Genome-Based In Silico Identification of New *Mycobacterium tuberculosis* Antigens Activating Polyfunctional CD8$^+$ T Cells in Human Tuberculosis

Sheila T. Tang,*,†,1 Krista E. van Meijgaarden,‡,1 Nadia Caccamo,§ Giuliana Guggino,§ Michèl R. Klein,‡,2 Pascale van Weeren,‡ Fatima Kazi,‡ Anette Stryhn,¶,‖ Alexander Zaigler,# Ugur Sahin,# Søren Buus,¶,‖ Francesco Dieli,§,3 Ole Lund,*,†,3 and Tom H. M. Ottenhoff‡,3

Although CD8$^+$ T cells help control *Mycobacterium tuberculosis* infection, their *M. tuberculosis* Ag repertoire, in vivo frequency, and functionality in human tuberculosis (TB) remains largely undefined. We have performed genome-based bioinformatics searches to identify new *M. tuberculosis* epitopes presented by major HLA class I supertypes A2, A3, and B7 (covering 80% of the human population). A total of 432 *M. tuberculosis* peptides predicted to bind to HLA-A*0201, HLA-A*0301, and HLA-B*0702 (representing the above supertypes) were synthesized and HLA-binding affinities determined. Peptide-specific CD8$^+$ T cell proliferation assays (CFSE dilution) in 41 *M. tuberculosis*-responsive donors identified 70 new *M. tuberculosis* epitopes. Using HLA/peptide tetramers for the 18 most prominently recognized HLA-A*0201-binding *M. tuberculosis* peptides, recognition by cured TB patients' CD8$^+$ T cells was validated for all 18 epitopes. Intracellular cytokine staining for IFN-γ, IL-2, and TNF-α revealed mono-, dual-, as well as triple-positive CD8$^+$ T cells, indicating these *M. tuberculosis* peptide-specific CD8$^+$ T cells were (poly)functional. Moreover, these T cells were primed during natural infection, because they were absent from *M. tuberculosis*-noninfected individuals. Control CMV peptide/HLA-A*0201 tetramers stained CD8$^+$ T cells in *M. tuberculosis*-infected and noninfected individuals equally, whereas Ebola peptide/HLA-A*0201 tetramers were negative. In conclusion, the *M. tuberculosis*-epitope/Ag repertoire for human CD8$^+$ T cells is much broader than hitherto suspected, and the newly identified *M. tuberculosis* Ags are recognized by (poly)functional CD8$^+$ T cells during control of infection. These results impact on TB-vaccine design and biomarker identification. ***The Journal of Immunology*****, 2011, 186: 1068–1080.**

One third of the world's population is latently infected with *Mycobacterium tuberculosis*, and each year, 1.8 million people die of tuberculosis (TB) (1, 2). Bacillus Calmette-Guérin (BCG) vaccination has been used for almost a century, but its protective efficacy is highly variable and incomplete, especially against pulmonary TB, the main and contagious form of the disease. BCG's effect wanes in adolescents, and revaccination does not confer additional protection. Currently, the TB problem is aggravated by HIV coinfection, and the emergence of multidrug-resistant and extensively drug-resistant *M. tuberculosis* strains, which threaten to set back TB control to the preantibiotic era (3, 4). New and better vaccines against TB are urgently needed, but to be able to develop these, a radically improved understanding of what constitutes protective host immunity is warranted. It is well established that CD4$^+$ T cell responses play a major role in acquired immunity against *M. tuberculosis* (5–7). There is, however, mounting evidence that CD8$^+$ T cells are also involved in the control of *M. tuberculosis* infection and in mediating optimal host defense in small animal models, nonhuman primates, and human infection (8–14).

*Center for Biological Sequence Analysis, Technical University of Denmark, 2800 Lyngby, Denmark; †Department of Systems Biology, Technical University of Denmark, 2800 Lyngby, Denmark; ‡Department of Infectious Diseases, Leiden University Medical Center, 2300 RC Leiden, The Netherlands; §Dipartimento di Biopatologia e Biottecnologie Mediche e Forensi, Università di Palermo, 90133 Palermo, Italy; ¶Division of Experimental Immunology, Institute of Medical Microbiology and Immunology, University of Copenhagen, 2200 Copenhagen N, Denmark ‖The Panum Institute 18.3.12, University of Copenhagen, 2200 Copenhagen N, Denmark; and #Ganymed Pharmaceuticals, DE-55131 Mainz, Germany

[1]S.T.T. and K.E.v.M. share first authorship for this work.

[2]Current address: National Institute for Public Health and the Environment, Bilthoven, The Netherlands.

[3]F.D., O.L., and T.H.M.O. share last authorship for this work.

Received for publication July 2, 2010. Accepted for publication November 12, 2010.

This work, project number LSHP-CT-2004-012175, was supported by the European Commission's Sixth Framework Program FP6 and by The Netherlands Leprosy Relief Foundation.

The text represents the authors' views and does not necessarily represent a position of the European Commission, which will not be liable for the use made of such information.

Address correspondence and reprint requests to Dr. Tom H. M. Ottenhoff, Prof. in Immunology, Head Group Immunology and Immunogenetics of Bacterial Infectious Diseases, Department of Infectious Diseases, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. E-mail address: T.H.M.Ottenhoff@lumc.nl

The online version of this article contains supplemental material.

Abbreviations used in this article: BCG, bacillus Calmette-Guérin; Bepi, proteins with B cell epitopes; BestPred, Best predicted epitopes; Cons, conserved candidate Ag; DOS/LAG, *Mycobacterium tuberculosis* DosR regulon encoded latency Ag; HC, healthy control; ICS, intracellular cytokine staining; IEDB, Immune Epitope Database; NCBI, National Center for Biotechnology Information; ORF, open reading frame; PPD, purified protein derivative; PredSecret, hypothetical proteins predicted to be secreted; Secret, secreted *M. tuberculosis* Ag; TB, tuberculosis; TB-CD8, *M. tuberculosis* CD8$^+$ T cell epitope expressing Ag; TB-VAC, tuberculosis vaccine Ag.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1002212

Following recognition of mycobacterial Ags on infected cells, $CD8^+$ T cells contribute to *M. tuberculosis* control through: 1) IFN-γ and TNF-α production (9, 15–17); 2) lysis of infected host cells (16–18); and 3) direct killing of mycobacteria (19–21). One study demonstrated clonal $CD4^+$ and $CD8^+$ T cell expansion in granulomas from subjects with latent TB infection (22), and similar changes in the TCR repertoire were reported in peripheral blood versus pleural fluid in TB patients (23). Furthermore, $CD8^+$ T cells specific for a number of mycobacterial Ags have been isolated from human and mouse models, consistent with the hypothesis that $CD8^+$ T cells are constantly being stimulated with Ag (18, 20). One of us reported that the frequency of *M. tuberculosis* Ag85A-specific $CD8^+$ T cells correlated with therapy-induced curative responses in children: Ag85A epitope-specific $CD8^+$ T cells during active TB produced low levels of IFN-γ and perforin, which normalized after therapy (24). In a later study, we reported similar findings for $CD8^+$ T cells directed against six *M. tuberculosis* epitopes (two of which were newly identified). In that study, it was also found that *M. tuberculosis* peptide-specific IL-2^+/IFN-γ^+ $CD8^+$ T cell responses were associated with natural protection against developing TB disease (15). In parallel studies, Kaufmann and colleagues (25) found clonal expansion of effector memory $CD8^+$ T cells in older children with TB, with potential impact on the course and severity of disease. Lewinsohn and colleagues (26) reported clonally expanded $CD8^+$ T cells that recognized a number of *M. tuberculosis* epitopes in the context of HLA-A and -B alleles, and we ourselves reported Ag85A, B, and C epitopes activating human $CD8^+$ T cells (13, 14, 27, 28). Despite these studies, little remains known about the size, quality, and specificity of *M. tuberculosis*-specific $CD8^+$ MHC-Ia–restricted T cell responses in TB patients (20) and their relevance to control of infection (i.e., prevention of progression to TB disease).

The complete genome sequence of *M. tuberculosis* encodes 3985 coding open reading frames (ORFs) (29). Surprisingly, *M. tuberculosis*-specific epitopes have been identified in only 270 ORFs, representing only 7% of the *M. tuberculosis* genome. In fact, 30 ORFs account for 65% of all epitopes reported (30). This indicates that our current knowledge of the *M. tuberculosis* antigenome/epitome is limited and incomplete and that many more relevant *M. tuberculosis* Ags and epitopes likely remain to be discovered, particularly for $CD8^+$ T cells (20). The search for Ags that stimulate human $CD8^+$ T cells requires new search strategies, because classical protein expression technologies are not easily amenable to unravel $CD8^+$ T cell responses. Recently developed new and powerful bioinformatics prediction tools may help to identify candidate epitopes and thus minimize the laborious screening of peptides for immunobiological characteristics of Ags capable of eliciting a protective immune response.

Using novel bioinformatics search tools in combination with functional immunological screening strategies, we have selected new *M. tuberculosis* proteins, which were likely to contain $CD8^+$ T cell-stimulating HLA class Ia-restricted epitopes (reverse Ag discovery). We also used unbiased forward Ag discovery, in which expression libraries representing the whole *M. tuberculosis* genome were screened for proteins that are targets for B cell responses in TB patients. Within the selected proteins, potential peptide epitopes were identified that were restricted by any three of the major HLA class Ia supertypes (A2, A3, and B7), which cover >80% of individuals from different ethnic groups (31). These predicted epitopes were validated using specific tetramers and peptide stimulation combined with intracellular cytokine staining (ICS) to quantify polyfunctional $CD8^+$ T cell responses in cured TB patients and immune donors.

Materials and Methods

Study subjects

Buffy coats from 41 in vitro purified protein derivative (PPD)-responsive (average IFN-γ responses to PPD: 1828 pg/ml, range 101 to >5000 pg/ml), HLA-typed, healthy anonymous blood bank donors (Sanquin, Leiden, The Netherlands) were used for the study. Their HLA class I types and their responses to PPD and ESAT6/CFP10 are shown in the Supplemental Table I. No clinical information is available for these donors other than that they were healthy and had no chronic viral infections or other contraindications for donating blood. BCG in The Netherlands is only administered to people at risk for TB exposure, and the TB incidence in The Netherlands is extremely low, such that the vast majority of our donors (>95%) is highly unlikely to have been vaccinated with BCG or to have had exposure to *M. tuberculosis*. All individuals gave written consent before blood donation. The study was approved by the Institutional Review Board of the Leiden University Medical Center.

In the second phase of the study, peripheral blood was obtained from 10 HLA-A*0201–positive adults with recently cured TB disease (six males, four females; age range 46–67 y) from the Dipartimento di Medicina Clinica e delle Patologie Emergenti, University Hospital, Palermo, Italy, and 10 tuberculin (PPD)-negative healthy subjects (seven males and three females, age range, 32–51 y). The cured TB patients had had clinical and radiological findings consistent with active pulmonary TB. Diagnosis had been confirmed by bacteriological isolation of *M. tuberculosis* and by clinical and radiological features. All patients had been treated in accordance with Italian guidelines and had received multidrug therapy for 6 mo. TB treatment was successful in all participants as evidenced by the absence of any clinical or radiographic evidence of active disease, the confirmed completion of anti-TB chemotherapy, and sterile mycobacterial cultures. Blood samples were taken at time points well after cessation of treatment. None of the cured TB patients had been vaccinated during infancy with BCG, had evidence of HIV infection, or was being treated with steroid or other immunosuppressive or anti-TB drugs at the time of their sampling. Tuberculin (PPD) skin tests were considered positive when the induration diameter was larger than 5 mm at 72 h since injection of 1 U PPD (Statens Serum Institute, Copenhagen, Denmark). All Italian subjects were HLA typed serologically, whereas the HLA-type A*0201 was confirmed to be A*0201 using sequence-specific oligonucleotide primers.

All individuals gave written consent before blood donation. The study was approved by the Ethical Committee of the Dipartimento di Medicina Clinica e delle Patologie Emergenti, University Hospital, Palermo. The 10 healthy controls had no symptoms or signs of active TB nor had they been vaccinated with BCG. They were tuberculin skin test negative as well as in vitro QuantiFERON-TB Gold test (QFT-G; Cellestis, Victoria, Australia) negative.

Bioinformatics strategy to identify $CD8^+$ T cell epitopes

Complete sequenced genome of *M. tuberculosis* strain H37Rv (National Center for Biotechnology Information [NCBI] Refseq Id: NC_000962) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). All 3918 candidate protein-encoding genomic sequences were submitted to the SubCell 1.0 (http://www.cbs.dtu.dk/services/SubCell/) and prediction performed using the option "gram positive bacteria" in the server. The SubCell server generated a list of 370 secreted proteins (the prediction program, SignalP, predicted 340 proteins with a signal peptide [the classical secretion pathway], SecretomeP predicted 10 proteins for the nonclassical secretion pathway, and lastly, TATP predicted 20 proteins to have a twin-Arg signal peptide cleavage site characteristic for bacteria). NetCTL 1.0 server (http://www.cbs.dtu.dk/services/NetCTL-1.0/) was used for predicting 9-mer $CD8^+$ T cell epitopes in *M. tuberculosis* protein sequences. Briefly, this method integrates prediction of MHC binding, proteasomal C-terminal cleavage and TAP transport efficiency (32). Prediction was performed for three main HLA supertypes: HLA-A2, -A3, and -B7. A threshold cutoff value of 0.42 in NetMHC corresponding to a predicted binding affinity of <500 nM was used.

Epitope selection criteria

Epitopes were selected using eight different selection criteria from proteins that were selected based on suspected immunogenicity. In bold below, the protein selections are shown; the numbers in parentheses represent the number of potential epitopes selected for further study within each protein group. A total of 498 peptides were selected initially but due to difficulties in peptide synthesis or purification, 66 peptides were either cancelled or discarded, leaving 432 peptides. A full list of proteins, which have been used

for the peptide prediction for each selection, is provided in Supplemental Table II.

*TB-vaccine Ags (*n = *27).* This group contains candidate epitopes from proteins being evaluated in TB-vaccine trials. These proteins were selected because they have been relatively well studied and are known to be immunogenic. The following proteins were used for the prediction: M. tuberculosis72f (Rv0125—NP_214639.1 [protein accession number according to NCBI] and Rv1196—[YP_177795.1]), TB10.4 (Rv0288—NP_214802.1), HBHA (Rv0475—NP_214989.1), Ag85B (Rv1186c—NP_216402.1), HspX (Rv2031c—NP_216547.1), Ag85A (Rv3804c—NP_218321.1), and ESAT-6 (Rv3875—YP_178023.1).

M. tuberculosis *CD8$^+$ T cell epitope-expressing Ags (*n = *60).* This group contains candidate epitopes from proteins with already known CD8 T cell epitopes (33). This selection is included because proteins containing immunogenic CD8 T cell epitopes have been reported to be enriched in other CD8 T cell epitopes in HIV (33).

Best predicted M. tuberculosis *epitopes (*n = *59).* This group contains candidate epitopes with the highest combined NetCTL score based on peptide MHC binding, proteasomal C-terminal cleavage, and TAP transport efficiency.

*Conserved candidate Ags (*n = *65).* This group contains candidate epitopes that are conserved among related or closely related organisms. One rationale to include this selection is that heterologous immunity may exist to cross-reactive epitopes in other organisms or strains of the same organism (reviewed in Ref. 34). Furthermore, there is a general belief that vaccines may be more effective if they focus on Ags that are under functional or structural constraints, as variation in these regions may affect the fitness of the pathogen. The best predicted peptides are 100% conserved in the following bacterial strains (NCBI Refseq Id in parentheses): *M. tuberculosis* CDC1551 (NC_002755), *M. bovis* AF2122/97 (NC_002945), *M. leprae* TN (NC_002677), *M. avium subsp. paratuberculosis* str. k10 (NC_002944), *Corynebacterium glutamicum* ATCC 13032 (NC_006958), *C. efficiens* YS-314 (NC_004369), *C. glutamicum* ATCC 13032 (NC_003450), *C. diphtheriae* NCTC 13129 (NC_002935), *Streptomyces coelicolor* A3(2) (NC_003888), *S. avermitilis* MA-4680 (NC_003155), and *Nocardia farcinica* IFM 10152 (NC_006361).

M. tuberculosis *DosR regulon-encoded latency Ags (*n = *63).* This group contains the best-predicted candidate epitopes from a list of recently discovered immunogenic proteins from the *M. tuberculosis* DosR regulon (28, 35). This selection was included because Ags of this dormancy regulon may contribute to the control of latent *M. tuberculosis* infection (28).

*Proteins with B cell epitopes (*n = *52).* This group contains candidate epitopes predicted from novel proteins with B cell epitopes discovered by generation of shotgun expression library (U. Sahin and A. Zaigler, unpublished observations). Sera from TB patient blood samples were used for immunoscreening. This particular selection was included because proteins containing B cell epitopes are likely also to host T cell epitopes (e.g., see Ref. 36).

Secreted M. tuberculosis *Ags (*n = *59).* This group contains candidate epitopes from potentially secreted *M. tuberculosis* Ags, because these are considered to be immunodominant and involved in protective immunity. Secreted proteins are readily available for Ag processing and presentation by MHC class I molecules on the infected host cell (37). Selection of peptides was based on a list of known secreted proteins obtained from the TubercuList database (http://genolist.pasteur.fr/TubercuList/).

*Hypothetical proteins predicted to be secreted (*n = *47).* This group contains candidate epitopes from unknown hypothetical proteins predicted to be secreted by both including proteins using the classical [with an NH2 terminal signal peptide (38)] and nonclassical secretion pathway (39). A prediction server, SubCell version 1.0, was used to predict various types of signal peptides and subcellular location in Gram-negative and Gram-positive bacteria (http://www.cbs.dtu.dk/services/SubCell; see above). Secreted proteins were then selected for *M. tuberculosis*.

Peptides and biochemical peptide-HLA class I binding assay

The 9-mer peptides were synthesized by standard 9-fluorenylmethyloxycarbonyl chemistry (Mimotope; Shafer-N, Copenhagen, Denmark). Peptides were distributed at 20 μg/vial and stored lyophilized at −20°C until use (for more details, see Ref. 32). Peptides were dissolved just before use. The biochemical peptide-HLA class I binding assay was performed as previously described (40).

Isolation of PBMCs

PBMC were isolated from buffy coats by density gradient centrifugation using Ficoll (Pharmacy, Leiden University Medical Center) and Leucosep tubes (Greiner, The Netherlands). Freshly isolated PBMC were cryopreserved for later use.

Proliferation assay

Proliferation was measured using CFSE dilution and flow cytometry. PBMC from study subjects were thawed, washed, and labeled with CFSE (Molecular Probes, Leiden, The Netherlands) at a final concentration of 5 μM for 10 min at 37°C. Washed, counted, and viable cells were seeded in six replicates in 96-well round-bottom plates at a concentration of 2×10^5 in the presence of control Ags (PPD, 5 μg/ml [Statens Serum Institute, Copenhagen, Denmark]), PHA 2 μg/ml (Remel; Oxoid, Haarlem, The Netherlands), or the test peptides (final concentration 10 μg/ml). Culture medium used was IMDM with glutamax supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Breda, The Netherlands), and 10% pooled human serum. After 7 d of incubation at 37°C in a 5% CO_2 humidified incubator, cells were harvested and stained for further analysis on the flow cytometer.

Flow cytometry for CFSE assay

Cells were stained for 30 min at 4°C using anti–CD8-allophycocyanin (DakoCytomation, Heverlee, Belgium), anti–CD3-PerCP, and anti–CD4-PE (BD Biosciences). Cells were then washed in PBS 0.1% BSA (Sigma-Aldrich, Zwijndrecht, The Netherlands), fixed in 1% paraformaldehyde (Pharmacy, Leiden University Medical Center) and analyzed on an LSRII with an HTS plate loader (BD Biosciences). Analysis was done using FACSDiva software (BD Biosciences). Cells gated on live lymphocytes combined with gating on CD3$^+$CD8$^+$ T cells were analyzed for CFSE proliferation. The Δ geometric mean was used as a measure of proliferation and calculated as follows: Δ geometric mean = geometric mean (non-proliferated cells) − geometric mean (total cells) (see Supplemental Fig. 1 for explanation). The Δ geometric mean was then used to calculate the relative proliferation that is the percentage of the maximal proliferation (PHA) corrected for spontaneous proliferation (medium): ([Δ geometric mean sample − Δ geometric mean control medium]/[Δ geometric mean PHA − Δ geometric mean control medium]) × 100% = percentage of maximal proliferation. The cutoff value for a positive proliferative response was arbitrarily set at 10% relative proliferation.

Tetramer staining

Tetramers were generated using a "one-pot, mix-and-read" strategy recently published by us (41) Tetramer staining was carried out as described in detail previously (15). PBMC (10^6/ml) were incubated in U-bottom 96-well plates, washed twice in PBS containing 1% FCS (Sigma-Aldrich), and stained for 30 min at 4°C with PE-labeled tetramers (35 μl each), washed, subsequently stained with FITC-labeled anti-CD8 mAb (clone HIT8a; BD Biosciences), and analyzed by flow cytometry on an FACSCalibur. Data were analyzed with the use of FlowJo software (Tree Star, Ashland, OR). Viable lymphocytes were gated by forward and side scatter, and the analysis was performed on 100,000 acquired CD8 events for each sample. A cutoff of 0.01% was used as described previously (15); values below this were set to zero.

Intracellular cytokine staining

ICS was carried out as described in detail previously (15). PBMC (10^6/ml) were stimulated with peptides (1 μg/ml, final concentration) in the presence of monensin for 6 h at 37°C in 5% CO_2. The cells were harvested, washed, and stained with PerCP Cy5-conjugated anti-CD8 mAb (BD Biosciences) in incubation buffer (PBS-1% FCS-0.1% Na azide) for 30 min at 4°C. The cells were washed twice in PBS-1% FCS and fixed with PBS-4% paraformaldehyde overnight at 4°C. Fixation was followed by permeabilization with PBS-1% FCS-0.3% saponin-0.1% Na azide for 15 min at 4°C. Staining of intracellular cytokines was performed by incubation of fixed permeabilized cells with PE-labeled anti–IFN-γ (clone B27), FITC-labeled IL-2 Ab (clone MQ1-17H12), and allophycocyanin-labeled anti–TNF-α (MAb11) or an isotype-matched control mAb (all from BD Biosciences). Cells were acquired and analyzed by FACS as described above. Analysis was performed on a minimum of 100,000 acquired CD8 events for each sample.

Negative controls were background staining obtained with PBMC incubated with medium, in the absence of any stimulant or with control peptide, or similar experiments using PBMC of PPD nonresponsive donors. Cutoff values for a positive response were predetermined to be in excess of

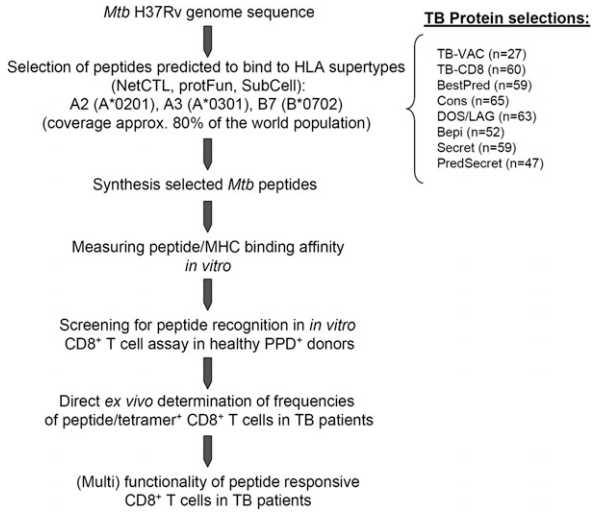

FIGURE 1. Schematic representation of study design. The complete genome sequence of *M. tuberculosis* strain H37Rv from GenBank was used for in silico CTL epitope prediction using NetCTL 1.0. *M. tuberculosis* proteins were included based on eight different selection criteria prior to peptide prediction. The numbers in each selection are given in parentheses. 1. TB-VAC: proteins used in TB vaccine trials; 2. TB-CD8: *M. tuberculosis* proteins with known CD8 T cell epitopes; 3. BestPred: proteins containing peptides with the best prediction values; 4. Cons: conserved *M. tuberculosis* proteins; 5. DOS/LAG: proteins encoded by the *M. tuberculosis* DosR regulon; 6. Bepi; 7. Secret: secreted proteins; and 8. PredSecret: *M. tuberculosis* proteins predicted to be secreted (see *Materials and Methods* for details). Epitope predictions were done for HLA-A2, A3, and B7. A total of 432 peptides were synthesized, and binding affinities were measured in an in vitro biochemical peptide-HLA class I binding assay (40). $CD8^+$ T cell proliferative responses were performed using a CFSE dilution assay on PBMC from PPD^+ donors in a flow cytometric analysis.

0.01% responsive cells. Results below this value were considered negative and set to zero.

Statistics

Nonparametric Mann–Whitney *U* test was used to determine statistical differences in the distribution of the results. The p values <0.05 were considered significant. Data were analyzed using statistical software SYSTAT 11 (Systat Software). The significance of a difference between two proportions was calculated as described by Armitage et al. (42).

Results

Selection of M. tuberculosis *Ags used for bioinformatics-based epitope identification*

The complete genome sequence of *M. tuberculosis* strain H37Rv was retrieved from GenBank for in silico epitope prediction using NetCTL. Proteins were selected using eight different criteria (as described in detail in *Materials and Methods*) to evaluate how proteins could be selected for epitope discovery. Fig. 1 gives an overview of the strategy employed.

Binding of predicted epitopes to HLA class Ia molecules

A total of 432 peptides were synthesized and binding to the respective HLA molecule determined as summarized in Table I. A total of 373 (86%) bound to their respective HLA molecules with a $K_D \leq 500$ nM, which is a normally accepted threshold for immunogenicity (43); 236 (55%) bound with high affinity with a $K_D \leq 50$ nM. Only 7% of the peptides bound with an affinity <5000 nM. The fraction of peptides binding to HLA-A2 and -B7 with high affinity (<50 nM) was significantly higher than for HLA-A3 ($p < 0.001$).

Immunogenicity of predicted new HLA class I-restricted M. tuberculosis *epitopes*

PBMC from HLA-A2–, HLA-A3–, and/or HLA-B7–positive PPD responsive donors were then screened for possible $CD8^+$ T cell proliferative responses toward the novel peptides. Ten donors with the appropriate HLA specificity were tested for each peptide (see also Supplemental Table III).

The results are summarized in Table II (see also Fig. 1, Supplemental Table III). Peptide-induced $CD8^+$ T cell responses were scored positive when exceeding 10% relative proliferation in two or more PPD^+ donors (as we have described before; see Ref. 44 and Supplemental Fig. 1). In case there was a relatively high (ranging between 10 and 20%) response against a given peptide in only one single donor, the peptide-induced response was repeated and only included as hit when reproducible in that donor. According to these criteria, a total number of 70 out of the 432 tested peptides (16%) were found to elicit a $CD8^+$ T cell response (Table II).

When the results were compared, 34% (44 out of 130) of the predicted HLA-A2 binding peptides were found capable of eliciting a proliferative $CD8^+$ T cell response in HLA-$A2^+$ PPD-responsive donors compared with 6% (9 out of 140) for HLA-A3 and 10% (17 out of 163) for HLA-B7. That predicted HLA-A2 binding peptides were more frequently recognized was highly significant ($p < 0.001$ in both cases) and likely reflects the more accurately defined peptide-binding motif for this allele. The difference between the fractions of predicted HLA-A3 and -B7 binding peptides recognized was not significant.

High-affinity HLA binding peptides are preferentially recognized compared with medium- or low-affinity binding peptides

A total of 19% (46 out of 236) of the peptides binding to an HLA class Ia molecule with an affinity >50 nM were recognized by the healthy immune donors (Table I). This fraction is significantly higher than that for the peptides with a lower binding affinity (>50 nM; $p < 0.02$). The $CD8^+$ T cell response frequencies to these latter peptides were 9% (13 out of 137) for intermediate-affinity binders, 14% (4 out of 29) for low-affinity binders, and,

Table I. Number of peptides in different intervals of binding affinity (K_D) values for *M. tuberculosis* peptides predicted to bind to three different major HLA class Ia alleles

HLA	High Binders ($K_D \leq 50$ nM)	Intermediate Binders (50 nM $< K_D \leq$ 500 nM)	Low Binders (500 nM $< K_D \leq$ 5000 nM)	Low or Nonbinders ($K_D >$ 5000 nM)	Total/Supertype	Discarded or Cancelled
A*0201	105 (33)	15 (5)	2 (1)	8 (5)	130	41
A*0301	18 (1)	82 (4)	20 (3)	19 (1)	139	16
B*0702	112 (12)	40 (4)	7 (0)	3 (1)	163	9
Total	236 (46)	137 (13)	29 (4)	30 (7)	432	66

The number of peptides that gave a positive $CD8^+$ T cell proliferative response are in parentheses.

Table II. Proliferative CD8$^+$ T cell responses against peptides selected to be restricted by each of the three supertypes: HLA-A2, -A3, and -B7

Selection	A2 Peptides			A3 Peptides			B7 Peptides			All Three Supertypes		
	No. Tested[a]	No. Plus Peptide[b]	Percent Reactivity[c]	No. Tested	No. Plus Peptide	Percent Reactivity	No. Tested	No. Plus Peptide	Percent Reactivity	No. Tested Total	No. Plus Peptide Total	Percent Reactivity Total
TB-VAC	8	1	13	9	2	22	10	4	40	27	7	26
TB-CD8	17	1	6	23	5	22	20	5	25	60	11	18
BestPred	16	1	6	20	2	10	23	3	13	59	6	10
Cons	18	1	6	22	0	0	25	3	12	65	4	6
DOS/LAG	19	9	47	23	0	0	21	0	0	63	9	14
Bepi	18	9	50	10	0	0	24	0	0	52	9	17
Secret	19	14	74	18	0	0	22	1	5	59	15	25
PredSecret	15	8	53	14	0	0	18	1	6	47	9	19
Total	130	44	34	139	9	6	163	17	10	432	70	16

Results are shown for each of the eight Ag selections.
[a]Number of peptides tested for this supertype.
[b]Number of peptides that gave a proliferative CD8$^+$ T cell response.
[c]Percentage of positive peptides that gave a proliferative CD8$^+$ T cell response.

somewhat unexpectedly, 23% (7 out of 30) for predicted nonbinders.

Frequency of the recognized peptides for each of the eight selections

Eight different selection criteria were used to select proteins for epitope prediction (Fig. 1). The results are shown in Table II. The highest overall proportional frequencies of CD8$^+$ T cell responses were found in the sets of proteins used in the TB-vaccine Ags set (TB-VAC; 26%) as well as in the secreted *M. tuberculosis* Ag set (Secret; 25%). Two other broad selection criterions, best predicted epitopes (BestPred) and conserved candidate Ag (Cons), had the lowest frequencies of CD8$^+$ T cell responses. BestPred and Cons together had a significantly lower response rate than the other more specific protein function-based selections combined (TB-VAC, *M. tuberculosis* CD8$^+$ T cell epitope-expressing Ags [TB-CD8], *M. tuberculosis* DosR regulon-encoded latency Ags [DOS/LAG], proteins with B cell epitopes [Bepi], Secret, and hypothetical proteins predicted to be secreted [PredSecret]) ($p < 0.01$). When analyzing HLA-A2–associated epitopes only, we found that the selections DOS/LAG, Bepi, Secret, and PredSecret yielded a higher frequency of CD8$^+$ T cell-stimulating epitopes compared with other selections, such as TB-VAC, TB-CD8, BestPred, and Cons ($p < 0.001$).

*The most frequently recognized HLA-A*0201–restricted peptides were selected for testing in cured TB patients.* The 18 most frequently recognized HLA-A*0201–restricted peptides were chosen for further studies. First, we selected 13 of the above-defined 44 HLA-A*0201–restricted peptides, namely those that were recognized in two or more donors (arbitrary cutoff was >10% relative CD8$^+$ T cell proliferation). In addition, we also included five peptides (B118, C250, C255, B130, and B134) that were recognized in a single donor but with a very high CD8$^+$ T cell response (>20% relative CD8$^+$ T cell proliferation). The characteristics of all 18 selected HLA-A*0201–restricted epitopes are summarized in Table III. Sixteen are among the high-affinity HLA-A*0201 binders ($K_D \leq 50$ nM), the majority having a binding affinity ≤ 5 nM ($n = 14$).

Validation and quantitation of M. tuberculosis *peptide-specific CD8$^+$ T cell responses in cured TB patients using tetramers*

Using HLA-A2/peptide tetramers for the 18 most frequently HLA-A*0201–restricted peptides, direct ex vivo recognition by cured TB patients' CD8$^+$ T cells was demonstrated for 16 of the 18 *M. tuberculosis* epitopes (Figs. 2, 3*A*). Of particular interest was that several epitopes were recognized by the majority of the cured TB patients: pmtb4 (A1) and pmtb15 (B182) were recognized both by 7 out of 10 cured TB patients, whereas several other peptides were recognized by a sizeable fraction of the patients as well. The epitopes studied in this paper thus constitute a significant expansion of the known antigenome for CD8$^+$ T cells during *M. tuberculosis* infection. In all individuals tested, specificity of tetramer staining was confirmed by the negative data obtained using tetramers of an irrelevant specificity (the HLA-A*0201/Ebola peptide; Fig. 3*B*), a tetramer of an irrelevant positive control specificity (the HLA-A*0201/CMV peptide, positive in many cured TB patients and healthy controls (HC) PBMC; Fig. 3*B*), as well as absence of staining among PBMC from normal, uninfected HLA-A*0201–positive donors using the same *M. tuberculosis* tetramers (Supplemental Fig. 2).

Mono-, dual-, and triple-cytokine–producing peptide-specific CD8$^+$ T cells at the single-cell level in TB

IFN-γ, IL-2, and TNF-α are relevant cytokines defining functional populations of Ag-specific CD4$^+$ and CD8$^+$ T cells (45, 46).

Table III. Characteristics of the 18 novel *M. tuberculosis* HLA-A2–restricted peptides

Peptide No.	Selection[a]	Peptide[b]	Protein Description[c]	Protein Id[d]	Gene	Rv No.[e]	Original Peptide No.	K_D (nM)[f]
pMtb1	TB-VAC	GLAGGAATA	Secreted Ag 85-B FBPB (85B) (Ag 85 complex B)	NP_216402.1	fbpB	Rv1886c	10851	308
pMtb2	BestPred	LLYDGSFAV	Hypothetical protein	NP_218356.1	NULL	Rv3839	11611	<1
pMtb3	Cons	AIYDTMQYV	ATP-dependent CIp protease proteolytic subunit	NP_216976.1	clpP2	Rv2460c	11679	<1
pMtb4	DOS/LAG	AMAGSIDLL	Probable trehalose-6-phosphate phosphatase OTSB1	YP_177855.1	otsB1	Rv2006	A-1	4
pMtb5	DOS/LAG	GMFANRWII	Probable metal cation transporter P-type ATPase	NP_216513.1	ctpF	Rv1997	A-4	27
pMtb6	Secret	YLPDPTVGV	Hypothetical protein	NP_217329.1	NULL	Rv2813	B-118	1
pMtb7	Secret	YVYPDNLPV	MCE-family protein MCE3A	YP_177852.1	mce3A	Rv1966	B-119	2
pMtb8	Secret	ALLGGLRPV	MCE-family protein MCE4B	NP_218015.1	mce4B	Rv3498c	B-130	1
pMtb9	Secret	HLDDVGFLV	Possible esterase lipoprotein LPQC	NP_217815.1	lpqC	Rv3298c	B-131	<1
pMtb10	Secret	SLIDLLHKI	MCE-family protein MCE4A	YP_177977.1	mce4A	Rv3499c	B-132	<1
pMtb11	Secret	SLRNWIATL	Possible MCE-family lipoprotein LPRL (MCE-family lipoprotein MCE2E)	NP_215107.1	lprL	Rv0593	B-134	66
pMtb12	PredSecret	FMYEGDTPL	Probable ATP-dependent helicase LHR (larger helicase-related protein)	NP_217813.1	lhr	Rv3296	B-178	<1
pMtb13	PredSecret	ALDEGLLPV	Possible conserved membrane protein	NP_218210.1	NULL	Rv3693	B-179	1
pMtb14	PredSecret	YLLADTFTV	Probable membrane-associated phospholipase C2	NP_216866.1	plcB	Rv2350c	B-181	1
pMtb15	PredSecret	WLYPGAQNL	Probable amino acid decarboxylase	YP_177889.1	NULL	Rv2531c	B-182	<1
pMtb16	TB-CD8	SLWKDGAPL	Glutamine synthetase GLNA1 (glutamine synthase)	NP_216736.1	glnA1	Rv2220	C-249	5
pMtb17	TB-CD8	KLQERLAKL	Chaperonin GroEL	NP_214954.1	groEL	Rv0440	C-250	18
pMtb18	TB-CD8	LLDSGTTSI	Secreted L-alanine dehydrogenase ALD (40 kDa Ag) (TB43)	NP_217296.1	ald	Rv2780	C-255	1

Peptide-induced T cell responses scored positive when they exceeded 10% $CD8^+$ T cell relative proliferation in multiple donors (two or more donors) or, in the case of 10–20% proliferation in one donor, were confirmed twice in the same donor. In addition, five peptides were included that induced a very strong $CD8^+$ T cell response (>20% relative proliferation) in a single donor.

[a]The type of protein the peptide is derived from (see Fig. 1).

[b]Peptide sequence.

[c]Description of protein.

[d]Protein accession number from GenBank (http://www.ncbi.nlm.nih.gov/).

[e]Rv names for *M. tuberculosis* (Tuberculist [http://genolist.pasteur.fr/TubercuList/] and Ref. 27).

[f]K_D (nM) is the binding affinity value for the epitope in nanomolars.

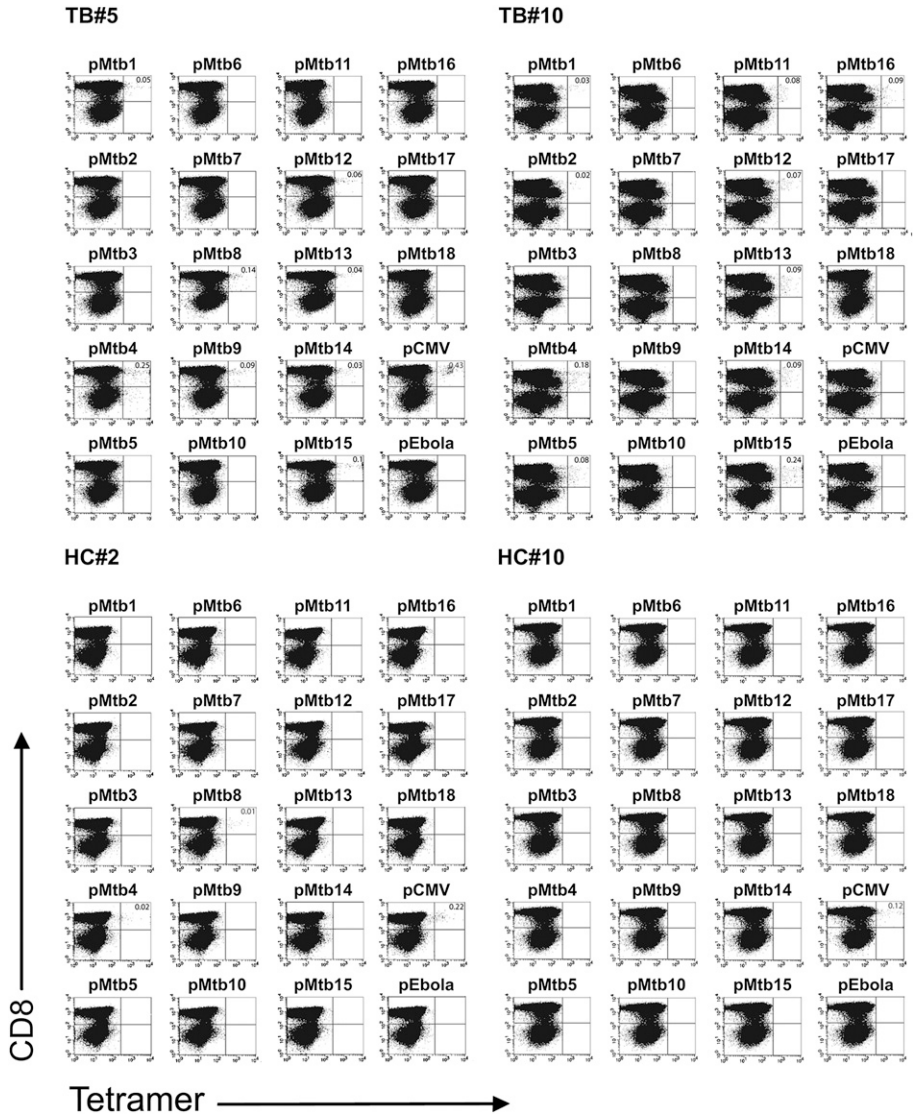

FIGURE 2. Tetramer analysis. Shown in the upper right quadrant of each FACS plot are the percentages of tetramer-positive CD8$^+$ T cells from two cured TB patients (TB#5 and TB#10) and two HC (HC#2 and HC#10) for all 18 *M. tuberculosis* peptide/HLA-A2 tetramers tested, as well as the negative control Ebola and the positive control CMV peptide. Values below 0 are not shown in the upper right quadrants. Data was analyzed by flow cytometry on an FACSCalibur and further analyzed with the use of the FlowJo software. Viable lymphocytes were gated by forward and side scatter, and the analysis was performed on ≥100,000 acquired CD8 events for each sample.

Mono-, dual-, and triple-functional CD8$^+$ T cells have been reported (45). The functionality of CD8$^+$ T cells was assessed by peptide/tetramer analysis in cured TB patients and as control HC were used. The data are shown in Fig. 4, which is a composite figure combining tetramer with cytokine expression data.

With the exception of *M. tuberculosis* peptide pMtb7 (B119) and pMtb6 (B118), single-, double-, and, in some cases, also triple-positive CD8$^+$ T cells could be detected in the cured TB patients. CMV peptide stimulation revealed responses in ≥60% of the cured TB patients, whereas the Ebola peptide induced very low if any responses as expected (all <0.2%). Of interest, some peptides induced rather strong responses (>0.3%) in the majority of TB patients, including pmtb14 (B181) (80% of the patients), pmtb15 (B182) (80%), and pmtb4 (A1) (70%). These were also the peptides that typically gave the strongest tetramer responses, such that functional and tetramer results for the specific peptides are well in agreement, as visualized in Fig. 4.

Positive tetramer staining and cytokine expression is an almost exclusive property of the cured TB patient group, with, as expected, mostly negative results in the healthy *M. tuberculosis* noninfected group (Figs. 4, 5). Control CMV-peptide/HLA-A2 tetramer CD8$^+$ T cell staining was similarly positive in the *M. tuberculosis*-infected and *M. tuberculosis* noninfected groups, whereas control Ebola-peptide/HLA-A2 tetramer staining was invariably negative.

Taken together, these results indicate that the newly identified *M. tuberculosis* epitopes and Ags are recognized by (poly)functional CD8$^+$ T cells during (control of) infection in TB. The results also reveal a strong correlation between the presence of *M. tuberculosis* peptide-tetramer–positive and polyfunctional CD8$^+$ T cells in natural *M. tuberculosis* infection in man.

Discussion

Whereas relatively much is known about the mycobacterial Ags recognized by CD4$^+$ T cells (20), surprisingly little is known about the *M. tuberculosis* Ags, which activate human CD8$^+$ T cells in TB. We applied both forward and reverse Ag discovery approaches to identify new *M. tuberculosis* epitopes for human CD8$^+$ T cells. In this study, we report 70 *M. tuberculosis* HLA-class Ia-restricted CD8$^+$ T cell-stimulating epitopes (44 HLA-A*0201, 9 HLA*0301, and 17 HLA-B*0702), of which 58 are new. The results were validated by using a panel of 20 HLA-A*0201 tetramers and by ICS, which revealed mono-, double-, and triple-functional CD8$^+$ T cell responses. There was a strong agreement between tetramer and cytokine positivity. Positive responses in the validation cohort were seen only in the cured TB patients, but absent from the

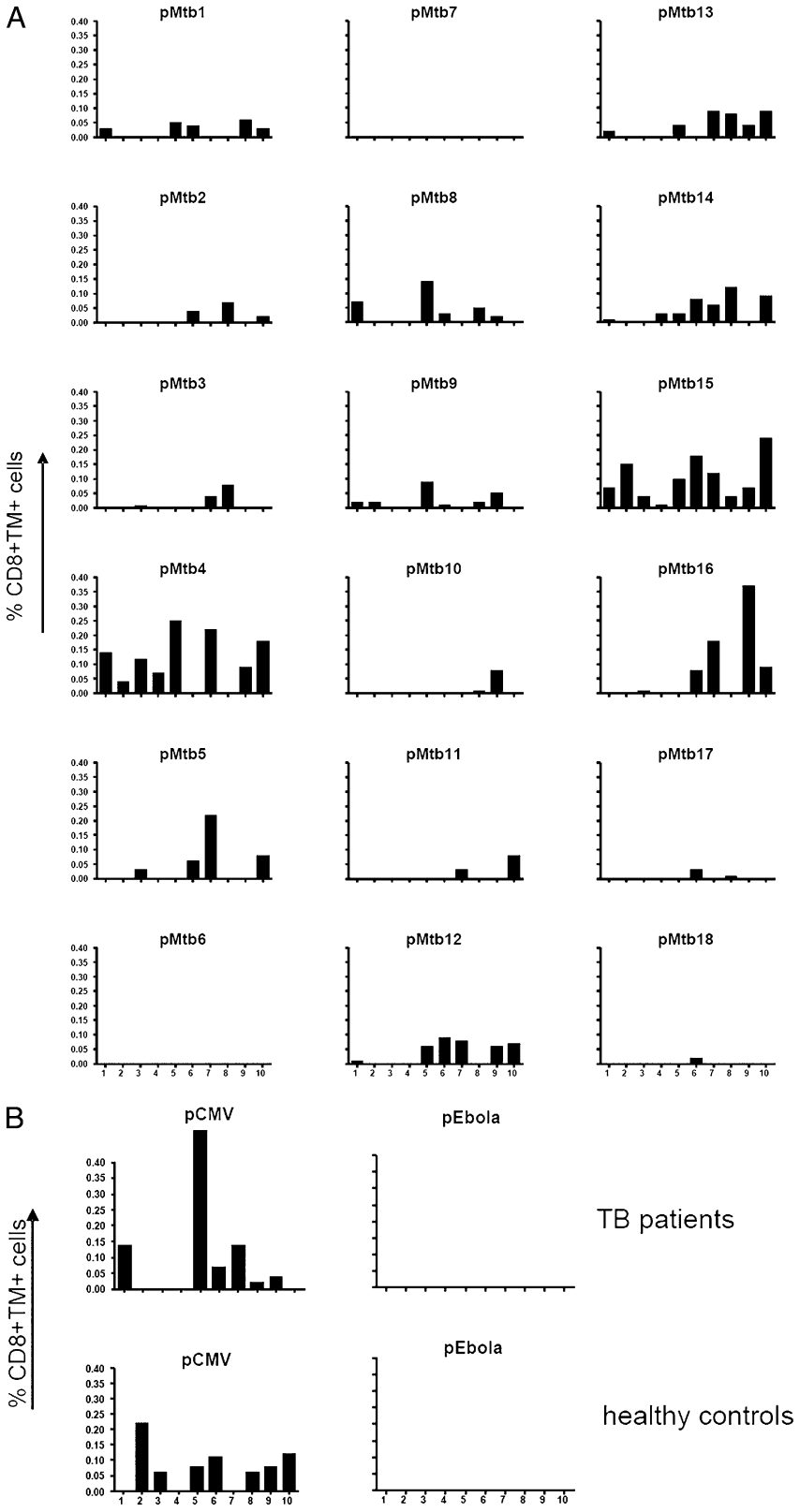

FIGURE 3. Representation of the percentage of $CD8^+$ T cells staining with each of the selected 18 *M. tuberculosis* peptide- or control Ebola or CMV peptide-loaded HLA-A2 tetramers. *A*, Tetramer staining data for all 10 HLA $A2^+$ cured TB patients' PBMC tested directly ex vivo. In brief, PBMC were cocultured for 30 min with PE-labeled tetramers, washed, stained for CD8, and analyzed on an FACSCalibur. The patient numbers, ranging from 1–10, are displayed on the *x*-axis, whereas the *y*-axis displays the percentage of positive $CD8^+$ T cells stained by the tetramer. Sixteen of the 18 selected peptides were recognized by one or more cured TB patients. The negative control Ebola peptide-tetramer was negative for all donors tested (*B*), both the cured TB patients and HC. The positive control CMV peptide-tetramer was recognized by 6 out of 10 donors in each group (*B*).

healthy non-*M. tuberculosis*-infected control individuals. In another recent study, we have reported the identification of a set of novel *M. tuberculosis* epitopes (>50) that are recognized by human $CD8^+$ T cells in the context of the nonclassical HLA class Ib molecule HLA-E (44). Those results and the ones reported in this study very significantly increase our understanding of the human immune response to *M. tuberculosis* by identifying large sets of $CD8^+$ T cell-recognized *M. tuberculosis* peptides.

We had expected newly discovered epitope frequencies to be highest for Ags from the TB vaccine Ag group and the *M. tuberculosis* $CD8^+$ T cell epitope-expressing Ag group, because these contained highly immunogenic proteins (e.g., from *M.*

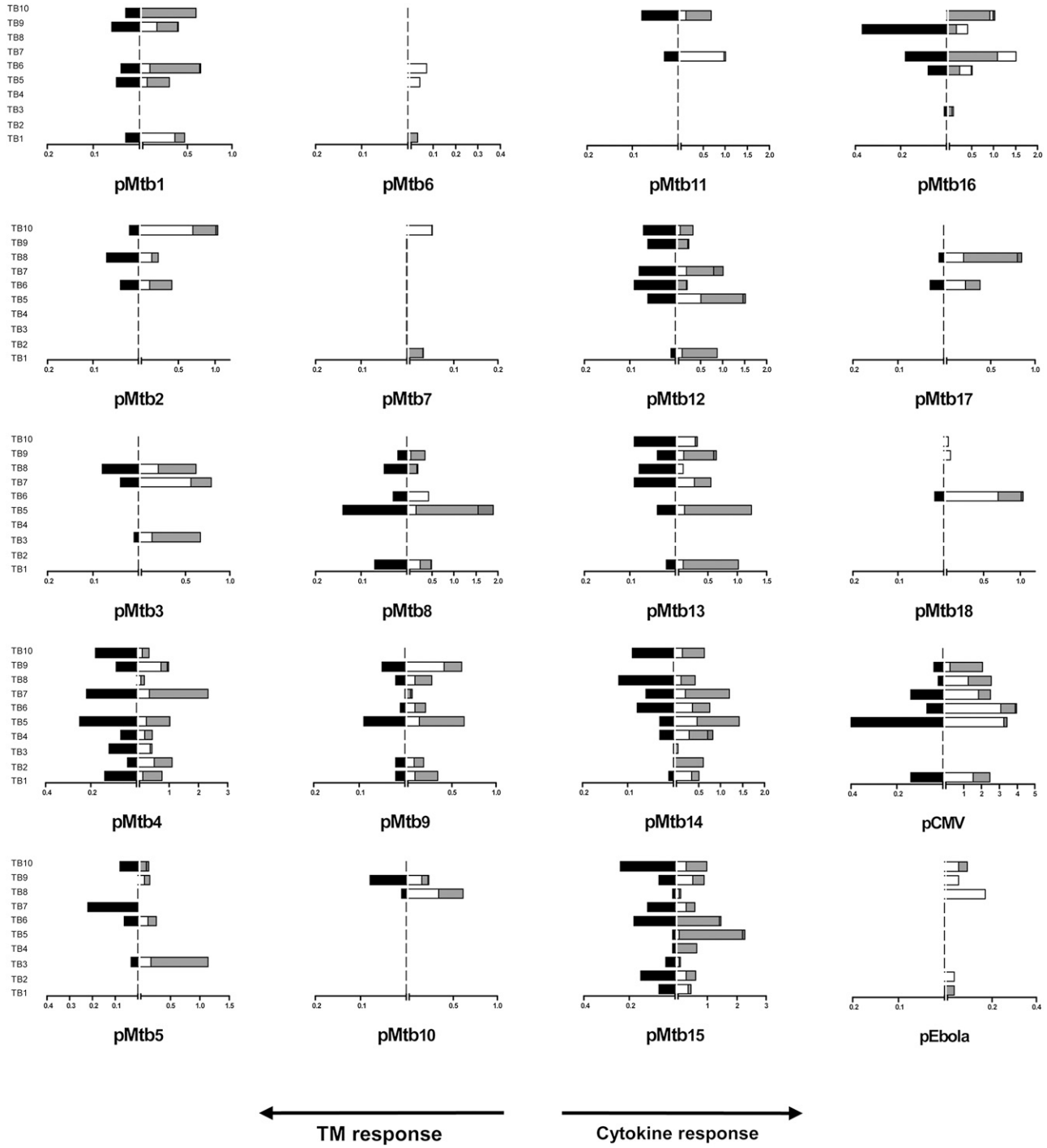

FIGURE 4. Tetramer staining and cytokine production by $CD8^+$ T cells upon peptide stimulation. The figure shows the combined tetramer staining and cytokine expression results for all 10 cured TB patients. On the *x*-axis, the percentage of tetramer$^+$ $CD8^+$ T cells is plotted on the left side of the figures (black bars), whereas the cytokine response is shown on the right. The *y*-axis shows the cured TB patients. For the cytokine production, PBMC from 10 cured HLA-$A2^+$ TB patients were cocultured for 6 h with peptide in the presence of monensin. Following incubation, cell surface staining was performed for CD8 followed by permeabilization and ICS for IFN-γ, IL-2, and TNF-α. The white bars represent the percentage of $CD8^+$ T cells producing only one cytokine: IFN-γ, IL-2, or TNF-α. The light gray bars represent the percentage of double-positive $CD8^+$ T cells (IFN-γ^+IL-2^+, IFN-γ^+TNF-α^+, and IL-2^+ TNF-α^+ cells). The dark bars represent the percentage of triple-positive $CD8^+$ T cells (IFN-γ^+IL-2^+TNF-α^+). The negative control Ebola peptide did not induce significant responses in any of the patients. The positive control CMV peptide did induce cytokine production and polyfunctional T cells in 6 out of 10 patients. This representation of the combined tetramer staining and cytokine expressions reveals the strong correlation between the presence of tetramer-positive $CD8^+$ T cells and the presence of polyfunctional $CD8^+$ T cells.

tuberculosis RD regions or culture filtrate proteins) (47–51) or proteins containing already known $CD8^+$ T cell epitopes reported by others (13, 16, 22, 45, 52–58). The known $CD8^+$ T cell epitopes were not deliberately deselected for, but our selection criteria were set to select those candidate peptide epitopes with the best predicted combined score and predicted binding affinity.

According to the Immune Epitope Database (IEDB), as of October 1, 2010, there were a total of 151 known MHC class I-

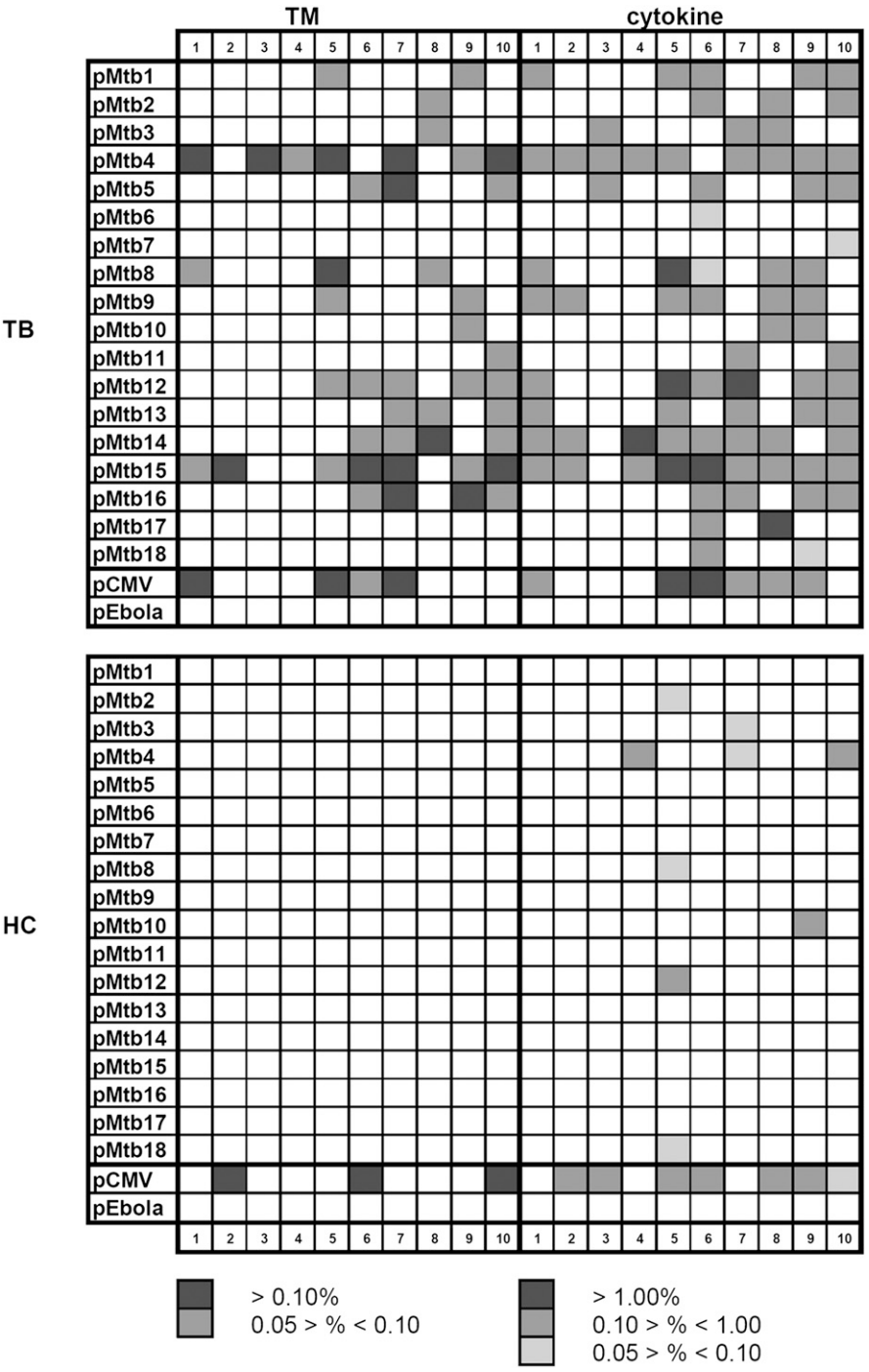

FIGURE 5. Representation of the combined tetramer staining and cytokine expression results for all cured TB patients and control individuals. In the table, results for the cured TB patients are shown in the *upper panel*, whereas those for the HC are shown in the *lower panel*. The left side shows the tetramer staining results of each individual donor with each of the 18 selected *M. tuberculosis* peptide/HLA-A2 tetramer, including the two control tetramers. White boxes, no detectable tetramer$^+$ CD8$^+$ T cells; light gray boxes, percentage of tetramer$^+$ CD8$^+$ T cells is between 0.05 and 0.10%; and dark gray boxes, percentage of tetramer$^+$ CD8$^+$ T cells is $>$0.10%. The right half of the figure shows the sum of the cytokine response. White boxes, no cytokine production could be measured; light gray boxes, the total percentage of cytokine$^+$ CD8$^+$ T cells ranges from 0.05–0.10%; dark gray boxes, the total percentage of cytokine$^+$ CD8$^+$ T cells ranges from 0.10–1.00%; and black boxes, the total percentage of cytokine$^+$ CD8$^+$ T cells is $>$1.00%).

restricted epitopes of *M. tuberculosis* (http://www.immuneepitope.org/). Ninety-three of these known epitopes were found in humans and are restricted by 6 different HLA-A and 10 HLA-B alleles. The majority were HLA-A2–restricted epitopes. Twelve of our predicted and tested peptides can be found in the IEDB and were found to be MHC class I-restricted CD8$^+$ T cell epitopes by other groups while this study was ongoing. These epitopes are highlighted in red in the arrayed tables. Four of these peptides (C228, 10853, 10861, and 10882) were confirmed as epitopes for CD8$^+$ T cells in our study, whereas the remaining eight were either not recognized at all in the study reported in this paper or did not meet the pre-established criteria for positivity: they only scored positive in one donor and/or induced $<$10% CD8$^+$ T cell proliferation. For example, the known epitope KLQERLAKL (#C250) was able to induce a very high response (55% CD8$^+$ T cell proliferation) but only in 1 of the 10 donors in our experiments. Based on this exceptionally high response, the #C250 peptide was nevertheless included for tetramer construction.

We found that a higher fraction (34%) of the predicted HLA-A2 binding *M. tuberculosis* peptides elicited proliferative CD8$^+$ T cell responses in HLA-A2$^+$ PPD-responsive donors, when compared with the peptides predicted to bind to HLA-A3 (6%) and HLA-B7 (10%). There are several possible explanations for this. One is related to the prediction server used, as NetCTL might have a poorer performance for HLA-A3 and -B7. It is also possible that fewer *M. tuberculosis* peptides are able to bind to these supertypes. However, more likely is that the lower frequency of HLA-A3–associated peptide hits is due to the larger diversity of

specificities among the alleles within the HLA-A3 supertype. Several studies have shown that peptides bind in larger numbers and at higher affinity to alleles of the B locus compared with alleles of the A and C loci (26, 59, 60). These studies therefore concluded that Ag presentation to CD8$^+$ T cells is dominated by B locus alleles. The design of epitope-based vaccines should then be directed toward HLA-B–presented peptides. Our findings do not directly support this, given the higher number of responses to *M. tuberculosis* peptides predicted to bind to HLA-A2 supertype molecules. Interestingly, a similar distribution of epitopes restricted by different supertypes was also reported by Pasquetto et al. (61), who performed a large scale screening for pox-virus epitopes. In that study, the frequencies were 30, 6, and 21% for HLA-A2, A3, and B7, respectively.

Determination of the peptide-binding affinities for the respective supertypes revealed a significant correlation between high-affinity ($<$50 nM) peptide/MHC binding and the peptides' ability to trigger CD8$^+$ T cell responses (Table I). In poxvirus systems, similar associations were reported (62, 63). Unexpectedly, however, in our study several of the nonbinding peptides (23%; 7 out of 30) were able to induce positive CD8$^+$ T cell responses (Table I). This may be because the cell-free peptide-MHC binding assay might have failed to capture key characteristics of (low-affinity) peptide/MHC binding (many other chaperones are involved next to MHC alone) or that these peptides actually bound to and were presented by HLA class I molecules other than A2/A3/B7.

We have used HLA-A2/peptide tetramers and peptide-specific single-cell multicytokine analyses to validate the above findings for the 18 most prominently recognized new *M. tuberculosis* HLA-A2–restricted epitopes. Importantly, direct ex vivo recognition by cured TB patients' CD8$^+$ T cells was found for 16 of the 18 *M. tuberculosis* epitopes (Figs. 2, 3*A*). The epitopes studied in this paper thus constitute a significant expansion of the known antigenome for CD8$^+$ T cells during *M. tuberculosis* infection. Of particular interest was that several epitopes were recognized by the majority of the cured TB patients: pmtb4, pmtb15, and pmtb14 were recognized by 70–80% of the cured TB patients, whereas several other peptides were recognized by a sizeable fraction of the patients as well. Of additional relevance, the peptides were not recognized by *M. tuberculosis* noninfected healthy individuals. In all individuals tested, specificity of tetramer staining was confirmed by lack of binding of irrelevant (Ebola) peptide/tetramers, whereas an unrelated positive control (CMV) peptide/tetramer visualized positive CD8$^+$ T cells both in multiple cured TB patients as well as uninfected HLA-A*0201–positive donors. These results indicate that several of these *M. tuberculosis* peptides are highly immunogenic in HLA-A2–positive TB patients. We do not know why these peptides appear particularly immunodominant, but speculate this is due to preferential processing/presentation separate from MHC binding affinity, as these peptides displayed high but not exceptional binding affinity compared with others.

A final aim of our study was to assess the capability of *M. tuberculosis*-specific CD8$^+$ T cells to produce IFN-γ, IL-2, and TNF-α as an indication of multifunctionality, which has been proposed as a possible correlate of protective immunity (45). However, recent work, among others from two of our groups in this study, reported multifunctional T cells to be associated with TB disease (64). Regardless, the functionality of CD8$^+$ T cells was detected using peptide/tetramers, and identical-peptide stimulated PBMC of the same cured TB patients and controls were studied to determine fractions of specific CD8$^+$ T cells producing IFN-γ, IL-2, and/or TNF-α at the single-cell level. With very few exceptions, single-, double-, and, in some cases, also triple-positive CD8$^+$ T cells could be detected in cured TB patients, but not controls. In line with the tetramer data, CMV peptide stimulation revealed responses in $\geq$60% of the cured TB patients as well as controls, whereas the Ebola peptide induced very low if any responses as expected. Of interest was that some peptides induced strong responses in the majority of the cured TB patients, including pmtb14 (B181) (80% of the patients), pmtb15 (B182) (80%), and pmtb4 (A1) (70%). These same peptides also showed the strongest tetramer responses. Thus, multifunctional cytokine production and tetramer results for the specific *M. tuberculosis* peptides are well in agreement (Fig. 4).

Although more extensive phenotyping of *M. tuberculosis*-specific IFN-γ–, IL-2–, and TNF-α–secreting CD8$^+$ T cells was beyond the scope of this study, previous studies have identified a relationship between the function and phenotype of memory CD4$^+$ T cells and have proposed that the IL-2 only-secreting cells are typical of central memory T cells that persist after Ag clearance, whereas the IFN-γ/IL-2– and IFN-γ only-secreting T cells are typical of effector memory T cells (42).

In conclusion, we have identified 70 *M. tuberculosis*-specific CD8$^+$ T cell epitopes in healthy PPD$^+$ individuals. Fifty-eight of these epitopes were novel and have not previously been described by others. This is a considerable expansion of the existing list of known *M. tuberculosis* CD8$^+$ T cell epitopes in the IEDB. Furthermore, our results indicate that the newly identified epitopes and Ags are recognized by (poly)functional CD8$^+$ T cells during (control of) infection in TB. Finally, the results reveal a strong correlation between the presence of *M. tuberculosis* peptide-tetramer–positive and polyfunctional CD8$^+$ T cells in natural *M. tuberculosis* infection in humans. These results provide a wealth of new *M. tuberculosis* Ags that may provide targets for TB vaccine development, particularly in the view of mounting evidence that CD8 T cells are important in controlling TB. Moreover, the epitopes we have identified may provide novel tools for monitoring the specific CD8$^+$ T cell response in TB cohorts, providing potential novel TB biomarkers, analogous to what we have reported for a limited set of other CD8$^+$ T cell epitopes in TB recently (15).

Disclosures

The authors have no financial conflicts of interest.

References

1. World Health Organization. 2008. WHO Report 2008: Global Tuberculosis Control—Surveillance, Planning, Financing. World Health Organization, Geneva, Switzerland.
2. Ottenhoff, T. H. 2009. Overcoming the global crisis: "yes, we can", but also for TB ...? *Eur. J. Immunol.* 39: 2014–2020.
3. Raviglione, M. C., and I. M. Smith. 2007. XDR tuberculosis—implications for global public health. *N. Engl. J. Med.* 356: 656–v659.
4. Shah, N. S., A. Wright, G. H. Bai, L. Barrera, F. Boulahbal, N. Martín-Casabona, F. Drobniewski, C. Gilpin, M. Havelková, R. Lepe, et al. 2007. Worldwide emergence of extensively drug-resistant tuberculosis. *Emerg. Infect. Dis.* 13: 380–387.
5. Caruso, A. M., N. Serbina, E. Klein, K. Triebold, B. R. Bloom, and J. L. Flynn. 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J. Immunol.* 162: 5407–5416.
6. Pathan, A. A., K. A. Wilkinson, P. Klenerman, H. McShane, R. N. Davidson, G. Pasvol, A. V. Hill, and A. Lalvani. 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.
7. Wallace, J. M., N. I. Hansen, L. Lavange, J. Glassroth, B. L. Browdy, M. J. Rosen, P. A. Kvale, B. T. Mangura, L. B. Reichman, P. C. Hopewell; Pulmonary Complications of HIV Infection Study Group. 1997. Respiratory disease trends in the Pulmonary Complications of HIV Infection Study cohort. *Am. J. Respir. Crit. Care Med.* 155: 72–80.
8. Ab, B. K., R. Kiessling, J. D. Van Embden, J. E. Thole, D. S. Kumararatne, P. Pisa, A. Wondimu, and T. H. Ottenhoff. 1990. 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.* 20: 369–377.
9. Flynn, J. L., M. M. Goldstein, K. J. Triebold, B. Koller, and B. R. Bloom. 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA* 89: 12013–12017.
10. Kamath, A. B., J. Woodworth, X. Xiong, C. Taylor, Y. Weng, and S. M. Behar. 2004. Cytolytic CD8+ T cells recognizing CFP10 are recruited to the lung after *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 200: 1479–1489.
11. Pathan, A. A., K. A. Wilkinson, R. J. Wilkinson, M. Latif, H. McShane, G. Pasvol, A. V. Hill, and A. Lalvani. 2000. High frequencies of circulating IFN-gamma-secreting CD8 cytotoxic T cells specific for a novel MHC class I-restricted *Mycobacterium tuberculosis* epitope in *M. tuberculosis*-infected subjects without disease. *Eur. J. Immunol.* 30: 2713–2721.
12. McShane, H., S. Behboudi, N. Goonetilleke, R. Brookes, and A. V. Hill. 2002. Protective immunity against *Mycobacterium tuberculosis* induced by dendritic cells pulsed with both CD8(+)- and CD4(+)-T-cell epitopes from antigen 85A. *Infect. Immun.* 70: 1623–1626.
13. Klein, M. R., S. M. Smith, A. S. Hammond, G. S. Ogg, A. S. King, J. Vekemans, A. Jaye, P. T. Lukey, and K. P. McAdam. 2001. HLA-B*35-restricted CD8 T cell epitopes in the antigen 85 complex of *Mycobacterium tuberculosis. J. Infect. Dis.* 183: 928–934.
14. Smith, S. M., R. Brookes, M. R. Klein, A. S. Malin, P. T. Lukey, A. S. King, G. S. Ogg, A. V. Hill, and H. M. Dockrell. 2000. Human CD8+ CTL specific for the mycobacterial major secreted antigen 85A. *J. Immunol.* 165: 7088–7095.
15. Caccamo, N., G. Guggino, S. Meraviglia, G. Gelsomino, P. Di Carlo, L. Titone, M. Bocchino, D. Galati, A. Matarese, J. Nouta, et al. 2009. Analysis of *Mycobacterium tuberculosis*-specific CD8 T-cells in patients with active tuberculosis and in individuals with latent infection. *PLoS ONE* 4: e5528.
16. Cho, S., V. Mehra, S. Thoma-Uszynski, S. Stenger, N. Serbina, R. J. Mazzaccaro, J. L. Flynn, P. F. Barnes, S. Southwood, E. Celis, et al. 2000. Antimicrobial activity of MHC class I-restricted CD8+ T cells in human tuberculosis. *Proc. Natl. Acad. Sci. USA* 97: 12210–12215.
17. Kaufmann, S. H., and A. J. McMichael. 2005. Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. *Nat. Med.* 11(4 Suppl): S33–S44.
18. Lalvani, A., R. Brookes, R. J. Wilkinson, A. S. Malin, A. A. Pathan, P. Andersen, H. Dockrell, G. Pasvol, and A. V. Hill. 1998. Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for *Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA* 95: 270–275.
19. Klein, M. R., and A. Fox. 2001. *Mycobacterium*-specific human CD8 T cell responses. *Arch. Immunol. Ther. Exp. (Warsz.)* 49: 379–389.
20. Ottenhoff, T. H. M., D. A. Lewinsohn, and D. M. Lewinsohn. 2008. *Human CD4 and CD8 T Cell Responses to Mycobacterium tuberculosis: Antigen Specificity, Function, Implications and Applications.* Wiley-VCH Verlag GmbH, Weinheim, Germany.
21. Stenger, S., R. J. Mazzaccaro, K. Uyemura, S. Cho, P. F. Barnes, J. P. Rosat, A. Sette, M. B. Brenner, S. A. Porcelli, B. R. Bloom, and R. L. Modlin. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science* 276: 1684–1687.
22. Tully, G., C. Kortsik, H. Höhn, I. Zehbe, W. E. Hitzler, C. Neukirch, K. Freitag, K. Kayser, and M. J. Maeurer. 2005. Highly focused T cell responses in latent human pulmonary *Mycobacterium tuberculosis* infection. *J. Immunol.* 174: 2174–2184.
23. Gambón-Deza, F., M. Pacheco Carracedo, T. Cerdá Mota, and J. Montes Santiago. 1995. Lymphocyte populations during tuberculosis infection: V beta repertoires. *Infect. Immun.* 63: 1235–1240.
24. Caccamo, N., S. Meraviglia, C. La Mendola, G. Guggino, F. Dieli, and A. Salerno. 2006. Phenotypical and functional analysis of memory and effector human CD8 T cells specific for mycobacterial antigens. *J. Immunol.* 177: 1780–1785.
25. Jacobsen, M., A. K. Detjen, H. Mueller, A. Gutschmidt, S. Leitner, U. Wahn, K. Magdorf, and S. H. Kaufmann. 2007. Clonal expansion of CD8+ effector T cells in childhood tuberculosis. *J. Immunol.* 179: 1331–1339.
26. Lewinsohn, D. A., E. Winata, G. M. Swarbrick, K. E. Tanner, M. S. Cook, M. D. Null, M. E. Cansler, A. Sette, J. Sidney, and D. M. Lewinsohn. 2007. Immunodominant tuberculosis CD8 antigens preferentially restricted by HLA-B. *PLoS Pathog.* 3: 1240–1249.
27. Geluk, A., K. E. van Meijgaarden, K. L. Franken, J. W. Drijfhout, S. D'Souza, A. Necker, K. Huygen, and T. H. Ottenhoff. 2000. 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.* 165: 6463–6471.
28. Leyten, E. M., M. Y. Lin, K. L. Franken, A. H. Friggen, C. Prins, K. E. van Meijgaarden, M. I. Voskuil, K. Weldingh, P. Andersen, G. K. Schoolnik, 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.
29. Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, III et al. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393: 537–544.
30. Blythe, M. J., Q. Zhang, K. Vaughan, R. de Castro, Jr., N. Salimi, H. H. Bui, D. M. Lewinsohn, J. D. Ernst, B. Peters, and A. Sette. 2007. An analysis of the epitope knowledge related to Mycobacteria. *Immunome Res.* 3: 10.
31. Sette, A., and J. Sidney. 1999. Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* 50: 201–212.
32. Larsen, M. V., C. Lundegaard, K. Lamberth, S. Buus, S. Brunak, O. Lund, and M. Nielsen. 2005. An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions. *Eur. J. Immunol.* 35: 2295–2303.
33. Yusim, K., C. Kesmir, B. Gaschen, M. M. Addo, M. Altfeld, S. Brunak, A. Chigaev, V. Detours, and B. T. Korber. 2002. Clustering patterns of cytotoxic T-lymphocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation. *J. Virol.* 76: 8757–8768.
34. Welsh, R. M., and R. S. Fujinami. 2007. Pathogenic epitopes, heterologous immunity and vaccine design. *Nat. Rev. Microbiol.* 5: 555–563.
35. Voskuil, M. I., K. C. Visconti, and G. K. Schoolnik. 2004. *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis (Edinb.)* 84: 218–227.
36. Ottenhoff, T. H., J. B. Haanen, A. Geluk, T. Mutis, B. K. Ab, J. E. Thole, W. C. van Schooten, P. J. van den Elsen, and R. R. de Vries. 1991. Regulation of mycobacterial heat-shock protein-reactive T cells by HLA class II molecules: lessons from leprosy. *Immunol. Rev.* 121: 171–191.
37. Mustafa, A. S. 2001. Biotechnology in the development of new vaccines and diagnostic reagents against tuberculosis. *Curr. Pharm. Biotechnol.* 2: 157–173.
38. Wiker, H. G., M. A. Wilson, and G. K. Schoolnik. 2000. Extracytoplasmic proteins of *Mycobacterium tuberculosis* - mature secreted proteins often start with aspartic acid and proline. *Microbiology* 146: 1525–1533.
39. Bendtsen, J. D., L. Kiemer, A. Fausbøll, and S. Brunak. 2005. Non-classical protein secretion in bacteria. *BMC Microbiol.* 5: 58.
40. Sylvester-Hvid, C., N. Kristensen, T. Blicher, H. Ferré, S. L. Lauemøller, X. A. Wolf, K. Lamberth, M. H. Nissen, L. O. Pedersen, and S. Buus. 2002. Establishment of a quantitative ELISA capable of determining peptide - MHC class I interaction. *Tissue Antigens* 59: 251–258.
41. Leisner, C., N. Loeth, K. Lamberth, S. Justesen, C. Sylvester-Hvid, E. G. Schmidt, M. Claesson, S. Buus, and A. Stryhn. 2008. One-pot, mix-and-read peptide-MHC tetramers. *PloS One* 3: e1678.
42. Armitage, P., G. Berry, and J. N. S. Matthews. 2002. *Statistical Methods in Medical Research.* Blackwell Science, Hoboken, NJ.
43. Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W. M. Kast, C. J. Melief, C. Oseroff, L. Yuan, J. Ruppert, et al. 1994. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J. Immunol.* 153: 5586–5592.
44. Joosten, S. A., K. E. van Meijgaarden, P. C. van Weeren, F. Kazi, A. Geluk, N. D. Savage, J. W. Drijfhout, D. R. Flower, W. A. Hanekom, M. R. Klein, and T. H. Ottenhoff. 2010. *Mycobacterium tuberculosis* peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS Pathog.* 6: e1000782.
45. Seder, R. A., P. A. Darrah, and M. Roederer. 2008. T-cell quality in memory and protection: implications for vaccine design. *Nat. Rev. Immunol.* 8: 247–258.
46. Yewdell, J. W., and J. R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu. Rev. Immunol.* 17: 51–88.
47. Dietrich, J., C. Aagaard, R. Leah, A. W. Olsen, A. Stryhn, T. M. Doherty, and P. Andersen. 2005. Exchanging ESAT6 with TB10.4 in an Ag85B fusion molecule-based tuberculosis subunit vaccine: efficient protection and ESAT6-based sensitive monitoring of vaccine efficacy. *J. Immunol.* 174: 6332–6339.
48. Horwitz, M. A., G. Harth, B. J. Dillon, and S. Maslesa-Galic. 2005. Enhancing the protective efficacy of *Mycobacterium* bovis BCG vaccination against tuberculosis by boosting with the *Mycobacterium tuberculosis* major secretory protein. *Infect. Immun.* 73: 4676–4683.
49. Olsen, A. W., A. Williams, L. M. Okkels, G. Hatch, and P. Andersen. 2004. Protective effect of a tuberculosis subunit vaccine based on a fusion of antigen 85B and ESAT-6 in the aerosol guinea pig model. *Infect. Immun.* 72: 6148–6150.
50. Roupie, V., M. Romano, L. Zhang, H. Korf, M. Y. Lin, K. L. Franken, T. H. Ottenhoff, M. R. Klein, and K. Huygen. 2007. Immunogenicity of eight dormancy regulon-encoded proteins of *Mycobacterium tuberculosis* in DNA-vaccinated and tuberculosis-infected mice. *Infect. Immun.* 75: 941–949.
51. Skeiky, Y. A., M. R. Alderson, P. J. Ovendale, J. A. Guderian, L. Brandt, D. C. Dillon, A. Campos-Neto, Y. Lobet, W. Dalemans, I. M. Orme, and S. G. Reed. 2004. Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. *J. Immunol.* 172: 7618–7628.
53. Dong, Y., S. Demaria, X. Sun, F. R. Santori, B. M. Jesdale, A. S. De Groot, W. N. Rom, and Y. Bushkin. 2004. HLA-A2-restricted CD8+-cytotoxic-T-cell responses to novel epitopes in *Mycobacterium tuberculosis* superoxide dismutase, alanine dehydrogenase, and glutamine synthetase. *Infect. Immun.* 72: 2412–2415.
54. Flyer, D. C., V. Ramakrishna, C. Miller, H. Myers, M. McDaniel, K. Root, C. Flournoy, V. H. Engelhard, D. H. Canaday, J. A. Marto, et al. 2002. Identification by mass spectrometry of CD8(+)-T-cell *Mycobacterium tuberculosis* epitopes within the Rv0341 gene product. *Infect. Immun.* 70: 2926–2932.
55. Hammond, A. S., M. R. Klein, T. Corrah, A. Fox, A. Jaye, K. P. McAdam, and R. H. Brookes. 2005. *Mycobacterium tuberculosis* genome-wide screen exposes multiple CD8 T cell epitopes. *Clin. Exp. Immunol.* 140: 109–116.
56. Lewinsohn, D. A., R. A. Lines, and D. M. Lewinsohn. 2002. Human dendritic cells presenting adenovirally expressed antigen elicit *Mycobacterium tuberculosis*—specific CD8+ T cells. *Am. J. Respir. Crit. Care Med.* 166: 843–848.
57. Marmiesse, M., P. Brodin, C. Buchrieser, C. Gutierrez, N. Simoes, V. Vincent, P. Glaser, S. T. Cole, and R. Brosch. 2004. Macro-array and bioinformatic analyses reveal mycobacterial 'core' genes, variation in the ESAT-6 gene family

and new phylogenetic markers for the *Mycobacterium tuberculosis* complex. *Microbiology* 150: 483–496.
58. Mohagheghpour, N., D. Gammon, L. M. Kawamura, A. van Vollenhoven, C. J. Benike, and E. G. Engleman. 1998. CTL response to *Mycobacterium tuberculosis*: identification of an immunogenic epitope in the 19-kDa lipoprotein. *J. Immunol.* 161: 2400–2406.
59. Bihl, F., N. Frahm, L. Di Giammarino, J. Sidney, M. John, K. Yusim, T. Woodberry, K. Sango, H. S. Hewitt, L. Henry, et al. 2006. Impact of HLA-B alleles, epitope binding affinity, functional avidity, and viral coinfection on the immunodominance of virus-specific CTL responses. *J. Immunol.* 176: 4094–4101.
60. Vani, J., M. S. Shaila, N. R. Chandra, and R. Nayak. 2006. A combined immunoinformatics and structure-based modeling approach for prediction of T cell epitopes of secretory proteins of *Mycobacterium tuberculosis. Microbes Infect.* 8: 738–746.
61. Pasquetto, V., H. H. Bui, R. Giannino, C. Banh, F. Mirza, J. Sidney, C. Oseroff, D. C. Tscharke, K. Irvine, J. R. Bennink, et al. 2005. HLA-A*0201, HLA-A*1101, and HLA-B*0702 transgenic mice recognize numerous poxvirus determinants from a wide variety of viral gene products. [Published erratum appears in 2005 *J. Immunol* 175: 8440.] *J. Immunol.* 175: 5504–5515.
62. Assarsson, E., J. Sidney, C. Oseroff, V. Pasquetto, H. H. Bui, N. Frahm, C. Brander, B. Peters, H. Grey, and A. Sette. 2007. A quantitative analysis of the variables affecting the repertoire of T cell specificities recognized after vaccinia virus infection. *J. Immunol.* 178: 7890–7901.
63. Tang, S. T., M. Wang, K. Lamberth, M. Harndahl, M. H. Dziegiel, M. H. Claesson, S. Buus, and O. Lund. 2008. MHC-I-restricted epitopes conserved among variola and other related orthopoxviruses are recognized by T cells 30 years after vaccination. *Arch. Virol.* 153: 1833–1844.
64. Caccamo, N., G. Guggino, S. A. Joosten, G. Gelsomino, P. Di Carlo, L. Titone, D. Galati, M. Bocchino, A. Matarese, A. Salerno, et al. 2010. Multifunctional CD4(+) T cells correlate with active *Mycobacterium tuberculosis* infection. *Eur. J. Immunol.* 40: 2211–2220.