Supplemental Materials and methods

Ethics Statement

The study was approved by the Institutional Review Board of Shenzhen Third People’s Hospital (Permit Number: 2010008) and informed consent was obtained from each participant or from their relatives if the patient could not provide consent.

Patients and samples

All clinical samples were collected from Shenzhen Third People’s Hospital, China and Beijing Chest Hospital-Beijing Tuberculosis and Thoracic Tumor Research Institute, China. Seven TB lung tissue samples were freshly collected post-surgery from patients diagnosed with pulmonary TB via roentgenographic imaging, sputum bacteriological examination and pathological section assessment. Meanwhile, three lung tissues from patients with fungal infections and two from lung adenocarcinoma were collected as controls (see Table I). Furthermore, peripheral blood mononuclear cells (PBMCs) were collected from 123 active TB patients and 34 healthy donors for function evaluation, including ELISPOT, T cell proliferation and cytotoxicity assay respectively. All subjects were over 18 years of age, sero-negative for HIV and HBV, and did not have autoimmune diseases. All TB patients were diagnosed and classified according to the 1990 edition of Diagnostic Standards and Classification of Tuberculosis. Diagnosis also included clinical signs and symptoms and radiographic findings (chest x-ray and/or HRCT). Moreover, all recruited individuals were also examined for antigen specific IFN-γ secretion using a well-established ELISPOT kit,
these patients were examined as positive IFN-γ ELISPOT, whose SFCs is >30 by either antigen A or B, while HD donors were <30.

**Laboratory Methods**

*Tissue lysate generation, chaperone protein enrichment and purification, and peptides stripping.*

Lung tissue was homogenized and centrifuged as previously described. In brief, 1g lung tissue (TB granulomatous lesion, tumor or fungal infection tissue) was homogenized at 4°C in a glass homogenizer and then was centrifuged at 10,000g for 30 min at 4°C. Then the supernatant was centrifuged at high speed of 100,000g for 90 min at 4°C, with subsequently supernatant being dialyzed against 5mM Tris-Cl (pH7.4), 5mM NaCl and 0.05% detergent (Triton X-100, Triton X-114, Nonidet P-40). Samples of homogenate from same group were mixed in equal parts and 25 mg of protein prepared for isoelectric focusing by adding urea to 6M, the detergents Triton X-100, Triton X-114 and Igepal each to 0.5%, ampholytes (pH3-10, Bio Rad Laboratories, USA) to 5%, and water to a total volume of 60ml in a Rotofor device (Bio Rad Laboratories, USA). FS-IEF was conducted for 4 hours at 15W constant power while the apparatus was cooled with re-circulating water at 4°C; the anode compartment contained 0.1 M H₃PO₄, while the cathode compartment contained 0.1 M NaOH. This resulted in 20 obtained fractions to be examined by SDS-PAGE and western blot assay for identifying Hsp70 and Gp96 immune reactive species. Fractions contained Hsp70 and Gp96 were pooled and concentrated. Peptides
associated with chaperones were stripped by trifluoroacetic acid elution (0.2% trifluoroacetic acid (TFA), pH 2.0). The low molecular mass fractions were separated by Amicon Ultra Centrifugal Filter Units (10KD cutoff; Merck Millipore) and then dried for subsequent Mass spectrometer (MS).

**Mass spectrometer and Epitope prediction.**

Peptide samples were resuspended in 0.1% formic acid and analyzed with an RP C18 capillary LC column (Michrom Bioresources). The eluted gradient was 5–30% buffer (0.1% formic acid, 99.9% ACN; flow rate, 0.5 μl/min) for 30 min. MS data were acquired using a LTQ-Orbitrap Velos mass spectrometer (Bremen, Germany); these were used for screening peptides. The MS/MS spectra were used to confirm origin of peptide candidates according to a merged database including protein sequences of Mycobacterium tuberculosis 02_1987 (*Taxonomy ID*: 515616), Mycobacterium tuberculosis 94_M4241A (*Taxonomy ID*: 515615), Mycobacterium tuberculosis T85 (*Taxonomy ID*: 520141), Mycobacterium tuberculosis str. Haarlem (*Taxonomy ID*: 395095), Mycobacterium tuberculosis F11 (*Taxonomy ID*: 336982) and Mycobacterium tuberculosis GM1503 (*Taxonomy ID*: 537209). All the sequences were downloaded from UniProtKB taxonomic databases on September, 2011. Bioworks software version 3.3.1 sp1 was used for MS/MS spectra. Other nine clinical *Mtb* strains (Table S1) whole protein sequences (NCBI and the UniProtKB taxonomic databases) were also used for making alignment of identified peptide sequence. Carbamidomethylation (C) was set as a fixed modification. The searches were performed using a peptide tolerance of 10 ppm and a product ion tolerance of
0.8 Da. The original mgf files as well as original raw files were submitted to the “PeptideAtlas” public website. (Official URL for this dataset: http://www.peptideatlas.org/PASS/PASS00634. Username: PASS00634. Password: MV5865m.) MHC-I T cell epitope prediction for HLA-A2 (*0201, *0206), HLA-A3 (*0301, *1101) and MHC-II epitope prediction for HLA-DRB1(01:01) were completed by online tools: IEDB (http://www.immuneepitope.org), SYFPEITHI (http://www.syfpeithi.de/) and MHCPred version 2.0 (http://www.ddg-pharmfac.net/mhc.pred/MHCPred/). For IEDB analysis Predicted Percentile Rank was given. The strong binders have been retrieved for low Percentile Rank, while the IC50 was also low. Combing with the Predicted IC50 Value from MHCPred version 2.0 and Predicted Score from SYFPEITHI methods, better MHC-I peptide binders were chosen. Likely, MHC-II T cell epitopes for HLA-DRB1*01:01 were predicted by IEDB and SYFPEITHI. All selected peptides (purity>95%) were synthesized by China Peptides Co. Ltd, Shanghai, China. High-performance liquid chromatography (HPLC) and mass spectrometry were used to analyze and verify the purity and molecular weight of these peptides.

*Experimental groups for function evaluating.*

In the following experiments, several groups were determined to examine functions of selected peptides. The normalized names were listed as below for better understanding: (1) full length of obtained peptide group: named as ABCp, Ch60p, CTp, FADp, NADp and PKAp (sequences shown in Table IIA); (2) pool of predicted MHC-I epitopes group: named as ABCp-I, Ch60p-I, CTp-I, FADp-I, NADp-I and PKAp-I
(sequences shown in Table IIIA); (3) pool of predicted MHC-II epitopes group: named ABCp-II, Ch60p-II, CTp-II, FADp-II, NADp-II and PKAp-II (sequences as Table IIIB) and (4) single MHC-I or MHC-II peptide, addressed by full length peptide’s name with a postfix of epitope sequence number (eg: PKAp9-17).

*Ex vivo IFN-γ ELISPOT.*

Testing was completed as previously described with a final concentration of antigen was 10μg/ml. Briefly, PBMCs from 116 TB patients or 28 healthy donors were obtained from whole blood respectively by centrifugation over Ficoll-Hypaque density gradient (Ficoll-Paque Plus; Amersham Biosciences). Cells were resuspended in Lympho-Spot medium (U-CyTech Bioscience, The Netherlands). 2×10^5 cells each well were seeded in duplicate in 96-well plates pre-coated with anti-IFN-γ capture monoclonal antibody (eBioscience). Cells were stimulated with the different antigens for 24 h at 37°C, 5% CO₂. PBMCs in medium alone or stimulated with phytohemagglutinin (Sigma) at 2.5μg/ml and antigen A and B were used as negative or positive controls, respectively. Biotinylated anti-IFN-γ detection monoclonal antibody (eBioscience) was added for 4 h, and followed by the addition of streptavidin-alkaline phosphatase conjugate (Pierce Biotechnology) for 1 h. After a wash, the nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma) chromogenic substrate was added. The individual spots were counted by use of an automated image analysis system ELISPOT reader (BioReader 4000 Pro-X; Biosys, Germany).
**T cell proliferation assay.**

PBMCs from four TB patients and four healthy donors were obtained from whole blood as described before and stained with CFSE (Cell Trace™ CFSE Cell Proliferation Kit, Invitrogen) before antigen stimulation. 3×10^5/well cells were incubated with or without antigens (10μg/ml) in U-bottom 96-well plate. Cells treated with OVA_{323-339} (10μg/ml) or untreated cells and an anti-CD3/CD28 mAbs cocktail (1μg/ml) were used as negative and positive controls respectively. After 4 days incubation in a 37°C, 5% CO2 incubator, cells were stained with PE-anti-CD4 and APC-anti-CD8 antibody (BD Pharmingen) and detected by FACS subsequently. For the FACS data analysis, SSC and FSC were used for gating lymphocytes. CD4^+ or CD8^+ cells proliferating cells were determined by low intensity of CFSE by compared to negative controls.

**Peptide binding assay.**

The T2 cell line was used as a reference to detect the peptide binding ability of HLA-A2. Briefly, 1×10^5/well T2 cells were incubate in 96-well plate with single peptide (MHC-I epitope, sequence shown in Table IIIA); or OVA_{257-264} and CAP-1 as controls, at a concentration 50μM. T2 cells cultured without peptides served as background control. After 18 hours incubation, the T2 cells were harvested and incubated with PE-conjugated anti-HLA-A2 antibody (BD Pharmingen) for 40 min at 4°C. Then cells were washed twice with PBS and analyzed by flow cytometer.

**PKA_r-targeted cytotoxicity assay and cytokine detection.**
CTL-induced apoptosis was made using FITC-anti-human active caspase-3 antibodies (BD) as previously described. Ratio of cytotoxicity were determined with the percentage of caspase-3-positive cells in the PKH-26-labeled target cell population. To examine PKA$_p$-induced cytotoxicity, *ex vivo* PKA$_p$-stimulated cells from healthy donors and patients were both used. First, PBMCs (2 ×10$^6$/ml, each) from HLA-A2$^+$ healthy donors were cultured with the pool of PKA$_p$ MHC-I epitopes (10μg/ml of each peptide) in RPMI 1640 medium containing 10% FCS and 20 U/ml recombinant human IL-2 (rhIL-2) in 24-well culture plate for 3 days. Cells were rested for another 4 days in completed culture medium with rhIL-2 (10U/ml). Another two rounds antigen stimulation was repeated and after the third time of stimulation, supernatant was obtained for cytokine detection and cells were used as effector cells for cytotoxic assay. A panel of 17 cytokines was measured using the Bio-Plex Pro Cytokine Assay, 17-Plex Group I kit (Bio-Rad Laboratories, USA) and data were analyzed using Luminex xPONENT software (Luminex Corporation, USA). In the cytotoxicity assay, T2 cells as target cells were pulsed overnight with the pool of PKA$_p$ MHC-I epitopes or the peptide control OVA$_{257-264}$ respectively. Cells were labeled with a fluorescent dye PKH-26 (sigma) according to the manufacturer’s protocol. Target cells were cultured with effector T cells at a ratio of 1:10 or 1:40 in 96-well, V-bottomed plate at 37°C. After 4 hours, all cells were harvest and incubated with FITC-anti-human active caspase-3 substrate for 30 minutes at room temperature in the dark. The percentage of caspase-3-positive cells in PKH-26-labeled target cell population was determined by the following formula: \[ \%\text{caspase-3 staining} = \frac{(\text{caspase-3}^+\text{PKH-26}^+ \text{cells})}{\text{total PKH-26}^+ \text{cells}} \]
(caspase-3’PKH-26’+caspase-3’PKH-26’) \times 100. In parallel, PKA_{p}\text{-stimulated cells from TB patients were also used for examining PKA}_{p}\text{-induced cytotoxicity and checking cytokine expression. Briefly, PBMCs from active TB patients were collected and incubated with antigens at 10\mu g/ml for 3 days (one round stimulation), supernatant were collected and cells were harvest respectively for cytokine detection and CTL assays as described above.}

*Mice immunization and intracellular IFN-\gamma-staining assay.*

Synthetic peptides (PKA_{p}, PKA_{p9-17}, PKA_{p19-27} and PKA_{p21-28}) or OVA protein conjugated peptides (PKA_{p}-OVA, PKA_{p9-17}-OVA, PKA_{p19-27}-OVA and PKA_{p21-28}-OVA) were synthesized by China Peptides Co. Ltd (Shanghai, China). CpG ODN 1826: 5’-TCCATGACGTTCCTGACGTT-3’ were synthesized with a nuclease-resistant phosphorothioate backbone (Invitrogen (Life Technologies), China) as adjuvant. Immunization was applied three times with antigens at 2 week intervals. Each group of three C57BL/6 female mice (8 weeks old) were respectively immunized intramuscular (i.m.) groups: (1) PKA_{p} peptide (PKA_{p}), (2) OVA conjugated PKA_{p} (PKA_{p}-OVA); (3) pool of PKA_{p} MHC-I epitopes (PKA_{p-I}); (4) OVA conjugated PKA_{p9-17}, OVA conjugated PKA_{p19-27} and OVA conjugated PKA_{p21-29}, pool (PKA_{p-I-OVA}); (5) PKA_{p} with CpG adjuvant at 5% (PKA_{p}+CpG); (6) pool of PKA_{p} MHC-I epitopes with CpG adjuvant at 5% (PKA_{p-I}+CpG); (7) 5% CpG alone (CpG) and (8) OVA protein alone (OVA); the concentration of each peptide/protein was 100\mu g/ml. Normal mice without immunization were the naïve control group. Seven days after the third immunization, mice were sacrificed and cell suspensions from lungs and spleens were collected
respectively. After antigen stimulation and BFA added incubation overnight, cells were washed with PBS. Then Fc receptors were blocked with anti-mouse CD16/CD32 antibodies (BD Pharmingen). IFN-γ intracellular staining of CD4⁺ or CD8⁺ cells was done according to the manufacture’s protocol. Anti-mouse IFN-γ (FITC), CD4 (PerCP-cy5.5) and CD8 (APC) antibodies were obtained from BD Pharmingen. Data were analyzed using a FACS Canto II flow cytometer.

**Hematoxylin-eosin staining of tissue sections.**

Lungs and spleens were fixed in 10% neutral buffered formalin. After paraffin embedding, 3-mm thick sections were cut, deparaffinized, rehydrated in graded alcohol water mixtures, and stained with hematoxylin and eosin (HE) for histomorphological analysis according to the manufacturer’s protocol. Photos were taken with an OLYMPUS BX61camera (Japan).

**Data analysis**

GraphPad Prism 5 (GraphPad software) and SPSS software were used to generate plots and perform statistical analyses on the data. One-way analysis of variance (ANOVA) and Newman-Keuls (compare all pairs of columns) served as a post-hoc test and the Mann-Whitney U test methods were used for statistical analysis. A p-value<0.05 was considered significant. FlowJo 7.6.1 was used to analyze the FACS data and export the figures.