb8.4) with BCG. Animals vaccinated with rBCG–AMM generated more antigen-specific CD4 and CD8 T cells than those vaccinated with BCG and showed a more efficient response that protected mice challenged with H37Rv Mtb strain. Moreover, rBCG–AMM was superior to BCG in reducing the severity of disease in the target organs such as lungs and spleen, indicating that rBCG–AMM could be a potential vaccine candidate for further studies. Again, this vaccine was not evaluated for memory induction.

Some rBCG vaccines designed over the past 5 years combined the ability to generate strong immune responses to Ag85 proteins using the antigen ESAT-6 (47, 52, 55). Of these studies, only one addressed protection and memory development. Deng et al. (52) constructed an rBCG expressing the fusion protein Ag85A–ESAT-6 (rBCG–AE) and this vaccine induced more potent immunogenicity than native BCG in mice and induced a shift toward a Th1 type immune response with an increase in the ratio of CD4 and CD8 T-cell subsets. Thus, rBCG–AE elicited long-lasting and stronger Th1 type cell-mediated immune responses than BCG. They further evaluated the protective efficacy conferred by rBCG–AE against Mtb infection in BALB/c mice (53). An rBCG vaccine expressing ESAT-6 alone did not exceed the parental BCG vaccine for

protection from Mtb H37Rv infection. The vaccine was developed using a BCG China strain, while others used BCG Tokyo, BCG Danish, or BCG Pasteur. As previously stated, vaccine construction over expressing proteins of the Ag85 Complex seem to have better protection efficacy than BCG, while rBCG–ESAT-6 induced protection similar or even inferior to BCG. However, using combined epitopes from proteins of the Ag85 complex or other proteins and ESAT-6 improved macrophage activation and antigen presentation (55), and strong humoral and cellular immune responses were induced (47), although protection or memory generation was not addressed.

The development of rBCG vaccines that re associated with or reintroduced genes lost during BCG attenuation appears to improve protection and memory most frequently when proteins from the Ag85 complex are associated with the fusion protein. This observation could be biased as Ag85 proteins are most frequently used in the development of rBCG vaccines. The combined results suggest that these genes are evolutionary maintained by Mtb to induce strong immune responses in animals and humans, independent of the type of BCG strain used. Most studies presented here did not evaluate functional memory, a crucial step for the development of a long-lasting protective vaccine.

rBCG VACCINE EXPRESSING MAMMALIAN CYTOKINES AND Mtb PROTEINS

Cytokines play a central role in the immune system and have multiple effects on different immune cells. IL-2, for example, has been used for the treatment of some diseases, including TB, but toxicity related to high doses has restricted its use. A solution to that problem was the expression of rIL-2 and other cytokines by BCG (71). Another example is IL-15, an important cytokine that maintains survival and proliferation of CD8+ T cells with a memory phenotype (72). To develop new vaccines capable of improving BCG vaccination, some research strategies have included the use of rBCG expressing IL-2, IL-12, IL-15, and GM-CSF, among others.

Recombinant vaccines expressing cytokines induced effector polyfunctional CD8 T cells and CD4 T cells (producing IFN-γ, IL-2, and TNF-α), as well as humoral immune responses with increased specific IgG2a/IgG1 levels [(32) rBCG–Ag85B–IL-15; (31) BCG: Ag85B–CFP10–IL-12; (56) rBCG: GMCSF–ESAT-6]. Among the types of vaccines, those that expressed IL-15 and IL-12, were most successful as they induced CD8+ T (CD8+ $CD44^{hi}$ $CD62L^{lo}$) and CD4+ T (CD4+ $CD44^{hi}$ $CD62L^{lo}$) memory cells (31, 32).

Although these vaccines showed better protection than BCG, the rBCG–Ag85B–IL-15 vaccine was more promising because it generated a greater induction of memory CD8 T cells than memory CD4 T cells, in support of the theory that CD8 T cells rather than CD4 T cells are important for long-lasting protection against TB (32). It is important to note that despite the positive influence of IL-15 in inducing memory cells, *in vivo* administration after priming with rBCG followed by challenge with Mtb, did not induce increased numbers of CD8 T memory cells, a phenomenon only seen when IL-15 is expressed by rBCG (32).

Thus, the induction of CD8+ T cells and polyfunctional CD8+ and CD4+ T cells (producing IFN-γ, IL-2, and TNF-α) are responsible for the improvement of protection generated by rBCG while the secretion of ILs might play an important role in the proliferation and maintenance of memory T cells.

BCG MODIFICATION: INDUCTION OF CD8 T IMMUNE RESPONSES

Mtb and BCG preferentially localize inside antigen presenting cell (APC) phagosomes, such as macrophages and dendritic cells. This localization dictates antigen traffic via MHC-II, which results in the preferential stimulation of CD4 T cells. CD8 T cytolytic lymphocytes (CTLs) are essential for the clearance of intracellular Mtb infection since CTLs kill cells and bacteria through secretion of cytolytic and antimicrobial effector molecules (perforin and granulysin). Mtb induced apoptosis in infected cells, resulting in vesicles that transport mycobacteria antigens, which can be captured by local dendritic cells that cross present MHC-I and MHC-II, that stimulate CD8 and CD4 T cells, respectively (73). It is also acknowledged that BCG is a weak inducer of apoptosis and thus activates CD8 T cells to a lesser extent (57, 73). Thus, in an attempt to improve BCG, rBCG vaccines have been developed to express listeriolysin (Hly) from *Listeria monocytogenes* (74) in the membrane, in combination with deletion of the Urease C (ureC) gene (rBCGΔureC:hly). One mechanism of BCG employs to survive phagosomes is pH neutralization through ureC activity. To induce apoptosis, Hly requires an acidic pH. The ureC mutant rBCG allows phagolysosome pH acidification to occur naturally (74). Using this vaccine protocol, Reece et al. (58) selected antigens based on their expression in response to nutrient deprivation (Rv2659c), hypoxia (Rv1733c), or disease reactivation (Rv3407) and transformed rBCGΔureC:hly with plasmids containing these antigens, rBCGΔureC:hly (pMPIIB01). The improved performance of this vaccine was demonstrated by a lower bacterial load in the spleen of infected mice (58). In addition, it induced Th-17, CD4+, and CD8+ T-cell responses and increased protection compared with BCG (23, 57). This vaccine is the most promising rBCG vaccine generated and it finished a phase I clinical trial for safety with great success. It is currently being tested in newborns in a phase II clinical trial (59). Although this vaccine aimed to improve T CD8 responses, the induction of specific CD4 T cells secreting IFN-γ as well as polyfunctional T CD4 responses were observed in vaccinated healthy humans.

In a similar approach to obtain a vaccine inducing increased CD8 T-cell responses, an rBCG, rBCG AFRO-1 (BCG expressing Ag85A, Ag85B, and TB10.4) was developed followed by two boosts with AERAS-402 [adenovirus vaccine 35 (rAd35) expressing Ag85A, Ag85B, and TB10.4]. AFRO-1 BCG expresses perfringolysin O, which allows BCG to escape to the cell cytosol, promoting antigen processing and presentation via MHC-I. After priming with rBCG AFRO-1, there was delayed but strong IFN-γ production 1 week after boost with AERAS-402, as well as strong proliferation of CD4 and CD8 T cells (48). This vaccine promoted longer survival and IFN-γ production; however, no difference in lung and spleen bacterial load between the groups vaccinated with BCG or AFRO-1 (also known as AERAS-422) was observed (43). Although a promising vaccine, AERAS-422 was terminated because of the development of shingles in some study participants that occurred during a phase I clinical trial (75).

The strategy of BCG modification for the induction of CD8 T specific immune responses has had a great impact, as the recombinant vaccine rBCGΔureC:hly is in a clinical trial[1].

CONCLUSION AND FUTURE PERSPECTIVES

Bacille Calmette–Guérin has been used for almost 100 years, with more than eight million doses used. However, TB incidence has shown a slow decrease during the last decade, mainly due to the increase of multi drug resistant strains and HIV co-infection (6). Two main cautions of BCG vaccine use are associated with its variable efficacy and immunity against Mtb infection resulting in a large pool of latently/persistently infected individuals. Furthermore, BCG might induce better protection among individuals from regions with lower environmental mycobacterial contaminations and lower TB rates. Development of a new vaccine or improvement of BCG to protect against TB is not an easy task, once the natural infection *per se* does not induce protection or long-lasting T or B functional memory cells since it does not avoid re-infection. It appears that the coevolution between mycobacteria and humans favors the mycobacteria. Over the past 5 years, several attempts were conducted to develop rBCGs (**Table 2**). Improvement of BCG remains the best choice for the rational design of a TB vaccine. This review sought to discuss recent TB studies advancing the rBCG strategy. The main purpose for developing rBCG is to design a vaccine capable of inducing long-lasting functional memory with protection similar or superior to that of BCG. In addition, BCG is a strong inducer of CD4+ T cells but it is an insufficient stimulator of CD8 T cells. The most effective rBCG vaccination strategies in animal models and human clinical trials to date were those that stimulated both CD4+ T and CD8+ T cells to produce Th1-associated cytokines and induce cytotoxic functions [(24, 76), see text footnote 1].

It is recognized that protein combinations, such as fusion proteins, as well as the expression of these proteins by different expression vectors are important strategies in the development of rBCG vaccines with an enhanced efficacy compared with BCG. Nevertheless, the vaccine approach of super expressing Mtb proteins in BCG, such as rBCG:30, which expresses only Ag85B, to induce central memory and more desirable protection than BCG, is useful (49). This plasmid-based vaccine passed a phase I clinical trial and is currently on hold awaiting the development of auxotrophic BCG strains to avoid the use of antibiotic resistance genes (see text footnote 1).

An intriguing point is that an rBCG vaccine currently in a phase II human clinical trial does not contain Mtb antigens or antigens lost by BCG during the attenuation process. The rBCGΔureC:hly vaccine improved BCG antigen presentation by dendritic cells and improved processing with the ultimate goal of activating CD8+ T cells. Therefore, this approach might have overcome some of the evolutionary mycobacteria immunological escape mechanisms and will allow protective long-lasting functional memory. In time, whether this vaccine induced better protection against TB will be determined (77).

Furthermore, the choice of parental BCG strain appears not to interfere with the recombinant vaccine outcome, because some vaccines using the same parental BCG strains had different outcomes depending on the selected antigen or fusion protein used. Likewise, the immune response profile of those vaccine candidates that showed better protection than BCG was based upon CD4 and CD8 T cells with polyfunctional activities. From all studies reviewed here, only six successfully evaluated immunological memory in animal models.

The animal models available to study TB vaccines (mice, guinea pig, or non-human primates) cannot predict the outcome among vaccinated humans. It is well-known that mice and guinea pigs are infected by BCG vaccination and the duration of the vaccination and the time until challenge are crucial to address the persistence of memory T cells. This premise could be used to justify the low number of studies that have addressed this issue over the past 5 years.

The real impact of these new vaccines using rBCG or other strategies that are currently in clinical trials will only be determined 5–10 years from now. Therefore, studies addressing new strategies to improve BCG need to be continued.

MATERIALS AND METHODS

STUDY SELECTION AND DATA COLLECTION PROCESS

The search for this review was conducted in April 2013, and was based on articles published in the previous 5 years (2008–2013). Articles were searched from the PubMed Database using the following key words: tuberculosis protection and rBCG vaccine with the intention to address publications showing studies on rBCG vaccine for tuberculosis. Then further manuscripts were selected using the key words: tuberculosis protection; rBCG vaccine and memory. Manuscripts lacking information of the BCG wild type strain and those that used the boost strategy without evaluating rBCG responses alone were not included in this review.

ACKNOWLEDGMENTS

This research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

1. Kamath AT, Fruth U, Brennan MJ, Dobbelaer R, Hubrechts P, Ho MM, et al. New live mycobacterial vaccines: the Geneva consensus on essential steps towards clinical development. *Vaccine* (2005) **23**(29):3753–61. doi:10.1016/j.vaccine.2005.03.001
2. World Health Organization (WHO). *Global Tuberculosis Control–Epidemiology, Strategy, Financing*. Geneva: WHO (2009).
3. World Health Organization. *Global Tuberculosis Control*. Geneva: WHO (1998).
4. Partnership WST. *The Global Plan to Stop TB 2011–2015: Transforming the Fight-Towards Elimination of Tuberculosis*. Geneva: WHO (2010).
5. Aronson NF, Santosham M, Comstock GW, Haward RS, Moulton LH, Rhoades ER, et al. Long-term efficacy of BCG vaccine in American Indians and Alaska Natives: a 60-year follow-up study. *JAMA* (2004) **5**:2086–91. doi:10.1001/jama.291.17.2086
6. Mangtani P, Abubakar I, Arti C, Beynon R, Pimpin L, Fine PE, et al. Protection by BCG vaccine against tuberculosis: a systemic review of randomized controlled trials. *Clin Infect Dis* (2014) **58**:470–80. doi:10.1093/cid/cit790
7. WHO. Tuberculosis prevention trials: Madras (1979). Trial of BCG vaccines in South India for tuberculosis prevention. *Bull World Health Organ* (1979) **57**:819–27.
8. Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, et al. Efficacy of BCG vaccine in the prevention of tuberculosis: meta-analysis of the published literature. *JAMA* (1994) **271**:698–702. doi:10.1001/jama.1994.03510330076038

[1] www.clinicaltrials.com

9. Trunz BB, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* (2006) **367**:1173–80. doi:10.1016/S0140-6736(06)68507-3
10. Calmette A, Guerin C, Ne'gre L, Boquet A. Premunition des nouveaux-nescontre la tuberculoseparlevaccin BCG, 1921–1926. *Ann Inst Pasteur (Paris)* (1926) **40**:89–133.
11. Brosch R, Gordon SV, Pym A, Eiglmeier K, Garnier T, Cole ST. Comparative genomics of the mycobacteria. *Int J Med Microbiol* (2000) **290**:143–52. doi:10.1016/S1438-4221(00)80083-1
12. Joung SM, Ryoo S. BCG vaccine in Korea. *Clin Exp Vaccine Res* (2014) **2**:83–91. doi:10.7774/cevr.2013.2.2.83
13. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* (1998) **393**:537–44. doi:10.1038/31159
14. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* (1999) **284**:1520–3. doi:10.1126/science.284.5419.1520
15. Honda I, Seki M, Ikeda N, Yamamoto S, Yano I, Koyama A, et al. Identification of two subpopulations of Bacillus Calmette-Guérin (BCG) Tokyo172 substrain with different RD16 regions. *Vaccine* (2006) **24**:4969–74. doi:10.1016/j.vaccine.2006.03.055
16. Leung AS, Tran V, Wu Z, Yu X, Alexander DC, Gao GF, et al. Novel genome polymorphisms in BCG vaccine strains and impact on efficacy. *BMC Genomics* (2008) **9**:413. doi:10.1186/1471-2164-9-413
17. Henao-Tamayo MI, Ordway DJ, Irwin SM, Shang S, Shanley C, Orme IM. Phenotypic definition of effector and memory T-lymphocyte subsets in mice chronically infected with *Mycobacterium tuberculosis*. *Clin Vaccine Immunol* (2010) **17**:618–25. doi:10.1128/CVI.00368-09
18. Junqueira-Kipnis AP, Turner J, Gonzalez-Juarrero M, Turner OC, Orme IM. Stable T-cell population expressing an effector cell surface phenotype in the lungs of mice chronically infected with *Mycobacterium tuberculosis*. *Infect Immun* (2004) **72**(1):570–5. doi:10.1128/IAI.72.1.570-575.2004
19. Kipnis A, Irwin S, Izzo AA, Basaraba RJ, Orme IM. Memory T lymphocytes generated by *Mycobacterium bovis* BCG vaccination reside within a CD4 CD44lo CD62 ligand (hi) population. *Infect Immun* (2005) **73**:7759–64. doi:10.1128/IAI.73.11.7759-7764.2005
20. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med* (1993) **178**:2243–7. doi:10.1084/jem.178.6.2243
21. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* (1993) **178**:2249–54. doi:10.1084/jem.178.6.2249
22. Ottenhoff TH. New pathways of protective and pathological host defense to mycobacteria. *Trends Microbiol* (2012) **20**:419–28. doi:10.1016/j.tim.2012.06.002
23. Desel C, Dorhoi A, Bandermann S, Grode L, Eisele B, Kaufmann SH. Recombinant BCG ΔureC hly+ induces superior protection over parental bcg by stimulating a balanced combination of type 1 and type 17 cytokine responses. *J Infect Dis* (2011) **204**:1573–84. doi:10.1093/infdis/jir592
24. Soares AP, Kwong Chung CK, Choice T, Hughes EJ, Jacobs G, van Rensburg RJ, et al. Longitudinal changes in CD4(+) T-cell memory responses induced by BCG vaccination of newborns. *J Infect Dis* (2013) **207**(7):1084–94. doi:10.1093/infdis/jis941
25. Jeong YH, Jeon BY, Gu SH, Cho SN, Shin SJ, Chang J, et al. Differentiation of antigen-specific T cells with limited functional capacity during *Mycobacterium tuberculosis* infection. *Infect Immun* (2014) **82**:132–9. doi:10.1128/IAI.00480-13
26. Behr MA, Small PM. Has BCG attenuated to impotence? *Nature* (1997) **389**:133–4. doi:10.1038/38148
27. Zhang W, Zhang Y, Zheng H, Pan Y, Liu H, Du P, et al. Genome sequencing and analysis of BCG vaccine strains. *PLoS One* (2013) **8**(8):e71243. doi:10.1371/journal.pone.0071243
28. Castillo-Rodal AI, Castañón-Arreola M, Hernándes-Pando R, Calva JJ, Sada-Díaz E, López-Vidal Y. *Mycobacterium tuberculosis* infection in a BALB/c model of progressive pulmonary tuberculosis. *Infect Immun* (2006) **74**:1718–24. doi:10.1128/IAI.74.3.1718-1724.2006
29. Davids V, Henakom WA, Mansoor N, Gamieldien H, Gelderbloem SJ, Hawkridge A, et al. The effect of bacilli Calmette-Guérin vaccine strain and rout of administration on induced immune responses in vaccinated infants. *J Infect Dis* (2006) **193**:531–6. doi:10.1086/499825
30. DasGupta SK, Jain S, Kaushal D, Tyagi AK. Expression systems for study of mycobacterial gene regulation and development of recombinant BCG vaccines. *Biochem Biophys Res Commun* (1998) **246**:797–804. doi:10.1006/bbrc.1998.8724
31. Lin CW, Su IJ, Chang JR, Chen YY, Lu JJ, Douh Y. Recombinant BCG coexpressing Ag85B, CFP10, and interleukin-12 induces multifunctional Th1 and memory T cells in mice. *APMIS* (2011) **120**:72–82. doi:10.1111/j.1600-0463.2011.02815.x
32. Tang C, Yamada H, Shibata K, Maeda N, Yoshida S, Wajjwalku W, et al. Efficacy of recombinant bacille Calmette-Guérin vaccine secreting interleukin-15/antigen 85B fusion protein in providing protection against *Mycobacterium tuberculosis*. *J Infect Dis* (2008) **197**:1263–74. doi:10.1086/586902
33. Sugawara I, Sun L, Mizuno S, Taniyama T. Protective efficacy of recombinant BCG Tokyo (Ag85A) in rhesus monkeys (*Macaca mulatta*) infected intratracheally with H37Rv *Mycobacterium tuberculosis*. *Tuberculosis* (2009) **89**:62–7. doi:10.1016/j.tube.2008.09.008
34. Sali M, Di Sante G, Cascioferro A, Zumbo A, Nicolo C, Dona V, et al. Surface expression of MPT64 as a fusion with the PE domain of PE_PGRS33 enhances *Mycobacterium bovis* BCG protective activity against *Mycobacterium tuberculosis* in mice. *Infect Immun* (2010) **78**:5202–13. doi:10.1128/IAI.00267-10
35. Tang C, Yamada H, Shibata K, Yoshida S, Wajjwalku W, Yoshikai Y. IL-15 protects antigen-specific CD8 T cell contraction after *Mycobacterium bovis* bacillus Calmette-Guérin infection. *J Leukoc Biol* (2009) **86**:187–94. doi:10.1189/jlb.0608363
36. Chapman R, Shephard E, Stutz H, Douglass N, Sambandamurthy V, Garcia I, et al. Priming with a recombinant pantothenate auxotroph of Mycobacterium bovis BCG and boosting with MVA elicits HIV-1 Gag specific CD8+ T cells. *PLoS One* (2012) **7**:e32769. doi:10.1371/journal.pone.0032769
37. Christy AJ, Dharman K, Dhandapaani G, Palaniyandi K, Gupta UD, Gupta P, et al. Epitope based recombinant BCG vaccine elicits specific TH1 polarized immune responses in BALB/c mice. *Vaccine* (2012) **30**:1364–70. doi:10.1016/j.vaccine.2011.12.059
38. Kong CU, Ng LG, Nambiar JK, Spratt JM, Weninger W, Triccas JA. Targeted induction of antigen expression within dendritic cells modulates antigen-specific immunity afforded by recombinant BCG. *Vaccine* (2011) **29**:1374–81. doi:10.1016/j.vaccine.2010.12.070
39. Shi C, Chen L, Chen Z, Zhang Y, Zhou Z, Lu J, et al. Enhanced protection against tuberculosis by vaccination with recombinant BCG over-expressing HspX protein. *Vaccine* (2010) **28**:5237–44. doi:10.1016/j.vaccine.2010.05.063
40. Rahman S, Magalhães I, Rahman J, Ahmed R, Sizemore DR, Scanga CA, et al. Prime-boost vaccination with rBCG/rAd35 enhances CD8+ cytolytic T-cell responses in lesions from *Mycobacterium tuberculosis* – infected primates. *Mol Med* (2012) **18**:647–58. doi:10.2119/molmed.2011.00222
41. Dey B, Jain R, Gupta UD, Katoch VM, Ramanathan VD, Tyagi AK. A booster vaccine expressing a latency-associated antigen augments BCG induced immunity and confers enhanced protection against tuberculosis. *PLoS One* (2011) **6**(8):e23360. doi:10.1371/journal.pone.0023360
42. Dey B, Jain R, Khera A, Gupta UD, Katoch VM, Ramanathan VD, et al. Latency antigen α-crystallin based vaccination imparts a robust protection against TB by modulating the dynamics of pulmonary cytokines. *PLoS One* (2011) **6**(4):e18773. doi:10.1371/journal.pone.0018773
43. Sun R, Skeiky YA, Izzo A, Dheenadhayalan V, Imam Z, Penn E, et al. Novel recombinant BCG expressing perfringolysin O and the over expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with *Mycobacterium tuberculosis*. *Vaccine* (2009) **27**:4412–23. doi:10.1016/j.vaccine.2009.05.048
44. Jain R, Dey B, Dhar N, Rao V, Singh R, Gupta UD, et al. Enhanced and enduring protection against tuberculosis by recombinant BCG-Ag85C and its association with modulation of cytokine profile in lung. *PLoS One* (2008) **3**(12):e3869. doi:10.1371/journal.pone.0003869
45. Qie YQ, Wang JL, Liu W, Shen H, Chen JZ, Zhu BD, et al. More vaccine efficacy studies on the recombinant bacille Calmette-Guérin co-expressing Ag85B, Mpt64 190–198 and Mtb8.4. *Scand J Immunol* (2009) **69**:342–50. doi:10.1111/j.1365-3083.2009.02231.x
46. Dey B, Jain R, Khera A, Rao V, Dhar N, Gupta UD, et al. Boosting with a DNA vaccine expressing ESAT-6 (DNAE6) obliterates the protection imparted by recombinant BCG (rBCGE6) against aerosol *Mycobacterium tuberculosis* infection in guinea pigs. *Vaccine* (2009) **28**:63–70. doi:10.1016/j.vaccine.2009.09.121

47. Lu Y, Xu Y, Yang E, Wang C, Wang H, Shen H. Novel recombinant BCG coexpressing Ag85B, ESAT-6 andRv2608 elicits significantly enhanced cellular immune and antibody responses in C57BL /6 Mice. *Scand J Immunol* (2012) **76**:271–7. doi:10.1111/j.1365-3083.2012.02726.x
48. Magalhães I, Sizemore DR, Ahmed RK, Mueller S, Wehlin L, Scanga C, et al. rBCG induces strong antigen-specific T cell responses in rhesus macaques in a prime-boost setting with an adenovirus 35 tuberculosis vaccine vector. *PLoS One* (2008) **3**(11):e3790. doi:10.1371/journal.pone.0003790
49. Hoft DF, Blazevic A, Abate G, Hanekom WA, Kaplan G, Soler JH, et al. A new recombinant bacille Calmette-Guérin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. *J Infect Dis* (2008) **198**:1491–501. doi:10.1086/592450
50. Tullius MV, Harth G, Maslesa-Galic S, Dillon BJ, Horwitz MA. A replication-limited recombinant *Mycobacterium bovis* BCG vaccine against tuberculosis designed for human immunodeficiency virus-positive persons is safer and more efficacious than BCG. *Infect Immun* (2008) **76**:5200–14. doi:10.1128/IAI.00434-08
51. Wang C, Fu R, Chen Z, Tan K, Chen L, Teng X, et al. Immunogenicity and protective efficacy of a novel recombinant BCG strain over expressing antigens Ag85A e Ag85B. *Clin Dev Immunol* (2012) **2012**:1–9. doi:10.1155/2012/563838
52. Deng YH, Sun Z, Yang XL, Bao L. Improved immunogenicity of recombinant *Mycobacterium bovis* bacillus Calmette-Guérin strains expressing fusion protein Ag85A-ESAT-6 of *Mycobacterium tuberculosis*. *Scand J Immunol* (2010) **72**:332–8. doi:10.1111/j.1365-3083.2010.02444.x
53. Deng YH, He HY, Zhang BS. Evaluation of protective efficacy conferred by a recombinant *Mycobacterium bovis* BCG expressing a fusion protein of Ag85A-ESAT-6. *J Microbiol Immunol Infect* (2012) **25**:S1684–1182. doi:10.1016/j.jmii.2012.11.005
54. Xu Y, Liu W, Shen H, Yan J, Qu D, Wang H. Recombinant *Mycobacterium bovis* BCG expressing the chimeric protein of antigen 85B and ESAT-6 enhances the Th1 cell-mediated response. *Clin Vaccine Immunol* (2009) **16**:1121–6. doi:10.1128/CVI.00112-09
55. Xu Y, Liu W, Shen H, Yan J, Yang E, Wang H. Recombinant *Mycobacterium bovis* BCG expressing chimeric protein of Ag85B and ESAT-6 enhances immunostimulatory activity of human macrophages. *Microbes Infect* (2010) **12**:683–689. doi:10.1016/j.micinf.2010.04.002
56. Yang X, Bao L, Deng Y. A novel recombinant *Mycobacterium bovis* bacillus Calmette-Guérin strain expressing human granulocyte macrophage colony-stimulating factor and *Mycobacterium tuberculosis* early secretory antigenic target 6 complex augments Th1 immunity. *Acta Biochim Biophys Sin* (2011) **43**:511–8. doi:10.1093/abbs/gmr045
57. Farinacci M, Weber S, Kaufmann SH. The recombinant tuberculosis vaccine rBCG ΔureC::hly(+) induces apoptotic vesicles for improved priming of CD4(+) and CD8(+) T cells. *Vaccine* (2012) **30**:7608–14. doi:10.1016/j.vaccine.2012.10.031
58. Reece ST, Nasser-Eddine A, Dietrich J, Stein M, Zedler U, Schommer-Leitner S, et al. Improved long-term protection against *Mycobacterium tuberculosis* Beijing/W in mice after intra-dermal inoculation of recombinant BCG expressing latency associated antigens. *Vaccine* (2011) **29**:8740–4. doi:10.1016/j.vaccine.2011.07.144
59. Grode L, Ganoza CA, Brohm C, Weiner J, Eisele B, Kaufmann SH. Safety and immunogenicity of the recombinant BCG vaccine VPM1002 in phase 1 open-label randomized clinical trial. *Vaccine* (2013) **18**:1340–8. doi:10.1016/j.vaccine.2012.12.053
60. Ohara N, Ohara-Wada N, Kitaura H, Nishiyama T, Matsumoto S, Yamada T. Analysis of the genes encoding the antigen 85 complex and MPT51 from *Mycobacterium avium*. *Infect Immun* (1997) **65**:3680–5.
61. Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* (1997) **276**:1420–2. doi:10.1126/science.276.5317.1420
62. Horwitz MA, Harth G, Dillon BJ, Maslesa-Galic' S. Recombinant bacillus Calmette-Guérin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proc Natl Acad Sci U S A* (2000) **97**:13853–8. doi:10.1073/pnas.250480397
63. Horwitz MA, Harth G. A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. *Infect Immun* (2003) **71**:1672–9. doi:10.1128/IAI.71.4.1672-1679.2003
64. Harth G, Lee BY, Wang J, Clemens DL, Horwitz MA. Novel insights into genetics, biochemistry, and immunocytochemistry of the 30-kilodalton major extracellular protein of *Mycobacterium tuberculosis*. *Infect Immun* (1997) **64**:3038–47.
65. Palma C, Iona E, Giannoni F, Pardini M, Brunori L, Fattorini L, et al. The LTK63 adjuvant improves protection conferred by Ag85B DNA-protein prime-boosting vaccination against *Mycobacterium tuberculosis* infection by dampening IFN-gamma response. *Vaccine* (2008) **26**:4237–43. doi:10.1016/j.vaccine.2008.05.050
66. Jackson M, Raynaud C, Lanéelle MA, Guilhot C, Laurent-Winter C, Ensergueix D, et al. Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. *Mol Microbiol* (1999) **31**:1573–87. doi:10.1046/j.1365-2958.1999.01310.x
67. Lozes E, Huygen K, Content J, Denis O, Montgomery DL, Yawman AM, et al. Immunogenicity and efficacy of a tuberculosis DNA vaccine encoding the components of the secreted antigen 85 complex. *Vaccine* (1997) **15**:830–3. doi:10.1016/S0264-410X(96)00274-5
68. Sugawara I, Udagawa T, Taniyama T. Protective efficacy of recombinant BCG Tokyo (Ag85A) BCG Tokyo with Ag85A peptide boosting against *Mycobacterium tuberculosis*-infected guinea pigs in comparison with that of DNA vaccine encoding Ag85A. *Tuberculosis* (2007) **87**:94–101. doi:10.1016/j.tube.2006.05.001
69. Sugawara I, Yamada H, Udagawa T, Huygen K. Vaccination of guinea pigs with DNA encoding Ag85A by gene gun bombardment. *Tuberculosis* (2003) **83**:331–7. doi:10.1016/S1472-9792(03)00054-4
70. Chang Z, Primm TP, Jakana J, Lee IH, Serysheva I, Chiu W, et al. *Mycobacterium tuberculosis* 16-kDa antigen (Hsp16.3) functions as an oligomeric structure in vitro to suppress thermal aggregation. *J Biol Chem* (1996) **12**:7218–23.
71. Kong D, Kunimoto DY. Secretion of human interleukin 2 by recombinant *Mycobacterium bovis* BCG. *Infect Immun* (1995) **63**:799–803.
72. McShane H, Behboudi S, Goonetilleke N, Brookes R, Hill AV. Protective immunity against *Mycobacterium tuberculosis* induced by dendritic cells pulsed with both CD8 (+)- and CD4(+)-T-cell epitopes from antigen 85A. *Infect Immun* (2002) **70**:1623–6. doi:10.1128/IAI.70.3.1623-1626.2002
73. Schaible UE, Winau F, Sieling PA, Fischer K, Collins HL, Hagens K, et al. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat Med* (2003) **9**:1039–46. doi:10.1038/nm906
74. Mandal M, Lee KD. Listeriolysin O-liposome mediated cytosolic delivery of macromolecule antigen in vivo: enhancement of antigen-specific cytotoxic T lymphocyte frequency, activity, and tumor protection. *Biochim Biophys Acta* (2002) **1563**:7–17. doi:10.1016/S0005-2736(02)00368-1
75. Kupferschmidt K. Infectious disease. Taking a new shot at a TB vaccine. *Science* (2011) **334**:1488–90. doi:10.1126/science.334.6062.1488
76. Hanekom WA. The immune response to BCG vaccination of newborns. *Ann NY Acad Sci* (2005) **1062**:69–78. doi:10.1196/annals.1358.010
77. World Health Organization (WHO). *Global Tuberculosis Report*. Geneva: WHO (2013).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 05 February 2014; accepted: 23 March 2014; published online: 07 April 2014. Citation: da Costa AC, Nogueira SV, Kipnis A and Junqueira-Kipnis AP (2014) Recombinant BCG: innovations on an old vaccine. Scope of BCG strains and strategies to improve long-lasting memory. Front. Immunol. **5**:152. doi: 10.3389/fimmu.2014.00152 This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 22 May 2014
doi: 10.3389/fimmu.2014.00219

The T-cell response to lipid antigens of *Mycobacterium tuberculosis*

***Gennaro De Libero*[1,2]* and *Lucia Mori*[1]**

[1] *Singapore Immunology Network, Agency for Science, Technology and Research (A*STAR), Singapore, Singapore*
[2] *Experimental Immunology, Department of Biomedicine, University Hospital Basel, Basel, Switzerland*

Edited by:
Juraj Ivanyi, Kings College London, UK

Reviewed by:
Roberto Nisini, Istituto Superiore di Sanità, Italy
Dirk Michael Zajonc, La Jolla Institute for Allergy and Immunology, USA

***Correspondence:**
Gennaro De Libero, 8A Biomedical Grove, Immunos, 138648 Singapore
e-mail: gennaro_delibero@immunol.a-star.edu.sg

T-cells recognize lipid antigens presented by dedicated antigen-presenting molecules that belong to the CD1 family. This review discusses the structural properties of CD1 molecules, the nature of mycobacterial lipid antigens, and the phenotypic and functional properties of T-cells recognizing mycobacterial lipids. In humans, the five CD1 genes encode structurally similar glycoproteins that recycle in and thus survey different cellular endosomal compartments. The structure of the CD1-lipid-binding pockets, their mode of intracellular recycling and the type of CD1-expressing antigen-presenting cells all contribute to diversify lipid immunogenicity and presentation to T-cells. Mycobacteria produce a large variety of lipids, which form stable complexes with CD1 molecules and stimulate specific T-cells. The structures of antigenic lipids may be greatly different from each other and each lipid may induce unique T-cells capable of discriminating small lipid structural changes. The important functions of some lipid antigens within mycobacterial cells prevent the generation of negative mutants capable of escaping this type of immune response. T-cells specific for lipid antigens are stimulated in tuberculosis and exert protective functions. The mechanisms of antigen recognition, the type of effector functions and the mode of lipid-specific T-cell priming are discussed, emphasizing recent evidence of the roles of lipid-specific T-cells in tuberculosis.

Keywords: CD1, lipid antigens, tuberculosis, T-cells, antigen presentation

INTRODUCTION

The discovery that T lymphocytes recognize lipid molecules as antigens (1) was a breakthrough in cellular immunology that opened unexpected horizons in the field of immune response to microbes, to cancer cells, and in autoimmunity.

Despite main structural differences with peptide antigens, lipid antigens are presented to T-cells following general rules that closely resemble those implicated in the presentation of peptide antigens. Indeed, only lipids forming stable complexes with dedicated antigen-presenting molecules, which belong to the CD1 protein family (2), are capable of interacting with the T-cell receptor (TCR) and may induce the activation of specific T-cells.

Many different types of lipids behave as antigens capable of stimulating specific T-cells and *Mycobacterium tuberculosis* is the bacterium with the largest number of antigenic lipids identified so far. Mycobacteria are characterized by a complex cellular envelope composed of a variety of unique lipids (3), which have important functions in survival of mycobacterial bacilli (4). Envelope lipids are also implicated in tissue-specific replication (5) and controlled penetration of engulfing cells (6) and in escaping immune response with different mechanisms (7). Therefore, Mycobacteria are reluctant in changing the lipid composition of their envelope. Mycobacterial lipids, glycolipids, and lipopeptides may bind to and form stable complexes with different CD1 molecules, and may interact with specific TCR.

The generation of stable CD1–lipid antigen complexes mostly occurs within the infected cell and follows a series of well coordinated events. These include the transport of lipid antigens by lipid chaperones in cellular membranes and endosomal compartments, the extraction of lipids from membranes, their processing, and subsequent insertion in the hydrophobic pockets of CD1 molecules. Stable CD1–lipid complexes are then routed to the plasma membrane where they become available for T-cell recognition (8).

Mycobacteria have evolved a series of tactics to interfere with lipid antigen presentation, showing the surprising capacity of inhibiting and hijacking different protective immune mechanisms. The existence of these evasion strategies is the indirect evidence that lipid-specific immune response represents an important mechanism of defense in tuberculosis. How lipid-specific T-cells participate in controlling mycobacterial infections is an important field of investigation in many laboratories that is providing new information on protective immune mechanisms and is suggesting novel approaches to vaccine development.

This chapter describes the nature, structure, and function of mycobacterial lipid antigens, the mechanisms of their recognition by T-cells, the biological properties of CD1 antigen-presenting molecules and the rules that govern presentation of lipids to T-cells. The relevance of lipid-specific T-cells in tuberculosis is also discussed.

CD1 ANTIGEN-PRESENTING MOLECULES

In humans five genes encode different CD1 isoforms, which are classified into three families (group 1, 2, and 3). CD1a, b, and c molecules belong to the group 1 family, whereas CD1d and CD1e are the only members of group 2 and 3, respectively. The separation into three families follows important structural differences,

cellular and tissue distribution of each CD1 protein. CD1a, b, and c are expressed on CD4, CD8 double positive thymocytes, and on peripheral antigen-presenting cells (APC), including dendritic cells (DC), monocytes, and a subset of B cells. Each of these molecules can be uniquely expressed. For example, CD1a, but not CD1b and c are expressed by Langerhans cells in the skin and represent a marker distinguishing this cell population. CD1b is expressed by a population of monocytes and is upregulated during differentiation into DC. CD1c is expressed on a large fraction of human B cells. CD1d is instead more extensively expressed on both hematopoietic and non-hematopoietic cells.

CD1e is the only CD1 protein that is not expressed on the plasma membrane and remains confined within the Golgi complex and late endosomes/lysosomes. All CD1 molecules are expressed by professional APC such as DC.

The structures of all CD1 molecules have been solved and showed important differences that illustrate their unique antigen-binding capacities.

STRUCTURE OF CD1 MOLECULES AND REPERTOIRE OF PRESENTED LIPID ANTIGENS

CD1 molecules have structures similar to those of MHC class I molecules. They are glycoproteins composed of a heavy chain non-covalently associated with β2-microglobulin. The heavy chain is made of three domains. The α1 and α2 domains, which assemble together in the membrane distal part of the molecule, are characterized by two anti-parallel α helices positioned above a β sheet plate. The α3 domain instead is membrane-proximal and associates with β2-microglobulin. It also continues with the transmembrane domain and a short intracytoplasmic tail. Each CD1 molecule shows a portal located in between the α1 and α2 helices, which allows access to two to four pockets. The pockets are surrounded by hydrophobic amino acids, thus representing ideal places to allocate the apolar alkyl chains of lipid antigens.

CD1a

Each CD1 molecule shows unique structural features.

CD1a has one portal located between the two α helices, which is connected with two pockets, called A′ and F′ (a nomenclature similar to that of MHC molecules). Around the portal a few charged amino acids stabilize and properly orient the antigen headgroup for TCR recognition. The F′ pocket has an incomplete roof thus being partially open on the surface of CD1a. The mode of lipid-binding of CD1a is unique, since the open F′ pocket permits ready insertion and replacement of lipid antigens. This feature optimally complements another unique characteristics of CD1a, i.e., its recycling through early endosomal stations (9, 10). Early endosomal compartments are slightly acidic environments that do not induce main conformational changes to the structure of recycling proteins. In addition, lipid transfer proteins that facilitate lipid antigen loading on CD1 molecules are localized in other endosomal compartments. Therefore, an open F′ pocket seems a natural evolution of CD1a to facilitate otherwise unfavorable conditions for loading of lipid antigens.

The co-crystal of CD1a with lipid antigens have also provided important information on another consequence of the unique open F′ pocket structure.

The CD1a-sulfatide structure revealed that the sphingosine occupies the A′ pocket whereas the fatty acid chain protrudes in the interface of the A′ and F′ pockets. The headgroup of sulfatide, made by a galactose and a sulfate is anchored in the A′–F′ junction and partially occupies the upper part of the F′ pocket in a position ideally suited for TCR recognition (11).

The crystal structure of CD1a with lipopeptide dideoxymycobactin (DDM) instead showed that while the single alkyl chain was inserted deep within the A′ pocket, the two peptidic moieties of the antigen were lying partially in the F′ pocket and at the surface of CD1a, an optimal position for recognition by the TCR (12).

MYCOBACTERIAL LIPID ANTIGENS PRESENTED BY CD1a

The overall size of CD1a grove is limited to 1350 Å^3, thus precluding binding of lipid antigens of large size. So far, only DDM has been identified as mycobacterial antigen presented by CD1a (13).

Dideoxymycobactin is the precursor of mycobactin, a siderophore with iron scavenging properties. The structure of DDM shows a complex head group made of unusual amino acids synthesized by non-ribosomal mycobacterial enzymes. The lysine of headgroup is linked to a single alkyl chain, which is 20 carbons long. One clone specific for CD1a–DDM was isolated and when it was tested the length and saturation of the DDM alkyl chain appeared very important for T-cell stimulation. This behavior was fully explained by the structural resolution of the CD1a–DDM complex, which showed a deep insertion of the acyl chain in the CD1a A′ pocket. As this pocket is closed at one end, the insertion of too short or too long acyl chains determines a different positioning of the peptidic residues, thus directly influencing the interaction with the TCR. This mode of binding led the authors to discuss a role as a ruler of the A′ CD1a pocket (12). During their intracellular growth, mycobacteria increase mycobactin synthesis to increase their capacity of iron capture. Thus presentation of DDM may be an efficient mechanism to identify cells harboring living mycobacteria.

CD1b

The molecule CD1b is expressed by activated monocytes and DC. CD1b presents most of the identified mycobacterial antigens and follows unique recycling and lipid loading rules. Within the group 1 CD1 family, CD1b has the largest pocket (~2200 Å^3) and is made by four interconnected pockets, which confer unique lipid antigen-binding properties. A large portal is located between the two α helices that is connected with the A′, C′, and F′ pockets. The A′ pocket continues into the F′ pocket through a tunnel called T′ pocket. The combination of the four pockets with the large size, allows CD1b to bind lipid antigens with very long alkyl chains, such as the mycobacterial mycolyl chains that can reach a length of 80 carbons. The structure of CD1b has been solved thus revealing how CD1b can accommodate either very long or short lipid antigens (14–17). Mycolic acid (MA), an antigen with long alkyl chain that can contain up to 80 carbons occupies the A′, T′, and F′ pockets, whereas the mycobacterial diacylated sulfoglycolipids (SGL) occupy the A′ and C′ pockets with their two alkyl chains. This flexibility in lipid-binding raised the question of why the CD1b T′ pocket does not collapse when antigens with short alkyl chains are bound. The reason for this stability was found when a soluble

CD1b molecule produced in eukaryotic cells and physiologically refolded *in vivo* was crystallized. This molecule showed the presence of different spacers within the T′ pocket, thus representing a filling lipid preventing the collapse of the molecule (18).

MYCOBACTERIAL LIPID ANTIGENS PRESENTED BY CD1b

Mycolic acids were the first antigens, which were identified as lipids capable of stimulating specific T-cells (19). These long and highly hydrophobic lipids represent very important components of the cell wall of several bacteria, including Mycobacteria, Corynebacteria, and *Nocardia*. MA are unique fatty acids of high-molecular-weight, with α-alkyl, β-hydroxyl groups. They may be present as such or as bound esters of sugars like arabinogalactan and trehalose. Activation assays using different types of MA, showed that the hydroxyl and carboxyl groups may represent important structural features sensed by T-cells (19).

Mycolic acid may also be considered as scaffold molecules that form stimulatory antigen when attached to other molecules. For example, glucose monomycolate (GMM) is capable of stimulating specific T-cells (20). In GMM, a glucose residue is attached to MA and is critical for T-cell recognition as the stereoisomers mannose- or galactose-monomycolate are not or poorly stimulatory. T-cells also discriminate the type of glycosidic bond, as GMM with glucose bound with 6-hydroxyl to MA is stimulatory, whereas glucose-3-monomycolate is not stimulatory (21).

Another antigen with modified MA is glycerol monomycolate (GroMM) (22) that is made of a glycerol moiety attached to a MA and is also produced by Mycobacteria, Corynebacteria, and *Nocardia*. The glycerol hydroxyl moiety and the fatty acid length of GroMM influence T-cell response. MA-related lipids are presented only by CD1b as this is the only CD1 molecule with an open T′ pocket allowing the insertion of long fatty acids.

A second family of lipid antigens presented by CD1b is those sharing a phosphatidyl-myo-inositol (PI). Phosphatidylinositol mannoside (PIM) and lipoarabinomannan (LAM) are two lipid antigens presented by CD1b (23). LAM and PIM share a core region, where the mannoses linked to PI are the stimulatory antigens. LAM contains a terminal part with many arabinoses in some instances capped with mannoses. Lipomannan (LM), which is similar to LAM, but does not contain arabinose, can also stimulate specific T-cells (23). T-cells discriminate lipoglycans made in different bacteria. T-cells recognizing LAM from *Mycobacterium leprae*, do not recognize LAM from *M. tuberculosis* (23). These LAM differ in the number and branching points of mannoses present in the core that are probably involved in interacting with the TCR.

All lipoglycans are too big to allocate within the CD1b pockets and interact with T-cells. They require internalization and processing in late endosomes (24). PIM_6, which is the hexamannosylated form of PIM, also requires processing with shortening of the hexamannoses in order to stimulate specific T-cells (25). Another aspect that is not clear yet is whether these antigens also require processing by lipases. Indeed naturally occurring PIM is characterized by four alkyl chains and they need *ad hoc* cleavage before forming stable complexes with CD1b (our unpublished data).

A third family of CD1b-presented lipid antigens is that of SGL (26). SGL are localized in the outer part of cell envelope and their abundance was associated with strain virulence (27), although mutant strains revealed that sulfolipid deficiency does not significantly affect the replication, persistence, and pathogenicity of *M. tuberculosis* H37Rv in mice and guinea pigs or in cultured macrophages (28). SGL contain a trehalose 2′ sulfate core, acylated by two to four fatty acids. This antigen isolated from virulent mycobacteria strains proved to a be a mixture of structurally related diacylated SGL (Ac_2SGL) containing hydroxyphtioceranoic and palmitic or stearic acid, both linked to the glucose without the sulfate in the trehalose. The tri- and tetra-acylated forms were not immunogenic, thus revealing that APC do not have the proper lipase apparatus to digest these forms and generate the diacylated immunogenic forms. Removal of the sulfate group completely abolished T-cell activation (26), suggesting that the fine antigen specificity of the TCR is also influenced by the strong negative charges of sulfate.

The structure of CD1b–SGL complex has been solved and showed a unique mode of interaction of this lipid antigen with the CD1b (17). Comparison of the CD1b crystals with and without SGL showed that upon antigen-binding, the endogenous spacers of CD1b, which consist of a mixture of diradylglycerols, move considerably within the lipid-binding groove. Spacer displacement was accompanied by F′ pocket closure and an extensive rearrangement of residues exposed to T-cell receptors. Such structural reorganization resulted in reduction of the A′ pocket capacity and led to incomplete embedding of the methyl-ramified portion of the phthioceranoyl chain of the antigen, explaining why such hydrophobic motifs are critical for T-cell receptor recognition. Mutagenesis experiments supported the functional importance of the observed structural alterations for T-cell stimulation. Thus, CD1b is a very plastic molecule, capable of important structural rearrangements that combine spacer repositioning and ligand-induced conformational changes. Both these changes endow CD1b with the capacity to present a broad range of structurally diverse antigens and to focus the TCR only on the antigen-filled CD1b molecules.

CD1c

The CD1c molecule is characterized by unique features that contribute to the important biological features of this CD1 isotype.

The central portal located in between the two alpha helices allows protrusion of the hydrophilic moiety of bound antigen, which is presumably involved also in contacting the TCR. This portal is connected with the A′ and F′ pockets. The A′ pocket may accommodate the alkyl chain of bound antigen (29). The A′ pocket is open to the external part of CD1c with an exit portal located underneath the α1 helix. Interestingly, the F′ pocket of CD1c showed an open cavity, which is similar to the antigen-binding groove of MHC-peptide-binding molecules and in some respects to the F′ pocket of CD1a. The F′ pocket is also open to the exterior with a second portal called E′. Whether these additional portals facilitate binding of very long alkyl chains, remains unclear, although this type of antigen interaction is energetically unfavorable.

CD1c is also characterized by a unique type of intracellular recycling. It may recycle in deep endosomal compartments,

where low pH may facilitate partial CD1 unfolding and antigen loading. Furthermore, in these compartments dedicated lipid antigen-binding proteins (LBP) are present, which behave as lipid chaperones and are involved in lipid antigen-binding, transport, and loading on CD1 molecules. This mode of antigen loading resembles that of lipids on CD1b and CD1d. In addition, some CD1c molecules recycle through the early endosomal compartments, where the most acidic pH is 6.5 and probably no LBP are present. In this cellular compartment, the partially open structure of the F′ pocket might facilitate antigen exchange and binding.

MYCOBACTERIAL LIPID ANTIGENS PRESENTED BY CD1c

Both the unique portals and structure of the F′ pocket explain the promiscuous antigen-binding behavior of CD1c. This molecule may present lipid antigens containing one alkyl chain (30), two alkyl chains (31), or lipopeptides formed by one alkyl chain linked to a short peptide (32). Furthermore, CD1c stimulates a population of T-cells expressing the gamma and delta chains of the TCR (TCR γδ) (33, 34). As the described CD1c-restricted TCR γδ cells are autoreactive, it is very probable that CD1c presents a self-antigen, which remains unknown.

Recently, we have identified the novel self lipid methyl-lysophosphatidic acids (mLPA), not described before, which accumulates in myeloid and lymphoid leukemia cells and induces a CD1c-restricted response directed against these human leukemias (35). mLPA-specific T-cells efficiently kill $CD1c^+$ acute leukemia cells, and protect immunodeficient mice against $CD1c^+$ human leukemia cells and poorly recognize non-transformed CD1c-expressing cells. Whether similar types of lipids are produced by mycobacteria or accumulate in infected cells during mycobacterial infection and induce CD1-restricted T-cell responses is matter of current investigation.

Mannosyl β-1-phosphomycoketides (MPM) were the first CD1c-restricted lipid antigens (30). MPM contain a single fully saturated alkyl chain with methyl branches at every fourth carbon. MPM are synthesized by Mycobacteria infecting human cells, including *M. tuberculosis*, BCG, and *Mycobacterium avium*, and not by rapidly growing saprophytes, including *Mycobacterium phlei*, *Mycobacterium fallax*, and *Mycobacterium smegmatis* (36). The structure of MPM is similar to that of phosphodolichols that in mammalian cells function as carbohydrate donors in glycan synthesis. However, differently from phosphodolichols they are not synthesized through an isoprenoid-independent pathway. Instead, the synthesis of MPM is accomplished by the enzyme Pks12 and requires the sequential condensation of malonate and methylmalonate units. Probably, these polyketides are involved in bacterial intracellular growth and in mannose transmembrane transport, according to their structural similarity with eukaryotic phosphodolichols. As found with other glycolipid-specific T-cells, also MPM-specific T-cell recognition is sensitive to structural changes in the hydrophilic and hydrophobic parts of MPM. Antigen recognition occurs when glucose and not mannose is present. Furthermore, the saturated α-prenyl like unit and the length of the prenyl chain are important for recognition. Moreover, variation in the number, length, and saturation of alkyl chains, and the precise chemistry and chirality of the lipid head group, also exert dominant influences on antigenicity (30, 36, 37), probably by affecting the mode of CD1 binding and interaction with the TCR (38).

CD1d

Like other CD1 molecules, also CD1d contains antigen-binding pockets, which are much deeper than the groove of peptide-binding MHC molecules. The two pockets are constituted by a non-polar or hydrophobic surface. The pockets converge in a portal open on the surface of CD1d and located in between the two anti-parallel helices. A series of TCR–CD1d–lipid antigen co-crystals have shown the importance of the polar antigen residues, which provide minimal but essential interactions with the TCR (39–42).

The A′ pocket of CD1d is closed and allows the insertion of alkyl chains with a defined length. In the presence of lipid antigens with too short alkyl chains, small fatty acids may also be present, which contribute to filling the entire A′ pocket probably behaving as rulers preventing CD1d collapse (43).

CD1d is expressed on mostly all hematopoietic cells. It is also present on the surface of gut epithelial cells, adipocytes, and some keratinocytes. Most of the cells retain a large fraction of properly assembled CD1d in late endosomes and lysosomes, in a manner similar to that of MHC class II molecules. Upon synthesis, CD1d rapidly traffics to the plasma membrane and then initiates a fast intracellular recycling. A fraction of CD1d molecules is assembled together with the invariant chain that promotes a direct traffic to late endosomes (44). The mode of intracellular trafficking of CD1d is dependent on the unique intracytoplasmic tail that facilitates the interaction with the AP3 proteins (45). Indeed, this association is necessary to properly load lipid antigens intracellularly (46).

Many microorganisms modulate surface CD1d, including *M. tuberculosis*. Several studies have outlines different modulation capabilities according to the type of cell, the triggered surface receptor, and the possible involvement of soluble factors. While several viruses, including vaccinia, herpes simplex, hepatitis, and lymphocytic choriomeningitis viruses decrease CD1d expression in myeloid cells (47–49), infections with *Salmonella typhimurium* or *E. coli* increase the plasma membrane display of CD1d (50). *M. tuberculosis* infection or mycobacterial lipids also upregulate CD1d on bone-marrow-derived macrophages (51). Instead *M. tuberculosis* infection prevents the upregulation of CD1d in monocytes that differentiate to DC (52, 53).

MYCOBACTERIAL LIPID ANTIGENS PRESENTED BY CD1d

CD1d-restricted T-cells are classified in two major groups. The most widely investigated are invariant natural killer T (iNKT) cells, which express an invariant human Vα24Jα18 or mouse Vα14Jα18 TCR chain. These cells recognize with high affinity the α-galactosylceramide (αGalCer) lipid extracted from the marine sponge *Agelas mauritianus* (54). iNKT cells are highly conserved in many species, thus suggesting that they exert important immunological functions conserved during evolution. iNKT cells also recognize other non-microbial antigens, including phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE), although with a weak affinity compared to the strong agonist αGalCer (55). Lysophosphatidylcholine, a lipid generated by lipid-dependent

signaling pathways, may also stimulate iNKT cells (56). Other CD1d-presented antigens are the ether-bonded mono-alkyl glycerophosphates that together with the precursors and degradation products of plasmalogens are required for the proper selection and maturation of iNKT cells in the thymus (57).

Very initial studies suggested that a tetramannosylated PIM form (PIM_4) present in a fraction of lipids purified from *Mycobacterium bovis* BCG was a potential CD1d-presented iNKT cell antigen (58). These findings have not been confirmed in subsequent studies and the potential role of PIM_4 as CD1d-presented antigen to iNKT cells still awaits confirmation. While CD1d mycobacterial antigens have not been identified so far, other bacteria produce strong iNKT stimulatory lipids, including the glycolipids with diacylglycerol moieties of *Borrelia burgdorferi* and *Streptococcus pneumoniae* (59, 60), and the glycosphingolipids present in the cell walls of bacteria belonging to the genera *Sphingomonas* and *Novosphingobium* (61). Surface glycophospholipids of the protozoal parasites *Leishmania* species are also iNKT stimulatory antigens (62). iNKT-deficient mice are more susceptible to *Leishmania* infection, indicating that iNKT cells have important functions in protection. However, it is still unknown how *Leishmania* lipophosphoglycan (LPG) stimulate these T-cells. While LPG binds to CD1d, CD1d–LPG complexes do not stimulate iNKT cells. DC pulsed with LPG can induce IFNγ release by iNKT cells, suggesting that LPG stimulates the production of endogenous lipid ligands, which in turn activate iNKT cells. This is not a remote possibility, since bacterial infections may induce important changes in the lipid metabolism of infected cells. This, in turn, may induce accumulation of endogenous lipids that stimulate T-cells restricted by either group 1 or group 2 CD1 molecules (63–65).

A second group of CD1d-restricted T-cells utilized non-invariant TCR and resemble other T-cells involved in classical adaptive immune responses. These T-cells recognize PG, diphosphatidylglycerol (DPG or cardiolipin), and PI from *M. tuberculosis* or *Corynebacterium glutamicum* as microbial antigens (66). Importantly, the same lipid molecules are present also in mammalian cells and thus they represent self-antigens. Thereby, the increased availability of these self-antigens within APC might also contribute to the activation of CD1d-restricted T-cells cross-reactive with microbial antigens.

CD1e

CD1e is a unique type of CD1 molecule as it remains intracellular and therefore cannot be considered a *bona fide* antigen-presenting molecule (67). CD1e is mostly expressed by DC where it accumulates in the Golgi stack of immature cells as pre-protein (68). Upon maturation, CD1e is ubiquitinated (69) and traffics to the lysosomes where it is cleaved in two places. In the amino-terminal region a propeptide is cleaved, that is responsible for the assembly of CD1e with β2-microglobulin. Upon its removal CD1e becomes fully active (70). CD1e is also cleaved in its membrane-proximal region, thus becoming a mature soluble protein. CD1e is apparently not secreted and does not accumulate in other intracellular compartments. These features make CD1e similar to the HLA-DM and HLA-DO molecules, which are also not expressed on the plasma membrane of APC. The acid pH of lysosomes promotes a partial alteration of CD1e structure together with the binding of lipid antigens through increased hydrophobic and ionic interactions (71).

The crystal structure of CD1e revealed a groove less intricate than in other CD1 proteins, with a significantly wider portal. The water-exposed CD1e groove probably allows loose contacts with lipid antigens as supported by the finding that lipid association and dissociation processes were faster with CD1e than with CD1b (72).

All these structural features support the function of CD1e as a LBP with the exquisite capability of facilitating lipid antigen presentation by other CD1 molecules. Indeed, CD1e mediates *in vitro* the transfer of lipids to CD1b and the displacement of lipids from stable CD1b-antigen complexes (72). CD1e is also required for the proper processing of complex mycobacterial lipid antigens such as the PIM_6 molecules. In the presence of CD1e, PIM_6 is cleaved to PIM_2 and is loaded on CD1b (25). CD1e may also positively or negatively affect lipid presentation by CD1b, CD1c, and CD1d (73). This effect is achieved by a rapid formation of CD1–lipid complexes in the presence of CD1e, and also by an accelerated turnover of formed CD1–lipid antigen complexes. These effects maximize and temporally narrow CD1-restricted responses. CD1e is therefore an important modulator of both group 1 and group 2 CD1-restricted responses influencing the lipid antigen availability as well as the generation and persistence of CD1–lipid complexes.

T-CELLS RECOGNIZING MYCOBACTERIAL LIPID ANTIGENS

T-cells stimulated by lipid antigens are divided into two categories according to their functional behavior and mechanism of antigen recognition. T-cells restricted by group 1 CD1 molecules and type II NKT cells are considered as arm of adaptive T-cell responses, while CD1d-restricted type II NKT or iNKT cells are considered as the typical representative of innate T-cell responses.

Lipid-specific T-cells that are restricted by group 1 CD1 molecules are present as naïve T-cells and expand after encountering the antigen, mostly during mycobacterial infections or BCG vaccination (26). They expand and may also differentiate into classical memory T-cells (74). These studies have been performed using human samples, as small rodents do not express group 1 CD1 molecules. Upon antigen recognition, lipid-specific T-cells release pro-inflammatory cytokines and may also kill intracellular Mycobacteria, thus participating in host protection (26). They also induce maturation of DC (75), thus facilitating priming of other mycobacteria-specific T-cells.

The TCR repertoire of lipid-specific T-cells is polymorphic and there is no evidence of unique TCR V genes imposing the recognition of antigens presented by CD1a, CD1b, or CD1c. There is also no evidence that type II NKT cells utilize a restricted TCR repertoire. Recently, using CD1b tetramers loaded with GMM, a rare population of T-cells was identified, which uses a nearly invariant TCR (76). These T-cells were defined as mycolyl lipid-reactive (GEM) cells. The TCR of these GMM-specific and CD1b-restricted T-cells is constituted by a TRAV1.2 gene rearranged to TRAJ19 segment. Furthermore, these T-cells showed a strong bias for usage of TRB6-2 gene and high affinity for the CD1b–GMM complex. The analysis of nucleotides in the CDR3 of the Vα chain showed that similar CDR3 amino acids were derived from different patterns of exonucleolytic trimming of the V and J regions, suggesting an antigen recognition-induced mechanism driving the

expansion of these cells. This conclusion was also supported by the finding of identical nucleotide sequences in GMM-specific T-cells. In addition, the same sequences were found several times in the same donors, indicating *in vivo* expansion of GEM T-cells. A second study also showed that CD1b–GMM tetramers may identify another population of T-cells, using the TRAV17 and TRBV4-1 genes, which is present in several donors, has intermediate affinity for the CD1b–GMM complexes and shows biased but different CDR3 motifs (77). Whether this second population is expanded in donors with active or latent tuberculosis remains to be defined.

The identification of lipid-specific T-cells has made an incredible technical jump by using the tetrameric forms of soluble CD1 molecules loaded with lipid antigens. In addition to the CD1b tetramers that have been discussed above, also CD1c (78) tetramers have been generated. With the use of these tetramers it was possible to identify a population of CD1c-restricted and mycoketide-specific T-cells that use a polyclonal TCR repertoire. The identification of CD1-restricted T-cells using antigen-loaded CD1 tetramers has important implications, since detection of lipid-specific T-cells might represent a novel approach to monitor the T-cell response in patients with active or latent forms of tuberculosis.

An important set of data indicates that group 1 CD1-restricted T-cells have important functions in animal models and in humans.

Immunization of guinea pigs (*Cavia porcellus*), a species expressing multiple CD1b and CD1c gene orthologs, induces strong proliferative and cytotoxic responses restricted by CD1b and CD1c (79, 80) and immunization with total *M. tuberculosis* lipids induced a significant reduction of lung lesions (81).

Cattle (*Bos taurus*) are sensitive to natural infection with *M. bovis*, causing bovine tuberculosis that shares many clinical and pathological characteristics with human tuberculosis. Polyclonal T-cells isolated from naturally infected animals showed specific response to challenge with lipids from *M. bovis* and *M. avium paratuberculosis* (82). Uninfected animals immunized with GMM also developed GMM-specific CD1b-restricted T-cells, which persisted 4 months after the last immunization (83).

Studies performed in CD1 transgenic mice showed lipid-specific T-cells upon immunization with *M. tuberculosis* lipids or infected with *M. tuberculosis* (84).

Several studies investigated the response of human T-cells to stimulation with mycobacterial lipids *ex vivo*. Using this approach, T-cells specific for the mycobacterial antigens GroMM (22), GMM (85), SGL (26), MA (74), and MPM (30) were identified. In several cases, these T-cells were detected only in tuberculosis patients and not in healthy individuals, indicating that priming with lipid antigens occurs during infection.

A series of important immunological questions on the role of lipid-specific T-cells in tuberculosis remain to be answered. Firstly, most of the studies were performed with established T-cell clones using different cellular assays including cell proliferation, IFNγ release, and cell cytotoxicity. These assays detect protective anti-microbial capacities and indicate potential anti-microbial protective effects of these cells. These studies should be complemented with the functional analysis of T-cells freshly isolated from tuberculosis patients and possibly by *in vivo* studies using CD1 transgenic mice. Secondly, the mechanisms of lipid-specific T-cell memory generation remain unknown. The experimental data showing absence of mycobacterial lipid-specific T-cells in non-infected individuals supports that naïve T-cells are primed by a specific antigen and expand *in vivo*. Whether these cells persist for a long period of time and rapidly expand upon challenge with the antigen is not clear. These issues are of paramount importance to understand the possible use of lipid antigens as novel sub-unit anti-mycobacterial vaccines. Thirdly, the tissue-specific expression of CD1 proteins might represent a limitation to the protective activity of CD1-restricted T-cells. Macrophages are the cells harboring Mycobacteria during infection and do not express detectable levels of CD1 molecules. Therefore, these cells might be invisible to lipid-specific T-cells, thus reducing their potential protective role. Another important issue is whether CD1-restricted T-cells massively migrate to infected tissues, mostly the lung, and therefore are rare in circulating blood. The accumulation of lipid-specific T-cells in the lung of tuberculosis patients has not been properly investigated yet.

The second population of lipid-specific T-cells is represented by iNKT cells, which are innate-like T-cells. These cells are constantly activated by a variety of self- and microbial antigens and show a phenotype of pre-activate cells. They contain in their cytoplasm preformed cytokines mRNA, including IFNγ mRNA. They readily secrete these cytokines upon antigen recognition at levels that are up to 200-fold higher than those of naïve peptide-specific T-cells (86, 87). They also rapidly induce DC maturation, which in turn facilitates priming of $CD8^+$ cytotoxic T-cells (88). Considering that this latter T-cell population is relevant in protection during mycobacterial infections (89), iNKT cell engagement might be also important for protection.

In vivo studies conducted in mice also showed that iNKT are activated during mycobacterial infections. Following BCG infection, there is an increase of iNKT cells in the lung that show signs of activation (90). iNKT cells were assumed to be important in development of lung granuloma following infection (90–92). Instead a series of studies performed using CD1d-deficient mice (lacking all types of CD1d-restricted T-cells) or Jα18-deficient animals (lacking mainly iNKT cells), did not show relevant difference in the mycobacterial colony forming units in the lung, nor in the survival of infected animals as compared to wild type mice (93–96).

Overall these studies showed that iNKT cells do not have an impact on chronic infection in mice and suggest that they are not involved in direct protective roles during tuberculosis. Thus, the potentially protective functions of iNKT cells, if any, may be present only in the early acute phases of microbial infections and be neglectable in the natural course of prolonged chronic infections such as tuberculosis.

Studies conducted in humans are observational and are limited to the numbers, phenotype, and *ex vivo* function of iNKT cells isolated from peripheral blood. None of these investigations supported an important role of iNKT cells in tuberculosis, although reduction in their number and phenotype has been observed in patients with active tuberculosis (97–99).

CONCLUSION

From the overview of the studies reported here, there are increasing evidences of the relevance of the immune response elicited by mycobacterial lipids. The identification of lipid molecules essential

for bacterial virulence together with the absence of functional CD1 polymorphisms is the basis for vaccine development. There is therefore increasing hope that the inclusion of lipid antigens in new vaccine formulations would lead to a successful vaccination against tuberculosis.

ACKNOWLEDGMENTS

European Union FP7 NEWTBVAC program, Swiss National Foundation Grant 310030_149571, A-STAR Australian NHMRC joint grant No. 12/1/82/24/6277, and BMRC-SERC grant No. 112 148 0006.

REFERENCES

1. Porcelli S, Morita CT, Brenner MB. CD1b restricts the response of human CD4-8- T lymphocytes to a microbial antigen. *Nature* (1992) **360**(6404):593–7. doi:10.1038/360593a0
2. Porcelli SA. The CD1 family: a third lineage of antigen-presenting molecules. *Adv Immunol* (1995) **59**:1–98. doi:10.1016/S0065-2776(08)60629-X
3. Karakousis PC, Bishai WR, Dorman SE. *Mycobacterium tuberculosis* cell envelope lipids and the host immune response. *Cell Microbiol* (2004) **6**(2):105–16. doi:10.1046/j.1462-5822.2003.00351.x
4. Neyrolles O, Guilhot C. Recent advances in deciphering the contribution of *Mycobacterium tuberculosis* lipids to pathogenesis. *Tuberculosis (Edinb)* (2011) **91**(3):187–95. doi:10.1016/j.tube.2011.01.002
5. Cox JS, Chen B, McNeil M, Jacobs WR Jr. Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. *Nature* (1999) **402**(6757):79–83. doi:10.1038/47042
6. Cambier CJ, Takaki KK, Larson RP, Hernandez RE, Tobin DM, Urdahl KB, et al. Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. *Nature* (2014) **505**(7482):218–22. doi:10.1038/nature12799
7. Ham H, Sreelatha A, Orth K. Manipulation of host membranes by bacterial effectors. *Nat Rev Microbiol* (2011) **9**(9):635–46. doi:10.1038/nrmicro2602
8. De Libero G, Mori L. Recognition of lipid antigens by T cells. *Nat Rev Immunol* (2005) **5**(6):485–96. doi:10.1038/nri1631
9. Sugita M, Grant EP, Van Donselaar E, Hsu VW, Rogers RA, Peters PJ, et al. Separate pathways for antigen presentation by CD1 molecules. *Immunity* (1999) **11**(6):743–52. doi:10.1016/S1074-7613(00)80148-X
10. Barral DC, Cavallari M, McCormick PJ, Garg S, Magee AI, Bonifacino JS, et al. CD1a and MHC class I follow a similar endocytic recycling pathway. *Traffic* (2008) **9**(9):1446–57. doi:10.1111/j.1600-0854.2008.00781.x
11. Zajonc DM, Elsliger MA, Teyton L, Wilson IA. Crystal structure of CD1a in complex with a sulfatide self antigen at a resolution of 2.15 A. *Nat Immunol* (2003) **4**(8):808–15. doi:10.1038/ni948
12. Zajonc DM, Crispin MD, Bowden TA, Young DC, Cheng TY, Hu J, et al. Molecular mechanism of lipopeptide presentation by CD1a. *Immunity* (2005) **22**(2):209–19. doi:10.1016/j.immuni.2004.12.009
13. Moody DB, Young DC, Cheng TY, Rosat JP, Roura-Mir C, O'Connor PB, et al. T cell activation by lipopeptide antigens. *Science* (2004) **303**(5657):527–31. doi:10.1126/science.1089353
14. Gadola SD, Zaccai NR, Harlos K, Shepherd D, Castro-Palomino JC, Ritter G, et al. Structure of human CD1b with bound ligands at 2.3 A, a maze for alkyl chains. *Nat Immunol* (2002) **3**(8):721–6. doi:10.1038/ni821
15. Batuwangala T, Shepherd D, Gadola SD, Gibson KJ, Zaccai NR, Fersht AR, et al. The crystal structure of human CD1b with a bound bacterial glycolipid. *J Immunol* (2004) **172**(4):2382–8. doi:10.4049/jimmunol.172.4.2382
16. Garcia-Alles LF, Versluis K, Maveyraud L, Vallina AT, Sansano S, Bello NF, et al. Endogenous phosphatidylcholine and a long spacer ligand stabilize the lipid-binding groove of CD1b. *EMBO J* (2006) **25**(15):3684–92. doi:10.1038/sj.emboj.7601244
17. Garcia-Alles LF, Collmann A, Versluis C, Lindner B, Guiard J, Maveyraud L, et al. Structural reorganization of the antigen-binding groove of human CD1b for presentation of mycobacterial sulfoglycolipids. *Proc Natl Acad Sci U S A* (2011) **108**(43):17755–60. doi:10.1073/pnas.1110118108
18. De Libero G, Mori L. Novel insights into lipid antigen presentation. *Trends Immunol* (2012) **33**(3):103–11. doi:10.1016/j.it.2012.01.005
19. Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. *Nature* (1994) **372**(6507):691–4. doi:10.1038/372691a0
20. Moody DB, Reinhold BB, Guy MR, Beckman EM, Frederique DE, Furlong ST, et al. Structural requirements for glycolipid antigen recognition by CD1b-restricted T cells. *Science* (1997) **278**(5336):283–6. doi:10.1126/science.278.5336.283
21. Moody DB, Guy MR, Grant E, Cheng TY, Brenner MB, Besra GS, et al. CD1b-mediated T cell recognition of a glycolipid antigen generated from mycobacterial lipid and host carbohydrate during infection. *J Exp Med* (2000) **192**(7):965–76. doi:10.1084/jem.192.7.965
22. Layre E, Collmann A, Bastian M, Mariotti S, Czaplicki J, Prandi J, et al. Mycolic acids constitute a scaffold for mycobacterial lipid antigens stimulating CD1-restricted T cells. *Chem Biol* (2009) **16**(1):82–92. doi:10.1016/j.chembiol.2008.11.008
23. Sieling PA, Chatterjee D, Porcelli SA, Prigozy TI, Mazzaccaro RJ, Soriano T, et al. CD1-restricted T cell recognition of microbial lipoglycan antigens. *Science* (1995) **269**(5221):227–30. doi:10.1126/science.7542404
24. Ernst WA, Maher J, Cho S, Niazi KR, Chatterjee D, Moody DB, et al. Molecular interaction of CD1b with lipoglycan antigens. *Immunity* (1998) **8**(3):331–40. doi:10.1016/S1074-7613(00)80538-5
25. De la Salle H, Mariotti S, Angenieux C, Gilleron M, Garcia-Alles LF, Malm D, et al. Assistance of microbial glycolipid antigen processing by CD1e. *Science* (2005) **310**(5752):1321–4. doi:10.1126/science.1115301
26. Gilleron M, Stenger S, Mazorra Z, Wittke F, Mariotti S, Bohmer G, et al. Diacylated sulfoglycolipids are novel mycobacterial antigens stimulating CD1-restricted T cells during infection with *Mycobacterium tuberculosis*. *J Exp Med* (2004) **199**(5):649–59. doi:10.1084/jem.20031097
27. Goren MB. Immunoreactive substances of mycobacteria. *Am Rev Respir Dis* (1982) **125**(3 Pt 2):50–69.
28. Rousseau C, Turner OC, Rush E, Bordat Y, Sirakova TD, Kolattukudy PE, et al. Sulfolipid deficiency does not affect the virulence of *Mycobacterium tuberculosis* H37Rv in mice and guinea pigs. *Infect Immun* (2003) **71**(8):4684–90. doi:10.1128/IAI.71.8.4684-4690.2003
29. Scharf L, Li NS, Hawk AJ, Garzon D, Zhang T, Fox LM, et al. The 2.5 A structure of CD1c in complex with a mycobacterial lipid reveals an open groove ideally suited for diverse antigen presentation. *Immunity* (2010) **33**(6):853–62. doi:10.1016/j.immuni.2010.11.026
30. Moody DB, Ulrichs T, Muhlecker W, Young DC, Gurcha SS, Grant E, et al. CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection. *Nature* (2000) **404**(6780):884–8. doi:10.1038/35009119
31. Shamshiev A, Gober HJ, Donda A, Mazorra Z, Mori L, De Libero G. Presentation of the same glycolipid by different CD1 molecules. *J Exp Med* (2002) **195**(8):1013–21. doi:10.1084/jem.20011963
32. Van Rhijn I, Young DC, De Jong A, Vazquez J, Cheng TY, Talekar R, et al. CD1c bypasses lysosomes to present a lipopeptide antigen with 12 amino acids. *J Exp Med* (2009) **206**(6):1409–22. doi:10.1084/jem.20082480
33. Faure F, Jitsukawa S, Miossec C, Hercend T. CD1c as a target recognition structure for human T lymphocytes: analysis with peripheral blood gamma/delta cells. *Eur J Immunol* (1990) **20**(3):703–6. doi:10.1002/eji.1830200336
34. Spada FM, Grant EP, Peters PJ, Sugita M, Melian A, Leslie DS, et al. Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *J Exp Med* (2000) **191**(6):937–48. doi:10.1084/jem.191.6.937
35. Lepore M, De Lalla C, Ramanjaneyulu GS, Gsellinger H, Consonni M, Garavaglia C, et al. A novel self-lipid antigen targets human T cells against CD1c+ leukaemias. *J Exp Med* (2014) (in press).
36. Matsunaga I, Bhatt A, Young DC, Cheng TY, Eyles SJ, Besra GS, et al. *Mycobacterium tuberculosis* pks12 produces a novel polyketide presented by CD1c to T cells. *J Exp Med* (2004) **200**(12):1559–69. doi:10.1084/jem.20041429
37. De Jong A, Arce EC, Cheng TY, Van Summeren RP, Feringa BL, Dudkin V, et al. CD1c presentation of synthetic glycolipid antigens with foreign alkyl branching motifs. *Chem Biol* (2007) **14**(11):1232–42. doi:10.1016/j.chembiol.2007.09.010
38. De Libero G, Mori L. The easy virtue of CD1c. *Immunity* (2010) **33**(6):831–3. doi:10.1016/j.immuni.2010.12.001
39. Borg NA, Wun KS, Kjer-Nielsen L, Wilce MC, Pellicci DG, Koh R, et al. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature* (2007) **448**(7149):44–9. doi:10.1038/nature05907
40. Patel O, Pellicci DG, Gras S, Sandoval-Romero ML, Uldrich AP, Mallevaey T, et al. Recognition of CD1d-sulfatide mediated by a type II natural killer T cell antigen receptor. *Nat Immunol* (2012) **13**(9):857–63. doi:10.1038/ni.2372
41. Girardi E, Maricic I, Wang J, Mac TT, Iyer P, Kumar V, et al. Type II natural killer T cells use features of both innate-like and conventional T cells to recognize sulfatide self antigens. *Nat Immunol* (2012) **13**(9):851–6. doi:10.1038/ni.2371

42. Rossjohn J, Pellicci DG, Patel O, Gapin L, Godfrey DI. Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol* (2012) **12**(12):845–57. doi:10.1038/nri3328
43. Zajonc DM, Cantu C III, Mattner J, Zhou D, Savage PB, Bendelac A, et al. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. *Nat Immunol* (2005) **6**(8):810–8. doi:10.1038/ni1224
44. Jayawardena-Wolf J, Benlagha K, Chiu YH, Mehr R, Bendelac A. CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. *Immunity* (2001) **15**(6):897–908. doi:10.1016/S1074-7613(01)00240-0
45. Sugita M, Cao X, Watts GF, Rogers RA, Bonifacino JS, Brenner MB. Failure of trafficking and antigen presentation by CD1 in AP-3-deficient cells. *Immunity* (2002) **16**(5):697–706. doi:10.1016/S1074-7613(02)00311-4
46. Cernadas M, Sugita M, Van der Wel N, Cao X, Gumperz JE, Maltsev S, et al. Lysosomal localization of murine CD1d mediated by AP-3 is necessary for NK T cell development. *J Immunol* (2003) **171**(8):4149–55. doi:10.4049/jimmunol.171.8.4149
47. Lin Y, Roberts TJ, Spence PM, Brutkiewicz RR. Reduction in CD1d expression on dendritic cells and macrophages by an acute virus infection. *J Leukoc Biol* (2005) **77**(2):151–8. doi:10.1189/jlb.0704399
48. Sanchez DJ, Gumperz JE, Ganem D. Regulation of CD1d expression and function by a herpesvirus infection. *J Clin Invest* (2005) **115**(5):1369–78. doi:10.1172/JCI24041
49. Yuan W, Dasgupta A, Cresswell P. Herpes simplex virus evades natural killer T cell recognition by suppressing CD1d recycling. *Nat Immunol* (2006) **7**(8):835–42. doi:10.1038/ni1364
50. Berntman E, Rolf J, Johansson C, Anderson P, Cardell SL. The role of CD1d-restricted NK T lymphocytes in the immune response to oral infection with *Salmonella typhimurium*. *Eur J Immunol* (2005) **35**(7):2100–9. doi:10.1002/eji.200425846
51. Skold M, Behar SM. Role of CD1d-restricted NKT cells in microbial immunity. *Infect Immun* (2003) **71**(10):5447–55. doi:10.1128/IAI.71.10.5447-5455.2003
52. Mariotti S, Teloni R, Iona E, Fattorini L, Romagnoli G, Gagliardi MC, et al. *Mycobacterium tuberculosis* diverts alpha interferon-induced monocyte differentiation from dendritic cells into immunoprivileged macrophage-like host cells. *Infect Immun* (2004) **72**(8):4385–92. doi:10.1128/IAI.72.8.4385-4392.2004
53. Gagliardi MC, Lemassu A, Teloni R, Mariotti S, Sargentini V, Pardini M, et al. Cell wall-associated alpha-glucan is instrumental for *Mycobacterium tuberculosis* to block CD1 molecule expression and disable the function of dendritic cell derived from infected monocyte. *Cell Microbiol* (2007) **9**(8):2081–92. doi:10.1111/j.1462-5822.2007.00940.x
54. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* (1997) **278**(5343):1626–9. doi:10.1126/science.278.5343.1626
55. Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T, et al. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* (2000) **12**(2):211–21. doi:10.1016/S1074-7613(00)80174-0
56. Fox LM, Cox DG, Lockridge JL, Wang X, Chen X, Scharf L, et al. Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS Biol* (2009) **7**(10):e1000228. doi:10.1371/journal.pbio.1000228
57. Facciotti F, Ramanjaneyulu GS, Lepore M, Sansano S, Cavallari M, Kistowska M, et al. Peroxisome-derived lipids are self antigens that stimulate invariant natural killer T cells in the thymus. *Nat Immunol* (2012) **13**(5):474–80. doi:10.1038/ni.2245
58. Fischer K, Scotet E, Niemeyer M, Koebernick H, Zerrahn J, Maillet S, et al. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc Natl Acad Sci U S A* (2004) **101**(29):10685–90. doi:10.1073/pnas.0403787101
59. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR, et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol* (2006) **7**(9):978–86. doi:10.1038/ni1380
60. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol* (2011) **12**(10):966–74. doi:10.1038/ni.2096
61. Kinjo Y, Pei B, Bufali S, Raju R, Richardson SK, Imamura M, et al. Natural *Sphingomonas* glycolipids vary greatly in their ability to activate natural killer T cells. *Chem Biol* (2008) **15**(7):654–64. doi:10.1016/j.chembiol.2008.05.012
62. Amprey JL, Im JS, Turco SJ, Murray HW, Illarionov PA, Besra GS, et al. A subset of liver NK T cells is activated during *Leishmania donovani* infection by CD1d-bound lipophosphoglycan. *J Exp Med* (2004) **200**(7):895–904. doi:10.1084/jem.20040704
63. De Libero G, Moran AP, Gober H-J, Rossy E, Shamshiev A, Chelnokova O, et al. Bacterial infections promote T cell recognition of self-glycolipids. *Immunity* (2005) **22**:763–72. doi:10.1016/j.immuni.2005.04.013
64. Paget C, Mallevaey T, Speak AO, Torres D, Fontaine J, Sheehan KC, et al. Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. *Immunity* (2007) **27**(4):597–609. doi:10.1016/j.immuni.2007.08.017
65. Salio M, Speak AO, Shepherd D, Polzella P, Illarionov PA, Veerapen N, et al. Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation. *Proc Natl Acad Sci U S A* (2007) **104**(51):20490–5. doi:10.1073/pnas.0710145104
66. Tatituri RVV, Watts GFM, Bhowruth V, Barton N, Rothchild A, Hsu F-F, et al. Recognition of microbial and mammalian phospholipid antigens by NKT cells with diverse tcrs. *Proc Natl Acad Sci U S A* (2013) **110**(5):1827–32. doi:10.1073/pnas.1220601110
67. Angenieux C, Salamero J, Fricker D, Cazenave JP, Goud B, Hanau D, et al. Characterization of CD1e, a third type of CD1 molecule expressed in dendritic cells. *J Biol Chem* (2000) **275**(48):37757–64. doi:10.1074/jbc.M007082200
68. Angenieux C, Fraisier V, Maitre B, Racine V, Van der Wel N, Fricker D, et al. The cellular pathway of CD1e in immature and maturing dendritic cells. *Traffic* (2005) **6**(4):286–302. doi:10.1111/j.1600-0854.2005.00272.x
69. Maitre B, Angenieux C, Salamero J, Hanau D, Fricker D, Signorino F, et al. Control of the intracellular pathway of CD1e. *Traffic* (2008) **9**(4):431–45. doi:10.1111/j.1600-0854.2008.00707.x
70. Maitre B, Angenieux C, Wurtz V, Layre E, Gilleron M, Collmann A, et al. The assembly of CD1e is controlled by an N-terminal propeptide which is processed in endosomal compartments. *Biochem J* (2009) **419**(3):661–8. doi:10.1042/BJ20082204
71. Bushmarina N, Tourne S, Giacometti G, Signorino-Gelo F, Garcia-Alles LF, Cazenave JP, et al. Increased flexibility and liposome-binding capacity of CD1e at endosomal pH. *FEBS J* (2011) **278**(12):2022–33. doi:10.1111/j.1742-4658.2011.08118.x
72. Garcia-Alles LF, Giacometti G, Versluis C, Maveyraud L, De Paepe D, Guiard J, et al. Crystal structure of human CD1e reveals a groove suited for lipid-exchange processes. *Proc Natl Acad Sci U S A* (2011) **108**(32):13230–5. doi:10.1073/pnas.1105627108
73. Facciotti F, Cavallari M, Angenieux C, Garcia-Alles LF, Signorino-Gelo F, Angman L, et al. Fine tuning by human CD1e of lipid-specific immune responses. *Proc Natl Acad Sci U S A* (2011) **108**(34):14228–33. doi:10.1073/pnas.1108809108
74. Montamat-Sicotte DJ, Millington KA, Willcox CR, Hingley-Wilson S, Hackforth S, Innes J, et al. A mycolic acid-specific CD1-restricted T cell population contributes to acute and memory immune responses in human tuberculosis infection. *J Clin Invest* (2011) **121**(6):2493–503. doi:10.1172/JCI46216
75. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* (2003) **198**(2):267–79. doi:10.1084/jem.20030324
76. Van Rhijn I, Kasmar A, De Jong A, Gras S, Bhati M, Doorenspleet ME, et al. A conserved human T cell population targets mycobacterial antigens presented by CD1b. *Nat Immunol* (2013) **14**(7):706–13. doi:10.1038/ni.2630
77. Van Rhijn I, Gherardin NA, Kasmar A, De Jager W, Pellicci DG, Kostenko L, et al. TCR bias and affinity define two compartments of the CD1b-glycolipid-specific T cell repertoire. *J Immunol* (2014) **192**(9):4054–60. doi:10.4049/jimmunol.1400158
78. Ly D, Kasmar AG, Cheng TY, De Jong A, Huang S, Roy S, et al. CD1c tetramers detect ex vivo T cell responses to processed phosphomycoketide antigens. *J Exp Med* (2013) **210**(4):729–41. doi:10.1084/jem.20120624
79. Hiromatsu K, Dascher CC, Leclair KP, Sugita M, Furlong ST, Brenner MB, et al. Induction of CD1-restricted immune responses in guinea pigs by immunization with mycobacterial lipid antigens. *J Immunol* (2002) **169**(1):330–9. doi:10.4049/jimmunol.169.1.330

80. Watanabe Y, Watari E, Matsunaga I, Hiromatsu K, Dascher CC, Kawashima T, et al. BCG vaccine elicits both T-cell mediated and humoral immune responses directed against mycobacterial lipid components. *Vaccine* (2006) **24**(29–30):5700–7. doi:10.1016/j.vaccine.2006.04.049
81. Dascher CC, Hiromatsu K, Xiong X, Morehouse C, Watts G, Liu G, et al. Immunization with a mycobacterial lipid vaccine improves pulmonary pathology in the guinea pig model of tuberculosis. *Int Immunol* (2003) **15**(8):915–25. doi:10.1093/intimm/dxg091
82. Van Rhijn I, Nguyen TK, Michel A, Cooper D, Govaerts M, Cheng TY, et al. Low cross-reactivity of T-cell responses against lipids from *Mycobacterium bovis* and *M. avium paratuberculosis* during natural infection. *Eur J Immunol* (2009) **39**(11):3031–41. doi:10.1002/eji.200939619
83. Nguyen TK, Koets AP, Santema WJ, Van Eden W, Rutten VP, Van Rhijn I. The mycobacterial glycolipid glucose monomycolate induces a memory T cell response comparable to a model protein antigen and no B cell response upon experimental vaccination of cattle. *Vaccine* (2009) **27**(35):4818–25. doi:10.1016/j.vaccine.2009.05.078
84. Felio K, Nguyen H, Dascher CC, Choi HJ, Li S, Zimmer MI, et al. CD1-restricted adaptive immune responses to mycobacteria in human group 1 CD1 transgenic mice. *J Exp Med* (2009) **206**(11):2497–509. doi:10.1084/jem.20090898
85. Ulrichs T, Moody DB, Grant E, Kaufmann SH, Porcelli SA. T-cell responses to CD1-presented lipid antigens in humans with *Mycobacterium tuberculosis* infection. *Infect Immun* (2003) **71**(6):3076–87. doi:10.1128/IAI.71.6.3076-3087.2003
86. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* (2003) **198**(7):1069–76. doi:10.1084/jem.20030630
87. Matsuda JL, Gapin L, Baron JL, Sidobre S, Stetson DB, Mohrs M, et al. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc Natl Acad Sci U S A* (2003) **100**(14):8395–400. doi:10.1073/pnas.1332805100
88. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, et al. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* (2003) **171**(10):5140–7. doi:10.4049/jimmunol.171.10.5140
89. Winau F, Weber S, Sad S, De Diego J, Hoops SL, Breiden B, et al. Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity* (2006) **24**(1):105–17. doi:10.1016/j.immuni.2005.12.001
90. Dieli F, Taniguchi M, Kronenberg M, Sidobre S, Ivanyi J, Fattorini L, et al. An anti-inflammatory role for V alpha 14 NK T cells in *Mycobacterium bovis* bacillus Calmette-Guerin-infected mice. *J Immunol* (2003) **171**(4):1961–8. doi:10.4049/jimmunol.171.4.1961
91. Ryll R, Watanabe K, Fujiwara N, Takimoto H, Hasunuma R, Kumazawa Y, et al. Mycobacterial cord factor, but not sulfolipid, causes depletion of NKT cells and upregulation of CD1d1 on murine macrophages. *Microbes Infect* (2001) **3**(8):611–9. doi:10.1016/S1286-4579(01)01416-2
92. Guidry TV, Olsen M, Kil KS, Hunter RL Jr, Geng YJ, Actor JK. Failure of CD1D-/- mice to elicit hypersensitive granulomas to mycobacterial cord factor trehalose 6,6'-dimycolate. *J Interferon Cytokine Res* (2004) **24**(6):362–71. doi:10.1089/107999004323142222
93. Behar SM, Dascher CC, Grusby MJ, Wang CR, Brenner MB. Susceptibility of mice deficient in CD1D or TAP1 to infection with *Mycobacterium tuberculosis*. *J Exp Med* (1999) **189**(12):1973–80. doi:10.1084/jem.189.12.1973
94. Szalay G, Zugel U, Ladel CH, Kaufmann SH. Participation of group 2 CD1 molecules in the control of murine tuberculosis. *Microbes Infect* (1999) **1**(14):1153–7. doi:10.1016/S1286-4579(99)00248-8
95. Sousa AO, Mazzaccaro RJ, Russell RG, Lee FK, Turner OC, Hong S, et al. Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. *Proc Natl Acad Sci U S A* (2000) **97**(8):4204–8. doi:10.1073/pnas.97.8.4204
96. Kawakami K, Kinjo Y, Uezu K, Yara S, Miyagi K, Koguchi Y, et al. Minimal contribution of Valpha14 natural killer T cells to Th1 response and host resistance against mycobacterial infection in mice. *Microbiol Immunol* (2002) **46**(3):207–10. doi:10.1111/j.1348-0421.2002.tb02687.x
97. Snyder-Cappione JE, Nixon DF, Loo CP, Chapman JM, Meiklejohn DA, Melo FF, et al. Individuals with pulmonary tuberculosis have lower levels of circulating CD1d-restricted NKT cells. *J Infect Dis* (2007) **195**(9):1361–4. doi:10.1086/513567
98. Im JS, Kang TJ, Lee SB, Kim CH, Lee SH, Venkataswamy MM, et al. Alteration of the relative levels of inkt cell subsets is associated with chronic mycobacterial infections. *Clin Immunol* (2008) **127**(2):214–24. doi:10.1016/j.clim.2007.12.005
99. Montoya CJ, Catano JC, Ramirez Z, Rugeles MT, Wilson SB, Landay AL. Invariant NKT cells from HIV-1 or *Mycobacterium tuberculosis*-infected patients express an activated phenotype. *Clin Immunol* (2008) **127**(1):1–6. doi:10.1016/j.clim.2007.12.006

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 April 2014; paper pending published: 30 April 2014; accepted: 30 April 2014; published online: 22 May 2014.
*Citation: De Libero G and Mori L (2014) The T-cell response to lipid antigens of Mycobacterium tuberculosis. Front. Immunol. **5**:219. doi: 10.3389/fimmu.2014.00219*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 11 August 2014
doi: 10.3389/fimmu.2014.00361

Toward understanding the essence of post-translational modifications for the *Mycobacterium tuberculosis* immunoproteome

Cécile A. C. M. van Els[1]*, Véronique Corbière[2], Kaat Smits[2], Jacqueline A. M. van Gaans-van den Brink[1], Martien C. M. Poelen[1], Francoise Mascart[2,3], Hugo D. Meiring[4†] and Camille Locht[5,6,7,8†]

[1] *Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, Netherlands*
[2] *Laboratory for Vaccinology and Mucosal Immunity, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium*
[3] *Immunobiology Clinic, Hôpital Erasme, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium*
[4] *Institute for Translational Vaccinology, Bilthoven, Netherlands*
[5] *Institut Pasteur de Lille, Center for Infection and Immunity of Lille, Lille, France*
[6] *INSERM U1019, Lille, France*
[7] *CNRS UMR8204, Lille, France*
[8] *Université Lille Nord de France, Lille, France*

Edited by:
Juraj Ivanyi, Kings College London, UK

Reviewed by:
Patrick Brennan, Colorado State University, USA
Raul Mancilla, National University of Mexico, Mexico

****Correspondence:***
Cécile A. C. M. van Els, Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, Netherlands
e-mail: cecile.van.els@rivm.nl

† Hugo D. Meiring and Camille Locht have contributed equally to this work.

CD4$^+$ T cells are prominent effector cells in controlling *Mycobacterium tuberculosis* (Mtb) infection but may also contribute to immunopathology. Studies probing the CD4$^+$ T cell response from individuals latently infected with Mtb or patients with active tuberculosis using either small or proteome-wide antigen screens so far revealed a multi-antigenic, yet mostly invariable repertoire of immunogenic Mtb proteins. Recent developments in mass spectrometry-based proteomics have highlighted the occurrence of numerous types of post-translational modifications (PTMs) in proteomes of prokaryotes, including Mtb. The well-known PTMs in Mtb are glycosylation, lipidation, or phosphorylation, known regulators of protein function or compartmentalization. Other PTMs include methylation, acetylation, and pupylation, involved in protein stability. While all PTMs add variability to the Mtb proteome, relatively little is understood about their role in the anti-Mtb immune responses. Here, we review Mtb protein PTMs and methods to assess their role in protective immunity against Mtb.

Keywords: post-translational modification, *Mycobacterium tuberculosis*, CD4+T cell epitope, proteomics, immunoproteome, T cell epitope repertoire, MHC ligands

INTRODUCTION

In the last few decades, the hallmarks of cell-mediated protection against *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), have been a subject of intense investigation. The production of the T helper cell type 1 cytokine IFNγ is considered key in Mtb immunity, since it is a central factor in activating macrophages to disarm intracellular mycobacteria (1, 2). A wide landscape of Mtb antigens targeted by human T cells is being uncovered, including proteins (3–6), lipoglycans (7–9), and lipoproteins (10–12) that are processed and exposed by antigen-presenting cells in the context of various presentation platforms. These can be either polymorphic classical MHC class I (HLA-A, -B, and -C) or MHC class II (HLA-DR, -DQ, and -DP) molecules (3–6, 10, 12), oligomorphic MHC class Ib molecules (HLA-E) (13–16) or CD1 isoforms (7–9, 11, 17–19). Relevant to the development of immunodiagnostic tests and vaccine candidates, strong human IFNγ responses consistently pointed at a range of immunodominant protein antigens, including members of the so-called PE/PPE and ESX protein families (5, 20–25). Whether these responses are for the greater part beneficial to the host by providing protection against Mtb or might actually help the pathogen to spread after damaging lung tissue is, for most of them, currently unanswered. Hyperconservation of human Mtb T cell peptide epitopes has been described, perhaps arguing for a beneficial effect of recognition by the host for the pathogen (26, 27), yet epitope sequence variability has also been reported (3, 28, 29).

Several genome-wide screens and bioinformatics-guided approaches further added to the identification of novel protein antigens and immunodominant epitopes for a number of antigen presentation platforms (5, 13, 24, 29–33). Altogether, the picture emerging from these studies is consistent with a multi-epitopic, multi-antigenic IFNγ response during Mtb infection. To investigate whether different protein classes have the same or diverse functional characteristics, Lindestam Arlehamn et al. combined genome-wide HLA class II binding predictions with high-throughput cellular screens of peptides to interrogate CD4+ T cell responses from latently infected individuals. A significant clustering was seen of the majority of targeted proteins, representing 42% of the total response to three broadly immunodominant antigenic islands, to only 0.55% of the total open reading frames (ORFs) (5). However, no quantitative, functional, or phenotypical distinction was observed between T cells elicited by the various protein classes involved, such as those assigned to be secreted or others belonging to secretion systems themselves, or to cell wall or

cellular processes. Hence, because of equal functionality, no antigen class could be implied in a more protective (or non-protective) profile over others.

Even though greatly informative, preselecting epitope candidates from the full Mtb proteome of approximately 4,000 ORFs based on bioinformatics has limitations. Binding algorithms may not be 100% effective and certain protective Mtb epitopes with weaker binding properties could perhaps rank too low in the assignment to be selected.

Moreover, the assumption that the immunoproteome is merely a direct translation of the coding genome is an oversimplification. As an additional level of proteome complexity, primary protein structures can be modified after translation. Multiple post-translational modifications (PTMs) occur in higher and lower organisms, involving proteolytic events or transfer of modifying groups to one or more amino acids of the proteins. These PTMs may influence the protein's active state, compartmentalization, turnover, and/or interactions with other proteins. The rich nature of PTMs of prokaryotic proteomes has started to become unraveled only recently (34), essentially through advances in mass spectrometry (MS) (35). However, their presence in the Mtb proteome and their role in virulence and immunity have not received sufficient attention yet. Here, we review PTMs currently known to occur in the Mtb proteome and discuss whether they modify the Mtb immunoproteome indirectly, by engaging eukaryotic innate receptor signaling or antigen-processing pathways, or directly by persisting as structural moieties in the immunogenic epitopes. In addition, we highlight technologies enabling the unbiased detection and identification of the Mtb T cell epitope repertoire, modified or unmodified.

POST-TRANSLATIONAL MODIFICATIONS OF Mtb PROTEINS

Current advances in MS-based proteomics have revealed that, like in eukaryotes, PTMs can create an enormous diversity and complexity of gene products in prokaryotes, as was reviewed recently elsewhere (34). PTMs are covalent-processing events chemically changing protein structure, often catalyzed by substrate-specific enzymes. Hundreds of types of PTMs are known, some of which can occur in parallel to create even more heterogeneity in the protein arsenal (36, 37). There are several technical obstacles still to overcome in PTM analysis. In proteome measurements, each protein can be identified based on combined mass and fragmentation patterns from various cleaved peptides. In PTM measurements, each modification site is only represented by a single peptide species. Modified peptides can be of low abundance and furthermore may have chemical properties requiring optimization of liquid chromatography (LC) separation techniques or fragmentation modules, used in MS identification. As a solution, robust MS-based proteomic workflows have been designed, including affinity-based enrichment strategies that can assist in the identification of, e.g., the phosphoproteome, the glycoproteome, or the acetylated proteome (35).

Over the last two decades, multiple proteomic studies were performed on Mtb. In one recent study, using dedicated subcellular fractionation combined with affinity enrichment and liquid chromatography mass spectrometry (LC-MS) based proteomics, Bell et al. were able to *bona fide* identify 1,051 protein groups present in the Mtb H37Rv proteome, including lipoproteins, glycoproteins, and glycolipoproteins (38). While data are accumulating, our insight into Mtb PTMs is still far from complete (see **Table 1** for summary and structure examples of PTMs discussed).

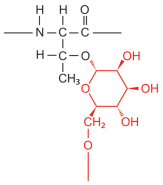
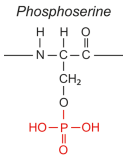
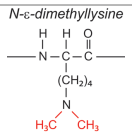
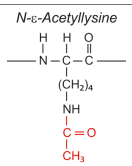
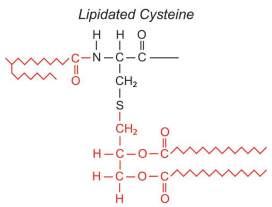
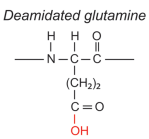
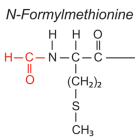
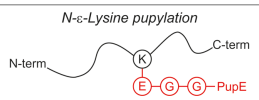
GLYCOSYLATION

Prokaryotes possess conserved N- and O-linked glycosylation pathways, capable of enzyme-catalyzed covalently coupling glycans (oligosaccharides) to proteins (65–67). N-linked glycosylation, in which oligosaccharide precursors are first assembled on a cytoplasmic carrier molecule before being transferred *en bloc* to the amide nitrogen of an Asn in the acceptor protein, has not been observed in Gram-positive bacteria or in pathogenic mycobacterial species. O-glycosylation in bacteria can proceed *en bloc* or stepwise, but for Mtb it is thought to be the latter. A model was proposed in which the initial glycosyl molecule is transferred to the hydroxyl oxygen of the acceptor Thr or Ser residue, a process catalyzed by the protein *O*-mannosyltransferase (PMT) (Rv1002c) (39). Hereafter, further sugars are added one at a time, but the enzymes involved in this elongation are unknown. While the precise role of O-glycosylation of Mtb proteins is still elusive (68), this PTM appears essential for Mtb virulence, since Rv1002c deficient strains are highly attenuated in immunocompromised mice (69). Initially, glycoproteins of Mtb were reported to contain glycan moieties based on their ability to bind the lectin concanavalin A (ConA), e.g., 38 kDa (PstS1) protein (40). MS then enabled assessment of glycosylation patterns of Mtb proteins, first the alanine-proline-rich 45–47 kDa antigen Apa (41, 70), followed by others, e.g., the lipoproteins (19 kDa) LpqH (42, 43) and SodC (44). Using ConA affinity capture or other sugar-based partitioning methods, and dedicated proteomics, Bell et al. reported a wealth of candidate Mtb glycoproteins, associated with membrane fractions and with culture filtrates (38), whereas others, comparing several fragmentation strategies, identified novel glycosylation sites directly from culture filtrate proteins (45, 71). These localizations corroborate with data suggesting that O-glycosylation and Sec-translocation, a process shuttling proteins across the bacterial cell envelope, are linked (39). As the number of *bona fide* identified Mtb glycoproteins is increasing, a glycosylation site motif is emerging, frequently observed at the protein C-terminus (45). Some O-glycosylated Mtb proteins constitute B cell antigens for serodiagnostics, such as the 38 kDa protein (72). Furthermore, they might contribute to the virulence of Mtb by binding as adhesins to innate immune receptors, promoting invasion of the host cells. The 19-kDa glycolipoprotein was shown to bind to the macrophage mannose receptor (MR) of monocytic THP-1 cells, hereby promoting the uptake of bacteria (73). Apa, secreted, as well as cell wall associated, binds to human pulmonary Surfactant Protein A (SP-A), an important lung C-type lectin (74). These two glycoproteins were also reported to be involved in Mtb binding to DC-SIGN on dendritic cells, although this needs further investigation (75).

PHOSPHORYLATION

Since Mtb can exist under various physiological states in the host, including dormancy and active replication, it makes use of a versatile mechanism to sense signals from the host and regulate cellular processes. Signal transduction through reversible

Table 1 | Post-translational modifications in the Mtb proteome.

PTM targeted aa[a] ΔM^b	Structure (example)	Function and notes	Mtb proteins exhibiting this PTM	Reference
O-glycosylation Thr, Ser e.g. +162 (mannose)	*O-(α-D-Mannosyl)-L-Threonine* 	Pathogenesis Immune decoy	Apa/Rv1860; Mpt83/Rv2873; 19 kDa LpqH/Rv3763; 38 kDa PstS1/Rv0934; SodC/Rv0432; WGA enriched candidate glycoproteins	(38–45)
Phosphorylation Ser, Thr, Tyr +80	*Phosphoserine* 	Regulation	301 proteins	(46, 47)
Methylation Lys, Arg, Gln, Glu +28	*N-ε-dimethyllysine* 	Protease resistance	HBHA/Rv0475; LBP/Rv2986c	(48)
Acetylation Ser, Thr, Lys (protein N-term) +42	*N-ε-Acetyllysine* 	Stability Compartmentalization	Esat-6 (N-terminal threonine)	(49)
Lipidation Cys, Ser, Thr +830	*Lipidated Cysteine* 	Compartmentalization Anchoring in membrane	99 Putative lipoproteins; 42 lipoproteins	(38, 42, 44, 50–55)
Deamidation Asn, Gln +1	*Deamidated glutamine* 	Regulator of protein-ligand interaction	Pup/Rv2111c	(56)
N-formylation[c] Met (startcodon) +28	*N-Formylmethionine* 	Start bacterial protein synthesis (fMet)	Rv0476, Rv0277C, Rv0749, Rv1686C	(57, 58)
Pupylation Lys +6,954	*N-ε-Lysine pupylation* 	Degradation signal (reversible)	1,305 proteins	(56, 59–64)

[a] *aa amino acid.*

[b] *Mass increment of modified aa (Da).*

[c] *Formally not a PTM but a modified aa.*

protein phosphorylation participates in this function. The Mtb genome encodes multiple serine/threonine protein kinases, and Ser/Thr/Tyr protein phosphorylation occurs extensively. In addition, Mtb makes extensive use of two-component signal transduction systems, which rely on a phosphorylation cascade involving His kinases (46). Using TiO_2-phosphopeptide enrichment, Prisic et al. assigned 301 phosphoproteins in Mtb grown under six different conditions and identified corresponding phosphorylation site motifs (47). These likely represent only a part of the Mtb phosphoproteome. However, little is known on the role of this PTM in

the function or pathogenicity of these proteins, with exception of the His kinases in two-component systems (46).

LIPIDATION

Lipidation of proteins is predicted for a small percentage (0.9–2.5%) of ORFs in mycobacterial genomes, and is required for their anchoring and sorting to the cell surface [reviewed in Ref. (50, 76)]. The first step in Mtb lipoprotein biogenesis occurs in the N-terminal leader of preprolipoproteins having a so-called lipobox motif, involving the attachment of diacylglycerol to the thiol group of a Cys, by Lgt (phosphatidylglycerol-pre-prolipoprotein diacylglyceryl transferase). Second, the signal peptide directly upstream of the modified Cys is cleaved off by LspA (prolipoprotein signal peptidase/signal peptidase II). Only recently, proof was found that slow-growing Gram-positive mycobacteria also share the third step in lipoprotein biosynthesis with Gram-negative bacteria, i.e., adding a third acyl residue to the free amino group of the modified Cys by Lnt (phospholipid-apolipoprotein *N*-acyltransferase) (51). Brulle et al. described the BCG_2070c as the major ORF in BCG to encode a functional Lnt using a mycobacteria-specific acyl substrate, tuberculostearic acid (52). Lipoprotein genesis is essential for Mtb. Deletion of lgt was not possible (77), while an lspA deletion mutant was viable but had an attenuated phenotype (78, 79). For Mtb, multiple (candidate) lipoproteins have been identified, and classified as components of transport systems, enzymes, or as molecules involved in cell adhesion or in signaling (38, 50), several of which were not only lipidated but also glycosylated (42, 44, 52). In line with the dogma that lipoproteins are pathogen associated molecular patterns (PAMPs) sensed by TLR2 (80), Sanchez et al. showed that the glycolipoprotein 38 kDa PstS1 triggers a TLR2 and caspase-dependent apoptotic pathway in human macrophages (53). Besides this mechanism, the 19-kDa glycolipoprotein LpqH was shown also to induce a caspase independent apoptotic mechanism, involving mitochondrial apoptosis-inducing factor (AIF), killing macrophages (54). Furthermore, TLR2-dependent inhibition of MHC class II function was observed for LpqH (81). The cumulative data on LpqH suggest that through its PTMs, this glycolipoprotein exploits multiple innate immune receptors and mechanisms to enter (73), incapacitate, and kill mononuclear phagocytes. Notably, Lopez et al. reported that the lipid moiety of LpqH was not required for the TLR2-dependent apoptosis of macrophages (82). As another innate feature, LpqH and the lipoprotein LprG were found to directly stimulate TLR2/TLR1 on memory $CD4^+$ T cells (55), presumably via engaging TLR2 and TLR1 pockets by their thioether-linked diacylglycerol and amide-linked third acyl chain, respectively (83).

FORMYLATION

Formylation/de-formylation of proteins is a typical hallmark of bacterial proteomes. Protein synthesis in bacteria is initiated with a formylated methionine (fMet) residue, which is then enzymatically cleaved by peptide deformylase (PDF) and methionine aminopeptidase to generate mature proteins. The human immune system can benefit from this unique formylation pathway to distinguish self from non-self proteins. Although formylation is not strictly a PTM, but comes with the first "modified" building block of protein synthesis, the presence of the formyl group can be considered a variation of plain translation of the genetic code. What might be the life span of the formylated state of proteins is unknown so far. However, short formylated Mtb protein fragments have been identified that can be presented as epitopes via non-classical murine MHC class Ib molecules of infected macrophages and appear to be protective in a Mtb challenge model (57, 58). This suggests that *in vivo*-formylated proteins can enter antigen-processing pathways before the enzymatic removal of the N-terminal fMet residue has occurred. Recently, N-formylated peptides of ESAT-6 and glutamine synthetase were found to have immunotherapeutic potential in a Mtb mouse infection model. A role for formyl peptide-receptor recognition in activation of innate immune cells was implied (84), but presentation via non-classical MHC molecules may also play a role.

PUPYLATION

Pupylation is a protein-to-protein modification, first identified in Mtb. It covalently attaches the C-terminal Glu of the 6.9-kDa "Protein Ubiquitin-like Protein" (Pup) to the ε-amine of Lys side chains of an interacting protein partner (59). Although the full purpose of the pupylation pathway in Actinobacteria remains to be elucidated, it is assumed that in Mtb, disposing of a proteasomal system, tagging proteins with Pup renders them susceptible for proteasomal degradation (60–62), similar to the well-known ubiquitin-initiated protein degradation pathway. The C-terminal Glu of Pup itself is generated by another PTM, i.e., deamidation of the C-terminal Gln (56). From various large-scale proteomic studies, a database of the mycobacterial "pupylome," containing > 150 verified pupylated proteins and >1,000 candidate pupylated proteins, was annotated (63). Depupylation activity also occurs (64), hence the modification can be reversed.

ACETYLATION AND ACETYL-LIKE MODIFICATIONS

Transferring an acetyl, propionyl, maloyl, or succinyl group to the ε-amine of lysines (N^{ε}-modification) or to the α-amines of protein N-termini (N^{α}-modification) are widely occurring PTMs in prokaryotes (34). Mtb encodes multiple proteins annotated as putative acetyl transferases acting on protein substrates (85). A well-studied N^{α}-acetylated Mtb protein is the virulence factor and immunodominant antigen, early secretory antigenic target 6 (Esat-6) (49). Acetylation presumably confers protein stability and compartmentalization, and occurs at Thr2, becoming the N-terminus after removal of the fMet residue at position 1.

METHYLATION

This PTM involves the addition of one or several methyl groups to either the ε-amine of lysines or to the side chain carboxyl of Glu. Although this PTM occurs in Mtb, genes encoding Mtb protein-methyltransferases have not been identified yet. Two Mtb adhesins, heparin-binding hemagglutinin (HBHA, Rv0475) and laminin-binding protein (LBP, Rv2986c) were shown to be methylated (48). HBHA is a 28-kDa multifunctional protein found on the surface and in culture filtrates of mycobacteria. Automated Edman degradation and mass spectrometric analysis indicate that at least 13 out of 16 Lys residues in the Lys-Ala-Pro rich C-terminal region of HBHA can be mono-or dimethylated, generating a

spectral envelop of isoforms (**Figure 1A**) (48). HBHA mediates mycobacterial adherence to epithelial cells via the interactions of this C-terminus with sulfated glycoconjugates on the surface of epithelial cells and methylation was implied to play a role in resistance to proteases present in bronchoalveolar lavage fluids (86–88). Recently, Sohn et al. showed that HBHA from Mtb also targeted murine macrophages and induced apoptosis via a mechanism involving mitochondria (89). Interestingly, HBHA purified from *Mycobacterium avium* subsp. *paratuberculosis* contains an N-terminal acetylated alanine residue in addition to the methylated lysines (90), whereas there is no evidence for acetylation of the N-terminal residue of Mtb HBHA (88).

POST-TRANSLATIONAL Mtb PROTEIN MODIFICATIONS IN PROTECTIVE IMMUNITY AND VACCINE CANDIDATES

The rich variety of PTMs to a large proportion of the Mtb proteome is likely to play a major role in the successful intracellular lifestyle of Mtb during chronic and sometimes lifelong infections. In the quest of novel vaccines, urgently needed to improve the limited protective capacity of BCG, it may be useful to understand the role of these PTMs in the host response to Mtb infection. Over thousands of years, a balance has been reached in which Mtb avoids excessive immunity allowing it to survive in the host, and in which a certain level of immunity allows the host not to succumb to the infection.

While the primary Mtb proteome shows features of hyperconservation, suggesting an evolutionary advantage to ensure stable epitope recognition by CD4+ T cells (26), PTMs superimpose a high level of complexity. This may complicate the identification of protective protein antigens based on *in silico* analyses and recombinant DNA technologies. Once protective protein antigens have been identified, the exact structural features need to be known for optimization and process development of the antigen. Furthermore, it will be important to know whether a particular PTM acts as an immune modulator, or/and whether it is part of the structural antigen moiety targeted by the adaptive immune system. Illustrative in this respect are three examples of Mtb protein antigens with PTMs, currently considered as vaccine candidates because of their immunodominance in humans and/or protective effect in animal models.

The 45–47 kDa secretory and cell-surface adhesin Apa is a major mycobacterial antigen with different O-mannosylation patterns in pathogenic versus non-pathogenic mycobacterial species that are critical for its T cell antigenicity *in vivo* and *in vitro* (70, 91). T cells from BCG-vaccinated PPD-responsive individuals recognize either both native mannosylated Apa (nApa) and recombinant non-mannosylated Apa (rApa), or nApa only. These latter T cells did, in contrast to the former, not recognize synthetic peptides corresponding to the Apa protein sequence. Together with the finding that recognition of nApa required active antigen processing, these data suggest that mannosylation does not induce alternate processing of nApa but rather that the carbohydrate moiety is an intrinsic part of the T cell epitope(s) (92). Protection by Apa was shown in guinea pig and mouse models in the context of various vaccine platforms (protein, DNA, and poxvirus boost) and routes (intanasal and subcutaneous), as a subunit or as a BCG-booster vaccine (70, 92–94). In a mouse model, adjuvanted nApa was found to induce higher frequencies of CD4+ T cells, producing more cytokines, compared to adjuvanted rApa. However, both antigens were equally protective against virulent Mtb infection when used as a subunit vaccine or as a BCG-booster vaccine (92). This indicates that O-mannosylation is not required for the protective effect in this model. However, understanding of the impact of the different immune responses evoked by nApa and rApa, as well as the nature of the putative naturally processed glycopeptide(s), need further investigation.

In contrast to Apa, the natural PTM of HBHA, methylation, is essential for providing high levels of protection against Mtb challenge in mice, in addition to its antigenicity in Mtb-infected human individuals (95, 96). However, immunization of mice with purified non-methylated HBHA induces antibodies and Th1 cytokines at levels similar to those induced by immunization with

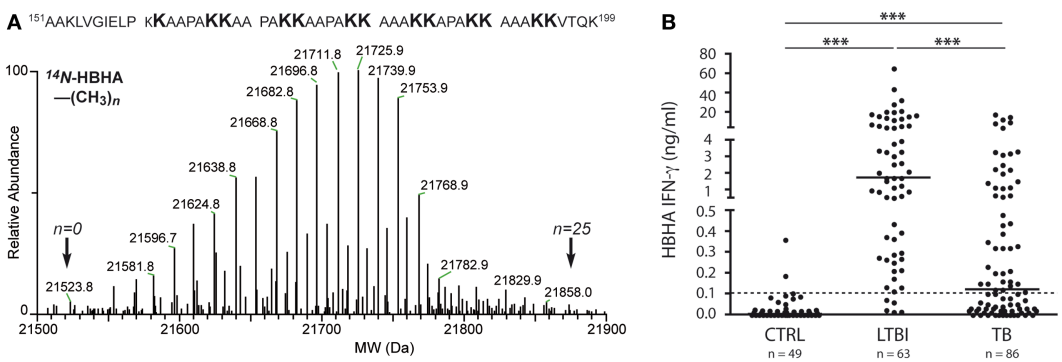


FIGURE 1 | Molecular and immunological hallmarks of naturally methylated HBHA. **(A)** LC-MS analysis (lower part) and summary of methylation pattern (upper part) of HBHA from BCG. Indicated by arrows are the masses of molecular variants in the mass envelope, the lowest and highest of which correspond to HBHA containing 0 or 25 methyl groups, respectively. Methylations are borne by the lysine residues of the C-terminal part. Data indicate that at least 13 out of the 16 C-terminal lysines can be mono- or dimethylated. **(B)** *In vitro* IFNγ release to methylated HBHA stimulation according to Mtb infection status. Shown are IFNγ concentrations in nanogram/milliter as measured in Elisa after stimulation with methylated HBHA for 24 h of PBMC from three groups of subjects: non-infected controls (CTRL), subjects with latent Mtb infection (LTBI), and patients with active tuberculosis (TB). The dotted line represents the positivity cut-off for the assay. For each group, the median of results is marked as a horizontal line. Statistical significance of differences: $***p \leq 0.0001$. Data are with licensed permission from Ref. (23).

methylated HBHA. Also, the antibody isotype profiles are similar in both instances. Interestingly, however, only splenocytes isolated from mice immunized with methylated HBHA, and not with non-methylated HBHA, induce IFNγ secretion upon incubation with Mtb-pulsed macrophages. Methylated HBHA-specific T cell responses are likely to participate in protection against disease in humans, since T cells from patients with active TB secrete significantly lower amounts of IFNγ after stimulation with methylated HBHA than subjects with latent Mtb infection (**Figure 1B**) (23, 97, 98). HBHA is being considered as a BCG-booster vaccine (99), as responses to methylated HBHA were found to be primed in BCG-vaccinated infants (100). It is not yet known whether the PTM affects the presentation of non-modified protective T cell epitopes via modulation of antigen uptake or processing, or whether methylation is part of the protective T cell epitope(s) involved.

The N-terminal-Thr acetylated antigen ESAT-6 is known as an immunological hotspot in humans (6). During natural infection or after subunit vaccination in mice, vigorous Th1 type $CD4^+$ T cell responses are directed to the N-terminal immunodominant epitope ESAT-$6_{1–15}$, whereas other epitopes are masked (101). These can be revealed by redesigning ESAT-6 analogs in which the dominant epitope is removed, resulting in the engagement of protective $CD4^+$ T cell responses that resist infection-driven terminal differentiation (102). To our knowledge, the role of the N-acetylation at Thr2 in generating the ESAT-6 peptide repertoire has not been interrogated, yet in view of ESAT-6's current status as a vaccine candidate in clinical testing (99, 103), such assessment may be important.

In order to fully characterize these candidate vaccine antigens, it will be important to elucidate the exact roles of the added glyco-, methyl-, or *N*-acetyl moieties, respectively. Does their presence modulate effective antigen processing, perhaps by steering proteolysis and immunodominance through masking certain enzyme cleavage sites as was shown for O-linked glycans (104), or are they part of the protective immunoproteome itself? Clearly more studies are needed, including epitope identification approaches to unravel, in these and other targeted vaccine candidates, the role of PTMs in the Mtb immunoproteome. Knowledge on the precise role of the PTM of Mtb vaccine candidates may be of great help to optimize vaccine candidates and potentially to simplify vaccine design and process development.

TOWARD UNBIASED ASSESSMENT OF THE Mtb IMMUNOPROTEOME

Protein antigens, modified or not, are translated for T cell surveillance into immunogens in antigen-processing pathways of antigen-presenting cells. This translation consists of enzymatic cleavage and rescue of protein fragments onto the molecules of a relevant antigen-presenting platform, such as classical class I or II MHC molecules (105), non-classical MHC molecules, including class Ib MHC molecules (16), or CD1 isoforms (17). The identification of the exact nature of the naturally processed and presented Mtb immunoproteome would require dedicated technologies such as LC-MS, first pioneered MHC class I ligands by Hunt et al. more than two decades ago (106, 107). Typically, cell lines would be grown at large scale ($>1 \times 10^9$ cells) and, after detergent solubilization and immunoaffinity purification of MHC-ligand complexes, bound peptide epitopes would be eluted. The purified endogenous MHC class I ligands were characterized by dedicated LC-MS and MS/MS sequencing.

Nowadays, ever evolving LC-MS/MS systems have greatly added to our understanding of the endogenous peptide repertoire and binding motifs of many MHC class I and II molecules (108–111), as well as of class1b MHC molecules (112). For the classical MHC pathways, the notion has emerged that antigen-presenting cells express approximately 100,000 MHC class I and II molecules at their surface, presenting thousands of different endogenous peptides, at widely divergent abundances (113). LC-MS/MS sequencing can unambiguously identify the epitopes as they are eluted from their antigen-presenting molecules in a qualitative and quantitative manner, revealing both primary epitope sequences, as well as any modifications to them (114). LC-MS/MS analyses have shown that processing inside antigen-presenting cells can generate modified or unpredictable MHC epitopes, such as deamidated (115), citullinated (116), or cysteinylated (117) ligands, as well as ligands arising from protein splicing (118–120) or from alternative reading frames or read-throughs of protein-encoding genes (121–123).

Pathogen-encoded immunoproteomes, including PTMs, generated from the proteome inside infected or antigen endocytosing antigen-presenting cells, should be detectable through LC-MS/MS sequencing approaches as well, although pathogen-derived ligands will be needles in the haystack of eluted *self* epitopes. To facilitate the identification of these *non-self* pathogen-derived antigens, targeted LC-MS/MS approaches have been developed (124–127). Foreign epitopes that originate from proteins synthesized during infection inside antigen-presenting cells, such as viral MHC class I epitopes during infection, can be traced using algorithms detecting isotopic patterns in the mass chromatograms of MHC immunoproteomes from carefully mixed infected and non-infected cell cultures that were metabolically labeled during growth (128). Alternatively, epitopes that arise from exogenous proteins endocytosed by antigen-presenting cells during infection, such as bacterial MHC class II epitopes, can be traced back in the MHC-bound peptide repertoire after metabolic labeling of antigen during the prokaryotic cell growth (126, 129). However, if PTMs are suspected in the foreign MHC immunoproteome, chromatography, ion fragmentation strategy, and even affinity enrichment strategies will have to be considered accordingly. Until now, only a single study has reported the identification of several Mtb epitopes presented by MHC class I via LC-MS (130). More approaches are underway to extend our knowledge on the naturally processed and MHC-presented Mtb epitopes, including those derived from methylated HBHA, using dedicated LC-MS. These studies include large-scale human monocyte or dendritic cell cultures and either *in vitro* Mtb infection or targeted antigen pulsing. Inhibition of MHC class II presentation upon incubation with live Mtb, mycobacterial lysates, or purified antigens may frustrate these attempts (131, 132). Dedicated isolation and analytical discovery procedures should then help to identify the Mtb epitope "needles" in the *self* "haystack," and increase our knowledge on the role of PTM in the Mtb immunoproteome.

CONCLUDING REMARKS

Fast developments in LC-MS/MS-based proteomics have enabled the detection of many types of PTMs in proteomes of prokaryotes, including Mtb. Elucidating the role of PTMs in the immunoproteome of protective Mtb protein antigens is important for the molecular optimization of vaccine candidates, and will also greatly benefit from technical advancements in LC-MS/MS.

ACKNOWLEDGMENTS

We thank Ad de Jong for stimulating discussions. Cécile A. C. M. van Els, Véronique Corbière, Kaat Smits, Hugo D. Meiring, Francoise Mascart, and Camille Locht received funding on Mtb epitope discovery by the EU FP7 Collaborative Project NEWTBVAC (grant no. HEALTH-F32009-241745).

REFERENCES

1. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol* (2001) **19**:93–129. doi:10.1146/annurev.immunol.19.1.93
2. Kaufmann SH. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol* (2001) **1**:20–30. doi:10.1038/35095558
3. Blythe MJ, Zhang Q, Vaughan K, de Castro R Jr, Salimi N, Bui HH, et al. An analysis of the epitope knowledge related to mycobacteria. *Immunome Res* (2007) **3**:10. doi:10.1186/1745-7580-3-10
4. Kunnath-Velayudhan S, Porcelli SA. Recent advances in defining the immunoproteome of *Mycobacterium tuberculosis*. *Front Immunol* (2013) **4**:335. doi:10.3389/fimmu.2013.00335
5. Lindestam Arlehamn CS, Gerasimova A, Mele F, Henderson R, Swann J, Greenbaum JA, et al. Memory T cells in latent *Mycobacterium tuberculosis* infection are directed against three antigenic islands and largely contained in a CXCR3+CCR6+ Th1 subset. *PLoS Pathog* (2013) **9**:e1003130. doi:10.1371/journal.ppat.1003130
6. Axelsson-Robertson R, Magalhaes I, Parida SK, Zumla A, Maeurer M. The immunological footprint of *Mycobacterium tuberculosis* T-cell epitope recognition. *J Infect Dis* (2012) **205**(Suppl 2):S301–15. doi:10.1093/infdis/jis198
7. Cala-De Paepe D, Layre E, Giacometti G, Garcia-Alles LF, Mori L, Hanau D, et al. Deciphering the role of CD1e protein in mycobacterial phosphatidyl-myo-inositol mannosides (PIM) processing for presentation by CD1b to T lymphocytes. *J Biol Chem* (2012) **287**:31494–502. doi:10.1074/jbc.M112.386300
8. Gilleron M, Stenger S, Mazorra Z, Wittke F, Mariotti S, Bohmer G, et al. Diacylated sulfoglycolipids are novel mycobacterial antigens stimulating CD1-restricted T cells during infection with *Mycobacterium tuberculosis*. *J Exp Med* (2004) **199**:649–59. doi:10.1084/jem.20031097
9. Layre E, Collmann A, Bastian M, Mariotti S, Czaplicki J, Prandi J, et al. Mycolic acids constitute a scaffold for mycobacterial lipid antigens stimulating CD1-restricted T cells. *Chem Biol* (2009) **16**:82–92. doi:10.1016/j.chembiol.2008.11.008
10. Bastian M, Braun T, Bruns H, Rollinghoff M, Stenger S. Mycobacterial lipopeptides elicit CD4+ CTLs in *Mycobacterium tuberculosis*-infected humans. *J Immunol* (2008) **180**:3436–46. doi:10.4049/jimmunol.180.5.3436
11. Kasmar AG, Van Rhijn I, Magalhaes KG, Young DC, Cheng TY, Turner MT, et al. Cutting edge: CD1a tetramers and dextramers identify human lipopeptide-specific T cells ex vivo. *J Immunol* (2013) **191**:4499–503. doi:10.4049/jimmunol.1301660
12. Seshadri C, Turner MT, Lewinsohn DM, Moody DB, Van Rhijn I. Lipoproteins are major targets of the polyclonal human T cell response to *Mycobacterium tuberculosis*. *J Immunol* (2013) **190**:278–84. doi:10.4049/jimmunol.1201667
13. Joosten SA, van Meijgaarden KE, van Weeren PC, Kazi F, Geluk A, Savage ND, et al. *Mycobacterium tuberculosis* peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS Pathog* (2010) **6**:e1000782. doi:10.1371/journal.ppat.1000782
14. Heinzel AS, Grotzke JE, Lines RA, Lewinsohn DA, McNabb AL, Streblow DN, et al. presentation of Mtb-derived antigen to human CD8+ T cells. *J Exp Med* (2002) **196**:1473–81. doi:10.1084/jem.20020609
15. Mir SA, Sharma S. Role of MHC class Ib molecule, H2-M3 in host immunity against tuberculosis. *Vaccine* (2013) **31**:3818–25. doi:10.1016/j.vaccine.2013.04.005
16. Rodgers JR, Cook RG. MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* (2005) **5**:459–71. doi:10.1038/nri1635
17. Adams EJ. Lipid presentation by human CD1 molecules and the diverse T cell populations that respond to them. *Curr Opin Immunol* (2014) **26C**:1–6. doi:10.1016/j.coi.2013.09.005
18. Moody DB. The surprising diversity of lipid antigens for CD1-restricted T cells. *Adv Immunol* (2006) **89**:87–139. doi:10.1016/S0065-2776(05)89003-0
19. Zajonc DM, Crispin MD, Bowden TA, Young DC, Cheng TY, Hu J, et al. Molecular mechanism of lipopeptide presentation by CD1a. *Immunity* (2005) **22**:209–19. doi:10.1016/j.immuni.2004.12.009
20. McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat Med* (2004) **10**:1240–4. doi:10.1038/nm1128
21. Mustafa AS, Al-Attiyah R, Hanif SN, Shaban FA. Efficient testing of large pools of *Mycobacterium tuberculosis* RD1 peptides and identification of major antigens and immunodominant peptides recognized by human Th1 cells. *Clin Vaccine Immunol* (2008) **15**:916–24. doi:10.1128/CVI.00056-08
22. Skjot RL, Brock I, Arend SM, Munk ME, Theisen M, Ottenhoff TH, et al. Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family. *Infect Immun* (2002) **70**:5446–53. doi:10.1128/IAI.70.10.5446-5453.2002
23. Hougardy JM, Schepers K, Place S, Drowart A, Lechevin V, Verscheure V, et al. Heparin-binding-hemagglutinin-induced IFN-gamma release as a diagnostic tool for latent tuberculosis. *PLoS One* (2007) **2**:e926. doi:10.1371/journal.pone.0000926
24. Bertholet S, Ireton GC, Kahn M, Guderian J, Mohamath R, Stride N, et al. Identification of human T cell antigens for the development of vaccines against *Mycobacterium tuberculosis*. *J Immunol* (2008) **181**:7948–57. doi:10.4049/jimmunol.181.11.7948
25. Vordermeier HM, Hewinson RG, Wilkinson RJ, Wilkinson KA, Gideon HP, Young DB, et al. Conserved immune recognition hierarchy of mycobacterial PE/PPE proteins during infection in natural hosts. *PLoS One* (2012) **7**:e40890. doi:10.1371/journal.pone.0040890
26. Comas I, Chakravartti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of *Mycobacterium tuberculosis* are evolutionarily hyperconserved. *Nat Genet* (2010) **42**:498–503. doi:10.1038/ng.590
27. McEvoy CR, Cloete R, Muller B, Schurch AC, van Helden PD, Gagneux S, et al. Comparative analysis of *Mycobacterium tuberculosis* pe and ppe genes reveals high sequence variation and an apparent absence of selective constraints. *PLoS One* (2012) **7**:e30593. doi:10.1371/journal.pone.0030593
28. Uplekar S, Heym B, Friocourt V, Rougemont J, Cole ST. Comparative genomics of Esx genes from clinical isolates of *Mycobacterium tuberculosis* provides evidence for gene conversion and epitope variation. *Infect Immun* (2011) **79**:4042–9. doi:10.1128/IAI.05344-11
29. Axelsson-Robertson R, Loxton AG, Walzl G, Ehlers MM, Kock MM, Zumla A, et al. A broad profile of co-dominant epitopes shapes the peripheral *Mycobacterium tuberculosis* specific CD8+ T-cell immune response in South African patients with active tuberculosis. *PLoS One* (2013) **8**:e58309. doi:10.1371/journal.pone.0058309
30. Commandeur S, van Meijgaarden KE, Prins C, Pichugin AV, Dijkman K, van den Eeden SJ, et al. An unbiased genome-wide *Mycobacterium tuberculosis* gene expression approach to discover antigens targeted by human T cells expressed during pulmonary infection. *J Immunol* (2013) **190**:1659–71. doi:10.4049/jimmunol.1201593
31. Cho S, Mehra V, Thoma-Uszynski S, Stenger S, Serbina N, Mazzaccaro RJ, et al. Antimicrobial activity of MHC class I-restricted CD8+ T cells in human tuberculosis. *Proc Natl Acad Sci U S A* (2000) **97**:12210–5. doi:10.1073/pnas.210391497
32. Hammond AS, Klein MR, Corrah T, Fox A, Jaye A, McAdam KP, et al. *Mycobacterium tuberculosis* genome-wide screen exposes multiple CD8 T cell epitopes. *Clin Exp Immunol* (2005) **140**:109–16. doi:10.1111/j.1365-2249.2005.02751.x
33. McMurry JA, Kimball S, Lee JH, Rivera D, Martin W, Weiner DB, et al. Epitope-driven TB vaccine development: a streamlined approach using immunoinformatics, ELISpot assays, and HLA transgenic mice. *Curr Mol Med* (2007) **7**:351–68. doi:10.2174/156652407780831584

34. Cain JA, Solis N, Cordwell SJ. Beyond gene expression: the impact of protein post-translational modifications in bacteria. *J Proteomics* (2014) **97**:265–86. doi:10.1016/j.jprot.2013.08.012
35. Olsen JV, Mann M. Status of large-scale analysis of post-translational modifications by mass spectrometry. *Mol Cell Proteomics* (2013) **12**:3444–52. doi:10.1074/mcp.O113.034181
36. Jensen ON. Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. *Curr Opin Chem Biol* (2004) **8**:33–41. doi:10.1016/j.cbpa.2003.12.009
37. Mann M, Jensen ON. Proteomic analysis of post-translational modifications. *Nat Biotechnol* (2003) **21**:255–61. doi:10.1038/nbt0303-255
38. Bell C, Smith GT, Sweredoski MJ, Hess S. Characterization of the *Mycobacterium tuberculosis* proteome by liquid chromatography mass spectrometry-based proteomics techniques: a comprehensive resource for tuberculosis research. *J Proteome Res* (2012) **11**:119–30. doi:10.1021/pr2007939
39. VanderVen BC, Harder JD, Crick DC, Belisle JT. Export-mediated assembly of mycobacterial glycoproteins parallels eukaryotic pathways. *Science* (2005) **309**:941–3. doi:10.1126/science.1114347
40. Espitia C, Mancilla R. Identification, isolation and partial characterization of *Mycobacterium tuberculosis* glycoprotein antigens. *Clin Exp Immunol* (1989) **77**:378–83.
41. Dobos KM, Khoo KH, Swiderek KM, Brennan PJ, Belisle JT. Definition of the full extent of glycosylation of the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. *J Bacteriol* (1996) **178**:2498–506.
42. Herrmann JL, O'Gaora P, Gallagher A, Thole JE, Young DB. Bacterial glycoproteins: a link between glycosylation and proteolytic cleavage of a 19 kDa antigen from *Mycobacterium tuberculosis*. *EMBO J* (1996) **15**:3547–54.
43. Herrmann JL, Delahay R, Gallagher A, Robertson B, Young D. Analysis of post-translational modification of mycobacterial proteins using a cassette expression system. *FEBS Lett* (2000) **473**:358–62. doi:10.1016/S0014-5793(00)01553-2
44. Sartain MJ, Belisle JT. N-terminal clustering of the O-glycosylation sites in the *Mycobacterium tuberculosis* lipoprotein SodC. *Glycobiology* (2009) **19**:38–51. doi:10.1093/glycob/cwn102
45. Smith GT, Sweredoski MJ, Hess S. O-linked glycosylation sites profiling in *Mycobacterium tuberculosis* culture filtrate proteins. *J Proteomics* (2014) **97**:296–306. doi:10.1016/j.jprot.2013.05.011
46. Kusebauch U, Ortega C, Ollodart A, Rogers RS, Sherman DR, Moritz RL, et al. *Mycobacterium tuberculosis* supports protein tyrosine phosphorylation. *Proc Natl Acad Sci U S A* (2014) **111**:9265–70. doi:10.1073/pnas.1323894111
47. Prisic S, Dankwa S, Schwartz D, Chou MF, Locasale JW, Kang CM, et al. Extensive phosphorylation with overlapping specificity by *Mycobacterium tuberculosis* serine/threonine protein kinases. *Proc Natl Acad Sci U S A* (2010) **107**:7521–6. doi:10.1073/pnas.0913482107
48. Pethe K, Bifani P, Drobecq H, Sergheraert C, Debrie AS, Locht C, et al. Mycobacterial heparin-binding hemagglutinin and laminin-binding protein share antigenic methyllysines that confer resistance to proteolysis. *Proc Natl Acad Sci U S A* (2002) **99**:10759–64. doi:10.1073/pnas.162246899
49. Okkels LM, Muller EC, Schmid M, Rosenkrands I, Kaufmann SH, Andersen P, et al. CFP10 discriminates between nonacetylated and acetylated ESAT-6 of *Mycobacterium tuberculosis* by differential interaction. *Proteomics* (2004) **4**:2954–60. doi:10.1002/pmic.200400906
50. Sutcliffe IC, Harrington DJ. Lipoproteins of *Mycobacterium tuberculosis*: an abundant and functionally diverse class of cell envelope components. *FEMS Microbiol Rev* (2004) **28**:645–59. doi:10.1016/j.femsre.2004.06.002
51. Okuda S, Tokuda H. Lipoprotein sorting in bacteria. *Annu Rev Microbiol* (2011) **65**:239–59. doi:10.1146/annurev-micro-090110-102859
52. Brulle JK, Tschumi A, Sander P. Lipoproteins of slow-growing Mycobacteria carry three fatty acids and are N-acylated by apolipoprotein N-acyltransferase BCG_2070c. *BMC Microbiol* (2013) **13**:223. doi:10.1186/1471-2180-13-223
53. Sanchez A, Espinosa P, Esparza MA, Colon M, Bernal G, Mancilla R. *Mycobacterium tuberculosis* 38-kDa lipoprotein is apoptogenic for human monocyte-derived macrophages. *Scand J Immunol* (2009) **69**:20–8. doi:10.1111/j.1365-3083.2008.02193.x
54. Sanchez A, Espinosa P, Garcia T, Mancilla R. The 19 kDa *Mycobacterium tuberculosis* lipoprotein (LpqH) induces macrophage apoptosis through extrinsic and intrinsic pathways: a role for the mitochondrial apoptosis-inducing factor. *Clin Dev Immunol* (2012) **2012**:950503. doi:10.1155/2012/950503
55. Lancioni CL, Li Q, Thomas JJ, Ding X, Thiel B, Drage MG, et al. *Mycobacterium tuberculosis* lipoproteins directly regulate human memory CD4(+) T cell activation via Toll-like receptors 1 and 2. *Infect Immun* (2011) **79**:663–73. doi:10.1128/IAI.00806-10
56. Striebel F, Imkamp F, Sutter M, Steiner M, Mamedov A, Weber-Ban E. Bacterial ubiquitin-like modifier Pup is deamidated and conjugated to substrates by distinct but homologous enzymes. *Nat Struct Mol Biol* (2009) **16**:647–51. doi:10.1038/nsmb.1597
57. Chun T, Serbina NV, Nolt D, Wang B, Chiu NM, Flynn JL, et al. Induction of M3-restricted cytotoxic T lymphocyte responses by N-formylated peptides derived from *Mycobacterium tuberculosis*. *J Exp Med* (2001) **193**:1213–20. doi:10.1084/jem.193.10.1213
58. Doi T, Yamada H, Yajima T, Wajjwalku W, Hara T, Yoshikai Y. H2-M3-restricted CD8+ T cells induced by peptide-pulsed dendritic cells confer protection against *Mycobacterium tuberculosis*. *J Immunol* (2007) **178**:3806–13. doi:10.4049/jimmunol.178.6.3806
59. Pearce MJ, Mintseris J, Ferreyra J, Gygi SP, Darwin KH. Ubiquitin-like protein involved in the proteasome pathway of *Mycobacterium tuberculosis*. *Science* (2008) **322**:1104–7. doi:10.1126/science.1163885
60. Barandun J, Delley CL, Weber-Ban E. The pupylation pathway and its role in mycobacteria. *BMC Biol* (2012) **10**:95. doi:10.1186/1741-7007-10-95
61. Cerda-Maira FA, Pearce MJ, Fuortes M, Bishai WR, Hubbard SR, Darwin KH. Molecular analysis of the prokaryotic ubiquitin-like protein (Pup) conjugation pathway in *Mycobacterium tuberculosis*. *Mol Microbiol* (2010) **77**:1123–35. doi:10.1111/j.1365-2958.2010.07276.x
62. Burns KE, Pearce MJ, Darwin KH. Prokaryotic ubiquitin-like protein provides a two-part degron to Mycobacterium proteasome substrates. *J Bacteriol* (2010) **192**:2933–5. doi:10.1128/JB.01639-09
63. Tung CW. PupDB: a database of pupylated proteins. *BMC Bioinformatics* (2012) **13**:40. doi:10.1186/1471-2105-13-40
64. Burns KE, Cerda-Maira FA, Wang T, Li H, Bishai WR, Darwin KH. "Depupylation" of prokaryotic ubiquitin-like protein from mycobacterial proteasome substrates. *Mol Cell* (2010) **39**:821–7. doi:10.1016/j.molcel.2010.07.019
65. Power PM, Jennings MP. The genetics of glycosylation in Gram-negative bacteria. *FEMS Microbiol Lett* (2003) **218**:211–22. doi:10.1111/j.1574-6968.2003.tb11520.x
66. Iwashkiw JA, Vozza NF, Kinsella RL, Feldman MF. Pour some sugar on it: the expanding world of bacterial protein O-linked glycosylation. *Mol Microbiol* (2013) **89**:14–28. doi:10.1111/mmi.12265
67. Nothaft H, Szymanski CM. Protein glycosylation in bacteria: sweeter than ever. *Nat Rev Microbiol* (2010) **8**:765–78. doi:10.1038/nrmicro2383
68. Espitia C, Servin-Gonzalez L, Mancilla R. New insights into protein O-mannosylation in actinomycetes. *Mol Biosyst* (2010) **6**:775–81. doi:10.1039/b916394h
69. Liu CF, Tonini L, Malaga W, Beau M, Stella A, Bouyssie D, et al. Bacterial protein-O-mannosylating enzyme is crucial for virulence of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* (2013) **110**:6560–5. doi:10.1073/pnas.1219704110
70. Horn C, Namane A, Pescher P, Riviere M, Romain F, Puzo G, et al. Decreased capacity of recombinant 45/47-kDa molecules (Apa) of *Mycobacterium tuberculosis* to stimulate T lymphocyte responses related to changes in their mannosylation pattern. *J Biol Chem* (1999) **274**:32023–30. doi:10.1074/jbc.274.45.32023
71. Ge Y, El-Naggar M, Sze SK, Oh HB, Begley TP, McLafferty FW, et al. Top down characterization of secreted proteins from *Mycobacterium tuberculosis* by electron capture dissociation mass spectrometry. *J Am Soc Mass Spectrom* (2003) **14**:253–61. doi:10.1016/S1044-0305(02)00913-3
72. Steingart KR, Dendukuri N, Henry M, Schiller I, Nahid P, Hopewell PC, et al. Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a meta-analysis. *Clin Vaccine Immunol* (2009) **16**:260–76. doi:10.1128/CVI.00355-08
73. Diaz-Silvestre H, Espinosa-Cueto P, Sanchez-Gonzalez A, Esparza-Ceron MA, Pereira-Suarez AL, Bernal-Fernandez G, et al. The 19-kDa antigen of *Mycobacterium tuberculosis* is a major adhesin that binds the mannose receptor of THP-1 monocytic cells and promotes phagocytosis of mycobacteria. *Microb Pathog* (2005) **39**:97–107. doi:10.1016/j.micpath.2005.06.002
74. Ragas A, Roussel L, Puzo G, Riviere M. The *Mycobacterium tuberculosis* cell-surface glycoprotein apa as a potential adhesin to colonize target cells via the

innate immune system pulmonary C-type lectin surfactant protein A. *J Biol Chem* (2007) **282**:5133–42. doi:10.1074/jbc.M610183200
75. Pitarque S, Herrmann JL, Duteyrat JL, Jackson M, Stewart GR, Lecointe F, et al. Deciphering the molecular bases of *Mycobacterium tuberculosis* binding to the lectin DC-SIGN reveals an underestimated complexity. *Biochem J* (2005) **392**:615–24. doi:10.1042/BJ20050709
76. Rezwan M, Grau T, Tschumi A, Sander P. Lipoprotein synthesis in mycobacteria. *Microbiology* (2007) **153**:652–8. doi:10.1099/mic.0.2006/000216-0
77. Tschumi A, Grau T, Albrecht D, Rezwan M, Antelmann H, Sander P. Functional analyses of mycobacterial lipoprotein diacylglyceryl transferase and comparative secretome analysis of a mycobacterial lgt mutant. *J Bacteriol* (2012) **194**:3938–49. doi:10.1128/JB.00127-12
78. Rampini SK, Selchow P, Keller C, Ehlers S, Bottger EC, Sander P. LspA inactivation in *Mycobacterium tuberculosis* results in attenuation without affecting phagosome maturation arrest. *Microbiology* (2008) **154**:2991–3001. doi:10.1099/mic.0.2008/018895-0
79. Sander P, Rezwan M, Walker B, Rampini SK, Kroppenstedt RM, Ehlers S, et al. Lipoprotein processing is required for virulence of *Mycobacterium tuberculosis*. *Mol Microbiol* (2004) **52**:1543–52. doi:10.1111/j.1365-2958.2004.04041.x
80. Zahringer U, Lindner B, Inamura S, Heine H, Alexander C. TLR2 – promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. *Immunobiology* (2008) **213**:205–24. doi:10.1016/j.imbio.2008.02.005
81. Noss EH, Pai RK, Sellati TJ, Radolf JD, Belisle J, Golenbock DT, et al. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J Immunol* (2001) **167**:910–8. doi:10.4049/jimmunol.167.2.910
82. Lopez M, Sly LM, Luu Y, Young D, Cooper H, Reiner NE. The 19-kDa *Mycobacterium tuberculosis* protein induces macrophage apoptosis through Toll-like receptor-2. *J Immunol* (2003) **170**:2409–16. doi:10.4049/jimmunol.170.5.2409
83. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* (2007) **130**:1071–82. doi:10.1016/j.cell.2007.09.008
84. Mir SA, Sharma S. Immunotherapeutic potential of N-formylated peptides of ESAT-6 and glutamine synthetase in experimental tuberculosis. *Int Immunopharmacol* (2014) **18**:298–303. doi:10.1016/j.intimp.2013.09.010
85. Vetting MW, Errey JC, Blanchard JS. Rv0802c from *Mycobacterium tuberculosis*: the first structure of a succinyltransferase with the GNAT fold. *Acta Crystallogr Sect F Struct Biol Cryst Commun* (2008) **64**:978–85. doi:10.1107/S1744309108031679
86. Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, Locht C, et al. The heparin-binding haemagglutinin of *M. tuberculosis* is required for extrapulmonary dissemination. *Nature* (2001) **412**:190–4. doi:10.1038/35084083
87. Lebrun P, Raze D, Fritzinger B, Wieruszeski JM, Biet F, Dose A, et al. Differential contribution of the repeats to heparin binding of HBHA, a major adhesin of *Mycobacterium tuberculosis*. *PLoS One* (2012) **7**:e32421. doi:10.1371/journal.pone.0032421
88. Menozzi FD, Rouse JH, Alavi M, Laude-Sharp M, Muller J, Bischoff R, et al. Identification of a heparin-binding hemagglutinin present in mycobacteria. *J Exp Med* (1996) **184**:993–1001. doi:10.1084/jem.184.3.993
89. Sohn H, Kim JS, Shin SJ, Kim K, Won CJ, Kim WS, et al. Targeting of *Mycobacterium tuberculosis* heparin-binding hemagglutinin to mitochondria in macrophages. *PLoS Pathog* (2011) **7**:e1002435. doi:10.1371/journal.ppat.1002435
90. Lefrancois LH, Bodier CC, Lecher S, Gilbert FB, Cochard T, Harichaux G, et al. Purification of native HBHA from *Mycobacterium avium* subsp. paratuberculosis. *BMC Res Notes* (2013) **6**:55. doi:10.1186/1756-0500-6-55
91. Romain F, Horn C, Pescher P, Namane A, Riviere M, Puzo G, et al. Deglycosylation of the 45/47-kilodalton antigen complex of *Mycobacterium tuberculosis* decreases its capacity to elicit in vivo or in vitro cellular immune responses. *Infect Immun* (1999) **67**:5567–72.
92. Nandakumar S, Kannanganat S, Dobos KM, Lucas M, Spencer JS, Fang S, et al. O-mannosylation of the *Mycobacterium tuberculosis* adhesin Apa is crucial for T cell antigenicity during infection but is expendable for protection. *PLoS Pathog* (2013) **9**:e1003705. doi:10.1371/journal.ppat.1003705
93. Kumar P, Amara RR, Challu VK, Chadda VK, Satchidanandam V. The Apa protein of *Mycobacterium tuberculosis* stimulates gamma interferon-secreting CD4+ and CD8+ T cells from purified protein derivative-positive individuals and affords protection in a guinea pig model. *Infect Immun* (2003) **71**:1929–37. doi:10.1128/IAI.71.4.1929-1937.2003
94. Sable SB, Cheruvu M, Nandakumar S, Sharma S, Bandyopadhyay K, Kellar KL, et al. Cellular immune responses to nine *Mycobacterium tuberculosis* vaccine candidates following intranasal vaccination. *PLoS One* (2011) **6**:e22718. doi:10.1371/journal.pone.0022718
95. Temmerman S, Pethe K, Parra M, Alonso S, Rouanet C, Pickett T, et al. Methylation-dependent T cell immunity to *Mycobacterium tuberculosis* heparin-binding hemagglutinin. *Nat Med* (2004) **10**:935–41. doi:10.1038/nm1090
96. Parra M, Pickett T, Delogu G, Dheenadhayalan V, Debrie AS, Locht C, et al. The mycobacterial heparin-binding hemagglutinin is a protective antigen in the mouse aerosol challenge model of tuberculosis. *Infect Immun* (2004) **72**:6799–805. doi:10.1128/IAI.72.12.6799-6805.2004
97. Masungi C, Temmerman S, Van Vooren JP, Drowart A, Pethe K, Menozzi FD, et al. Differential T and B cell responses against *Mycobacterium tuberculosis* heparin-binding hemagglutinin adhesin in infected healthy individuals and patients with tuberculosis. *J Infect Dis* (2002) **185**:513–20. doi:10.1086/338833
98. Loxton AG, Black GF, Stanley K, Walzl G. Heparin-binding hemagglutinin induces IFN-gamma(+) IL-2(+) IL-17(+) multifunctional CD4(+) T cells during latent but not active tuberculosis disease. *Clin Vaccine Immunol* (2012) **19**:746–51. doi:10.1128/CVI.00047-12
99. Kaufmann SH. Fact and fiction in tuberculosis vaccine research: 10 years later. *Lancet Infect Dis* (2011) **11**:633–40. doi:10.1016/S1473-3099(11)70146-3
100. Smith SG, Lecher S, Blitz R, Locht C, Dockrell HM. Broad heparin-binding haemagglutinin-specific cytokine and chemokine response in infants following *Mycobacterium bovis* BCG vaccination. *Eur J Immunol* (2012) **42**:2511–22. doi:10.1002/eji.201142297
101. Aagaard CS, Hoang TT, Vingsbo-Lundberg C, Dietrich J, Andersen P. Quality and vaccine efficacy of CD4+ T cell responses directed to dominant and subdominant epitopes in ESAT-6 from *Mycobacterium tuberculosis*. *J Immunol* (2009) **183**:2659–68. doi:10.4049/jimmunol.0900947
102. Woodworth JS, Aagaard CS, Hansen PR, Cassidy JP, Agger EM, Andersen P. Protective CD4 T cells targeting cryptic epitopes of *Mycobacterium tuberculosis* resist infection-driven terminal differentiation. *J Immunol* (2014) **192**:3247–58. doi:10.4049/jimmunol.1300283
103. van Dissel JT, Soonawala D, Joosten SA, Prins C, Arend SM, Bang P, et al. Ag85B-ESAT-6 adjuvanted with IC31(R) promotes strong and long-lived *Mycobacterium tuberculosis* specific T cell responses in volunteers with previous BCG vaccination or tuberculosis infection. *Vaccine* (2011) **29**:2100–9. doi:10.1016/j.vaccine.2010.12.135
104. Hanisch FG, Schwientek T, Von Bergwelt-Baildon MS, Schultze JL, Finn O. O-Linked glycans control glycoprotein processing by antigen-presenting cells: a biochemical approach to the molecular aspects of MUC1 processing by dendritic cells. *Eur J Immunol* (2003) **33**:3242–54. doi:10.1002/eji.200324189
105. Vyas JM, Van der Veen AG, Ploegh HL. The known unknowns of antigen processing and presentation. *Nat Rev Immunol* (2008) **8**:607–18. doi:10.1038/nri2368
106. Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* (1992) **255**:1261–3. doi:10.1126/science.1546328
107. Henderson RA, Michel H, Sakaguchi K, Shabanowitz J, Appella E, Hunt DF, et al. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science* (1992) **255**:1264–6. doi:10.1126/science.1546329
108. Hassan C, Kester MG, de Ru AH, Hombrink P, Drijfhout JW, Nijveen H, et al. The human leukocyte antigen-presented ligandome of B lymphocytes. *Mol Cell Proteomics* (2013) **12**:1829–43. doi:10.1074/mcp.M112.024810
109. Mommen GP, Frese CK, Meiring HD, van Gaans-van den Brink J, de Jong AP, van Els CA, et al. Expanding the detectable HLA peptide repertoire using electron-transfer/higher-energy collision dissociation (EThcD). *Proc Natl Acad Sci U S A* (2014) **111**:4507–12. doi:10.1073/pnas.1321458111
110. Rammensee HG. Chemistry of peptides associated with MHC class I and class II molecules. *Curr Opin Immunol* (1995) **7**:85–96. doi:10.1016/0952-7915(95)80033-6
111. Rammensee HG, Friede T, Stevanoviic S. MHC ligands and peptide motifs: first listing. *Immunogenetics* (1995) **41**:178–228. doi:10.1007/BF00172063

112. Lampen MH, Hassan C, Sluijter M, Geluk A, Dijkman K, Tjon JM, et al. Alternative peptide repertoire of HLA-E reveals a binding motif that is strikingly similar to HLA-A2. *Mol Immunol* (2013) **53**:126–31. doi:10.1016/j.molimm.2012.07.009
113. van Els CA, Herberts CA, van der Heeft E, Poelen MC, van Gaans-van den Brink JA, van der Kooi A, et al. A single naturally processed measles virus peptide fully dominates the HLA-A*0201-associated peptide display and is mutated at its anchor position in persistent viral strains. *Eur J Immunol* (2000) **30**:1172–81. doi:10.1002/(SICI)1521-4141(200004)30:4<1172::AID-IMMU1172>3.0.CO;2-J
114. Hillen N, Stevanovic S. Contribution of mass spectrometry-based proteomics to immunology. *Expert Rev Proteomics* (2006) **3**:653–64. doi:10.1586/14789450.3.6.653
115. Skipper JC, Hendrickson RC, Gulden PH, Brichard V, Van Pel A, Chen Y, et al. An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. *J Exp Med* (1996) **183**:527–34. doi:10.1084/jem.183.2.527
116. Ireland JM, Unanue ER. Autophagy in antigen-presenting cells results in presentation of citrullinated peptides to CD4 T cells. *J Exp Med* (2011) **208**:2625–32. doi:10.1084/jem.20110640
117. Pierce RA, Field ED, den Haan JM, Caldwell JA, White FM, Marto JA, et al. Cutting edge: the HLA-A*0101-restricted HY minor histocompatibility antigen originates from DFFRY and contains a cysteinylated cysteine residue as identified by a novel mass spectrometric technique. *J Immunol* (1999) **163**:6360–4.
118. Hanada K, Yewdell JW, Yang JC. Immune recognition of a human renal cancer antigen through post-translational protein splicing. *Nature* (2004) **427**:252–6. doi:10.1038/nature02240
119. Vigneron N, Stroobant V, Chapiro J, Ooms A, Degiovanni G, Morel S, et al. An antigenic peptide produced by peptide splicing in the proteasome. *Science* (2004) **304**:587–90. doi:10.1126/science.1095522
120. Engelhard VH. Creating new peptide antigens by slicing and splicing proteins. *Nat Immunol* (2004) **5**:128–9. doi:10.1038/ni0204-128
121. Bullock TN, Patterson AE, Franlin LL, Notidis E, Eisenlohr LC. Initiation codon scanthrough versus termination codon readthrough demonstrates strong potential for major histocompatibility complex class I-restricted cryptic epitope expression. *J Exp Med* (1997) **186**:1051–8. doi:10.1084/jem.186.7.1051
122. Malarkannan S, Horng T, Shih PP, Schwab S, Shastri N. Presentation of out-of-frame peptide/MHC class I complexes by a novel translation initiation mechanism. *Immunity* (1999) **10**:681–90. doi:10.1016/S1074-7613(00)80067-9
123. Mandic M, Almunia C, Vicel S, Gillet D, Janjic B, Coval K, et al. The alternative open reading frame of LAGE-1 gives rise to multiple promiscuous HLA-DR-restricted epitopes recognized by T-helper 1-type tumor-reactive CD4+ T cells. *Cancer Res* (2003) **63**:6506–15.
124. Dengjel J, Decker P, Schoor O, Altenberend F, Weinschenk T, Rammensee HG, et al. Identification of a naturally processed cyclin D1 T-helper epitope by a novel combination of HLA class II targeting and differential mass spectrometry. *Eur J Immunol* (2004) **34**:3644–51. doi:10.1002/eji.200425510
125. Lemmel C, Weik S, Eberle U, Dengjel J, Kratt T, Becker HD, et al. Differential quantitative analysis of MHC ligands by mass spectrometry using stable isotope labeling. *Nat Biotechnol* (2004) **22**:450–4. doi:10.1038/nbt947
126. Meiring HD, Kuipers B, van Gaans-van den Brink JA, Poelen MC, Timmermans H, Baart G, et al. Mass tag-assisted identification of naturally processed HLA class II-presented meningococcal peptides recognized by CD4+ T lymphocytes. *J Immunol* (2005) **174**:5636–43. doi:10.4049/jimmunol.174.9.5636
127. Meiring HD, Soethout EC, Poelen MC, Mooibroek D, Hoogerbrugge R, Timmermans H, et al. Stable isotope tagging of epitopes: a highly selective strategy for the identification of major histocompatibility complex class I-associated peptides induced upon viral infection. *Mol Cell Proteomics* (2006) **5**:902–13. doi:10.1074/mcp.T500014-MCP200
128. Soethout EC, Meiring HD, de Jong AP, van Els CA. Identifying the epitope-specific T cell response to virus infections. *Vaccine* (2007) **25**:3200–3. doi:10.1016/j.vaccine.2007.01.029
129. Stenger RM, Meiring HD, Kuipers B, Poelen M, van Gaans-van den Brink JA, Boog CJ, et al. *Bordetella pertussis* proteins dominating the MHC Class II-presented epitope repertoire in human monocyte derived dendritic cells. *Clin Vaccine Immunol* (2014) **21**:641–50. doi:10.1128/CVI.00665-13
130. Flyer DC, Ramakrishna V, Miller C, Myers H, McDaniel M, Root K, et al. Identification by mass spectrometry of CD8(+)-T-cell *Mycobacterium tuberculosis* epitopes within the Rv0341 gene product. *Infect Immun* (2002) **70**:2926–32. doi:10.1128/IAI.70.6.2926-2932.2002
131. Harding CV, Boom WH. Regulation of antigen presentation by *Mycobacterium tuberculosis*: a role for Toll-like receptors. *Nat Rev Microbiol* (2010) **8**:296–307. doi:10.1038/nrmicro2321
132. Hava DL, van der Wel N, Cohen N, Dascher CC, Houben D, Leon L, et al. Evasion of peptide, but not lipid antigen presentation, through pathogen-induced dendritic cell maturation. *Proc Natl Acad Sci U S A* (2008) **105**:11281–6. doi:10.1073/pnas.0804681105

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 April 2014; accepted: 14 July 2014; published online: 11 August 2014.
Citation: van Els CACM, Corbière V, Smits K, van Gaans-van den Brink JAM, Poelen MCM, Mascart F, Meiring HD and Locht C (2014) Toward understanding the essence of post-translational modifications for the Mycobacterium tuberculosis immunoproteome. Front. Immunol. 5:361. doi: 10.3389/fimmu.2014.00361
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 27 May 2014
doi: 10.3389/fimmu.2014.00236

The PGRS domain from PE_PGRS33 of *Mycobacterium tuberculosis* is target of humoral immune response in mice and humans

Ingrid Cohen[1], Cristina Parada[1], Enrique Acosta-Gío[2] and Clara Espitia[1]*

[1] *Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico*
[2] *Facultad de Odontología, Universidad Nacional Autónoma de México, Mexico City, Mexico*

Edited by:
Juraj Ivanyi, Kings College London, UK

Reviewed by:
Diego A. Vargas-Inchaustegui, National Cancer Institute, USA
Juraj Ivanyi, Kings College London, UK

****Correspondence:***
Clara Espitia, Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Apartado Postal 70-228, 04510 D.F. México, México
e-mail: espitia@biomedicas.unam.mx

The PE_PGRS33 protein is a member of the PE family, which encompasses the PE and the PE_PGRS subfamilies. Among PE_PGRS's, this protein is one of the most studied antigens and its immunomodulatory properties are influence by both PE and PGRS domains. However, the contribution of these domains to the host immune recognition of the PE_PGRS33 protein and their potential role in latent tuberculosis infection in humans is still unknown. In this study, the immunogenic properties of the complete PE_PGRS33 protein and each domain separately were evaluated in BALB/c mice and latent tuberculosis infected (LTBI) humans. In mice, PE_PGRS33 and its domains induced similar antibody production and secretion of IFN-γ. PE_PGRS33 and the PE domain stimulated higher $CD4^+$ and $CD8^+$ T-cell proliferation compared to the PGRS domain. This demonstrated that the principal difference in the immune recognition of the domains is the higher activation of T-cell subpopulations involved in the control of tuberculosis. In humans, the secretion of IFN-γ in response to PE_PGRS33 was detected in both LTBI and in non-infected vaccinated individuals. The same was observed for antibody response, which targets epitopes located in the PGRS domain but not in the PE domain. These observations suggest that T and B cell responses to PE_PGRS33 are induced by BCG vaccination and can be maintained for many years in non-infected individuals. This also indicates that the IFN-γ response detected might not be associated with latent tuberculosis infection. These results contribute to the elucidation of the role of the PE_PGRS33 protein and its PE and PGRS domains in the immune response against *Mycobacterium tuberculosis*.

Keywords: ***Mycobacterium tuberculosis*, PE_PGRS33, PE domain, PGRS domain, latent tuberculosis infection**

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of human tuberculosis, is one of the most successful pathogens known. This bacterium is able to elude the host immune system and starts the disease after the infection or remains latent during long time (1). Many factors that could explain these characteristics were elucidated after the genome sequencing of the *M. tuberculosis* H37Rv (2). The genome sequence of these bacteria also revealed the presence of the PE family, which encompasses the PE and PE_PGRS subfamilies with about 100 genes scattered throughout the genome. Around 61 of these genes encodes for members of the PE_PGRS subfamily. These proteins are characterized by a highly conserved PE domain of approximately 110 amino acid residues that contains the motif Pro–Glu (PE) near the N-terminus. This domain is followed by the PGRS (polymorphic GC-rich-sequence) domain, which varies in size from 100–1400 amino acid residues and is rich in repetitive Gly–Gly–X motifs (2).

Some PE_PGRS proteins are exposed at the bacterial surface, where they can interact with the host immune system (3–5). Antibodies against PE_PGRS51, PE_PGRS62, PE_PGRS33, and the PGRS domain of Wag22 ($Rv1759c^{PE\_PGRS}$) are present in sera from patients with tuberculosis or during experimental tuberculosis in mice (6–10). Several PE_PGRS elicit T-cell responses in humans and are recognized by major histocompatibility complex-I (MHC-I)-restricted $CD8^+$ T cells in mice, suggesting that many members of the PE_PGRS subfamily are highly immunogenic (11, 12).

PE_PGRS proteins are also involved in latency. Mutations in *pe_pgrs* genes of other mycobacterial species have shown decreased persistence in granulomas (13). The PE_PGRS33 protein is a member of the PE_PGRS subfamily that stimulates tumor necrosis factor-α (TNF-α) production, one of the cytokines involved in the induction and maintenance of latent tuberculosis infection in animal models mimicking human latency (14–16). The $Rv1759c^{PE\_PGRS}$ antigen induces immune response maintaining the latent infection in a murine model of chronic tuberculosis (17). *M. tuberculosis* clinical strains harboring big genetic variations in the *rv1818c* that codifies the PE-PGRS33 have been associated with clustering of tuberculosis cases and absence of cavitations in the lungs. This suggests that this protein plays a role in the establishment or maintenance of latent infection (18). Until now, the immune response against the PE_PGRS proteins has not been described in *M. tuberculosis* latent-infected individuals.

Additionally, the PE_PGRS33 protein plays an important and may be non-redundant role in the pathogenesis of *M. tuberculosis*

(19). The sequence of the PE domain of this protein is highly conserved among *M. tuberculosis* clinical isolates (18, 20). This domain directs the cell wall localization of PE_PGRS33 (21). It has been reported that mutations in the PE domain affect the pro-inflammatory properties of the protein (22). On the other hand, the PGRS domain exhibits the major sequence variations in clinical *M. tuberculosis* strains (20). The PGRS fragment mediates the interaction with toll-like receptor 2 (TLR2) triggering host-cell death (14, 22). Deletions inside this domain can modulate the secretion of TNF-α induced by the PE_PGRS33 (14). The immunogenic properties of the PE domain have been evaluated in a murine model (9). The contribution of the PGRS domain to the immune response generated by PE_PGRS33 has been inferred from the study of the complete protein and the PE domain. However, the effect of the PGRS single domain has not been reported.

In this work, the immunogenic properties of the PE_PGRS33 protein and the PE and PGRS domains were studied in mice. This study was extended to humans where the secretion of IFN-γ and antibodies levels in latent tuberculosis-infected (LTBI) and non-infected individuals were evaluated.

MATERIALS AND METHODS

PREPARATION OF ANTIGENS

Cloning the PE and PGRS domains of PE_PGRS33

The full-length *rv1818c* gene, which codifies for the PE_PGRS33 was cloned into the pET15b vector (Novagen Inc., Madison, WI, USA) fused to a histidine (His) tag was kindly provided by Dr. M. J. Brennan [CBER, FDA, Bethesda, MD, USA (13)]. The *rv1818c* gene from pET15b was inserted into the plasmid pcDNA3 (Invitrogen, Carlsbad, CA, USA). An 1172 bp fragment (from nucleotide 339 to 1494) encoding the PGRS region of the *rv1818c* gene was amplified by PCR from pcDNA3 using the forward primer 5′-GGAATTCCATATGGGGCGCCCACTGATCGGT-3′ (which includes an NdeI site) and the reverse primer 5′-ATGGATCCCTAC GGTAACCCGTTCATCCCGTTC-3′ containing a BamHI site and a stop codon. The NdeI–BamHI fragment was then cloned in pET15b. The PE coding region of the *rv1818c* gene (from nucleotide 1 to 339) was amplified using the forward primer 5′-CGGGATCCATGTCATTTGTGGTCACGATCC-3′ holding a BamHI site and the reverse primer 5′-CGGAATTCACAACAGCGCCAGGGCG-3′ including an EcoRI site and a stop codon. The EcoRI–BamHI fragment was then cloned into the multiple cloning site of the plasmid pGEX-4T-2 (Amersham Pharmacia, Piscataway, NJ, USA) to create a fusion product with the coding sequence for glutathione *S*-transferase (GST).

Expression and production of PE_PGRS33, PE, and PGRS domains

PE_PGRS33 and its PGRS domain were expressed in *Escherichia coli* C41 (DE3). Bacteria were cultivated at 37°C in Luria–Bertani broth with 100 μg/ml ampicillin. All the chemicals were obtained from SIGMA Aldrich, St. Louis, MO, USA unless otherwise stated. Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD = 0.6. Cells were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS) with 1× complete ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor (Roche Applied Science, Mannheim, Germany), and disrupted by sonication. After centrifugation, the inclusion bodies were washed sequentially with 2% Triton X-100, 1% Triton X-100, and PBS. The inclusion bodies were dissolved in buffer A [50 mM Na_2HPO_4 (pH 8.0), 8 M urea, 300 mM NaCl, 10 mM Imidazole] at 4°C for 14 h. The PE_PGRS33 protein was purified by metal affinity chromatography in an Akta-Prime (GE Healthcare Biosciences, Pittsburgh, PA, USA). The solution of proteins was bound to a His–Trap HP column (GE Healthcare Biosciences, Pittsburgh, PA, USA) previously equilibrated with buffer A. The protein elution was performed using 500 mM Imidazole in buffer. The purified protein was dialyzed and buffer was exchanged for 50 mM Tris–HCl (pH 8.0), 150 mM NaCl.

The PE domain fusion to GST was expressed in *E. coli* BL21 (DE3) grown in Luria–Bertani broth with 100 μg/ml carbenicillin at 37°C. Protein expression was induced with 0.25 mM IPTG at OD = 0.6. Cells were harvested by centrifugation and disrupted by sonication. Inclusion bodies were dissolved in 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 8 M urea, 1 mM dithiothreitol (DTT), 1 mM EDTA. The supernatant was dialyzed against 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 1 mM DTT (union buffer) containing 4 and 2 M urea in sequential steps. The final dialysis step was performed against union buffer. A final concentration of 1% Triton X-100, 20 μg/ml PMSF, and 1 mM EDTA was added to the dialyzed sample. The suspension was bound to glutathione agarose at 0.5 ml resin per 2 ml sample by the batch method with gentle agitation at 4°C for 12 h. The resin was washed with 20 bed volumes of union buffer/1% Triton X-100 and then with 20 bed volumes of union buffer. The PE protein was eluted by incubation with elution buffer [50 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 20 mM reduced glutathione] at 4°C for 90 min. The glutathione was removed by dialysis against 50 mM Tris–HCl (pH 8.0), 100 mM NaCl.

MURINE MODEL

Preparation of PE_PGRS33 for the immunization of mice

A total amount of 100 μg of recombinant PE_PGRS33 was resolved in 12% polyacrylamide gels containing SDS according to the discontinuous buffer system of Laemmli (23). Proteins were transferred to nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ, USA). The protein band was identified by temporary staining with Ponceau S solution. The portion of the membrane with the identified protein was cut and converted into antigen-bearing particles using a previously described method (24). Briefly, the protein band was excised from the nitrocellulose sheet, cut in small pieces, and dissolved in dimethyl sulfoxide. The PE_PGRS33-bearing nitrocellulose was precipitated with 0.05 M carbonate/bicarbonate buffer (pH 9.6). After washing three times with PBS, the product was resuspended in 500 μl of sterile PBS. Nitrocellulose particles without protein were prepared under same conditions to be used as control. This methodology minimized the presence of undesirable proteins allowing the immunization to be performed only with the protein of interest.

Immunization of mice

The 6–7 weeks old female BALB/c mice were obtained from Harlan, Mexico. These mice were housed under standard

pathogen-free conditions. All animal studies were carried out in strict accordance with the recommendations from the current Institutional Guidelines for the Care and Use of Laboratory Animals. The animal study was previously approved by the Committee for the Care and Use of Laboratory Animals of Instituto de Investigaciones Biomédicas. Two groups of mice ($n = 4$ per group) were used in each of three independent experiments. Mice were immunized by intraperitoneal injection of 20 μg of recombinant PE_PGRS33 on antigen-bearing nitrocellulose (100 μl of PBS containing 20 μg of protein per mouse). Control mice received 100 μl of PBS containing only nitrocellulose particles by the same route. Intraperitoneal booster injections with same amount of antigen-bearing nitrocellulose were administered on days 21 and 42 to immunized mice. Control mice were injected with nitrocellulose particles on days 21 and 42.

Carboxyfluorescein diacetate succinimidyl ester proliferation assay

Spleen cells were obtained from immunized and control mice 2 weeks after the last immunization. Cells were extracted by tissue disruption and suspended in RPMI 1640 medium. All chemicals were obtained from GIBCO, Gran Island, NY, USA, unless otherwise stated. The erythrocytes were lysed by mixing the cells with 0.15 M ammonium chloride (NH_4Cl), 1.5 mM buffer HEPES (SIGMA Aldrich, St. Louis, MO, USA), 1 mM sodium bicarbonate ($NaHCO_3$) at room temperature for 5 min. The reaction was stopped by adding 10 volumes of Dulbecco's PBS (DPBS). Later 10^7 splenocytes were incubated at room temperature in the dark with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA, USA) for 5 min. The reaction was stopped by adding nine volumes of RPMI 1640 plus 10% fetal bovine serum (FBS). CFSE-stained cells were centrifuged at 1500 rpm for 5 min, washed twice in DPBS with 10% FBS, and resuspended in RPMI supplemented with 10% FBS, 1.5 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids, 20 mM HEPES buffer, and 50 μM 2-mercaptoethanol. Viable cells were counted by trypan blue exclusion, and 1.5 million cells were stimulated with 25 μg of PE_PGRS33, PE, PGRS, or GST protein plus 10 μg/ml of polymyxin B (Calbiochem, Pacific Center, CA, USA) in 24-well plates at 37°C in 5% CO_2 for 96 h. Polymyxin B was added to rule out the possibility of contamination with lipopolysaccharide (LPS). As positive control, 1.5 million CFSE-labeled spleen cells from control and immunized mice were stimulated with 2 μg/ml of concanavalin A (SIGMA Aldrich, St. Louis, MO, USA) at 37°C in 5% CO_2 for 72 h. The cells were harvested, washed with DPBS with 2% FBS and 0.09% sodium azide (NaN_3), and incubated with an anti-mouse FcγR antibody (CD16–CD32, CALTAG, Burlingame, CA, USA) diluted 1:500 to block the non-antigen-specific binding of conjugated antibodies. CFSE-labeled cells were then incubated with phycoerythrin-conjugated anti-CD4 antibody (BD Pharmingen, San Diego, CA, USA) diluted 1:200 and allophycocyanin-conjugated anti-CD8 antibody diluted 1:200 (CALTAG, Burlingame, CA, USA) on ice for 15 min. The cells were washed with DPBS with 2% FBS and 0.09% NaN_3 and resuspended in 0.5 ml DPBS. Three-color flow cytometry was performed in a fluorescence-activated cell sorting (FACS) Calibur cytometer (BD, Mountain View, CA, USA). Lymphocytes and blasts were identified by forward scatter (FCS) and side scatter characteristics (SSC) in 10,000 events acquired. The percentage of proliferating cells was determined by gating on $CD4^+$ or $CD8^+$ cells and comparing the proliferating population ($CFSE^{dim}$) with lineage positive cells that had not divided ($CFSE^{bright}$). Cellquest™ software was used to acquire and analyze the data. Samples and controls were analyzed under same conditions.

Mouse cytokine assay

The supernatants from mice cultured spleen cells used in the CFSE proliferation assay were evaluated for IFN-γ production using a Murine IFN-γ ELISA development kit (PEPROTECH, Mexico City, Mexico) following the instructions of manufacturer. Briefly, ELISA plates containing 1 μg/ml capture antibody per well were incubated at 4°C overnight. After addition of standards and samples, plates were incubated at room temperature for 2 h. The detection was performed with 0.5 μg/ml detection antibody. The plates were incubated at room temperature for 2 h. Avidin peroxidase diluted 1:2000 was added and incubated at room temperature for 30 min. A color reaction was developed using ABTS liquid substrate and absorbance values were measured at 405 nm using an ELISA plate reader.

Antibody detection in mice

Two weeks after the last immunization blood was collected from the tail veins of the immunized and control mice. The titers of Immunoglobulin G (IgG), IgG1 and IgG2a in sera were determined using the ELISA assay. Briefly, 96-well plates (Maxisorp Nunc Immunoplates) were coated with 5 μg/ml of recombinant PE_PGRS33, PE, PGRS, or GST at 4°C overnight. The plates were blocked with bovine serum albumin (BSA. SIGMA, St. Louis, MO, USA) and incubated with different dilutions of the mouse sera (3×10^2–1×10^5). Goat anti-mouse IgG–horseradish peroxidase (HRP) conjugate, IgG1–HRP and IgG2a–HRP antibody (all from ZYMED, San Francisco, CA, USA) were used as secondary antibodies. A color reaction was developed with *o*-phenylenediamine tetrahydrochloride (SIGMA, St. Louis, MO, USA), and absorbance values were measured at 492 nm using an ELISA plate reader.

STUDY IN HUMANS

Human donors

All the 88 volunteers who participated in this study were graduate students from the School of Dentistry, Universidad Nacional Autónoma de México. All participants had been vaccinated with *Mycobacterium bovis* Bacillus Calmette–Guérin (*M. bovis* BCG) when infants. The individuals were between 25 and 32 years old, clinically healthy, and had chest radiography negative for tuberculosis. The study in humans was performed in accordance with the Guidelines for Scientific Research with Humans of the Instituto de Investigaciones Biomédicas. The protocol was previously approved by the Ethics Committee of the same Institute. All participants signed consent forms detailing all relevant information about the nature of the study. All individuals participated voluntarily and their identities will remain undisclosed. No incentives were offered to participants.

IFN-γ release assay to detect LTBI and non-infected individuals

Blood samples were collected in heparinized tubes from each of the 88 participants. Aliquots of 1 ml of heparinized blood were incubated in the presence of the QuantiFERON®-TB Gold Kit (Cellestis Limited, Carnegie, VIC, Australia) antigens ESAT-6, CFP-10, and mitogen in 24-well tissue culture plates at 37°C in 5% CO_2 for 16 h. Plasma was obtained by centrifugation and the samples were evaluated for IFN-γ production using the Human IFN-γ ELISA kit provided with the QuantiFERON®-TB Gold Kit following the instructions of manufacturer. Briefly, human IFN-γ standards and plasma samples were added to 96-well microplates coated with murine anti-human IFN-γ. The conjugate murine anti-human IFN-γ HRP was incorporated immediately after samples, standards, and conjugates were thoroughly mixed in a microplate shaker and incubated at room temperature for 2 h. After the addition of the enzyme substrate solution, the plate was incubated at room temperature for 30 min. The reaction was stopped with enzyme stopping solution. The optical density was read after 5 min using a microplate reader. The results were analyzed using the QuantiFERON® Analysis software. The results of this analysis were used to divide the group of participants as LTBI and non-infected individuals.

IFN-γ release assay to detect responders to the PE_PGRS33 complete protein

An aliquot of 1 ml of heparinized blood obtained from each of the 88 participants was incubated with 25 μg/ml of PE_PGRS33 in 24-well tissue culture plates at 37°C in 5% CO_2 for 16 h. Plasma was obtained by centrifugation and the samples were evaluated for IFN-γ production using the Human IFN-γ ELISA kit provided with the QuantiFERON®-TB Gold Kit following the instructions of manufacturer, as above described. The results of this assay identified responders to the complete PE_PGRS33 protein among the LTBI and non-infected individuals.

IFN-γ release assay to study the response to the PE_PGRS33 protein and its PE and PGRS domains in LTBI and non-infected individuals

A volume of 5 ml of blood was collected in heparinized tubes and diluted 1:10 with RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from GIBCO BRL). An aliquot of 1 ml of diluted blood was incubated in a 24-well plate with 25 μg of PE_PGRS33, PE, PGRS, or GST protein plus 10 μg/ml polymyxin B at 37°C in 5% CO_2 for 6 days. The concentration of IFN-γ in supernatants was quantified using the Human IFN-γ ELISA kit provided with the QuantiFERON®-TB Gold Kit according to the instructions of manufacturer, as above described.

Antigen-specific antibody detection in LTBI and non-infected humans

Antibodies against PE_PGRS33 and their domains were detected in 63 out of 88 sera of the individuals by ELISA assay. This assay was carried out as above described for antibody detection in mice with the following modifications: human sera were diluted 1:100, 1:300, and 1:500. All samples were incubated with anti-human IgG HRP conjugate. In addition, three samples from LTBI and four from non-infected individuals that produce IFN-γ in response to PE_PGRS33 were also tested with IgG1 HRP conjugates (Caltag, Burlingame, CA, USA). The color reaction was developed with *o*-phenylenediamine tetrahydrochloride. Absorbance values were measured at 492 nm using an ELISA plate reader.

STATISTICAL ANALYSIS

Data between groups were compared using the Mann–Whitney *U* test at the 0.05 significance level.

RESULTS

HUMORAL AND CELLULAR IMMUNE RESPONSE TO PE_PGRS33, THE PE, AND PGRS DOMAINS IN MICE

Humoral immune response in mice

Sera from immunized and control mice were tested using ELISA assay to determine the levels of antigen-specific IgG antibodies against PE_PGRS33 and its PGRS and PE domains. Similar titers of IgG were observed in both immunized and control mice (**Figure 1A**). Titers of IgG1 and IgG2a were quantified to identify the differences between these IgG subclasses. Similar titers of IgG1 against the complete protein and its domains were detected in all dilutions tested (**Figure 1B**). In contrast, the levels of IgG2a subclass against the PE domain were lower than the levels against the PGRS domain or the complete PE_PGRS33 protein (**Figure 1C**). This indicated that IgG1 antibodies in sera from the immunized mice targeted the complete PE_PGRS33 and its domains, whereas the IgG2a antibodies recognized epitopes located exclusively in the PGRS domain.

Antigen-specific T-cell proliferation in mice

The proliferation of $CD4^+$ and $CD8^+$ T cells was measured in splenocytes of the mice immunized with the PE_PGRS33 protein to determine the contribution of PE_PGRS33 and its PE and PGRS domains to the activation of T cells (for flow cytometry histograms, see Figure S1 in Supplementary Material). The PE_PGRS33 complete protein similarly activated the proliferation of $CD4^+$ and $CD8^+$ T cells (**Figure 2**). Both the PE and PGRS domains stimulated the proliferation of $CD4^+$ and $CD8^+$ T cells. However, the response of the two cell subpopulations against the PE domain was significantly higher than that from the PGRS domain (**Figure 2**). The proliferative responses of lymphocytes from the control and immunized mice were statistically different (**Figure 2**). The GST co-expressed as a fusion product with the PE domain did not impact cell proliferation (**Figure 2**).

INF-γ secretion in mice

The PE_PGRS33 protein, the PE, and PGRS domains induced significantly higher IFN-γ responses compared with unstimulated cells (**Figure 3**). The concentration of IFN-γ in the immunized mice was also higher than that from the controls (**Figure 3**). The levels of INF-γ secretion after stimulation with PE_PGRS33 and the domains were similar (**Figure 3**). This indicated that the PE_PGRS33, the PE, and PGRS domain are inducers of cellular immune response in mice.

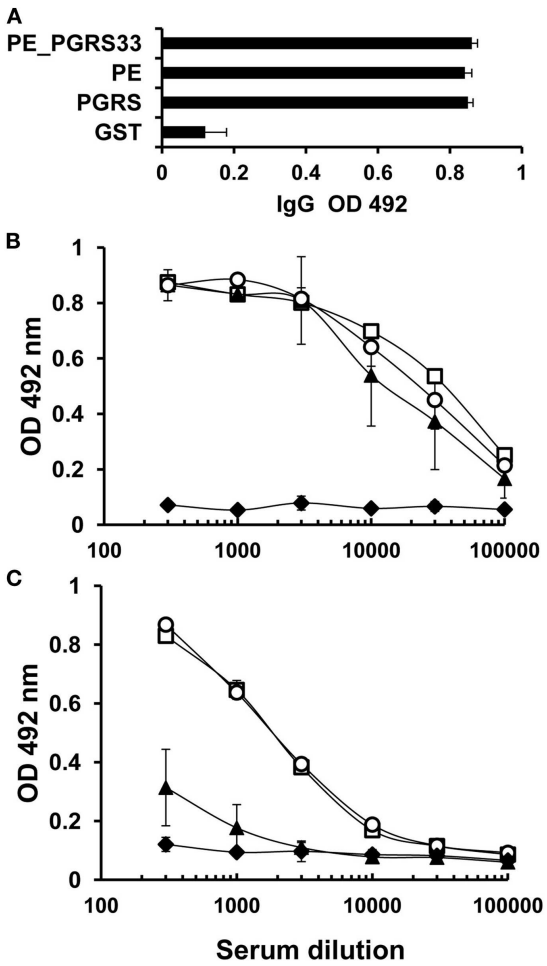


FIGURE 1 | Humoral immune response to PE_PGRS33, PGRS, and PE in immunized mice. BALB/c mice were immunized with PE_PGRS33 and sera were collected 15 days after the last immunization. IgG **(A)**, IgG1 **(B)**, and IgG2a **(C)** responses to PE_PGRS33 (□), PE (▲), PGRS (◯), and the control protein GST (♦), were evaluated by ELISA assay. For total IgG determination, each serum was diluted 1:100 and for IgG1 and IgG2a determination, mice sera were diluted as indicated in the figure. Bars **(A)** and datum points **(B,C)** are the average readings ± SD of data from four mice in the group, and the results showed are representative of three independent experiments.

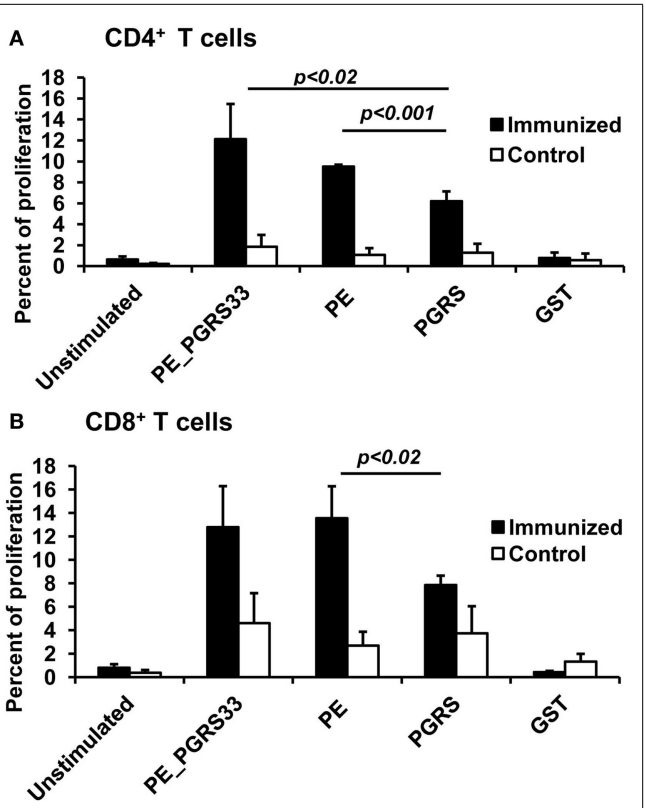


FIGURE 2 | Antigen-specific proliferation of CD4+ and CD8+ T cells in immunized mice. Splenocytes from PE_PGRS33-immunized mice were stained with CFSE and incubated with 25 μg of PE_PGRS33, PGRS, PE, or GST protein plus 10 μg of Polymyxin B for 4 days. Spleen cells from mice injected with only nitrocellulose were also cultured with antigens (control). Cells without the antigens were incubated for the same length of time (unstimulated). Splenocytes were then labeled with anti-CD4-phycoerythrin **(A)** or anti-CD8-allophycocyanin **(B)** monoclonal antibodies and the percentage of proliferating cells were determined by CFSE dilution and flow cytometry. Each bar represents the mean ± SD of data from four mice per group, and the results are representative of those obtained from three independent experiments.

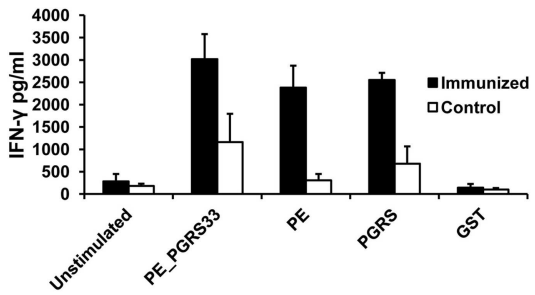


FIGURE 3 | Antigen-specific IFN-γ secretion in immunized mice. Spleen cells from mice immunized with PE_PGRS33 or from control mice were incubated with 25 μg of PE_PGRS33, PGRS, PE, or GST recombinant purified proteins plus 10 μg/ml of polymyxin B for 4 days. The culture supernatants were evaluated for IFN-γ production using ELISA assay. Each bar represents the mean ± SD of data from four mice per group. Representative results of three independent experiments are shown.

IMMUNE RESPONSE IN HUMANS

INF-γ responses to the complete PE_PGRS33 protein in LTBI and non-infected individuals

From the 88 individuals that participated in the study, 14 were identified as LTBI and 74 as non-infected (**Table 1**). To determine whether PE_PGRS33 is inducing an immunological response in humans, the secretion of IFN-γ by whole blood cells stimulated with the PE_PGRS33 protein was measured. The results obtained showed that the blood cells from 28.5% of LTBI secreted IFN-γ in response to the PE_PGRS33 compared to the 21.6% from the non-infected individuals (**Table 1**). This suggests that the IFN-γ response to the PE_PGRS33 protein is not associated with latent tuberculosis infection.

Table 1 | IFN-γ release assay to detect responders to the PE_PGRS33 complete protein in LTBI and non-infected individuals.

Status of individuals	No. of individuals	% IFN-γ response to PE_PGRS33	
		Positive	Negative
LTBI	14	(4/14) 28.5	(10/14) 71.5
Non-infected	74	(16/74) 21.6	(58/74) 78.4

INF-γ response to PE_PGRS33 and its PE and PGRS domains in LTBI and non-infected individuals

From the 20 individuals (4 from LTBI and 16 from non-infected) that showed positive IFN-γ response to the PE_PGRS33 protein, only 7 were further tested for immune response to the PE and PGRS domains (**Table 1**). This group of participants that agreed to continue participating in the study included three LTBI and four non-infected individuals.

The secretion of IFN-γ in response to the PE_PGRS33 protein and its PE and PGRS domains was quantified in whole blood cells from the seven participants with positive IFN-γ response to PE_PGRS33. The results indicated a higher secretion of IFN-γ in response to the PE_PGRS33 protein in non-infected individuals than in LTBI individuals (**Figure 4**). A tendency for IFN-γ secretion to be higher in response to the PE domain than the PGRS domain was observed (**Figure 4**).

Antigen-specific humoral immune response in LTBI and non-infected humans

The serum from each of the seven individuals from LTBI and from non-infected individuals that produce IFN-γ in response to PE_PGRS33 was tested for antibody response using the ELISA assay. Human anti-IgG and IgG1 antibodies were used to detect specific antibodies against the PE_PGRS33 protein and its PE and PGRS domains. The IgG response against the PE domain was significantly lower compared to that from the PE_PGRS33 complete protein in the non-infected ($p < 0.05$) and in the LTBI individuals ($p < 0.05$) (**Figure 5A**). The IgG response against the PE was lower than that from the PGRS ($p < 0.05$) in non-infected individuals. On the other hand, the IgG response against the PE was as low as the response against the negative control (GST) in the LTBI individuals (**Figure 5A**). The IgG1 response against the PE domain was significantly lower than that against the PGRS domain and the PE_PGRS33 protein in non-infected and LTBI individuals (**Figure 5B**).

Furthermore, levels of IgG against PE_PGRS33 and their PGRS and PE domains were detected by ELISA in 63 sera from LTBI and non-infected individuals. Based in the above assay, only individuals with OD 600 nm over 0.3 were considered to have a significant amount of antibodies. It is worth of note, that 58.4% of non-infected individuals showed antibodies against the complete protein and a higher number of sera 66.6% recognized the PGRS domain. Results are shown in **Table 2**.

Together these results indicated that the humoral immune responses against PE_PGRS33 targets epitopes mainly located in the PGRS domain.

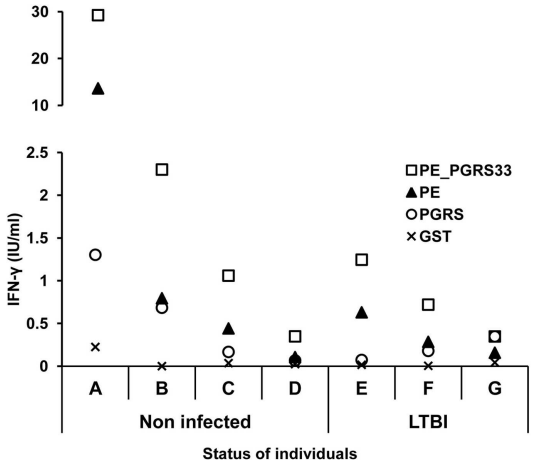


FIGURE 4 | INF-γ response to the PE_PGRS33 and its PE and PGRS domains in LTBI and non-infected individuals. Diluted whole blood cells were stimulated with 25 μg of PE_PGRS33, PE, PGRS, or GST protein plus 10 μg/ml of polymyxin B for 6 days. IFN-γ in the supernatants was quantified using the Human IFN-γ ELISA kit provided with the QuantiFERON®-TB Gold Kit. Letters represent each individual tested and symbols correspond to the amount of IFN-γ produced by each of them.

DISCUSSION

The PE_PGRS33 protein has been involved in the pathogenesis of *M. tuberculosis* (18) and it is known that the PE domain is required for the protein translocation through the mycobacterial cell wall and the induction of primary necrosis (21, 25). The PGRS domain interacts with the TLR2-inducing apoptosis, targets the mitochondria triggering necrosis, and is responsible of the immunomodulatory properties of the entire protein (14, 22, 25). Even though the mentioned characteristics of the PE and PGRS domains have been elucidated, their contribution to the immunogenicity of the complete PE_PGRS33 protein has not been described.

In this study, the immunization of mice with the PE_PGRS33 protein stimulated $CD4^+$ and $CD8^+$ T-cell proliferation as well as IFN-γ secretion. This indicated that PE_PGRS33 is highly immunogenic. These results agreed with previous reports describing the immunogenic properties of the PE_PGRS33 (12). The $CD4^+$ and $CD8^+$ T cells are crucial in the protective host response against *M. tuberculosis.* These T-cell subsets migrate to the site of infection to produce the cytokines involved in the control of the disease (26, 27). The proliferation of $CD8^+$ T cells caused by the immunization with the gene *rv1818c*, which codifies for PE_PGRS33 has been reported (12). The activation of $CD4^+$ T cells in response to PE_PGRS33 presented in this work contribute to the knowledge of T cells subpopulations involved in the immunological response against this protein. These findings support the potential use of PE_PGRS33 as a vaccine candidate for tuberculosis (12).

The absence of secretion of IFN-γ in response to the full-length PE_PGRS33 protein has been reported in C57Bl/6 mice (9). In contrast, high concentrations of IFN-γ secreted using BALB/c

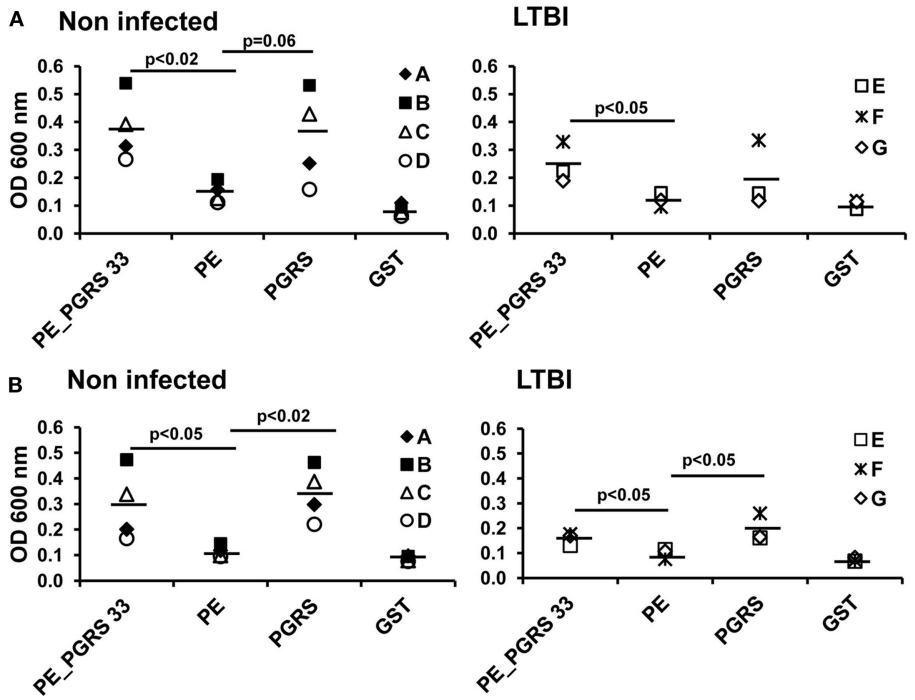


FIGURE 5 | Antigen-specific antibody response in LTBI and non-infected individuals. Sera from LTBI and non-infected vaccinated individuals were diluted 1:300 and incubated with PE_PGRS33, PE, PGRS, and the control protein GST. Antigen-specific IgG **(A)** and IgG1 **(B)** were evaluated by ELISA. Letters in legend represent each individual tested and symbols correspond to OD readings. Mean values are showed as horizontal bars.

Table 2 | IgG levels against PE_PGRS33 and their PGRS and PE domains detected by ELISA in LTBI and non-infected individuals.

Status of individuals	No. of individuals	% Individuals with IgG levels over 0.3 of OD 600 nm		
		PE_PGRS	PGRS	PE
LTBI	10	(2/10) 2	(4/10) 4	(0/10) 0
Non-infected	53	(31/53) 58.4	(35/53) 66.06	(0/53) 0

Sera from LTBI and non-infected vaccinated individuals were diluted 1:300.

mice were obtained. These results agreed with those published by Chaitra et al. (12) in same mice strain. The BALB/c mice were immunized with protein in the present study, while DNA and DNA prime-protein boosted were used in published works (12). Therefore, the immunization method does not explain the contrasting results. Such discrepancy is probably due to the dissimilar strains of mice used. This indicates that differences in the MHC might have an impact in the immune recognition of the PE_PGS33 protein.

The results of the cellular immune response in mice indicated that the PE and PGRS domains triggered the proliferation of $CD4^+$ and $CD8^+$ T cells. In agreement, Chaitra et al. (12) reported epitopes in the PE and PGRS domains presented to MHC-I and inducing effectors functions in $CD8^+$ T cells. A MHC-II-restricted epitope found in the PE_PGRS53 is capable of stimulating $CD4^+$ T-cell responses in human reactors to PPD (28). A comprehensive analysis of MHC-II epitopes has not been performed in PE_PGRS33. However, the activation of $CD4^+$ T cells observed in this work suggested that both domains carry peptides inducers of MHC-II-dependent responses. According to the results obtained, both domains stimulated comparable IFN-γ secretion levels while the PE domain was the main inducer of proliferation of $CD4^+$ and $CD8^+$ T cells. This suggested that the PE domain could be stimulating a higher proliferation rate in these subpopulations to perform other functions besides the production of IFN-γ, as described previously (27).

When the PE_PGRS33 was used as immunogen in mice, the induced IgG levels to the full-length protein and to its domains was very similar. A possible explanation to this observation could be that when the domains are separated, they lose their original conformation and expose cryptic epitopes that are recognized by the antibodies generated in the immunized animal. On the other hand, in the complete protein these antigenic determinants remain hidden. This demonstrated that both domains are as highly antigenic as the entire protein in mice. The levels of IgG subclasses were further analyzed to detect differences in response to the domains studied. The IgG2a antibodies were directed to the complete protein and the PGRS domain whereas the IgG1 targeted all three antigens. The titration of these IgG subclasses revealed higher IgG1 levels than IgG2a. This showed that the IgG2a response is masked by the high IgG1 titer, which is the mayor contributor to the total IgG. These results indicated that PE and PGRS are antigenic in BALB/c mice with differences in the recognition at IgG subclass levels.

The role of some PE_PGRS proteins in mycobacterial persistence has been described (13, 17, 18). The identification of antigens interacting with the immune system during the latent infection will be essential in the development of immunological markers for this particular condition. One of the hypotheses of the present study was that PE_PGRS33 could be an important antigen in *M. tuberculosis* latency in humans. For this reason, a cellular immune response to PE_PGRS was expected in LTBI individuals. The results obtained in humans indicated that the IFN-γ response to the PE_PGRS33 protein might not be associated with latent tuberculosis infection. The high number of LTBI individuals with negative response to the PE_PGRS33 might be explained by the possible infection with strains not expressing *rv1818c* gene. Another reason could be the infection by strains containing large variations in the gene sequence. This genetic variation would result in significant changes in the PE_PGRS33 leading to the non-recognition by the immune system. Both mechanisms have been described to be a source of polymorphism for PE_PGRS members in clinical isolates of *M. tuberculosis* (20, 29–32).

The IgG1 subclass has been reported to be the predominant isotype in tuberculosis infection (33). For this reason, the humoral immune response to the PE_PGRS33 protein and the PE and PGRS domains was evaluated in LTBI and non-infected individuals by determination of total IgG and IgG1. The results showed that the antibody response was directed against the PE_PGRS33 protein targeting specifically the PGRS domain. The PGRS domain of PE_PGRS33 is rich in Gly–Gly–Ala–Gly–Gly repeats. These sequences could be the target of the antibody response observed in this study because proteins with repetitive amino acid sequences have been identified as immunodominant in rabbits and humans (7). In agreement, the PE_PGRS62 protein induced a strong antibody response against the full-length protein and a weak response to its PE domain in LTBI and non-infected humans (8). This supports the pattern of antibody recognition observed in this study for PE_PGRS33. The antibody response to the PE_PGRS33 in non-infected individuals can be attributed to *M. bovis* BCG vaccination. This indicates that sera reactivity to this protein in healthy individuals is independent of the infection with *M. tuberculosis*. In the PE_PGRS33 responders who participated in the second stage of the study, the cellular immune recognition of PE_PGRS33 showed a tendency to be higher in non-infected individuals compared with LTBI individuals. For the protein domains a clear tendency was not observed. In studies involving large populations a more evident trend might be obtained.

In conclusion, it was demonstrated that the PE and the PGRS domains have a role in the cellular and humoral immune response stimulated by the PE_PGRS33 protein in BALB/c mice. The PE_PGRS33 also induced the activation of T-cell subpopulations involved in the control of tuberculosis and secretion of IFN-γ. This confirmed the potential use of the PE_PGRS33 protein as candidate vaccine for tuberculosis and further increased the understanding of the immunogenicity of this protein. The IFN-γ response in humans to PE_PGRS33 protein might not be associated with latent tuberculosis infection. In this context, the PE_PGRS33 will not be suitable as immunological biomarker for this condition. The IFN-γ response and the sera reactivity to the PE_PGRS33 protein in healthy individuals is independent of the infection with *M. tuberculosis*. These observations suggest that T and B cell responses to PE_PGRS33 could be induced by BCG vaccination and can be maintained for many years in non-infected individuals. Additionally, the humoral immune response against PE_PGRS33 in humans targets epitopes located in the PGRS domain. All the findings reported here contribute to the elucidation of the role of the PE_PGRS33 protein in the immune response against *M. tuberculosis*.

AUTHOR CONTRIBUTIONS

Ingrid Cohen carried out the experimental procedures unless otherwise stated, performed statistical analysis, participated in the experiment design, and wrote the manuscript. Cristina Parada carried out ELISA assays in human studies and helped in mice experiments. Enrique Acosta-Gío participated in the design and coordination of the human sampling. Clara Espitia conceived and coordinated the study, participated in the experiment design, and wrote part of the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

Authors acknowledge Carlos Castellanos for his help in the FACS data acquisition, Erika Segura for technical assistance, and Dr. Alejandro Negrete for critical editorial assistance. Ingrid Cohen was a Ph.D. student in the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México and recieved a scholarship from Universidad Nacional Autónoma de México. This work was supported by grants from Consejo Nacional de Ciencia y Tecnología (CONACyT) No. 81214 and Dirección General de Asuntos del Personal Académico (DGPA) IN221107.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00236/abstract

REFERENCES

1. Parrish NM, Dick JD, Bishiai WR. Mechanism of latency in *Mycobacterium tuberculosis*. *Trends Microbiol* (1998) **6**:107–12. doi:10.1016/S0966-842X(98)01216-5
2. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* (1998) **393**:537–44. doi:10.1038/31159
3. Brennan MJ, Delogu G, Chen Y, Bardarov S, Kriakov J, Alavi M, et al. Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. *Infect Immun* (2001) **69**:7326–33. doi:10.1128/IAI.69.12.7326-7333.2001
4. Delogu G, Pusceddu C, Bua A, Fadda G, Brennan MJ, Zanetti S. *Rv1818c*-encoded PE_PGRS protein of *Mycobacterium tuberculosis* is surface exposed and influences bacterial cell structure. *Mol Microbiol* (2004) **52**:725–33. doi:10.1111/j.1365-2958.2004.04007.x
5. Banu S, Honore N, Saint-Joanis B, Philpott D, Prevost MC, Cole ST. Are the PE-PGRS proteins of *Mycobacterium tuberculosis* variable surface antigens? *Mol Microbiol* (2002) **44**:9–19. doi:10.1046/j.1365-2958.2002.02813.x
6. Espitia C, Laclette JP, Mondragon-Palomino M, Amador A, Campuzano J, Martens A, et al. The PE-PGRS glycine-rich proteins of *Mycobacterium tuberculosis*: a new family of fibronectin-binding proteins? *Microbiology* (1999) **145**:3487–95.
7. Singh KK, Zhang X, Patibandla AS, Chien P Jr, Laal S. Antigens of *Mycobacterium tuberculosis* expressed during preclinical tuberculosis: serological immunodominance of proteins with repetitive amino acid sequences. *Infect Immun* (2001) **69**:4185–91. doi:10.1128/IAI.69.6.4185-4191.2001

8. Koh KW, Soh SE, Seah GT. Strong antibody responses to *Mycobacterium tuberculosis* PE-PGRS62 protein are associated with latent and active tuberculosis. *Infect Immun* (2009) **77**:3337–43. doi:10.1128/IAI.01175-08
9. Delogu G, Brennan MJ. Comparative immune response to PE and PE_ PGRS antigens of *Mycobacterium tuberculosis*. *Infect Immun* (2001) **69**:5606–11. doi:10.1128/IAI.69.9.5606-5611.2001
10. Narayana Y, Joshi B, Katoch VM, Mishra KC, Balaji KN. Differential B-cell responses are induced by *Mycobacterium tuberculosis* PE antigens Rv1169c, Rv0978c, and Rv1818c. *Clin Vaccine Immunol* (2007) **14**:1334–41. doi:10.1128/CVI.00181-07
11. Chaitra MG, Shaila R, Nayak R. Detection of Interferon gamma-secreting $CD8^+$ T lymphocytes in humans specific for three PE/PPE proteins of *Mycobacterium tuberculosis*. *Microbes Infect* (2008) **10**:858–67. doi:10.1016/j.micinf.2008.04.017
12. Chaitra MG, Shaila R, Nayak R. Evaluation of T-cell responses to peptides with MHC class I-binding motifs derived from PE_PGRS33 protein of *Mycobacterium tuberculosis*. *J Med Microbiol* (2007) **56**:466–74. doi:10.1099/jmm.0.46928-0
13. Ramakrishnan L, Federspiel NA, Falkow S. Granuloma-specific expression of *Mycobacterium* virulence proteins from the glycine-rich PE_PGRS family. *Science* (2000) **288**:1436–8. doi:10.1126/science.288.5470.1436
14. Basu S, Pathak SK, Banerjee A, Pathak S, Bhattacharyya A, Yang Z, et al. Execution of macrophage apoptosis by PE_PGRS33 of *Mycobacterium tuberculosis* is mediated by toll-like receptor 2-dependent release of tumor necrosis factor-alpha. *J Biol Chem* (2007) **282**:1039–50. doi:10.1074/jbc.M604379200
15. Flynn JL, Scanga CA, Tanaka KE, Chan J. Effects of aminoguanidine on latent murine tuberculosis. *J Immunol* (1998) **160**:1796–803.
16. Arriaga AK, Orozco EH, Aguilar LD, Rook GA, Hernandez-Pando R. Immunological and pathological comparative analysis between experimental latent tuberculous infection and progressive pulmonary tuberculosis. *Clin Exp Immunol* (2002) **128**:229–37. doi:10.1046/j.1365-2249.2002.01832.x
17. Campuzano J, Aguilar D, Arriaga K, Leon JC, Salas-Rangel LP, González-y-Merchand J, et al. The PGRS domain of *Mycobacterium tuberculosis* PE_PGRS Rv1759c antigen is an efficient subunit vaccine to prevent reactivation in a murine model of chronic tuberculosis. *Vaccine* (2007) **25**:3722–9. doi:10.1016/j.vaccine.2006.12.042
18. Talarico S, Cave MD, Foxman B, Marrs CF, Zhang L, Bates JH, et al. Association of *Mycobacterium tuberculosis* PE_PGRS33 polymorphism with clinical and epidemiological characteristics. *Tuberculosis* (2007) **87**:338–46. doi:10.1016/j.tube.2007.03.003
19. Brennan MJ, Espitia C, Gey van Pitius N. The PE and PPE multigene families of *Mycobacterium tuberculosis*. 2nd ed. In: Cole S, McMurray DN, Eisenach K, Gicquel B, Jacobs WR, editors. *Tuberculosis*. Washington, DC: American Society for Microbiology (2004). p. 513–25.
20. Talarico S, Cave MD, Marrs CF, Foxman B, Zhang L, Yang Z. Variation of the *Mycobacterium tuberculosis* PE_PGRS33 gene among clinical isolates. *J Clin Microbiol* (2005) **43**:4954–60. doi:10.1128/JCM.43.10.4954-4960.2005
21. Cascioferro A, Delogu G, Colone M, Sali M, Stringaro A, Arancia G, et al. PE is a functional domain responsible for protein translocation and localization on mycobacterial cell wall. *Mol Microbiol* (2007) **66**:1536–47. doi:10.1111/j.1365-2958.2007.06023.x
22. Zumbo A, Palucci I, Cascioferro A, Sali M, Ventura M, D'Alfonso P, et al. Functional dissection of protein domains involved in the immunomodulatory properties of PE_PGRS33 of *Mycobacterium tuberculosis*. *Pathog Dis* (2013) **69**:232–9. doi:10.1111/2049-632X.12096
23. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (1970) **227**:680–5. doi:10.1038/227680a0
24. Filley E, Abou-Zeid C, Waters M, Rook G. The use of antigen-bearing nitrocellulose particles derived from Western blots to study proliferative responses to 27 antigenic fractions from *Mycobacterium leprae* in patients and controls. *Immunology* (1989) **67**:75–80.
25. Cadieux N, Parra M, Cohen H, Maric D, Morris SL, Brennan MJ. Induction of cell death after localization to the host cell mitochondria by the *Mycobacterium tuberculosis* PE_PGRS33 protein. *Microbiology* (2011) **157**:793–804. doi:10.1099/mic.0.041996-0
26. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol* (2001) **19**:93–119. doi:10.1146/annurev.immunol.19.1.93
27. Zuñiga J, Torres-García D, Santos-Mendoza T, Rodrígez-Reyna TS, Granados J, Yunis EJ. Cellular and humoral mechanisms involved in the control of tuberculosis. *Clin Dev Immunol* (2012) **2012**:193923. doi:10.1155/2012/193923
28. Wang M, Tang ST, Stryhn A, Justesen S, Larsen MV, Dziegiel MH, et al. Identification of MHC class II restricted T-cell-mediated reactivity against MHC class I binding *Mycobacterium tuberculosis* peptides. *Immunology* (2011) **132**:482–91. doi:10.1111/j.1365-2567.2010.03383.x
29. Gao Q, Kripke KE, Saldanha AJ, Yan W, Holmes S, Small PM. Gene expression diversity among *Mycobacterium tuberculosis* isolates. *Microbiology* (2005) **151**:5–14. doi:10.1099/mic.0.27539-0
30. Flores J, Espitia C. Differential expression of PE and PE_PGRS genes in *Mycobacterium tuberculosis* strains. *Gene* (2003) **318**:75–81. doi:10.1016/S0378-1119(03)00751-0
31. McEvoy CR, Cloete R, Müller B, Schürch AC, van Helden PD, Cagneux S, et al. Comparative analysis of *Mycobacterium tuberculosis pe* and *ppe* genes reveals high sequence variation and an apparent absence of selective constraints. *PLoS One* (2012) **7**:e30593. doi:10.1371/journal.pone.0030593
32. Talarico S, Zhang L, Marrs CF, Foxman B, Cave MD, Brennan MJ, et al. *Mycobacterium tuberculosis* PE_PGRS16 and PE_PGRS26 genetic polymorphism among clinical isolates. *Tuberculosis* (2008) **88**:283–94. doi:10.1016/j.tube.2008.01.001
33. Sousa AO, Henry S, Marója FM, Lee FK, Brum L, Singh M, et al. IgG subclass distribution of antibody responses in leprosy and tuberculosis patients. *Clin Exp Immunol* (1998) **111**:48–55. doi:10.1046/j.1365-2249.1998.00452.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 February 2014; accepted: 06 May 2014; published online: 27 May 2014.
*Citation: Cohen I, Parada C, Acosta-Gío E and Espitia C (2014) The PGRS domain from PE_PGRS33 of Mycobacterium tuberculosis is target of humoral immune response in mice and humans. Front. Immunol. **5**:236. doi: 10.3389/fimmu.2014.00236*
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology.

frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 08 October 2014
doi: 10.3389/fmicb.2014.00517

Immunogenicity of 60 novel latency-related antigens of *Mycobacterium tuberculosis*

Mª del Mar Serra-Vidal[1,2,3], Irene Latorre[1,2,3], Kees L. C. M. Franken[4], Jéssica Díaz[1,2,3], Maria Luiza de Souza-Galvão[2,5], Irma Casas[2,6], José Maldonado[7], Cèlia Milà[5], Jordi Solsona[5], M. Ángeles Jimenez-Fuentes[5], Neus Altet[5], Alícia Lacoma[1,2,3], Juan Ruiz-Manzano[2,3,8], Vicente Ausina[1,2,3], Cristina Prat[1,2,3], Tom H. M. Ottenhoff[4] and José Domínguez[1,2,3]*

[1] Department of Microbiology, Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Hospital Universitari Germans Trias i Pujol, Badalona, Spain
[2] Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, Bellaterra, Spain
[3] CIBER Enfermedades Respiratorias, Instituto de Salud Carlos III, Badalona, Spain
[4] Department of Immunohematology and Blood Transfusion/Department of Infectious Diseases, Leiden University Medical Center, Leiden, Netherlands
[5] Unitat de Tuberculosi de Drassanes, Hospital Universitari Vall d'Hebron, Barcelona, Spain
[6] Department of Preventive Medicine, Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Hospital Universitari Germans Trias i Pujol, Badalona, Spain
[7] Serveis Clínics de Barcelona, Barcelona, Spain
[8] Department of Pneumology, Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Hospital Universitari Germans Trias i Pujol, Badalona, Spain

Edited by:
Juraj Ivanyi, Kings College London, UK

Reviewed by:
Jieliang Li, Temple University, USA
Kris Huygen, Scientific Institute of Public Health, Belgium

****Correspondence:***
José Domínguez, Servei de Microbiologia, Hospital Universitari Germans Trias i Pujol, Carretera del Canyet s/n, Badalona 08916, Barcelona, Spain
e-mail: jadomb@gmail.com

The aim of our work here was to evaluate the immunogenicity of 60 mycobacterial antigens, some of which have not been previously assessed, notably a novel series of *in vivo*-expressed *Mycobacterium tuberculosis* (IVE-TB) antigens. We enrolled 505 subjects and separated them in individuals with and without latent tuberculosis infection (LTBI) vs. patients with active tuberculosis (TB). Following an overnight and 7 days stimulation of whole blood with purified recombinant *M. tuberculosis* antigens, interferon-γ (IFN-γ) levels were determined by ELISA. Several antigens could statistically significantly differentiate the groups of individuals. We obtained promising antigens from all studied antigen groups [dormancy survival regulon (DosR regulon) encoded antigens; resuscitation-promoting factors (Rpf) antigens; IVE-TB antigens; reactivation associated antigens]. Rv1733, which is a probable conserved transmembrane protein encoded in DosR regulon, turned out to be very immunogenic and able to discriminate between the three defined TB status, thus considered a candidate biomarker. Rv2389 and Rv2435n, belonging to Rpf family and IVE-TB group of antigens, respectively, also stood out as LTBI biomarkers. Although more studies are needed to support our findings, the combined use of these antigens would be an interesting approach to TB immunodiagnosis candidates.

Keywords: tuberculosis, latent tuberculosis infection, immune response, antigenic stimulation, interferon-γ

INTRODUCTION

Tuberculosis (TB) remains one of the most death-causing microorganism worldwide (World Health Organization, 2013). The increasing numbers of drug-resistant TB cases evidence that there is an urgent need for effective diagnosis, drugs and vaccines (Mwaba et al., 2011; Abubakar et al., 2013). The control of latent TB, a stage in which a person is infected with *Mycobacterium tuberculosis* (*Mtb*) but does not currently have active disease, plays an important role for disease control, since dormant bacilli are an enormous reservoir of potential TB cases (Rustad et al., 2009).

M.tb can live in a latent stage without causing any clinical symptom and has a potential of reactivation during all the infected individual lifetime. In fact, about one third of the world population is considered to be latently infected (Corbett et al., 2003). The diagnosis of latent tuberculosis infection (LTBI) through the classic tuberculin skin test (TST) has a lack of specificity, and its sensitivity is low in high-risk groups of progression to active TB. The new interferon (IFN)-γ release assays (IGRAs) are immunodiagnostic methods based on the *in vitro* quantification of the cellular immune response. The detection of IFN-γ released by sensitized T cells stimulated with specific *M.tb* antigens enables the identification of infected individuals. The main antigens used in IGRAs, the 6-kDa *M.tb* early-secreted antigenic target (ESAT)-6 protein, 10-kDa culture filtrate protein (CFP-10), coded in the region of difference (RD) 1, and TB7.7, coded in RD11, are present in *M.tb* but not in any *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine strain nor in the majority of non-tuberculous mycobacteria (Andersen et al., 2000). Although their specificity is better than in TST, IGRAs do not discriminate between active disease and LTBI (Latorre et al., 2009) and do not clearly distinguish between a recently acquired infection and remote LTBI (Esmail et al., 2012; Pollock et al., 2013). Moreover, their sensitivity barely exceed 80%, and the response level against the antigens used does not seem to indicate high risk of progression to active TB.

There is a need of new TB antigens as biomarkers for LTBI immunodiagnosis.

During LTBI, *M.tb* is contained within granulomas, which are formed by activated macrophages and other host components that isolate the infected cells in an organized structure and create an environment that suppresses *M.tb* replication (Esmail et al., 2012). Bacilli must adapt to a variety of environment stresses including reduced oxygen tension, iron limitation, nutrient deprivation, low pH and production of host factors such as nitric oxide and carbon monoxide. Some *in vitro* models demonstrated that *M.tb* is capable of an extensive repertoire of metabolic realignments to enter a defined non-replicating state. The initial response of *M.tb* is encoded by the dormancy survival regulon (DosR, also called DevR, Rv3133c), which leads to induction of a set of ∼50 genes, many of unknown function. DosR controls the expression of genes that allow the bacteria to use alternative energy sources, especially lipids, and genes encoding factors that are selectively recognized by T cells from humans with LTBI (Ernst, 2012). This initial response is followed by a more extensible and more stable response called Enduring Hypoxic Response (EHR), which is comprised of 230 genes involved in the control of the regulatory factors and enzymatic machines of the long-term bacteriostasis program of non-replicating *M.tb* (Rustad et al., 2008). The antigens expressed by *M.tb* vary during the continued pressure mounted by host immune response in the course of the infection (Honer zu Bentrup and Russell, 2001; Demissie et al., 2006). Using *in vitro* models which mimic the conditions that the tubercle bacillus encounter within the host as infection progresses from latency to active disease, some infection phase-dependent genes have been identified and believed to be candidates for immunodiagnostics or for future vaccines (Mukamolova et al., 1998; Zvi et al., 2008; Ottenhoff and Kaufmann, 2012).

DosR regulon is crucial for rapid resumption of growth by involving resuscitation-promoting factors (Rpf) once *M.tb* exits the hypoxic non-respiring state. *M.tb* contains five Rpf -like proteins that are implicated in resuscitation of this microorganism from dormancy to reactivation via a mechanism involving hydrolysis of the peptidoglycan by Rpfs and partnering proteins (Ernst, 2012).

The *in vitro* models mentioned above supposed to recapitulate relevant environmental stress conditions that *M.tb* encounters upon host infection, which allow to identify differentially regulated *M.tb* genes. However, they present some limitations: many of these environmental stress factors may not be well known yet; there may be additive or synergistic effects between multiple stress factors *in vivo* that may easily be missed when studied in isolation *in vitro*; and certain key features of host response-induced stress cannot readily be recapitulated *in vitro*, including granuloma formation and TB necrosis (Commandeur et al., 2013).

For these reasons, different approaches have been developed to analyze the gene expression profiles of intracellular *M.tb* using infected human or murine macrophages, infected murine tissue or artificial granuloma mouse models (Schnappinger et al., 2003; Karakousis et al., 2004; Talaat et al., 2004; Cappelli et al., 2006). Specifically, several *M.tb* genes have been found to be differentially expressed in the lungs of mice strains with high susceptibility to TB during *in vivo* infection, the so-called *in vivo*-expressed *M.tb* (IVE-TB) genes. Interestingly, some of these IVE-TB genes had been also described as induced for nutrient deprivation in *in vitro* models (Commandeur et al., 2013).

Some *M.tb* infection phase-dependent antigens have already been tested in whole-blood assays or in peripheral blood mononuclear cells and may be differentially recognized in individuals with different TB status, that is, subjects with no risk of *M.tb* infection, LTBI individuals and active TB patients (Leyten et al., 2006; Lin et al., 2007, 2009; Black et al., 2009; Schuck et al., 2009; Goletti et al., 2010; Commandeur et al., 2013). The response is commonly measured through the release of IFN-γ. Additionally, IFN-γ plays a central role in the protection against *M.tb* (Cooper et al., 1993; Flynn et al., 1993; Kaufmann, 2001).

However the studies conducted *in vivo*, mice models of latency, seem to merely recapitulate primary disease and are closer to human HIV-TB co-infection. The infectious forms of TB arise after an adequate immune response, which itself may contribute to tissue destruction and cavitation. Hence, there has been a move away from considering IFN-γ as protective, except in the first encounter with the tubercle bacillus, with a renewed emphasis on polyfunctional T cells to contain TB infection (Ernst, 2012; Kaufmann, 2012). However, the ease of measuring IFN-γ gives it applicability to diagnostic tests.

The study of the immune response to the potential immunogenic *M.tb* antigens described above will enlarge our knowledge and will get us closer to the validation of a diagnostic LTBI candidate antigen. We hypothetized that these antigens expressed in latency conditions and involved in reactivation of the dormant bacterial will mainly induce IFN-γ response in LTBI infected patients, and not in no LTBI individuals.

This prospective study aims to evaluate the whole blood IFN-γ response to 60 *M.tb* recombinant antigens, the immunogenicity of some of them has not yet been assessed: 6 DosR regulon-encoded antigens, 12 TB reactivation-associated antigens, 1 Rpf antigen, 1 starvation antigen, 6 other stress response-associated TB antigens and 34 IVE-TB antigens (2 of those were EHR and 3 were EHR/starvation), in order to identify potential candidates for new LTBI diagnostic methods. We enrolled subjects with LTBI, active TB patients and controls not *M.tb* infected.

MATERIALS AND METHODS

STUDY POPULATION

We prospectively recruited 578 patients from contact-tracing studies, LTBI screening (such as immigrants from endemic areas or health-care workers) and active TB patients between October 2010 and May 2013. A detailed questionnaire from each subject was collected, including age, birth country, previous TST, BCG vaccination status, history of prior active TB, chest radiography, and other medical conditions (**Table 1**). A total of 8 mL of whole blood was collected in a heparinized tube from each participant. The study obtained approval of the Hospital Universitari Germans Trias i Pujol Ethics Committees (Ref. 10/00214-28/03/2010). They supervised that all the experiments were performed according to the regulatory standards. All the study participants gave written informed consent before entering the study.

Table 1 | Demographic characteristics and clinical details for individuals in this study.

	No TB infected individuals ($n = 97$)	LTBI infected ($n = 306$)	Active TB patients ($n = 102$)
GENDER			
Male	61 (62.9)	131 (42.8)	73 (71.6)
Female	36 (37.1)	175 (57.2)	29 (28.4)
ORIGIN			
Spain	31 (32)	171 (55.9)	43 (42.2)
Africa	2 (2.1)	15 (4.9)	15 (14.7)
America	16 (16.5)	40 (13.1)	13 (12.7)
South-East Asia	15 (15.5)	16 (5.2)	5 (4.9)
Europe	2 (2.1)	11 (3.6)	0 (0)
Eastern Mediterranean	21 (21.6)	41 (13.4)	23 (22.5)
Western pacific	10 (10.3)	12 (3.9)	3 (2.9)
BCG VACCINATION			
Yes	71 (73.2)	138 (45.1)	36 (35.3)
No	25 (25.8)	167 (54.6)	57 (55.9)
Unknown	1 (1.0)	1 (0.3)	9 (8.8)
QFN RESULT			
Positive	0 (0)	182 (59.5)	21 (20.6)
Negative	97 (100)	123 (40.2)	5 (4.9)
Indeterminate	0 (0)	0 (0)	0 (0)
Not done	0 (0)	1 (0.3)	76 (74.5)
PREVIOUS TB			
Yes	0 (0)	1 (0.3)	11 (10.8)
No	96 (99)	238 (77.8)	91 (89.2)
Unknown	1 (1.0)	67 (21.9)	0 (0)
IMMUNOSUPPRESSION			
No	94 (96.9)	300 (98)	94 (92.2)
HIV+	1 (1.0)	2 (0.7)	2 (2.0)
Other	2 (2.1)	4 (1.3)	6 (5.9)
TB CLINICAL FORM			
Pulmonar	0 (0)	0 (0)	84 (82.4)
Ganglionar	0 (0)	0 (0)	7 (6.9)
Pleural	0 (0)	0 (0)	1 (1.1)
Pulmonar and ganglionar	0 (0)	0 (0)	2 (2.0)
Pulmonar and pleural	0 (0)	0 (0)	2 (2.0)
Disseminated	0 (0)	0 (0)	5 (4.9)
Erythema nodosum	0 (0)	0 (0)	1 (1.0)

Number of individuals and percentage (%) are indicated.

Participants were classified, following Spanish Society of Respiratory Pathology (SEPAR) guidelines (Ruiz-Manzano et al., 2008) and also Centers for Disease Control and Prevention (CDC) recommendations (Centers for Disease Control and Prevention, 2000), in four groups depending on the TB status, as is described in detail below. The method used for diagnosing LTBI was TST (PPD RT23, Statens Serum Institute, Copenhagen, Denmark) and QuantiFERON-TB Gold *In Tube* (QFN; QIAGEN, Düsseldorf, Germany).

The following individuals were included as no LTBI: (a) individuals from LTBI screening studies, who tested QFN negative and TST under 10 or 15 mm (depending on the absence or presence of BCG vaccination, respectively); and (b) individuals who reported contact with a TB patient and with negative QFN, whose TST was under 5 mm. All patients included were HIV negative.

As LTBI were included: (a) individuals who reported a contact with a TB patient or from LTBI screening studies, who tested QFN positive; (b) individuals who reported an intense contact with a TB patient, with negative QFN and TST higher than 5 mm (if the subject is BCG vaccinated, the index case has to be smear-positive; if it is smear-negative, TST has to be higher than 15 mm); (c) individuals from LTBI screening studies, who tested QFN negative and whose TST converted (by definition, from under 10 mm to above 10 mm with a change of 6 mm); and (d) individuals from LTBI screening studies, who tested QFN negative and TST positive (higher than 10 mm in non BCG-vaccinated and recent immigrants; and higher than 15 mm in BCG-vaccinated).

Individuals with pulmonary or extrapulmonary active TB, clinically, radiologically and/or microbiologically diagnosed (World Health Organization, 2013) were included.

MYCOBACTERIUM TUBERCULOSIS ANTIGENS

A total of 60 *M.tb* recombinant latency-related antigens were evaluated (**Table 2**): 6 DosR regulon-encoded antigens, 12 TB reactivation-associated antigens, 1 Rpf antigen, 1 starvation antigen, 6 other stress response-associated TB antigens and 34 IVE-TB antigens (two of them were EHR and three were EHR/starvation). They were previously produced at the Department of Infectious Diseases, Leiden University Medical Center following the methododology previously described (Franken et al., 2000). Briefly, antigens were selected from RNA microarray studies after inducing hypoxic conditions in a *M.tb* liquid culture. The selected genes were cloned in *Escherchia coli* and antigens were overexpressed and purified by immobilized metal chelate affinity chromatography. Some antigens were prepared as two or three recombinant protein fragments owing to their large sizes (C, middle [M], and N termini). For IVE-TB genes, mice were infected with *M.tb* and RNA was isolated from mouse lung tissue (Commandeur et al., 2013). After a RT-PCR, highly or differentially expressed genes were selected and cloned by Gateway technology (Invitrogen, Carlsbad, US) in *E. coli* and antigens were obtained as explained before.

Apart from those *M.tb* recombinant antigens, we used 4 control antigens for which immunogenicity and specificity to *M.tb* is well defined: the fusion protein ESAT-6 [Rv3875]/CFP-10[Rv3874], Ag85A[Rv3804c], TB10.4[Rv0288], and PPD (PPD RT 23, Serum Institute, Copenhagen, Denmark).

Antigens were reconstituted in sterile phosphate buffered saline, to a concentration of 50 μg/mL and stored at −20°C. The 60 latency-related antigens were randomly grouped into 10 batches of 6, and the individuals tested randomly selected. Thus, the whole blood from each patient was stimulated with six antigens, and the four control antigens as well.

WHOLE BLOOD ASSAY

400 μL of whole blood were transferred to a 48 well culture plate (Nunc, St. Louis, US) and control antigens were added at a final concentration of 10 μg/mL except for PPD, that was

Table 2 | Description of the 4 control and 60 *M.tb* recombinant antigens tested, included DosR regulon-encoded ($n = 6$), TB reactivation-associated ($n = 12$), Rpf ($n = 1$), starvation ($n = 1$), other stress response-associated ($n = 6$), and IVE-TB antigens ($n = 34$) (Function information source: http://www.ncbi.nlm.nih.gov/nuccore).

Antigen name	Function
CONTROL ANTIGENS ($n = 4$)	
PPD	Purified protein derivative
Rv0288 (TB10.4)	Low molecular weight protein antigen belongs to the ESAT-6 (esx) family
Rv3875/3874 (ESAT-6/CFP-10)	6-kDa early secretory antigenic target/10 kDa culture filtrate (fusion protein)
Rv3804c (Ag85A)	Secreted antigen. Fibronectin binding protein acyltransferase activity
***M.tb* RECOMBINANT ANTIGENS ($n = 60$)**	
DosR	
Rv0570c	Probable ribonucleoside-diphosphate reductase C-ter (aa 333-692)
Rv0570n	Probable ribonucleoside-diphosphate reductase N-ter (aa 1-354)
Rv1733	Probable conserved transmembrane protein
Rv2626	Conserved hypothetical protein
Rv2627	Conserved hypothetical protein
Rv2628	Hypothetical protein
Reactivation	
Rv0140	Conserved hypothetical protein
Rv0251	Possible heat shock protein
Rv0384	Heat shock protein F84.1
Rv0753	Methylmalmonate semialdehyde dehydrogenase
Rv1471	Thioredoxin reductase
Rv1874	Hypothetical protein
Rv1875	Conserved hypothetical protein
*Rv2465**	Phosphopentose isomerase
Rv2466	Conserved hypothetical protein
Rv2662	Hypothetical protein
Rv3223	ECF subfamily sigma subuint
Rv3862	Possible transcriptional regulatory protein WHIB6
Rpf	
Rv2389	Possible resuscitation promoting factor D
Starvation	
Rv2660	Hypothetical protein
Other *M.tb* stress induced	
Rv0244	Probable Acyl-coA dehydrogenase
Rv0767	Conserved hypothetical protein
Rv1909	Ferric uptake regulation protein
Rv2745	Possible transcriptional regulatory protein
Rv2913	Possible D-amino acid amonohydrolase
Rv3406	Probable dioxygenase
IVE-TB	
Rv0847	Probable LpqS, lipoprotein
Rv0967	Copper-sensitive operon repressor
Rv0990	Hypothetical protein
Rv0991	Conserved serine rich protein
Rv1170	N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy-alpha-D-glucopyranoside deacetylase MshB
Rv1284≠*	Conserved hypothetical protein
Rv1363	Possible membrane protein
Rv1403	Putative methyltransferase
Rv1806	PE family protein PE20
Rv1955	Possible toxine HigB
Rv1956≠*	Possible antitoxin HigA
Rv1957	Hypothetical protein
Rv2034≠*	ArsR repressor protein
Rv2035	Conserved hypothetical protein
Rv2225	3-methyl-2-oxobutanoate hydroxymethyltransferase (panB)
*Rv2324**	Probable transcriptional regulatory protein (probably AsnC-family)
Rv2380c	Peptide synthetase mbtE C-ter (aa 1120-1682)
Rv2380M	Peptide synthetase mbtE middle part (aa 560-1140)
Rv2380N	Peptide synthetase mbtE N-ter (aa 1-580)
Rv2435c	Probable cyclase (adenylate or guanylate cyclase) C-ter (aa 340-730)
Rv2435n	Probable cyclase (adenylate or guanylate cyclase) N-ter (aa 1-360)
Rv2558	Conserved protein
Rv2642	Possible transcriptional regulatory protein
Rv2643	Probable arsenic-transport integral membrane protein ArsC
Rv2658	Possible prophage protein
Rv2737c	Recombination protein recombinase A (recA) C-ter (aa 400-790)
Rv2737n	Recombination protein recombinase A (recA) N-ter (aa 1-420)
Rv2838	Probable ribosome-binding factor A (P15B protein)
Rv2982	Probable glycerol-3-phosphate dehydrogenase (gpdA2)
Rv3353	Conserved hypothetical protein
Rv3420	Ribosomal-protein-alanine acetyltransferase rimI
*Rv3515**	Fatty-acid-CoA synthase
Rv3536	Probable hidratase
Rv717	30S ribosomal protein S14 RpsN1

M.tb recombinant antigens with Rv designation in bold induced a relevant IFN-γ response in this study.

**also EHR.*

≠also starvation

added at 1 μg/mL. *M.tb* recombinant antigens were tested at a final concentration of 10 μg/mL. A negative (RPMI medium; PAA, Pasching, Austria) and a positive control of immunity (phytohemagglutinn, Invitrogen, Carlsbad, US) were included.

This procedure was performed in two different plates: one plate was incubated in a 5% CO_2 incubator a 37°C overnight (18 h, short-term stimulation) and the other for 7 days (long-term stimulation). In the long-term incubation plate, blood was previously diluted 1:5 with RPMI 1640 medium supplemented with L-glutamine, penicillin and streptomycin (Weir et al., 2003). After incubation time, supernatants were then collected and stored at −80°C until tested.

DETERMINATION OF IFN- γ BY ELISA

The measurement of the amount of IFN-γ released following the antigenic stimulation was evaluated by the commercial ELISA included in the QFN kit and data are presented as pg/mL after subtraction of the negative control. We considered a valid result when the value of the negative control was under 50 pg/ml. The cut-off value for high level of IFN-γ response was arbitrarily set at 20 pg/ml, taking as reference the QFN cut-off.

STATISTICAL ANALYSIS

The production level of IFN-γ was compared between the groups included in the study. Median and range of the cytokine production was calculated and Mann Withney test was used for pair-wise comparisons and Kruskall Wallis test was used for multiple comparisons. A *P*-value <0.05 was considered significant. Data were analyzed using SPSS statistical software (IBM SPSS Statistics 20; IBM Corporation, NY, USA). Graphical representation is based on GraphPad Prism version 4 (GraphPad Software, Inc., San Diego, CA).

RESULTS

From the 578 participants, 60 were not tested for the determination of IFN-γ because of insufficient samples, eight subjects did not fulfill the inclusion criteria and five subjects were excluded from the study because the amount of IFN-γ in the negative control was too high. In the patients PHA induced high responses, which ratifies the validity of our methodology.

IMMUNOGENICITY OF CONTROL TB ANTIGENS

We included four control antigens in our study. Significant differences in the IFN-γ responses elicited by all of them could be observed between the three study groups after short-term and after long-term stimulation (**Tables 3**, **4**). The antigens that elicited a higher response were PPD and the fusion protein ESAT-6/CFP-10, followed by TB10.4 and Ag85A.

IMMUNOGENICITY OF DosR REGULON-ENCODED ANTIGENS

We evaluated six different DosR regulon-encoded antigens (**Figure 1**). Two of them were the C-ter and N-ter domain of a latency antigen (**Table 2**). There were three antigens which presented a differentiated response depending on the TB status group: Rv1733, Rv2627, and Rv0570c (**Tables 3**, **4**). While Rv1733 discriminated between groups when the stimulation was either short-term or long-term, the discrimination of Rv2627 only was significant after short-term stimulation, and Rv0570c was only after long-term stimulation (although with a low IFN-γ response). The best discriminatory response was elicited when whole blood was stimulated overnight with Rv1733 ($p = 0.001$), where the infected individuals response was clearly much higher

Table 3 | Median levels of IFN-γ (pg/ml), minimum and maximum values (in brackets) elicited in no TB infected individuals, subjects with LTBI and active TB patients by the antigens, when tested after short-term stimulation.

Antigen	No TB infection		TB infection		Active TB		*p*-value
	n	Median	*n*	Median	*n*	Median	
CONTROL							
PHA	95	907.0 (26.3, 5396.5)	294	807.5 (19.5, 12132.0)	98	139.3 (0.0, 2943.5)	0.000
PPD	90	25.5 (0.0, 502.5)	275	57.3 (0.0, 1299.0)	95	19.5 (0.0, 1198.0)	0.000
TB10.4	94	3.5 (0.0, 125.5)	288	9.3 (0.0, 581.5)	91	3.0 (0.0, 355.7)	0.003
ESAT6/CFP10	94	6.5 (0.0, 591.0)	280	20.3 (0.0, 2758.5)	91	5.5 (0.0, 752.0)	0.005
Ag85A	88	0.0 (0.0, 69.5)	262	1.0 (0.0, 145.0)	78	0.5 (0.0, 311.0)	0.011
DosR							
Rv2627	16	0.8 (0.0, 5.0)	54	1.0 (0.0, 34.5)	6	0.0 (0.0. 0.5)	0.044
Rv1733	4	32.8 (12.0, 66.5)	20	69.8 (0.5, 733.0)	9	3.0 (0.0, 32.5)	0.001
REACTIVATION							
Rv1471	4	3.8 (0.0, 9.0)	19	1.5 (0.0, 19.0)	8	0.0 (0.0, 0.5)	0.010
Rv1874	4	2.3 (0.0, 3.0)	19	1.0 (0.0, 9.5)	7	0.0 (0.0, 0.5)	0.009
Rv3862	7	2.0 (0.0, 14.0)	21	4.5 (0.0, 32.5)	9	0.0 (3.0, 4.0)	0.019
IVE-TB							
Rv0967	8	1.8 (0.0, 13.0)	19	3.0 (0.0, 253.0)	10	0.0 (0.0, 1.5)	0.017
Rv1806	7	5.0 (0.0, 35.5)	15	7.5 (0.0, 56.5)	7	1.0 (0.0, 3.5)	0.023
Rv1957	4	2.8 (0.0, 5.5)	19	0.5 (0.0, 18.5)	8	0.0 (0.0, 0.5)	0.020

N indicates the number of subjects in each group. Data were analyzed by Kruskal Wallis test for the comparison of no TB infection, TB infection, and active TB groups of patients. Only significant differences (p < 0.05) were included.

Table 4 | Median levels of IFN-γ (pg/ml), minimum and maximum values (in brackets) elicited in no TB infected individuals, subjects with LTBI and active TB patients by the antigens, when tested after long-term stimulation.

Antigen	No TB infection		TB Infection		Active TB		*p*-value
	n	Median	*n*	Median	*n*	Median	
CONTROL							
PHA	94	1136.0 (189.8, 6443.0)	293	1013.9 (296.5, 12135.5)	102	854.3 (0.0, 4026.3)	0.000
PPD	89	13.5 (0.0, 925.5)	275	59.5 (0.0, 2246.0)	99	60.0 (0.0, 1681.2)	0.000
TB10.4	94	1.6 (0.0, 115.0)	288	3.2 (0.0, 1154.0)	100	1.5 (0.0, 675.0)	0.004
ESAT6/CFP10	93	86.0 (0.0, 2818.5)	280	163.5 (0.0, 6440.0)	100	29.7 (0.0, 3966.0)	0.000
DosR							
Rv0570c	8	0.0 (0.0, 4.5)	19	0.0 (0.0, 5.0)	5	1.5 (0.5, 5.5)	0.012
Rv1733	4	345.3 (178.5, 2766.5)	20	356.5 (3.5, 2150.0)	10	7.3 (1.0, 394.5)	0.010
REACTIVATION							
Rv1471	3	2.0 (1.5, 38.5)	15	1.0 (0.0, 25.5)	8	10 (0.0, 1.5)	0.028
Rpf, OTHER STRESS-INDUCED							
Rv0244	8	17.8 (5.0, 50.0)	24	7.3 (0.0, 63.5)	8	0.3 (0.0, 4.5)	0.005
Rv2389	7	148.5 (12.0, 257.0)	16	135.8 (1.5, 610.5)	5	22.5 (5.0, 59.5)	0.046
IVE-TB							
Rv0847	7	6.5 (2.5, 182.5)	21	3.5 (0.0, 298.5)	9	1.0 (0.0, 111.0)	0.011
Rv2558	4	1.0 (0.0, 2.5)	17	0.0 (0.0, 2.0)	9	0.5 (0.0, 6.5)	0.038
Rv2642	3	23.5 (16.5, 315.0)	15	33.5 (1.5, 191.0)	8	1.0 (0.0, 3.5)	0.002

N indicates the number of subjects in each group. Data were analyzed by Kruskal Wallis test for the comparison of no TB infection, TB infection, and active TB groups of patients. Only significant differences ($p < 0.05$) were included.

than in TB patients and higher than the response produced by non-infected individuals. When incubated for 7 days, Rv1733 also induced a high amount of IFN-γ released in infected individuals, being the response much higher than in TB patients. Rv1733 turned out to be a strong immunoresponse inducer, a promising LTBI biomarker and a promising antigen in discriminating between LTBI individuals, active TB patients and non-infected subjects. The Rv1733, considering the 20 pg/ml as a cut-off, accurately predicted 85% (17/20) of LTBI patients in short-term stimulation; and 95% (19/20) in long-term stimulation.

IMMUNOGENICITY OF TB REACTIVATION-ASSOCIATED ANTIGENS

A total of 12 TB reactivation-associated antigens were evaluated in the study (**Figure 2**). In general, recognition of these antigens was poor in subjects with LTBI and TB patients. However, there were several antigens which showed different IFN-γ production depending on the group of individuals (**Tables 3**, **4**). While Rv1471, Rv1874, Rv1875, Rv2662, and Rv3862 induced differentiate response in infected individuals in short-term stimulation; Rv1471, Rv2622, and Rv3862 induced differentiate and high response in infected individuals in long-term stimulation.

IMMUNOGENICITY OF Rpf, STARVATION AND OTHER STRESS RESPONSE-ASSOCIATED ANTIGENS

We tested 1 Rpf, 1 starvation, and 6 other stress response-associated antigens (**Figure 3**). The response to some of those antigens did present statistical differences when compared among groups (**Tables 3**, **4**). Rv2389, and Rv0244 in short and long-term estimulation, and Rv1909 in long-term stimulation induce high response in infected individuals. However, in some of them relevant production of IFN-γ after the stimulation in non-infected individuals was observed. Rv2389 accurately predicted 81% (13/16) of LTBI patients in long-term stimulation.

IMMUNOGENICITY OF IVE-TB ANTIGENS

We evaluated the immunogenicity of 34 IVE-TB antigens (**Figure 4**). The LTBI individuals were the group that most recognized IVE-TB antigens, although the response level was not very high (**Tables 3**, **4**). In addition to the antigens that induce a significant IFN-γ response (Rv0967, Rv1806, and Rv1957 after short-term stimulation; and Rv0847, Rv2558, and Rv2642 after long-term stimulation), several antigens obtained IFN-γ response in infected individuals: Rv0847, Rv0990, Rv0991, Rv1363, Rv1955, Rv2034, Rv2035, Rv2435n, Rv2642, Rv2643, Rv2658, Rv3420, and Rv3536 after short-term stimulation; and Rv1363, Rv1806, Rv2435n, Rv2643, Rv25658, and Rv3536 after long-term stimulation. However, in some of them the amount of IFN-γ was not very high, and the regions of response overlapped with the response obtained in non-infected individuals.

COMPARISON BETWEEN QFN-POSITIVE AND QFN-NEGATIVE LTBI INDIVIDUALS

Given that the lack of specificity of the TST, we evaluated if the response of LTBI individuals to the 60 novel latency-related mycobacterial antigens varied according to the result of their QFN. There were four antigens to which the response was statistically different depending on the QFN result (**Table 5**) when used in short-term stimulation. Latently infected individuals responded to Rv2389 ($p = 0.029$) and Rv2435n ($p = 0.050$) in higher amounts when the individual presented a positive QFN, thus indicating to be promising LTBI biomarkers. However, the

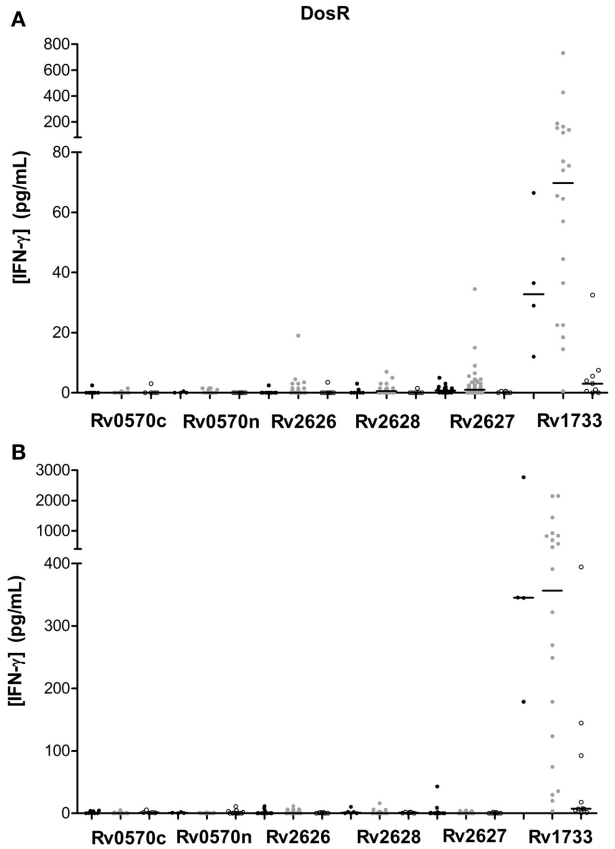


FIGURE 1 | DosR regulon-encoded antigen stimulated IFN-γ response (pg/ml) after short (A) or long-term (B) incubation of whole blood from individuals without LTBI (black dots), with LTBI (gray dots), and active TB patients (empty dots). The horizontal line represents the median.

response to Rv2435n overlapped between the two groups. The other two antigens, Rv2660 and Rv2380M, induced a significant response in individuals with a negative QFN ($p = 0.046$ and $p = 0.030$, respectively), indicating to be possible candidates for remote infection. The response to Rv2380M barely overlapped in the two groups, whereas the response to Rv2660 clearly overlapped. Some of these antigens could be good candidates to be used in combination with the QFN.

EFFECT OF BCG VACCINATION IN THE RESPONSE TO STUDIED ANTIGENS IN INDIVIDUALS WITH AND WITHOUT LTBI

Among non-infected individuals, there were five antigens in which the response was different according to the vaccination status. Specifically, Rv717, Rv0570n, Rv2658, and Rv2643 induced a significant response in BCG vaccinated individuals ($p = 0.043$, $p = 0.009$, $p = 0.041$, $p = 0.041$, respectively). In contrast, it was the non-BCG vaccinated individuals who most responded to Rv2627 ($p = 0.019$). Interestingly, while the higher response to antigens in BCG vaccinated individuals was produced after short-term stimulation, the higher response elicited by non-vaccinated individuals was only produced after long-term stimulation.

Regarding the LTBI subjects, BCG vaccinated individuals elicited a significant response to the following antigens: Rv2035, Rv1471, Rv1957, and Rv2435n ($p = 0.016$, $p = 0.020$, $p = 0.020$, $p = 0.015$, respectively) when compared to non-BCG subjects. In all four antigens the significance was after overnight stimulation.

DISCUSSION

In this study we evaluated whether latency antigens induced a response which varied according to the group of individuals. Each category of antigens, that is, DosR regulon-encoded, TB reactivation-associated, Rpf, starvation, IVE-TB antigens and other stress response-associated TB antigens, contained at least one antigen whose statistical analysis was significative.

Regarding the control antigens we studied, in general, the response they induced was higher after 7 days of incubation. PPD and ESAT-6/CFP-10 were the antigens which induced a highest response, followed by the TB10.4 and finally Ag85A, which is in concordance with what Kassa et al. (2012) found. According to our finding, Chegou et al. (2012) observed that the majority of response to *M.tb* infection is largely driven by ESAT6/CFP-10, not by the other antigens that they used as controls (TB7.7, Ag85A/B, and HSP65), where the recognition was poor. Generally speaking, it was observed that infected individuals provided higher responses than those with the disease. In a study performed by Sutherland et al. (2013) it was found, instead, that PPD and ESAT-6/CFP-10 generated dominant responses but very few differences between active TB and LTBI subjects. In the present study, some antigens induced a lower response in active TB patients when compared with non-TB infected subjects. This fact could be explained by the criteria selection followed for including individuals in the non-infected group. In our study, patients from LTBI screening with negative QFN, but TST results under 10 mm (non BCG-vaccinated) or 15 mm (BCG-vaccinated) were considered non-infected, but in some cases some cross-reactivity with the antigens used as a control (including ESAT-6/CFP-10) and the latency antigens could not be rejected. Indeed, in general, in the group of non-infected individuals the IFN-γ responses against ESAT-6/CFP-10 are lower in patients with TST under 5 mm, than in patients with TST over 5 mm (data not shown). On the other hand, results in the literature regarding IFN-γ responses to the these antigens in active TB patients are inconsistent. Possible differences may reside in variations in host genetic makeup, *M.tb* strains, study methodologies or the extent of TB progression, with diminished IFN-γ production during advanced disease (Weir et al., 2003; Jabado and Gros, 2005; Tsenova et al., 2007; Winek et al., 2008; Day et al., 2011).

Among all *M.tb* recombinant antigens we studied, there is one which stands up significantly: Rv1733, which is a probable conserved transmembrane protein and is part of the DosR regulon. As it can be observed in **Figure 1**, this antigen induces a differentiated response between non-infected subjects, infected individuals and TB patients. Specifically, infected individuals are the ones that generate a highest response, followed by the non-infected, and by the patients with the active disease at the end. Rv1733 immunogenity has been previously analyzed, and a genomic study from Zvi et al. (2008) describes it as an immunodominant T cell antigen. Moreover, many authors agree that there is

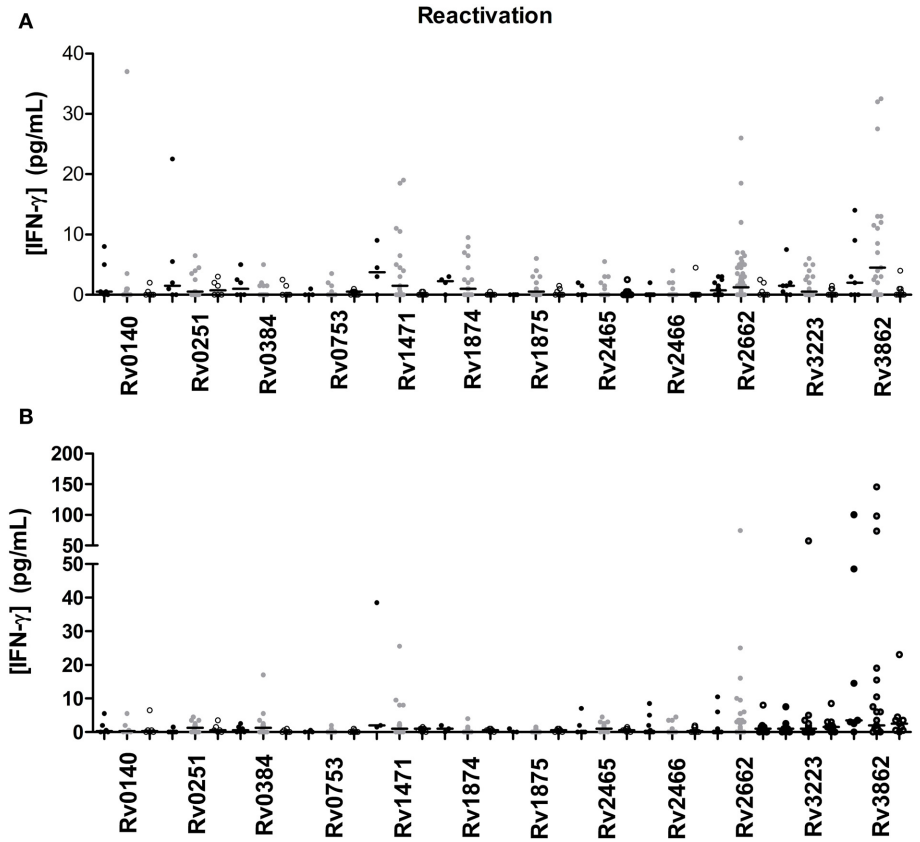


FIGURE 2 | TB reactivation-associated antigen stimulated IFN-γ response (pg/ml) after short (A) or long-term (B) incubation of whole blood from individuals without LTBI (black dots), with LTBI (gray dots), and active TB patients (empty dots). The horizontal line represents the median.

significantly higher T cell response in LTBI as compared to TB patients (Vordemeier et al., 1991; Leyten et al., 2006; Black et al., 2009; Schuck et al., 2009; Commandeur et al., 2011; Sutherland et al., 2013), and Rv1733 is one of the DosR antigens that induces a highest response. Interestingly enough, this response pattern against Rv1733 is produced after the stimulation during 24 h, which makes it very appealing to be used for diagnostic purposes. In contrast with our finding, Riaño et al. (2012) observed that LTBI and TB patients did not react to Rv1733. In another study with TB patients, a high response to Rv1733 was also obtained (Kassa et al., 2012).

Even though Rv1733 turned out to be a very immunogenic antigen, Rv2389, which belongs to the Rpf family, induced a high response in most of the individuals as well. Kassa et al. (2012) described that the Rv2389 was able to induce a high IFN-γ response in active TB patients. In fact, the immunoresponse to Rpf may play a protective role against bacilli reactivation (Riaño et al., 2012). Rv2389 was able to differentiate between non-infected individuals, individuals with LTBI and active TB patients when incubated with whole blood for 7 days, even though some overlapping is present. As observed by Chegou et al. (2012), active TB patients response was much lower than in non-TB individuals. Commandeur et al. (2011), demonstrated Rv2389 specific T cell response in long-term *M.tb* nonprogressors to active TB patients. Riaño et al. (2012), observed higher levels of IFN-γ in the supernatant of stimulated cells from LTBI compared to active TB patients. Huang et al. (2013) demonstrated that LTBI infected through household contacts possessed higher IFN-γ production to Rv2389c than did the community exposed individuals. In addition, QFN-positive individuals responded in a higher level to Rv2389 when compared to QFN-negative individuals. The Rv2389 ability of discriminate between these two groups of patients have been confirmed by receiver operating characteristics curve (ROC) analysis (area under curve = 0.877). Altogether, these findings indicate that Rv2389 would be a good biomarker of LTBI.

Concerning the recently identified IVE-TB antigens, the only research group that has studied their immunogenicity *in vitro* found that some of them induced high levels of IFN-γ (Commandeur et al., 2013). In our study, some antigens showed a certain difference when comparing the three groups of individuals. While the median value was quite low in all groups, a great number of antigens such as Rv0967, Rv1363, Rv1957, Rv2034, Rv3420, or Rv2642 among others were able to induce outstanding IFN-γ responses in some individuals. In concordance with the study performed by Commandeur and coworkers, it was the active TB patients the group who showed a lowest response. They observed that the individuals who generated a

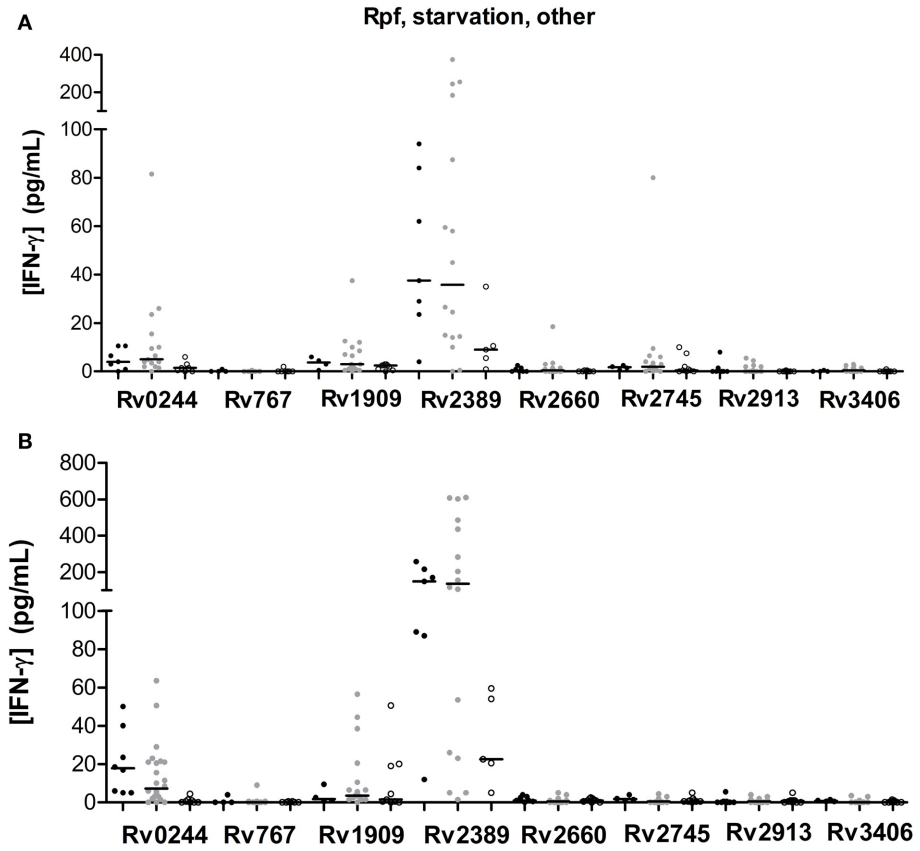


FIGURE 3 | Rpf, starvation and other stress response-associated antigen stimulated IFN-γ response (pg/ml) after short (A) or long-term (B) incubation of whole blood from individuals without LTBI (black dots), with LTBI (gray dots), and active TB patients (empty dots). The horizontal line represents the median.

highest response were those that did it with ESAT-6/CFP-10 as well. They also observed that controls not exposed to *M.tb* and individuals with positive TST and negative response to ESAT-6/CFP-10 did not respond to IVE-TB antigens, which shows that there is a specificity linked to *M.tb* exposure. In our study, although some non-TB infected individuals produced IFN-γ after being stimulated with IVE-TB antigens, individuals with LTBI also responded as well. BCG vaccine was ruled out as the response trigger in subjects without LTBI, as T cells from non-BCG vaccinated individuals generated a response as well. However, due the difficulty of totally rule out the infection in the no LTBI individuals, we cannot reject that, alternatively, those responder non-BCG vaccinated individuals were really *M.tb* infected; and that the responder BCG-vaccinated individuals were, in fact, responding to the shared BCG antigens.

Interestingly, the response to Rv2380M and Rv2660 was higher in individuals with a negative QFN than subjects with a positive QFN, indicating that both antigens could be possible biomarkers for remote infection. In the opposite way, Rv2435n induced a higher response in subjects with positive QFN. It may be, therefore, a possible biomarker for recent infection.

Activated lymphocytes and effector T cells that produce IFN-γ from *M.tb* antigens sensitized individuals, persist for a limited time in the circulation once the antigen is cleared (Pathan et al., 2001). It is thought that central memory T cells, but not effector ones, may take several days (rather than hours) to produce effector cytokines (Kaech et al., 2002; Dheda et al., 2007). This is because, the commercial IGRAs are thought to reflect more recent, rather than remote infections. Therefore, contrary to the findings of the TST, in cases of remote infection, the IFN-γ level did not increase during the short period of exposure to the antigen in the *ex vivo* IFN-γ assay at baseline. For these reasons we chose to stimulate short and long term the blood samples with the different latency-related antigens. Interestingly, the higher IFN-γ responses have been obtained after long-term stimulation instead of short-term stimulation: Rv1733, Rv3862, Rv2662, Rv0244, Rv2389, Rv1909, Rv2435n, Rv0847, Rv0967, Rv1806, and Rv2642.

As far as we know, only Goletti et al. (2010) assessed the comparison between individuals recently and remotely infected to five latency mycobacterial antigens. They found that Rv2628 was able to differentiate recent from remote infection, being the individuals with remote infection the group that showed significantly higher IFN-γ whole blood responses. In a very preliminary results, using well TB status characterized individuals, we have observed that responses to some antigens (Rv2380M, Rv0967,

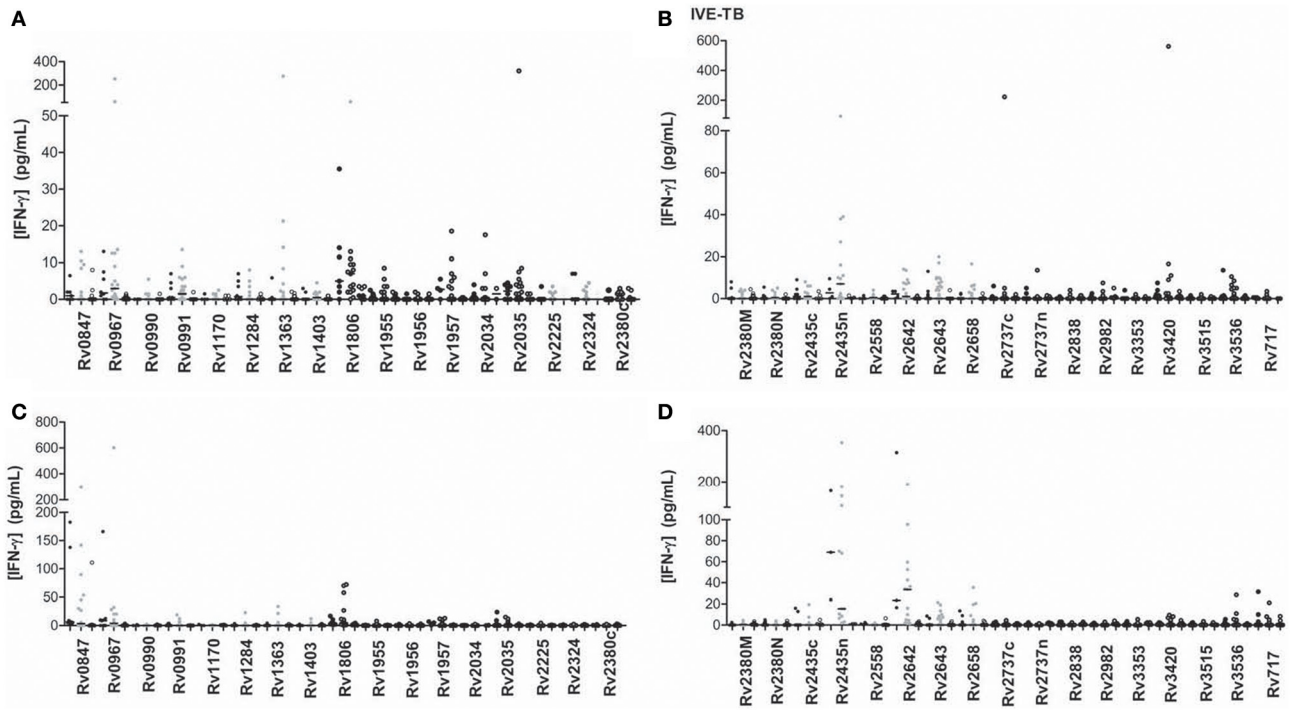


FIGURE 4 | IVE-TB antigen stimulated IFN-γ response (pg/ml) after short (A,B) or long-term (C,D) incubation of whole blood from individuals without LTBI (black dots), with LTBI (gray dots), and active TB patients (empty dots). The horizontal line represents the median.

Table 5 | Median levels of IFN-γ (pg/ml), minimum, and maximum values (in brackets) elicited in latently infected individuals depending on the QFN result.

	Positive QFN		Negative QFN		
Antigen	***n***	**Median**	***n***	**Median**	***p*-value**
Rv2389	13	9.0 (0.0, 95.5)	5	0.5 (0.0, 2.5)	0.029
Rv2660	15	0.0 (0.0, 18.5)	6	1.0 (0.0, 3.5)	0.046
Rv2380M	13	0.0 (0.0, 1.0)	6	3.3 (1.0, 4.5)	0.003
Rv2435n	10	10.5 (0.0, 92.5)	7	1.0 (0.0, 39.0)	0.05

N indicates the number of subjects in each group. Data were analyzed by Mann Whitney test for the comparison of patients with positive QFN and negative QFN. Only significant differences ($p < 0.05$) were included.

Rv2435n, and Rv2913) could differentiate between recent and remote infection (data not shown).

The fact of not obtaining response to an antigen that other studies identify as immunogenic, can be due to different host immune responses, *M.tb* strains and variations in the methodology used (Ottenhoff et al., 1998; Caws et al., 2008; Homolka et al., 2010), and also some factors such as ethnicity (host genetics), nutritional status, and microbial environment (Sutherland et al., 2013). The discordance in results between studies could be also attributed to the lack of gold standard for defining LTBI, and the consequent heterogenicity in the study population included in the different studies. The difficulty of establishing a group of LTBI is demonstrated by the criteria followed by the different authors: Leyten et al. (2006) included both patients from contact-tracing studies and also from screening studies; Chegou et al. (2012) included contact-tracing studies individuals, where neither TST nor QFN results were available; Commandeur et al. (2013) included TST positive patients, with exposure to *M.tb* and or with history of traveling to high TB incidence countries; and Sutherland et al. (2013) included household contacts of TB patients or by random community selection or from HIV care clinics with TST higher than 10 mm in HIV negative, and higher than 5 in HIV positive (independently of the BCG status). According to a recent study (Sutherland et al., 2013), which includes individuals from different sub-Saharan African countries, despite possible differences in the criteria of study subjects, there were variations between sites in regards to antigen reactivity, suggesting that need to be considered.

In order to ensure the validity of the promising antigens, we decided to study if some antigens induced a different response depending on whether the individual had been vaccinated with BCG or not. Among the antigens that distinguished between non-infected individuals, infected individuals and patients with TB disease, three of them were also identified when we analyze the effect of the BCG: Rv2627, Rv1471, and Rv1957. In order to measure the magnitude of the BCG influence, the response of non-vaccinated individuals was assessed. Being $p > 0.05$ and the charts showing an overlapping of the response between the three groups it seems that BCG has a considerable influence in the results (data not shown).

The effect of the BCG on the immune response against latency *M.tb* antigens has been studied by other authors. Lin et al. (2007) found that, although the homology between the DosR

regulon from the BCG strain and from *M.tb* was very high, BCG-vaccinated individuals did not present immune response against DosR. Instead, individuals exposed to *M.tb* did respond to DosR. Thus, it seems that the response to antigens linked to the control of LTBI is only generated when there is an exposure to *M.tb*, and it does not depend on whether the individual has been immunized by the BCG, probably because BCG fails to establish long-lived latent infections, and therefore it may not express (or under express) these antigens *in vivo* following vaccination (Honaker et al., 2008). However, this issue warrants further investigation.

The current study presents certain limitations which are worth mentioning. In the first place, it is worth highlighting the difficulty found in the classification of the individuals according to their TB status, specially among BCG-vaccinated individuals, since there is no gold standard assay for LTBI diagnosis. We therefore cannot rule out in some cases a misclassification. Secondly, it seems that some of the studied antigens could present certain lack of specificity; they could be shared in BCG strain and also in other mycobacteria (Lin et al., 2009), since some non-infected and non BCG-vaccinated individuals responded. Anyway, it is not clear whether the cross-reactivity to latency antigens in *M.tb* naive people contributes to the natural protection developed in 90% of the individuals who are infected but do not progress to active TB (Fine, 1995; Brandt et al., 2002). Thirdly, the sample size we could include was certainly limited for some antigens, including some antigens found as promising. Another limitation of our work lies in the fact that we only evaluated the immunoresponse in terms of IFN-γ production by T cells. Combination of other cytokines with IFN-γ can strengthen the diagnostic potential of *M.tb* antigen (Goldsack and Kirman, 2007). However, despite these limitations, this work obtained strong conclusions identifying potential antigens as candidates for further validation studies.

In conclusion, after screening the potential antigenicity in subjects across the spectrum of TB, we could identify promising antigens in all groups of antigens studied. Rv1733, which is encoded in DosR regulon, turned out to be very immunogenic and able to discriminate between the three defined TB status, thus considered a candidate biomarker. Rv2389 and Rv2435n, belonging to Rpf family and IVE-TB group of antigens, respectively, also stood out as LTBI biomarkers. Further work needs to be done in order to support our hypothesis and to have a pattern of host responses available so that by testing the response to a set of *M.tb* antigens we can define the TB status and make a clinical decision.

AUTHOR CONTRIBUTIONS

All authors listed contributed substantially in the conception or design of the work (Tom H. M. Ottenhoff, José Domínguez) or the adquisition of data (Irene Latorre, Kees L. C. M. Franken, Jéssica Díaz, Maria Luiza de Souza-Galvão, Irma Casas, José Maldonado, Cèlia Milà, Jordi Solsona, M. Ángeles Jimenez-Fuentes, Neus Altet, Alícia Lacoma, Juan Ruiz-Manzano, Cristina Prat), analysis (Vicente Ausina, M[a]del Mar Serra-Vidal, José Domínguez, Tom H. M. Ottenhoff), or interpretation of data (M[a]del Mar Serra-Vidal, José Domínguez, Tom H. M. Ottenhoff); in drafting the work or revising it critically for important intellectual content; in doing final approval of the version to be published; and in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

The research was supported by grants from the Instituto de Salud Carlos III and Sociedad Española de Patología Respiratoria (SEPAR). M[a]del Mar Serra-Vidal is a PhD student funded from Instituto de Salud Carlos III and José Domínguez is a researcher funded from the Miguel Servet programme of the Instituto de Salud Carlos III.

REFERENCES

Abubakar, I., Zignol, M., Falzon, D., Raviglione, M., Ditiu, L., Masham, S., et al. (2013). Drug-resistant tuberculosis: time for visionary political leadership. *Lancet Infect. Dis.* 13, 529–539. doi: 10.1016/S1473-3099(13)70030-6

Andersen, P., Munk, M. E., Pollock, J. M., and Doherty, T. M. (2000). Specific immune-based diagnosis of tuberculosis. *Lancet* 356, 1099–1104. doi: 10.1016/S0140-6736(00)02742-2

Black, G. F., Thiel, B. A., Ota, M. O., Parida, S. K., Adegbola, R., Boom, W. H., et al. (2009). Immunogenicity of novel DosR regulon-encoded candidate antigens of *Mycobacterium tuberculosis* in three high-burden populations in Africa. *Clin. Vaccine Immunol.* 16, 1203–1212. doi: 10.1128/CVI.00111-09

Brandt, L., Feino Cunha, J., Weinreich Olsen, A., Chilima, B., Hirsch, P., Appelberg, R., et al. (2002). Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect. Immun.* 70, 672–678. doi: 10.1128/IAI.70.2.672-678.2002

Cappelli, G., Volpe, E., Grassi, M., Liseo, B., Colizzi, V., and Mariani, F. (2006). Profiling of *Mycobacterium tuberculosis* gene expression during human macrophage infection: upregulation of the alternative sigma factor G, a group of transcriptional regulators, and proteins with unknown function. *Res. Microbiol.* 157, 445–455. doi: 10.1016/j.resmic.2005.10.007

Caws, M., Thwaites, G., Dunstan, S., Hawn, T. R., Lan, N. T., Thuong, N. T., et al. (2008). The influence of host and bacterial genotype on the development of disseminated disease with *Mycobacterium tuberculosis*. *PLoS Pathog.* 4:e1000034. doi: 10.1371/journal.ppat.1000034

Centers for Disease Control and Prevention. (2000). *Targeted Tuberculin Testing and Treatment of Latent Tuberculosis Infection*. MMWR;49(No. RR-6), Atlanta, GA.

Chegou, N. N., Black, G. F., Loxton, A. G., Stanley, K., Essone, P. N., Klein, M. R., et al. (2012). Potential of novel *Mycobacterium tuberculosis* infection phase-dependent antigens in the diagnosis of TB disease in a high burden setting. *BMC Infect. Dis.* 12:10. doi: 10.1186/1471-2334-12-10

Commandeur, S., van Meijgaarden, K. E., Lin, M. Y., Franken, K. L., Friggen, A. H., Drijfhout, J. W., et al. (2011). Identification of human T-cell responses to *Mycobacterium tuberculosis* resuscitation-promoting factors in long-term latently infected individuals. *Clin. Vaccine Immunol.* 18, 676–683. doi: 10.1128/CVI.00492-10

Commandeur, S., van Meijgaarden, K. E., Prins, C., Pichugin, A. V., Dijkman, K., van den Eeden, S. J., et al. (2013). An unbiased genome-wide *Mycobacterium tuberculosis* gene expression approach to discover antigens targeted by human T cells expressed during pulmonary infection. *J. Immunol.* 190, 1659–1671. doi: 10.4049/jimmunol.1201593

Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G., and Orme, I. M. (1993). Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J. Exp. Med.* 178, 2243–2247. doi: 10.1084/jem.178.6.2243

Corbett, E. L., Watt, C. J., Walker, N., Maher, D., Williams, B. G., Raviglione, M. C., et al. (2003). The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch. Intern. Med.* 163, 1009–1021. doi: 10.1001/archinte.163.9.1009

Day, C. L., Abrahams, D. A., Lerumo, L., Janse van Rensburg, E., Stone, L., O'rie, T., et al. (2011). Functional capacity of *Mycobacterium tuberculosis*-specific T cell responses in humans is associated with mycobacterial load. *J. Immunol.* 187, 2222–2232. doi: 10.4049/jimmunol.1101122

Demissie, A., Leyten, E. M., Abebe, M., Wassie, L., Aseffa, A., Abate, G., et al. (2006). Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with *Mycobacterium tuberculosis*. *Clin. Vaccine Immunol.* 13, 179–186. doi: 10.1128/CVI.13.2.179-186.2006

Dheda, K., Pooran, A., Pai, M., Miller, R. F., Lesley, K., Booth, H. L., et al. (2007). Interpretation of *Mycobacterium tuberculosis* antigen-specific IFN-gamma release assays (T-SPOT.TB) and factors that may modulate test results. *J. Infect.* 55, 169–173. doi: 10.1016/j.jinf.2007.02.005

Ernst, J. D. (2012). The immunological life cycle of tuberculosis. *Nat. Rev. Immunol.* 12, 581–591. doi: 10.1038/nri3259

Esmail, H., Barry, C. E. 3rd., and Wilkinson, R. J. (2012). Understanding latent tuberculosis: the key to improved diagnostic and novel treatment strategies. *Drug Discov. Today* 17, 514–521. doi: 10.1016/j.drudis.2011.12.013

Fine, P. E. (1995). Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 346, 1339–1345. doi: 10.1016/S0140-6736(95)92348-9

Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A., and Bloom, B. R. (1993). An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 178, 2249–2254. doi: 10.1084/jem.178.6.2249

Franken, K. L., Hiemstra, H. S., van Meijgaarden, K. E., Subronto, Y., den Hartigh, J., Ottenhoff, T. H., et al. (2000). Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expr. Purif.* 18, 95–99. doi: 10.1006/prep.1999.1162

Goldsack, L., and Kirman, J. R. (2007). Half-truths and selective memory: interferon gamma, CD4(+) T cells and protective memory against tuberculosis. *Tuberculosis (Edinb)* 87, 465–473. doi: 10.1016/j.tube.2007.07.001

Goletti, D., Butera, O., Vanini, V., Lauria, F. N., Lange, C., Franken, K. L., et al. (2010). Response to Rv2628 latency antigen associates with cured tuberculosis and remote infection. *Eur. Respir. J.* 36, 135–142. doi: 10.1183/09031936.00140009

Homolka, S., Niemann, S., Russell, D. G., and Rohde, K. H. (2010). Functional genetic diversity among *Mycobacterium tuberculosis* complex clinical isolates: delineation of conserved core and lineage-specific transcriptomes during intracellular survival. *PLoS Pathog.* 6:e1000988. doi: 10.1371/journal.ppat.1000988

Honaker, R. W., Stewart, A., Schittone, S., Izzo, A., Klein, M. R., and Voskuil, M. I. (2008). *Mycobacterium bovis* BCG vaccine strains lack narK2 and narX induction and exhibit altered phenotypes during dormancy. *Infect. Immun.* 76, 2587–2593. doi: 10.1128/IAI.01235-07

Honer zu Bentrup, K., and Russell, D. G. (2001). Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiol.* 9, 597–605. doi: 10.1016/S0966-842X(01)02238-7

Huang, W., Qi, Y., Ren, C., Wen, H., Franken, K. L., Ottenhoff, T. H., et al. (2013). Interferon-gamma responses to *Mycobacterium tuberculosis* Rpf proteins in contact investigation. *Tuberculosis (Edinb)* 93, 612–617. doi: 10.1016/j.tube.2013.08.005

Jabado, N., and Gros, P. (2005). Tuberculosis: the genetics of vulnerability. *Nature* 434, 709–711. doi: 10.1038/434709a

Kaech, S. M., Wherry, E. J., and Ahmed, R. (2002). Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol.* 2, 251–262. doi: 10.1038/nri778

Karakousis, P. C., Yoshimatsu, T., Lamichhane, G., Woolwine, S. C., Nuermberger, E. L., Grosset, J., et al. (2004). Dormancy phenotype displayed by extracellular *Mycobacterium tuberculosis* within artificial granulomas in mice. *J. Exp. Med.* 200, 647–657. doi: 10.1084/jem.20040646

Kassa, D., Ran, L., Geberemeskel, W., Tebeje, M., Alemu, A., Selase, A., et al. (2012). Analysis of immune responses against a wide range of *Mycobacterium tuberculosis* antigens in patients with active pulmonary tuberculosis. *Clin. Vaccine Immunol.* 19, 1907–1915. doi: 10.1128/CVI.00482-12

Kaufmann, S. H. (2001). How can immunology contribute to the control of tuberculosis? *Nat. Rev. Immunol.* 1, 20–30. doi: 10.1038/35095558

Kaufmann, S. H. (2012). Tuberculosis vaccine development: strength lies in tenacity. *Trends Immunol.* 33, 373–379. doi: 10.1016/j.it.2012.03.004

Latorre, I., De Souza-Galvao, M., Ruiz-Manzano, J., Lacoma, A., Prat, C., Fuenzalida, L., et al. (2009). Quantitative evaluation of T-cell response after specific antigen stimulation in active and latent tuberculosis infection in adults and children. *Diagn. Microbiol. Infect. Dis.* 65, 236–246. doi: 10.1016/j.diagmicrobio.2009.07.015

Leyten, E. M., Lin, M. Y., Franken, K. L., Friggen, A. H., Prins, C., van Meijgaarden, K. E., et al. (2006). Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*. *Microbes Infect.* 8, 2052–2060. doi: 10.1016/j.micinf.2006.03.018

Lin, M. Y., Geluk, A., Smith, S. G., Stewart, A. L., Friggen, A. H., Franken, K. L., et al. (2007). Lack of immune responses to *Mycobacterium tuberculosis* DosR regulon proteins following *Mycobacterium bovis* BCG vaccination. *Infect. Immun.* 75, 3523–3530. doi: 10.1128/IAI.01999-06

Lin, M. Y., Reddy, T. B., Arend, S. M., Friggen, A. H., Franken, K. L., van Meijgaarden, K. E., et al. (2009). Cross-reactive immunity to *Mycobacterium tuberculosis* DosR regulon-encoded antigens in individuals infected with environmental, nontuberculous mycobacteria. *Infect. Immun.* 77, 5071–5079. doi: 10.1128/IAI.00457-09

Mukamolova, G. V., Kaprelyants, A. S., Young, D. I., Young, M., and Kell, D. B. (1998). A bacterial cytokine. *Proc. Natl. Acad. Sci. U.S.A.* 95, 8916–8921. doi: 10.1073/pnas.95.15.8916

Mwaba, P., McNerney, R., Grobusch, M. P., O'grady, J., Bates, M., Kapata, N., et al. (2011). Achieving STOP TB Partnership goals: perspectives on development of new diagnostics, drugs and vaccines for tuberculosis. *Trop. Med. Int. Health* 16, 819–827. doi: 10.1111/j.1365-3156.2011.02777.x

Ottenhoff, T. H., and Kaufmann, S. H. (2012). Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathog.* 8:e1002607. doi: 10.1371/journal.ppat.1002607

Ottenhoff, T. H., Kumararatne, D., and Casanova, J. L. (1998). Novel human immunodeficiencies reveal the essential role of type-I cytokines in immunity to intracellular bacteria. *Immunol. Today* 19, 491–494. doi: 10.1016/S0167-5699(98)01321-8

Pathan, A. A., Wilkinson, K. A., Klenerman, P., McShane, H., Davidson, R. N., Pasvol, G., et al. (2001). Direct *ex vivo* analysis of antigen-specific IFN-gamma-secreting CD4 T cells in *Mycobacterium tuberculosis*-infected individuals: associations with clinical disease state and effect of treatment. *J. Immunol.* 167, 5217–5225. doi: 10.4049/jimmunol.167.9.5217

Pollock, L., Basu Roy, R., and Kampmann, B. (2013). How to use: interferon gamma release assays for tuberculosis. *Arch. Dis. Child. Educ. Pract. Ed.* 98, 99–105. doi: 10.1136/archdischild-2013-303641

Riaño, F., Arroyo, L., Paris, S., Rojas, M., Friggen, A. H., van Meijgaarden, K. E., et al. (2012). T cell responses to DosR and Rpf proteins in actively and latently infected individuals from Colombia. *Tuberculosis (Edinb)* 92, 148–159. doi: 10.1016/j.tube.2011.12.005

Ruiz-Manzano, J., Blanquer, R., Calpe, J. L., Caminero, J. A., Caylà, J., Domínguez, J., et al. (2008). SEPAR Guidelines. Diagnostic and treatment of tuberculosis. *Arch. Bronconeumol.* 44, 551–566. doi: 10.1157/13126836

Rustad, T. R., Harrell, M. I., Liao, R., and Sherman, D. R. (2008). The enduring hypoxic response of *Mycobacterium tuberculosis*. *PLoS ONE* 3:e1502. doi: 10.1371/journal.pone.0001502

Rustad, T. R., Sherrid, A. M., Minch, K. J., and Sherman, D. R. (2009). Hypoxia: a window into *Mycobacterium tuberculosis* latency. *Cell Microbiol.* 11, 1151–1159. doi: 10.1111/j.1462-5822.2009.01325.x

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., et al. (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J. Exp. Med.* 198, 693–704. doi: 10.1084/jem.20030846

Schuck, S. D., Mueller, H., Kunitz, F., Neher, A., Hoffmann, H., Franken, K. L., et al. (2009). Identification of T-cell antigens specific for latent *Mycobacterium tuberculosis* infection. *PLoS ONE* 4:e5590. doi: 10.1371/journal.pone.0005590

Sutherland, J. S., Lalor, M. K., Black, G. F., Ambrose, L. R., Loxton, A. G., Chegou, N. N., et al. (2013). Analysis of host responses to *Mycobacterium tuberculosis* antigens in a multi-site study of subjects with different TB and HIV infection states in sub-Saharan Africa. *PLoS ONE* 8:e74080. doi: 10.1371/journal.pone.0074080

Talaat, A. M., Lyons, R., Howard, S. T., and Johnston, S. A. (2004). The temporal expression profile of *Mycobacterium tuberculosis* infection in mice. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4602–4607. doi: 10.1073/pnas.0306023101

Tsenova, L., Harbacheuski, R., Sung, N., Ellison, E., Fallows, D., and Kaplan, G. (2007). BCG vaccination confers poor protection against *M. tuberculosis* HN878-induced central nervous system disease. *Vaccine* 25, 5126–5132. doi: 10.1016/j.vaccine.2006.11.024

Vordemeier, H. M., Harris, D. P., Roman, E., Lathigra, R., Moreno, C., and Ivanyi, J. (1991). Identification of T cell stimulatory peptides from the 38-kDa protein of *Mycobacterium tuberculosis. J. Immunol.* 147, 1023–1029.

Weir, R. E., Fine, P. E., Nazareth, B., Floyd, S., Black, G. F., King, E., et al. (2003). Interferon-gamma and skin test responses of schoolchildren in south-east England to purified protein derivatives from *Mycobacterium tuberculosis* and other species of mycobacteria. *Clin. Exp. Immunol.* 134, 285–294. doi: 10.1046/j.1365-2249.2003.02272

Winek, J., Rowinska-Zakrzewska, E., Demkow, U., Szopinski, J., Szolkowska, M., Filewska, M., et al. (2008). Interferon gamma production in the course of *Mycobacterium tuberculosis* infection. *J. Physiol. Pharmacol.* 59, 751–759.

World Health Organization. (2013). *Global Tuberculosis Report (WHO/HTM/TB/2013.11)*. Geneva: World Health Organization.

Zvi, A., Ariel, N., Fulkerson, J., Sadoff, J. C., and Shafferman, A. (2008). Whole genome identification of *Mycobacterium tuberculosis* vaccine candidates by comprehensive data mining and bioinformatic analyses. *BMC Med. Genomics* 1:18. doi: 10.1186/1755-8794-1-18

Conflict of Interest Statement: Tom H. M. Ottenhoff is coinventor of a *M.tb* latency antigen patent, which is owned by Leiden University Medical Center, but receives no financial benefits from this. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 April 2014; accepted: 15 September 2014; published online: 08 October 2014.

Citation: Serra-Vidal M, Latorre I, Franken KLCM, Díaz J, de Souza-Galvão ML, Casas I, Maldonado J, Milà C, Solsona J, Jimenez-Fuentes MÁ, Altet N, Lacoma A, Ruiz-Manzano J, Ausina V, Prat C, Ottenhoff THM and Domínguez J (2014) Immunogenicity of 60 novel latency-related antigens of Mycobacterium tuberculosis. Front. Microbiol. ***5****:517. doi: 10.3389/fmicb.2014.00517*

This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology.