A Novel Tuberculosis Antigen Identified from Human Tuberculosis Granulomas*

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Tuberculosis is a global infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). Although novel *Mtb* biomarkers from both the pathogen and host have been studied, more breakthroughs are still needed to meet different clinic requirements. In an effort to identify *Mtb* antigens, chaperone-peptide complexes were purified from TB infected lungs using free-solution isoelectric focusing combined with high resolution LTQ Orbitrap Velos mass spectrometry. Antigen specific cellular immune responses in vitro were then examined. Those efforts led to the identification of six *Mtb* peptides only identified in Tuberculosis lung samples and that were not found in the control samples. Additionally, antigen-specific IFN-γ secretion, T-cell proliferation, cytokine expression, and a cytotoxic assay were also evaluated. Among the peptides isolated, we identified a 34 amino acid peptide named PKAp belonging to a serine/threonine–protein kinase, as being able to generate *Mtb*-specific cellular immune responses as noted by elevated antigen-specific cytokine secretion levels, increased CD8+ T-cell proliferation and a strong cytotoxic lymphocyte (CTL) response. Moreover, the immune stimulating abilities of PKAp were further validated in vivo, with target peptide immunized mice showing an increased cellular IFN-γ in both the lungs and spleen without causing immunopathogenesis. In conclusion, we identified novel functional *Mtb* antigens directly from the granulomatous lesions of Tuberculosis patients, inducing not only significant antigen-specific IFN-γ secretion but also a marked cytotoxic lymphocyte functional response. These findings indicated that PKAp has potential as a novel antigen biomarker for vaccine development. *Molecular & Cellular Proteomics* 14: 10.1074/mcp.M114.045237, 1093–1103, 2015.

*Mycobacterium tuberculosis* (*Mtb*), the infectious agent that causes tuberculosis, is associated with an estimated 1.4 million deaths per year and remains a major global health concern (1). Current research and diagnostics have focused on antigen screening and biomarker discovery, with most antigen screening methods focused on the bacterial pathogen itself, with less focus on the *Mtb* infected host (2). The pathogenic progression of TB occurs in the lungs, making the characterization of any functional antigens existing in the lungs during infection potentially useful for immunotherapy or vaccine development. The immune response to an *Mtb* infection results in the formation of a granuloma that initially contains bacterial expansion, but may fail to eliminate the pathogen (3, 4). This immune response brings with the possibility of identifying *Mtb* functional antigens in the lung tissue and to gain a clearer understanding of the immune mechanisms (5, 6). Although it has been well studied that a T-cell mediated adaptive immune response plays a central role during *Mtb* infection and is crucial in both protection and pathogenesis, a better understanding of the antigen induced immune response and correlations to pathogenicity is necessary (2, 7).

It has been reported that heat shock proteins (such as the HSP70 family members) and other chaperones such as Gp96 can specifically bind many hydrophobic sequences, enabling them to bind foreign peptides associated with intra-

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1 The abbreviations used are: *Mtb*, *Mycobacterium tuberculosis*; TB, Tuberculosis; LC, patients with lung adenocarcinoma; FP, patients with fungal infections; FS-IEF, free-solution isoelectric focusing; CTL, cytotoxic lymphocyte; HSP, heat shock proteins; LC-MS/MS, Liquid chromatography/tandem mass spectrometry; HLA, human leukocyte antigen; CFSE, carboxyfluorescein succinimidyl amino ester; HE stain, Hematoxylin-eosin stain; ABC, hypothetical drugs-transport transmembrane ATP-binding protein ABC transporter protein; Ch60, 60 kDa chaperonin protein; CT, Conserved transmembrane protein; FAD, Oxidoreductase fadB5 protein; NAD, NADH dehydrogenase subunit D protein; PKA, transmembrane serine/threonine-protein kinase A pknA protein.
Antigen Identified in TB Granulomas

cellular bacterial or viral challenge (8), such as Gp96 associating with a HBV-specific peptide (9). Previous studies have shown that chaperone-peptide complexes can induce a disease-specific immune response (10–12), with the gp96-peptide complex from H37Rv infected cells able to induce a protective antigen specific immune response (13). Currently, no Mtb chaperone-associated peptides have been isolated directly from patients, thus the present study explores the possible existence of these complexes in TB lung tissue.

To achieve this objective, the free-solution isoelectric focusing (FS-IEF) technique, which has been reported to enrich chaperones in cell lysates or tissue samples, was combined with Linear Trap Quadrupole (LTQ) OrbitrapVelos mass spectrometry, which was used to identify the associated Mtb peptides. Using these techniques, we obtained chaperone-rich cell lysates from the granulomatous lung lesions of active TB patients and identified six Mtb-associated peptides not noted in the control samples. Among them, a peptide (PKAp) derived from Mtb Protein Kinase A not only contributed to significant antigen-specific IFN-γ secretion, but also contributed to CTL function and T-cell proliferation. Importantly, murine immunization with PKAp derived peptides elicited an antigen-specific cellular activation without the occurrence of immune pathogenesis.

EXPERIMENTAL PROCEDURES

Patients and Samples—All clinical samples were collected from the Shenzhen Third People’s Hospital, China and the 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 (14), with three lung samples obtained from patients with fungal infections and two from patients with lung adenocarcinomas used as controls (Table I). Additionally, peripheral blood mononuclear cells (PBMCs) were collected from 123 active TB patients and 34 healthy donors for function evaluation via enzyme-linked immunospot (ELISPOT), T-cell proliferation and cytotoxicity assays. Whole blood was collected from healthy donors and patients were used. Detection of CTL-induced apoptosis was made using cleaved caspase-3 antibodies as previously described with the percentage of caspase-3-positive cells within the PKH-26-labeled target cell population used to determine the ratio of cytotoxicity (23, 24). Supernatants from three times antigen stimulated PBMCs from HLA-A2* healthy donors and of one time stimulated PBMCs from TB donors were collected for cytokine detection. A panel of 17 cytokines was measured using the Bio-Plex Pro cytokine assay and 17-Plex Group I kit (Bio-Rad) according to the manufacturer’s protocols, and the data analyzed using the Luminex xPONENT software.

Mice Immunization, Intracellular IFN-γ Staining Assay, and Hemeoxytin-eosin Staining of Tissue Sections—Immunizations were administered with different antigens a total of three times in 2 week intervals. Each group was comprised of three C57BL/6 female mice (8 weeks old), with immunizations administered intramuscular (i.m.) with or without CpG ODN 1826: 5′-TTATGACGTTCCTGACGTT-3′ adjuvant. Seven days after the third immunization, the mice were sacrificed, lung and spleen cellular suspensions were collected. IFN-γ intracellular staining of CD4+ or CD8+ cells was done according to the manufacturer’s protocol and analyzed via flow cytometer. Additionally, lung and spleen tissues were sectioned, fixed, and stained with hematoxylin and eosin (HE) for histomorphological analysis.

Data Analysis—Plots and statistical analysis were performed using the GraphPad Prism 5 (GraphPad software) and SPSS software. One-way analysis of variance (ANOVA) and Newman-Keuls served as a post-hoc test, with the Mann-Whitney U test used for statistical analysis and a p value <0.05 considered significant. FlowJo 7.6.1 was used to analyze the FACS data and export the figures.

More details of Material Methods are in the supplemental Materials and Methods.

RESULTS

Mtb Specific Chaperone (Hsp70/Gp96)-associated Peptides Identified in Lung Tissues—Clinical pulmonary TB samples, along with control lung adenocarcinoma and pulmonary fungal infection samples (Table I), were lysed to verify the presence of Hsp70 and Gp96 (Fig. 1A). Lysates were sub-
Antigen Identified in TB Granulomas

| Group | PTB | FP | LC |
|-------|-----|----|----|
| Number | 7 | 3 | 2 |
| Female/male | 2/5 | 2/1 | 1/1 |
| Average age | 43 | 47 | 61 |
| Acid-fast stain test | + | – | – |
| Clinical diagnosis | Pulmonary tuberculosis | Pulmonary fungal infection | Lung adenocarcinoma |

*a Stands for a group as control to PTB (pulmonary TB).

Table I: Study sample information

Fig. 1. Hsp70 or Gp96 chaperone proteins in lung tissues and concentrated by free-solution isoelectric focusing (FS-IEF). A. Western blot analysis of Hsp70 (left square) and Gp96 (right square) from tissue lysates from pulmonary TB (n = 7), fungal infection (n = 3), or lung cancer (n = 2) patients, with arrows indicating the Hsp70 or Gp96 bands. B. All 20 fractions were further analyzed via SDS-PAGE in two gel plates, with the gel pictures combined and separately shown in different gray squares with the pH values of each fraction under each gel picture. C. Fractions from number 1 to number 9 analyzed via Western blot were shown with arrows indicating Hsp70 or Gp96 bands.

Liquid chromatography/tandem mass spectrometry (LC-MS/MS) was used to screen for TB-specific peptides from a merged database of clinical Mtb strains with the Bioworks software. Protein sequences of all Mtb strains were obtained from the NCBI and UniProtKB taxonomic databases to form an indexing database for MS data processing. There were two Beijing strains and other clinical strains from East Asia, Europe, America, West Africa, the Philippines, and rim of the Indian Ocean (27) as shown in supplemental Table S1. LTQ-OrbitrapVelos mass spectrometry was used to identify peptides with a high confidence as defined by an XC (X Core) parameter above 2.0 and a Delta Cn above 0.1 (Table II), with a whole indexed peptide rank (Rsp) of 1 was used to select target peptides. Of the six selected peptides, four peptides, Ch60p, FADp, NADp, and PKAp, were identified and fully meet the three parameters, whereas two peptides, ABCp and CTp, had an Rsp value of 1, but had a lower XC value (Table II, more detail are shown in supplemental Table S2). For LC and FP control samples, there were no related Mtb peptides identified.

Prediction of T-cell Epitopes and Related Immune Functions of Selected Peptides—To evaluate whether the selected peptides contain a useful MHC-I or MHC-II epitope, the T-cell epitope of each peptide was predicted by IEDB, SYFPEITHI, and MHCpred version 2.0. Herein, epitopes for HLA-A2 (*0201) and HLA-A3 (*1101) (MHC-I) and HLA-DR1 (*0101) (MHC-II) were predicted and studied because of these super-types being reported as the most common worldwide and present in an elevated frequency in China (28–30). Selected
peptide regions within the six identified *Mtb* peptides that displayed a low percentile rank or low predicted IC50 value or high SYFPEITHI score were assigned a high predictive score, meaning that the associated peptide could provide an effective binding to specific HLA supertype molecules (Table IIIA). MHC-II epitopes with high predictive scores were also determined using the IEDB and SYFPEITHI methods (Table IIIB). All six peptides including the MHC epitopes were then synthesized for immune function evaluation. An *Mtb* antigen-specific IFN-γ ELISPOT assay is broadly used for indicating *Mtb* infection, with the response to specific *Mtb* antigen (ESAT-6, CFP-10 with or without Tb7.7) used in the IFN-γ release assays as a biomarker. Thus, selected peptide stimulation of PBMCs from diagnosed TB patients were evaluated via ELISPOT (Totally 116 TB PBMCs when using ESAT-6 peptides pool as Ag-A stimulator the Spot Forming cells, SFCs average was 92.7 and CFP-10 peptides pool as Ag-B stimulator the average was 133.4. Either Ag-A or Ag-B SFCs >30, it means TB positive. (Supplemental Fig. S2). The results showed that the PKAp peptide-stimulated PBMCs from clinical TB patients secreted significantly higher (p<0.01) levels of IFN-γ compare with other peptide candidates and control samples (1.9–4 fold higher; Fig. 2A). Based on the epitope peptide predictions, MHC-I and MHC-II epitope peptides were also analyzed via ELISPOT assay, with the pooled PKAp MHC-I epitopes inducing a greater number of IFN-γ secreting cells compared with the other samples (p<0.001, Fig. 2B: 9.4–14.8 fold higher). The pooled PKAp MHC-II epitope peptides also induced higher IFN-γ levels compared with other candidates groups, but showed comparable IFN-γ lev-
PKA<sub>p</sub> MHC-I Epitope Peptides Promote Significant CD8<sup>+</sup> T-cell Proliferation—T-cell activation triggers proliferation and cytokine production. To measure proliferation CFSE-labeled lymphocytes were stimulated with the pooled MHC-I epitopes or MHC-II epitopes (sequences identified in Table IIIA and IIIb) to promote CD4<sup>+</sup>/CD8<sup>+</sup> T-cell proliferation. Following FACS analysis, we found that PKA<sub>p</sub>-derived peptides displayed significant activation abilities on CD8<sup>+</sup> T-cell proliferation. The pooled PKA<sub>p</sub> MHC-I epitopes (PKA<sub>p-I</sub>, with a 21.5% higher proliferation rate compared with normal controls) and the pooled PKA<sub>p</sub> MHC-II epitopes (PKA<sub>p-II</sub>, with 17.5% higher proliferation rate compared with normal controls) significantly induced CD8<sup>+</sup> T-cell proliferation (Fig. 3, CD8<sup>+</sup> cells selected), with these groups displaying significant differences (p < 0.05) when compared with the HD control and other peptide-treated groups. Furthermore, although the pooled FAD MHC-II epitopes (FAD-II, with a 9.6% higher proliferation rate compared with normal controls) generated a marked increase in proliferation, no significant differences were noted. For CD4<sup>+</sup> T cells, the proliferation changes were not significant (data not shown). These data indicate that PKA<sub>p</sub> peptides may mainly contribute to activating CD8<sup>+</sup> T-cell functions.

The PKA<sub>p</sub> MHC-I Epitope Shows Higher HLA-A*0201 Affinity, Higher CD8<sup>+</sup> T-cell Proliferation, and a Strong CTL Response—As the PKA<sub>p</sub> peptide appeared to be significantly implicated in CD8<sup>+</sup> T-cell activation, the cellular functions of the predicted PKA<sub>p</sub> MHC-I epitopes were examined. To evaluate the peptide binding affinity to MHC molecules, the T2 cell line was used, whose HLA-A2 cell surface molecules could be stabilized and maintain significantly longer half-lives via peptide binding. The MHC-I epitopes PKA<sub>p</sub>-9–17, PKA<sub>p</sub>-19–27, and PKA<sub>p</sub>-21–29 had the highest affinity levels (FI<sub>p</sub> = 2.27, 3.21, 1.72; Table IV), whereas the other examined peptides had an FI score <1 indicating low affinity.

Next, antigen-specific proliferation and cytotoxicity was examined showing that the MHC-I epitopes PKA<sub>p</sub>-19–27 and PKA<sub>p</sub>-21–29 stimulated significant higher CD8<sup>+</sup> T-cell proliferation in TB samples relative to healthy controls (increased 20.8% and 10.8%, respectively, Fig. 4A), whereas no proliferation was noted in CD4<sup>+</sup> T cells (data not shown). These findings were consistent with the results showing that both MHC-I epitopes had higher HLA-A2 affinity (Table IV). Next, an ex vivo PKA<sub>p</sub>-antigen induced CTL assay was performed following three-rounds of pooled PKA<sub>p</sub> MHC-I epitope stimulation, with cells from HLA-A2 healthy donors used as effector cells and T2 cells used as target cells. Anti-Caspase-3 antibody staining followed by FACS was used to measure antigen-specific cytotoxic ability, with the PKH-26 and CFSE double-positive cell percentage indicating the antigen-specific CTL induced apoptosis. The pooled PKA<sub>p</sub> MHC-I epitopes activated effector cells from HDs (at effector/target ratios of 10:1 and 40:1) induced cytotoxicity ~six and four times higher in target cells respectively than induced by OVA<sub>257–264</sub> peptide control (Fig. 4B). Additionally, pooled
PKAp-pool associated MHC-I Epitopes Promote Cytokine Production—We further examined cytokine secretion levels in cells from HD and TB patients PBMCs following stimulation with the pooled PKAp-MHC-I epitopes. Levels of IL-6, IFN-γ, TNF-α, GM-CSF, G-CSF, and IL-17 in the TB group were significantly higher compared with unstimulated patient PBMCs (Fig. 5A). Similar results were obtained from HD samples following three rounds of antigen stimulation, showing IL-6, IFN-γ, GM-CSF, and G-CSF levels to be significant higher, whereas TNF-α, and IL-17 levels were not significantly different (Fig. 5B). These data are consistent with our previous study (31) that showed IL-6, GM-CSF, and G-CSF cytokines to have significantly higher plasma levels in TB patients and H37Rv lysate supernatants upon PBMCs or PFMCs stimulation, indicating that the PKAp-MHC-I epitopes induced an Mtbb antigen-specific cellular immune response.

C57BL/6 Mice Immunized with PKAp-MHC-I Epitopes Showed an Increased Cellular IFN-γ Expression—To evaluate PKAp-associated immunological functions in vivo, we immunized eight groups of mice: Group 1 and 2, immunization with peptides alone without OVA protein conjugation or CPG adjuvant, including PKAp peptide (PKAp) and pooled PKAp-MHC-I epitopes (PKAp-I); Group 3 and 4, peptides conjugated with OVA protein, including PKAp-OVA protein (PKAp-OVA); OVA conjugated with three PKAp-MHC-I epitopes (PKAp19–27, PKAp21–29, and PKAp19–29, PKAp21–29, PKAp-I-OVA); Group 5 and 6: administration with or without Cpg adjuvant (PKAp+Cpg or PKAp-I+Cpg). C57BL/6 mice were immunized three times in 2 week intervals with immunization with OVA protein alone or CpG alone used as controls (Group 7 and 8). Mice were sacrificed 7 days after the third immunization, lung and spleen secretions from CD4+ and CD8+ cells harvested and cellular IFN-γ secretions from CD4+ and CD8+ cells examined. The results showed that the mice immunized with the pooled PKAp epmopes (PKAp-I) had increased IFN-γ expression in both lung and spleen CD4+ and

PKAp-derived MHC-I epitope-activated cells from TB patient PBMCs were also used for the CTL assay, with only one-round of stimulation with the pooled PKAp-MHC-I epitopes or PKAp19–27 peptide carried out prior to effector cell collection. Pooled PKAp-MHC-I epitopes and PKAp19–27 peptide on target cells led to significantly higher levels of cytotoxicity relative to the OVA peptide controls, at effector/target ratios of 10:1 (Fig. 4C, 4D). The presence of PKAp19–27 on target cells increased cytotoxicity about seven times over that of the OVA257–264 control, and about four times higher with the pooled PKAp peptides. PKAp19–27 apoptosis was highly elevated, suggesting that it is an epitope candidate for the activation of CD8+ T-cell functions. Granzyme B (GZMB) is present in the granules of CTLs and is involved in targeted cellular apoptosis; thus GZMB expression levels in TB and HD PBMCs were examined following pooled MHC-I epitope stimulation via quantitative real-time PCR (qRT-PCR). Only the pooled PKAp-MHC-I epitopes increased GZMB expression significantly compared with the other group epitope peptides and HD controls (supplemental Fig. S3).
**FIG. 4.** PKA$_\alpha$ MHC-I epitopes stimulate CD8$^+$ T-cell proliferation and CTL response. A, PBMCs from HD ($n = 4$) or TB patients ($n = 4$) were CFSE-labeled and incubated with PKA$_{9-17}$, PKA$_{19-27}$, or PKA$_{21-29}$. After 96 h, the cells were harvested and stained with APC-anti-CD8 antibodies and evaluated by FACS. Horizontal bars represent median values, boxes represent the interquartile range (25–75%) and whiskers represent the highest and the lowest values.

B, Ex vivo CTL assay: HLA-A2 positive HD PBMCs ($n = 2$) were stimulated with three-rounds of pooled PKA$_\alpha$ MHC-I epitopes and collected as effector cells. Antigen pre-treated T2 cells were used as target cells and stained with PKH26. Ratio differences between effector and target cells were examined, with the average cytotoxicity ratios displayed. HLA-A2 TB patient ($n = 3$) PBMCs were stimulated once with, C, pooled PKA$_\alpha$ MHC-I epitopes, or D, PKA$_{19-27}$ peptide with the cytotoxicity ability examined. The associated number displayed the average cytotoxicity ratio of antigen-stimulated T2 cells. Mann-Whitney $U$ test methods, * $p < 0.05$.

*Antigen Identified in TB Granulomas*
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In this study, new peptide antigens were identified through chaperone-peptide complex targeting, with previous studies indicating that chaperones can carry antigen-specific peptides (13). Although antigen-specific peptides associated with chaperone proteins have been reported in some infectious disease such as HBV (9), whether Mtb peptides associated with chaperones from TB patients had not yet been examined. Therefore, we screened Mtb-peptides from chaperone complexes harvested from the damaged lungs TB patients, with lung cancer and fungi infected patients serving as controls. First, chaperone/peptide complexes were enriched from tissue lysates using FS-IEF via a Rotofor cell to capture Hsp70 and Gp96, which are two chaperones reported to carry peptides (13). Although antigen-specific peptides associated with chaperones from TB patients had not yet been examined. Therefore, we screened Mtb-peptides from chaperone complexes harvested from the damaged lungs TB patients, with lung cancer and fungi infected patients serving as controls. First, chaperone/peptide complexes were enriched from tissue lysates using FS-IEF via a Rotofor cell to capture Hsp70 and Gp96, which are two chaperones reported to carry peptides. Only human chaperones were detected, without Mtb-derived Hsp70 in tissue lysate (Data not shown). This led to the identification of six Mtb peptides from the lungs of TB patients.

CD8$^+$ T cells (Fig. 6A, 6B), with the addition of OVA protein conjugation or CpG adjuvant enhancing the response. Mice immunized with the PKAp peptide alone only showed an increased IFN-γ secretion in lung CD4$^+$ T cells but not in spleen cells, with the addition of conjugated OVA protein only enhancing IFN-γ secretions in lung CD4$^+$ and CD8$^+$ T cells. These results identified candidate PKAp epitope peptides (PKAp$_{9-17}$, PKAp$_{19-27}$, and PKAp$_{21-28}$) that can act as potential antigen stimulators in vivo. To exclude the possibility of PKAp-antigen induced lung tissue damage, lung and spleen samples were obtained from the immunized mice and histologically examined via HE stain. These stains showed no pathogenic damage in lungs (supplemental Fig. S4) or spleen (data not shown), suggesting that the antigen activation is not pathological in vivo.

**DISCUSSION**

A proper preventive or therapeutic vaccine is urgently needed for TB control, with BCG being the only vaccine in clinic use and its protective efficacy controversial. According to a 2013 WHO report, there are 12 TB vaccine candidates in clinical trials, including one for immunotherapy (1), with antigen based vaccines being limited. However, the most hopeful candidate vaccine, MVA85A, was proven unable to confer significant protection against TB or Mtb infection in phase 2b trials (32). Thus numerous unsolved problems relating to TB vaccine development remain. Current antigen targeted research has focused on the pathogen using such approaches as genomic DNA screening from a field strain of Mtb, genome-wide ORF-protein screenings or the use of membrane proteins from *Mycobacterium bovis* to screen for human T-cell stimulatory activity (33–35). Besides, Jungblut et al. have elucidated antigens of *Borrelia garinii* by two-dimensional electrophoresis (2-DE) and immunoblotting with sera of patients based on antigen specific antibodies (36). However, tuberculosis is a cellular immune response mediated disease, including antigen specific IFN-γ and CD8$^+$ T-cell mediated cytotoxic response. This is well-known that antibody-mediated immune response may be dispensable in protective immunity in Tuberculosis. Moreover, aim of our study is to obtain Mtb antigens directly from damaged lung tissue, which may be involved in inducing strong adaptive immunity. According to previous studies, we believe the strategy on combining enrichment of chaperone/peptide complex via free-solution isoelectric focusing (FS-IEF) and analysis by mass spectrometry technique could be a proper way to quickly find functional antigens in directly mediating cellular immune response in the lung. Therefore, both proteomic and immunological analysis were utilized to identify functional Mtb antigens naturally existing in the lungs of TB patients in this study. This approach generating fractionated chaperone protein-based samples, six Mtb-related peptides found only in TB lungs, but not in control samples. Of these, peptides from PKAp, a serine/threonine-protein kinase, were found to stimulate T-cell proliferation and antigen specific IFN-γ secretion. Importantly, the PKAp MHC-I epitope was found to induce Mtb specific CD8$^+$ T-cell cytotoxic functions ex vivo and induce elevated IFN-γ/CD4$^+$ or IFN-γ/CD8$^+$ cell frequencies in vivo without inducing lung tissue immunopathogenesis in immunized mice.

In this study, new peptide antigens were identified through chaperone-peptide complex targeting, with previous studies indicating that chaperones can carry antigen-specific peptides (13). Although antigen-specific peptides associated with chaperone proteins have been reported in some infectious disease such as HBV (9), whether Mtb peptides associated with chaperones from TB patients had not yet been examined. Therefore, we screened Mtb-peptides from chaperone complexes harvested from the damaged lungs TB patients, with lung cancer and fungi infected patients serving as controls. First, chaperone/peptide complexes were enriched from tissue lysates using FS-IEF via a Rotofor cell to capture Hsp70 and Gp96, which are two chaperones reported to carry peptides. Only human chaperones were detected, without Mtb-derived Hsp70 in tissue lysate (Data not shown). This led to the identification of six Mtb peptides from the lungs of TB patients.

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patients, which were importantly not present in the lung control samples.

A cellular immune response to Mtb is indispensable in disease development and pathogenesis. CD4$^+$ T cells have been highlighted in numerous research efforts regarding their importance in protection against TB by secreting high levels of IFN-γ and other cytokines. Our results showed that PKA peptide immunization can increase IFN-γ secretions in CD4$^+$ T cells in both lung and spleen of mice. Moreover, recent studies have found that an effective Mtb immunity depends on both CD4$^+$ and CD8$^+$ T cells, with recent attention focusing on the role of MHC-I-restrict CD8$^+$ T cells as means to design an effective vaccine or preventative therapy (37, 38). In these studies, both ex vivo and in vivo data support that CD8$^+$ T cells can generate antigen specific CTL functions to suppress bacterial growth (39, 40). Furthermore, cancer research has demonstrated that chaperon-peptide complexes can stimulate antigen specific T-cell proliferation, CTL activation, dendritic cell activation, and promote regulatory T cells inactivation, thus enabling the development of a protective immune response (41–43). It has also been reported that a Gp96-Mtb peptide complex based vaccination could confer

Fig. 6. CD4$^+$ and CD8$^+$ T-cell intracellular IFN-γ detection. A, Mixed lung suspension cells, or B, Splenocytes from different mice immunization groups (n = 3 of each immunization group) were separated and activated with antigen overnight. IFN-γ intracellular staining was carried out and detected by FACS. Plot numbers reflect the percentage of IFN-γ$^+$CD4$^+$ or IFN-γ$^+$CD4$^+$ T cells. The arrows means higher level detected groups.
Mtb resistance in mice, but the associated peptides remain unknown (13). The present study found that not only single PKA peptide could increase IFN-γ expression, but also the ex vivo combining of Hsp70 and the PKA peptide effectively increased cellular IFN-γ expression in TB patients (data not shown), suggesting that the chaperone-peptide complex participates in eliciting an antigen-specific immune response against an Mtb infection. Furthermore, MHC-I and MHC-II (HLA-A2 (*0201), A3 (*1101), and HLA-DR*0101 supertypes) T-cell epitopes from selected peptides were examined. Among these peptide candidates, interestingly, PKA, and its MHC-I epitopes induced increased T-cell IFN-γ secretion levels. To our knowledge, this is the first report of the immune function of the PKA (Rv0015c), a serine/threonine-protein kinase (44, 45). This candidate PKA peptide is 34 amino acids long, highly hydrophobic and is part of the transmembrane domain, and yet is still able to activate a T-cell response. When examining the PKA MHC-I epitope, it was found to possess the ability to induce an antigen specific CTL immune response leading to an increased secretion of IL-6, IFN-γ, TNF-α, G-CSF, GM-CSF, and IL-17. Previous studies examining Mtb infected mice have indicated that CD8+ T cells contributed to the successful resolution of an Mtb infection by releasing IFN, thus killing cells harboring the bacilli (46, 47). Therefore, a PKA induced CD8+ T-cell response may confer protection or elicit an immune response in the presence of bacteria, making it a potential antigen for a novel TB-related vaccine. To exclude potential effect of PKA induced pathogenesis, C57BL/6 mice were immunized with PKA epitope peptides to evaluate PKA in vivo antigenicity. The results of both ex vivo and in vivo experimentation showed that the PKA epitope peptides increased cellular IFN-γ secretions in the lungs and spleen without causing immune pathogenesis. It has been well-studied that secreted Mtb proteins such as early secretory antigen target-6 (ESAT-6), Antigen 85 (Ag85), and culture filtrate protein-10 (CFP-10) can induce a potentially strong CD4+ mediated immunity in both mice and humans (48). Although few publications have reported on the functions on CD8+ T cells, their presence is clearly required to confer Mtb protection. Some reports have delineated that the effector mechanisms of CD8+ T cells might contribute to the control of Mtb infection, and once these cells are activated, their release of cytokines or cytotoxic molecules may cause target cell apoptosis (49). The identification of PKA may expand the types of Mtb antigen, which could provide a novel functional membrane antigen different from the currently explored secreted protein antigens. Collectively, our study reveals the potential benefits of PKA on not only a TB biomarker but also vaccine antigen to further contribute to improving TB treatments.

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