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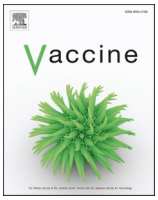

Immunoproteomic discovery of *Mycobacterium bovis* antigens, including the surface lipoprotein Mpt83 as a T cell antigen useful for vaccine development

Karuna P. Karunakaran [a,c,1], Hong Yu [a,1], Xiaozhou Jiang [a], Queenie W.T. Chan [b], Lynette Sigola [c], Leonard A. Millis [c], Jiaqi Chen [b], Patrick Tang [d], Leonard J. Foster [b], Robert C. Brunham [a,*]

[a] *Vaccine Research Laboratory, University of British Columbia Centre for Disease Control, Vancouver, B.C., Canada*
[b] *Department of Biochemistry and Molecular Biology, Michael Smith Laboratories, University of British Columbia, Vancouver, B.C., Canada*
[c] *Department of Biology, Faculty of Science and Technology, Douglas College, New Westminster, B.C., Canada*
[d] *Weill-Cornell Medical College, Doha, Qatar*

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ABSTRACT

Tuberculosis (TB) is one of the leading causes of death from infectious diseases, killing approximately 1.3 million people worldwide in 2022 alone. The current vaccine for TB contains a live attenuated bacterium, *Mycobacterium bovis* BCG (Bacille Calmette-Guérin). The BCG vaccine is highly effective in preventing severe forms of childhood TB but does not protect against latent infection or disease in older age groups. A new or improved BCG vaccine for prevention of pulmonary TB is urgently needed.

In this study, we infected murine bone marrow derived dendritic cells from C57BL/6 mice with *M. bovis* BCG followed by elution and identification of BCG-derived MHC class I and class II-bound peptides using tandem mass spectrometry. We identified 1436 MHC-bound peptides of which 94 were derived from BCG. Fifty-five peptides were derived from MHC class I molecules and 39 from class II molecules. We tested the 94 peptides for their immunogenicity using IFN- γ ELISPOT assay with splenocytes purified from BCG immunized mice and 10 showed positive responses. Seven peptides were derived from MHC II and three from MHC class I. In particular, MHC class II binding peptides derived from the mycobacterial surface lipoprotein Mpt83 were highly antigenic. Further evaluations of these immunogenic BCG peptides may identify proteins useful as new TB vaccine candidates.

1. Introduction

Tuberculosis (TB), a leading infectious cause of mortality worldwide is caused by *Mycobacterium tuberculosis* (Mtb) and is a serious global health problem with 10 million new cases and 1.3 million deaths annually [1]. Furthermore about 2.2 billion people, close to one-third of the world's population, are thought to live with latent Mtb infection and represent a potential source of future active pulmonary TB [2]. Reactivated pulmonary TB is the main route for transmission and a vaccine for latently infected persons that prevents pulmonary reactivation would have major public health impact. The current vaccine for TB, Bacille Calmette and Guérin (BCG), is efficient in preventing severe forms of childhood TB but fails to afford protection against adolescent and adult pulmonary TB [3]. Because almost all infants receive BCG vaccine in TB endemic countries, most persons with reactivated pulmonary TB have been previously vaccinated with BCG.

Protective immunity against Mtb depends on a wide range of innate and adaptive immune mechanisms with T cell-mediated immune responses being of major importance. The ability of CD4 T cells to produce interferon-γ (IFN-γ), which activates macrophages to contain the intracellular pathogen, is central to protection. CD8 T cells also contribute to adaptive immunity but may be of lesser importance than CD4 T cells [4,5].

Antigen presenting cells such as dendritic cells (DCs) play an

* Corresponding author.
E-mail address: robert.brunham@bccdc.ca (R.C. Brunham).
[1] K.P.K. and H.Y. contributed equally to this study.

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essential role in initiating the adaptive immune response by T cells. DCs capture antigens in the peripheral tissues and migrate to regional lymph nodes where they present processed antigens by MHC class I and class II molecules to naïve CD8 or CD4 T cells. The activated effector CD4 T cells undergo proliferation and produce IFN-γ to activate macrophage-dependent inhibition of intracellular Mtb growth. The activated macrophages generate effector molecules such as nitric oxide (NO) that control and contain Mtb infection [6]. Although individuals infected with Mtb or vaccinated with BCG acquire a cell-mediated immune response, most hosts do not achieve complete elimination of Mtb. Instead, the immune response restricts Mtb to granulomas in the lung, which can persist for decades in a state of latency. However, the bacteria may eventually start replicating, leading to active TB disease, in up to 10 % of these individuals over their lifetime [2].

Immunoproteomics is an approach where peptides presented by MHC molecules are identified by tandem mass spectrometry (MS/MS) [7–9]. Advances in MS/MS technology provide sensitivity limits near one femtomole (fmol) and are able to measure peptide masses to within one part-per-million accuracy. This brings the detection technology into a range compatible with the levels of microbial peptides that can reasonably be purified from MHC molecules presented on the surface of antigen-presenting cells such as DCs. The DC-based immunoproteomic approach that is used in this program of research directly identifies T cell epitopes presented by MHC molecules resulting in vast improvement in the positive validation rate. An advantage of using the immunoproteomic approach is that the identified peptides are the result of physiological processing and presentation pathways and are based on both the peptide affinity for the MHC molecules as well as the frequency of their presentation.

Our laboratory pioneered an immunoproteomics approach to identify *Chlamydia trachomatis* and *Salmonella enterica* T cell antigens [10–13]. Results demonstrated that T cell antigens identified by immunoproteomics can be successfully exploited as a T cell protein-based subunit vaccine against *Chlamydia* infection [14–16]. Identification of *Salmonella* T cells epitopes allowed identification of the T cell maturation pathway that generated protective T cells against *Salmonella* infection [17,18]. We hypothesized that proteins containing MHC-bound peptides derived from BCG and which are completely conserved between *M. bovis* BCG and Mtb are vaccine candidates that can boost protective immunity in BCG immunized individuals.

In this study, we generated dendritic cells from the bone marrow of C57BL/6 mice infected with BCG followed by elution of MHC class I and class II-bound peptides. The purified peptides were then identified using tandem mass spectrometry. We identified 1436 MHC-bound peptides of which 94 were BCG derived peptides, 55 from class I molecules and 39 from class II molecules. We tested the 94 peptides for their immunogenicity using IFN-γ ELISPOT assay with splenocytes purified from BCG immunized mice. Among the 94 BCG derived MHC bound peptides tested, 10 showed positive response, of which seven originated from MHC class II and three from MHC class I. Further evaluations of the source proteins of these immunodominant peptides may identify proteins useful as new TB vaccine candidates in outbred populations.

2. Materials and methods

2.1. BCG

Mycobacterium bovis BCG (Bacillus of Calmette and Guérin) Tokyo strain 172 (BCG) was prepared by inoculation of stock culture in Middlebrook 7H10 supplemented with 10 % oleic albumin dextrose catalase enrichment and incubating for three weeks at 37 °C. The whole genome of this strain is already sequenced [19], and the transcriptional profile is available in public domains.

2.2. Mice

Six to 8 weeks old female C57BL/6 (H2-K^b, D^b, and IA^b) mice were purchased from Charles River Laboratories - Saint Constant, QC, Canada. The University of British Columbia's rigorous rules for animal care were strictly followed when caring for and using the mice.

2.3. Generation of BMDCs

Bone marrow-derived dendritic cells (BMDCs) were produced as previously described [10]. In brief, 4×10^7 cells in 50 ml of DC media were grown in Falcon petri dishes using bone marrow cells that had been removed from the femurs of female C57BL/6 mice. DC medium was IMDM supplemented with 10 % FCS, 0.5 mM 2-ME, 4 mM L-glutamine, 50 μg/ml gentamicin, and 5 % of culture supernatant of murine GM-CSF-transfected plasmacytoma X63-Ag8 and 5 % of culture supernatant of murine IL-4 transfected plasmacytoma X63-Ag8 which contained 10 ng/ml GM-CSF and 10 ng/ml IL-4, respectively. On day 3, fresh DC medium was introduced, and half of the culture supernatants were withdrawn. On day 5, non-adherent cells with purity of approximately 50 % CD11c + were isolated and grown in new DC medium in preparation for BCG infection.

2.4. Optimization of BCG infection of BMDCs

BMDCs were infected with BCG at varying multiplicity of infection (MOI) and incubated for various times to allow the cells to phagocytize the bacteria in order to find the best conditions for antigen presentation. Cell viability was assessed through trypan blue staining. Expression levels of CD11c (a dendritic cell marker), MHC I, and MHC II in BMDC cultures were analyzed using flow cytometry with anti-CD11c-APC (clone N418), anti-MHC I-FITC (clone AF6–88.5.3), and anti-MHC II-PE (clone M5/114.15.2) antibodies. Cytokine production (IL-12 and IL-10) in BMDC cultures was quantified by ELISA.

2.5. Purification of MHC-bound peptides

The procedure for peptide purification is based on Cox et al. [20]. In brief, the 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) lysis buffer was used to lyse BMDCs. Inhibitors of proteases were applied to reduce peptide breakdown. Allele-specific anti-MHC monoclonal antibody affinity columns were used to purify MHC molecules (mAb AF6–88.5.3 or HB158 specific to H2-Kb class I allele, Y—3P or HB183 specific to I-A class II allele). Purified MHC molecules were ultra-filtered via 10-kDa cut-off membranes after being washed and eluted with acetic acid.

2.6. Identification of MHC-bound peptides

The MHC class I and class II-bound peptides were further purified, concentrated, filtered and desalted using STop And Go Extraction tips. Peptides were analyzed by LC/MS/MS using an LTQ-Orbitrap Velos (Thermo Electron) on-line coupled to Agilent 1200 Series nanoflow HPLCs using nanospray ionization sources (Proxeon Biosystems, Odense, Denmark). Analytical columns were packed into 15 cm long, 50 μm inner diameter fused silica emitters using 3 μm diameter ReproSil Pur C18 AQ beads (Dr. Maisch, www.Dr-Maisch.com), joint with 2-cm-long, 100-μm-inner diameter fused silica trap column packed with 5 μm-diameter Aqua C-18 beads (Phenomenex, www.phenomenex.com) and a 20-μm-inner diameter fused silica gold coated spray tip with 6-μm-diameter opening. LC buffer A consisted of 0.5 % acetic acid and buffer B consisted of 0.5 % acetic acid and 80 % acetonitrile in water. Gradients were run from 10 % B to 32 % B over 51 min, then from 32 % B to 40 % B in the next 5 min, then increased to 100 % B over 2 min period, held at 100 % B for 2.5 min, and then dropped to 0 % B for another 20 min to recondition the column. The Velos was set to acquire a full range scan at

60,000 resolution in the Orbitrap, from which the ten most intense multiply-charged ions per cycle were isolated for fragmentation in the LTQ. Centroided fragment peak lists were processed with Proteome Discoverer v. 1.2 (ThermoFisher Scientific). The search was performed with Mascot algorithm v. 2.4 against a database comprised of the protein sequences from the mouse and BCG proteome. The estimated false discovery rate (FDR) was below 2 %. Even though 1 % FDR rate is typically applied for proteins, empirically, setting a 1 % FDR protein cut off was too stringent for our approach since some peptides eliminated at a 1 % FDR but included at a 2 % FDR scored positive in the T cell assay. For a peptide to be assigned to a particular protein, the peptide must have an exact match to the protein sequence.

2.7. Synthesis of BCG-derived peptides

A total of 55 MHC class I and 39 MHC class II BCG peptides (scoring >20) were synthesized at the Leonard J. Foster Lab, Department of Biochemistry and Molecular Biology, University of British Columbia. Peptides were synthesized using Fmoc solid phase synthesis in an automated multiple peptide synthesizer (MultiPep RS, Intavis Bioanalytical Instruments AG, Cologne, Germany) on amide resin (TentaGel S RAM, Rapp Polymers, Tübingen, Germany), following the procedure previously described [21]. Briefly, the resin was primed using dimethylformamide (DMF) followed by reaction with Fmoc protected amino acids (Sigma) dissolved in DMF. The reaction proceeded by the addition of 4-methylmorpholine in DMF and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorophosphate in DMF were added per well. This was done twice for 30 min each. Unreacted N-termini were capped with acetic anhydride in DMF. This was repeated until peptide synthesis was completed. After that, the resin was deprotected with piperidine in DMF and the N-termini were capped again with acetic anhydride. The resin was dried by vacuum then cleaved in a cocktail containing trifluoroacetic acid, triisobutylsilane and water. The resulting peptides were precipitated using cold tert-butyl methyl ether and cyclohexane. Peptides were pelleted by centrifugation then dried, and stored in -80°C until used.

2.8. ELISPOT assay

The IFN-γ ELISPOT assay was used to screen BCG-derived peptides identified in this study. C57BL/6 mice received subcutaneous vaccination twice with 2.5 × 10^7 viable colony-forming units (CFUs) of *M. bovis* BCG in 100 µl PBS with a 4-week interval. Fourteen days post the final immunization, spleens from five vaccinated mice were harvested and pooled for the subsequent IFN-γ ELISPOT assay. Two independent experiments were conducted to evaluate IFN-γ secreting cells to individual peptides.

MultiScreen-HA filter 96-well plates (Millipore #MAHAS4510) were coated overnight at 4 °C with 2 µg/ml of anti-mouse IFN-γ antibody (BD PharMingen, Clone R4-6A2). Splenocytes pooled from five BCG-immune mice were then seeded into the coated plates (10^6 splenocytes per well) in the presence of individual BCG peptides at 2 µg/ml (with two replicates for each peptide). Purified protein derivative (PPD) served as a positive control and an irrelevant ovalbumin (OVA) peptide was used as a negative control. After 20 h incubation at 37 °C and 5 % CO_2, the plates were thoroughly washed and subsequently incubated with biotinylated anti-mouse IFN-γ antibodies (BD PharMingen, Clone XMG1.2) at 2 µg/ml for 2 h at room temperature. This was followed by incubation with streptavidin–alkaline phosphatase (BD PharMingen) at a 1:1000 dilution for 1 h at room temperature. The spots were then visualized using a substrate comprising 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (NCIP/NBT tablet, Sigma–Aldrich) and counted under a dissecting microscope.

2.9. Statistical analysis

All data were analyzed with the aid of the Graph-Pad Prism software program. Differences between the means of experimental groups were analyzed using an independent, two-tailed *t*-test. A p value <0.05 was considered significant. Data are presented as means ± standard deviation (SD).

3. Results

3.1. Optimization of BCG infection of BMDCs

BMDCs infected with BCG at different MOI were incubated for various periods of time and were evaluated to identify optimal conditions for antigen presentation. First, BMDCs were exposed to BCG at MOI of 0, 1, 2.5, 5, 7.5, or 10 for a fixed period (16 h), and cell viability, CD11c + cells, IL 10 and IL 12 in BMDC cultures were assessed (Fig. 1). Our findings revealed a significant decline in cell viability with increasing MOI in BMDC cultures (Fig. 1A). The percentage of CD11c + cells exhibited a significant reduction with MOI 7.5 or 10, not with MOI 1, 2.5, or 5, compared to the non-infected treatment (Fig. 1B). Importantly, BMDCs produced the highest level of IL-12, a critical cytokine that stimulates the growth and function of T cells, when they were infected with BCG at MOI of 2.5. The increase of MOI did not raise the production of IL-12 (Fig. 1C) but enhanced the production of IL-10 (Fig. 1D), a cytokine that down regulates the expression of Th1 cytokines and MHC class II antigens. Based on these results, the optimal MOI of BCG was determined as 2.5.

MOI of 2.5 was then utilized to infect BMDCs with BCG for varying durations to establish the optimal infection time for antigen presentation. BMDCs were infected with BCG at MOI of 2.5 for 12, 16, 24, 36, 42, or 48 h, and cell viability, CD11c + cell population, and expression levels of MHC I and MHC II (MFI) on CD11c + cells were evaluated

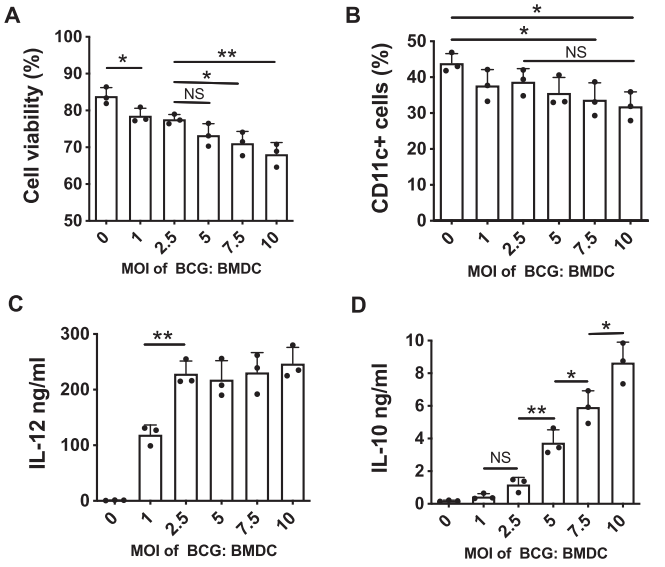


Fig. 1. BMDCs infected with BCG at various multiplicities of infection (MOI). C57 mouse bone marrow cultures were stimulated with GM-CSF and IL-4 for 4 days and subsequently exposed to BCG organisms at MOIs of 0.1, 2.5, 5, 7.5, and 10 for 16 h. (A) Cell viability (%) in BMDC culture. (B) CD11c + cells (%) in BMDC culture. (C) IL-12 production by BMDCs following 16 h of infection with BCG. (D) IL-10 production by BMDCs following 16 h of infection with BCG. The results represent the average of three replicates for each treatment and are expressed as the means ± SD. * and ** indicate *P*-values of <0.05 and < 0.01, respectively, in comparison between two groups. The data are representative of two similar experiments.

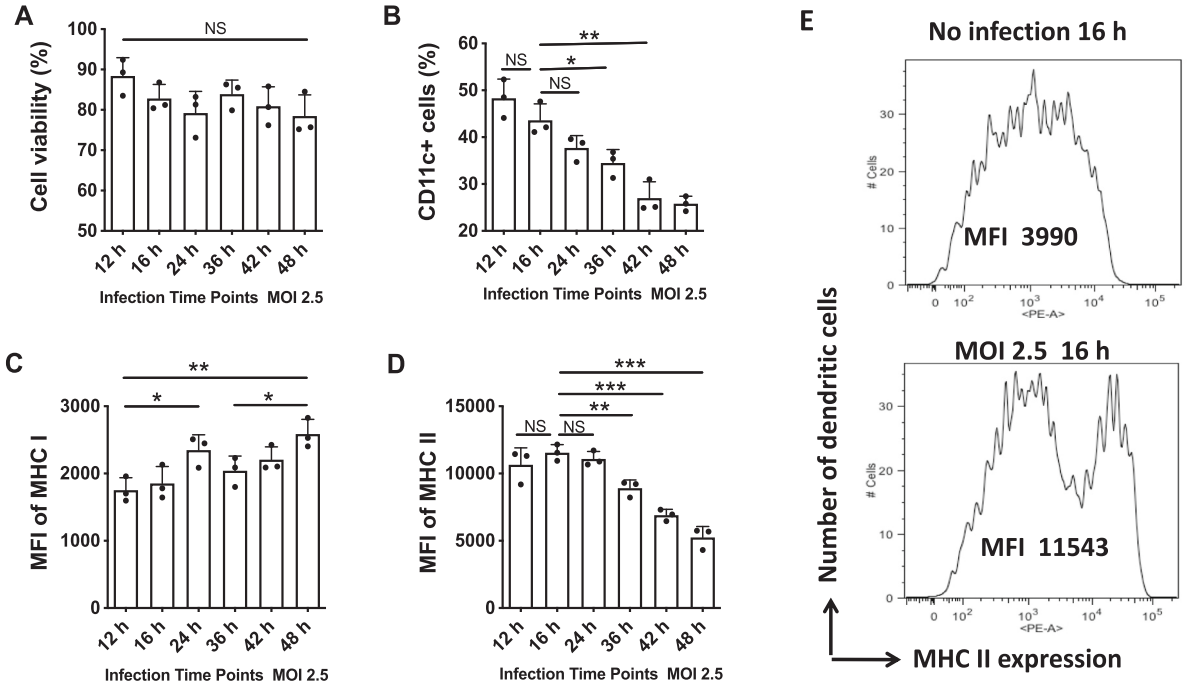


Fig. 2. BMDC infected with BCG at various time points with MOI of 2.5. C57 mouse bone marrow cultures were stimulated with GM-CSF and IL-4 for 4 days and subsequently exposed to BCG at MOI of 2.5 for 12, 16, 24, 36, 42, or 48 h. (A) Cell viability (%) in BMDC culture. (B) CD11c + cells (%) in BMDC culture. (C) Mean fluorescence intensity (MFI) of MHC I on CD11c + cells. (D) MFI of MHC II on CD11c + cells. (E) MHC II expression (MFI) on CD11c + cells in BMDC culture with BCG infection (MOI 2.5 for 16 h) versus no infection, illustrated by flow cytometry plot. The results represent the average of three replicates for each treatment and are expressed as the means ± SD. *, **, and *** indicate *P* values of <0.05, < 0.01, and < 0.001, respectively, in comparison between two groups. The data are representative of two similar experiments.

(Fig. 2). The results indicated that prolonging the infection duration at MOI of 2.5 did not remarkably impact cell viability (Fig. 2A) but did lead to a significant reduction in CD11c + cell population (Fig. 2B). Interestingly, the expression of MHC I (MFI) on CD11c + cells increased with prolonged infection time (Fig. 2C), whereas the MFI of MHC II exhibited a significant decrease. Notably, MHC II expression reached its peak at the 16-h infection (Fig. 2C). As illustrated in Fig. 2E, BMDCs infected with BCG for 16 h at MOI of 2.5 showed a significant increase in MHC class II expression compared to non-infected DCs. We therefore chose 16 h of infection time at 2.5 MOI as the optimal condition for DCs infected with BCG.

3.2. Identification of MHC-bound peptides generated from BCG

BMDCs were exposed to BCG at a 1:2.5 multiplicity for 16 h. To determine the number of BMDCs used in our experiment we assumed that each antigen-presenting cell presents as few as one molecule of a particular antigen; yet in practice the current sensitivity of mass spectrometers has only been in the range of ~6 billion molecules or ~ 10 fmol. To collect five to ten billion antigen-presenting DCs required around 100 mice.

After the infection, BCG-infected BMDCs were lysed, and MHC molecules were extracted using monoclonal antibodies that were specific to the MHC class I and class II alleles. Low pH was used to disrupt protein-protein and peptide-peptide interactions in the column, and ultrafiltration was used to extract peptides from high molecular weight material. The purified MHC-bound peptides were subsequently identified using a hybrid linear trapping quadrupole/Fourier transform-ion cyclotron resonance mass spectrometer and nano-flow liquid chromatography/tandem mass spectrometry. Repeated tests led to the identification of 1436 MHC binding peptides, of which 94 were derived from BCG and 1342 peptides were derived from murine proteome (Table 1). Less than 7 % of all the peptides presented by MHC molecules on the surface of BCG-infected murine BM-DCs were BCG-derived, 55 were MHC class I binding and 39 were MHC class II binding peptides.

Table 1
Summary of BCG and murine derived MHC class I and II-bound peptides identified in this study.

		Number of peptides identified
Mouse-derived	Class I	695
	Class II	647
BCG-derived	Class I	55
	Class II	39

3.3. Properties of peptides bound to MHC molecules

To verify the accuracy of these MHC-bound peptides, we examined the characteristics of both BCG and mouse-derived peptides. According to previous studies, the length of class I-bound peptides ranged from 8 to 13 amino acids, with the majority being 8 or 9 amino acids long [22]. In contrast, class II peptides ranged in length from 9 to 24 amino acids (Tables 2 & 3, Supplemental Tables S1 & S2).

In order to assess whether any particular protein group was presented by MHC molecules preferentially, we determined the putative source of the proteins which contained the detected peptides. According to the LOCATE database, the cellular location of each source protein was categorized as cytoplasmic, membrane, extracellular or cell wall using Entrez accession numbers. The majority of the BCG source proteins were cytoplasmic in origin followed by membranous location (Fig. 3A). Using Logo plot, we also determined the consensus anchor residues of BCG derived class I and class II peptides (Fig. 3B, C).

To determine if the source proteins of BCG derived MHC-bound peptides are sequence-conserved between BCG and Mtb, we identified their corresponding Mtb homolog and aligned them. More than 50 % of

Table 2
MHC class I-bound BCG peptides and source proteins identified using immunoproteomics (ELISPOT positives are in bold).

Protein ID	BCG T cell antigens	MHC class I-bound peptides	Ion Score	Mtb homolog	Identity
gi|224,990,812|ref.|YP_002645499.1|	ribokinase	AAAAIDAALR	21.75	NP_216952.1	99
gi|224,988,855|ref.|YP_002643542.1|	3-hydroxybutyryl-CoA dehydrogenase	AATKQPQR	25.65	6HRD_A	100
gi|224,991,133|ref.|YP_002645822.1|	hypothetical protein JTY_2773	AAVVVGGVAEPGSAGP	27.74	NP_217278.1	100
gi|224,989,435|ref.|YP_002644122.1|	two component transcriptional regulator	ADGSPIRVLL	20.35	NP_215549.1	100
gi|224,991,244|ref.|YP_002645933.1|	1-deoxy-D-xylulose 5-phosphate reductoisomerase	AFLAGRI	29.79	NP_217386.2	100
gi|224,988,813|ref.|YP_002643500.1|	exodeoxyribonuclease III protein	AIASRVGLDDV	20.48	NP_214941.1	100
gi|224,990,110|ref.|YP_002644797.1|	hypothetical protein JTY_1741	AIITFSTV	21.85	NP_216243.1	99
gi|224,991,338|ref.|YP_002646027.1|	phosphopantetheine adenylyltransferase	AILVNPAK	23.94	6G6V_A	100
gi|224,991,755|ref.|YP_002646444.1|	penicillin-binding protein	AVEGTKVGVNTGGTYTV	23.49	AUQ09650.1	99
gi|224,989,069|ref.|YP_002643756.1|	transmembrane transport protein	AVIVGTVVLS	20.16	NP_215190.1	99
gi|224,991,937|ref|YP_002646626.1|	**hypothetical protein JTY_3586**	**DPATGKPTS**	**21.78**	**NP_218038.1**	**99**
gi|224,990,788|ref.|YP_002645475.1|	hypothetical protein JTY_2423	EAGKDAAAVI	30.16	NP_216929.3	100
gi|224,992,313|ref.|YP_002647003.1|	poly(A) polymerase	ELDKLLVGEDPAAGIDL	26.81	YP_178026.1	100
gi|224,990,674|ref.|YP_002645361.1|	heat shock protein 90	ELISNASDALDK	71.4	NP_216815.1	100
gi|224,991,189|ref|YP_002645878.1|	**hypothetical protein JTY_2831**	**ELSAEFPDRTILLNTSSGT**	**22.38**	**P71635.5**	**100**
gi|224,990,970|ref.|YP_002645657.1|	PE-PGRS family protein	EQNLLAVINAPA	32.37	YP_177891.1	99
gi|224,991,886|ref.|YP_002646575.1|	acetolactate synthase large subunit	FVATSLGI	20.54	NP_217987.1	100
gi|224,988,772|ref.|YP_002643459.1|	PPE family protein	GDLPEVGGRVT	23.82	WP_003401914.1	99
gi|224,990,477|ref.|YP_002645164.1|	PE-PGRS family protein	GGAGGNGGLGGLLLG	20.12	AFN50042.1	99
gi|224,991,774|ref.|YP_002646463.1|	PPE Family protein	GIANSGNVNTGAFISGN	20.16	YP_177964.1	99
gi|224,992,221|ref.|YP_002646911.1|	UDP-galactopyranose mutase	GLGLVSQFF	31.83	NP_218326.1	100
gi|224,990,292|ref.|YP_002644979.1|	sialic acid-transport integral membrane protein	GLLQEGYAFGYLLAS	22.94	NP_216418.1	100
gi|224,988,667|ref.|YP_002643354.1|	PE-PGRS family protein	GSSPDGGGGAGGI	27.82	AFN48133.1	99
gi|224,991,603|ref.|YP_002646292.1|	RNA polymerase sigma factor RpoE	GTNLKAWL	22.28	NP_217739.1	100
gi|224,989,087|ref.|YP_002643774.1|	L-lactate dehydrogenase	HDLSPADILVPTGF	23.52	NP_215208.1	100
gi|224,989,605|ref.|YP_002644292.1|	transferase	HLAPGTTVMH	21.91	NP_215717.1	100
gi|224,992,298|ref.|YP_002646988.1|	PPE family protein	IAAFETANAAMRPAP	20.39	YP_178024.1	99
gi|224,989,140|ref.|YP_002643827.1|	PE-PGRS family protein	IGGASTVLGGTGGGGGVG	40.44	WP_032073221.1	99
gi|224,992,205|ref.|YP_002646895.1|	integral membrane indolylacetylinositol arabinosyltransferase	ILAAATVTAIPI	21.05	NP_218310.1	99
gi|224,991,218|ref.|YP_002645907.1|	integral membrane efflux protein	ILGYSALRA	32.49	NP_217362.1	99
gi|224,990,445|ref.|YP_002645132.1|	CobI-CobJ fusion protein	IPVVIGRN	21.91	NP_216582.1	100
gi|224,988,960|ref.|YP_002643647.1|	ribonucleoside-diphosphate reductase large subunit	IQDCVEARL	26.61	NP_215084.1	100
gi|224,989,776|ref.|YP_002644463.1|	hypothetical protein JTY_1407	KRALAGLPYRAAE	24.75	YP_177803.1	99
gi|224,990,940|ref.|YP_002645627.1|	hypothetical protein JTY_2578	LAGVDVILVH	20.13	P9WL99.1	99
gi|224,991,569|ref.|YP_002646258.1|	hypothetical protein JTY_3212	LALIEGWV	23.92	NP_217711.1	100
gi|224,991,492|ref.|YP_002646181.1|	transposase	LDALTDKLP	20.54	NP_215715.1	100

(*continued on next page*)

Table 2 (*continued*)

Protein ID	BCG T cell antigens	MHC class I-bound peptides	Ion Score	Mtb homolog	Identity
gi|224,989,764|ref.|YP_002644451.1|	transcriptional regulatory protein	LSPIVLRI	29.09	NP_215875.1	100
gi|224,991,791|ref.|YP_002646480.1|	PE-PGRS family protein	NGLTGNDGVN	20.65	WP_009938654.1	99
gi|224,992,044|ref.|YP_002646733.1|	UDP-glucose 4-epimerase	NLEHLADN	21.46	NP_215015.2	100
gi|224,992,227|ref.|YP_002646917.1|	acyltransferase	QIGRAKVPVTVQVGRPL	25.86	NP_218332.1	100
gi|224,989,585|ref.|YP_002644272.1|	polyketide beta-ketoacyl synthase	QLPFGLRGF	20.27	A0A089QRB9.2	99
gi|224,991,626|ref|YP_002646315.1|	**lipoprotein**	**QVILAGVEQTQAGQF**	**31.83**	**NP_217761.1**	**99**
gi|224,990,659|ref.|YP_002645346.1|	hypothetical protein JTY_2294	RRYLQELGDLPT	22.95	NP_216801.1	100
gi|224,989,831|ref.|YP_002644518.1|	esterase	SAGGHLCALAALT	22.31	NP_215942.1	100
gi|224,991,809|ref.|YP_002646498.1|	PE-PGRS family protein	SAGTGGVGASGGTGGNAGLIG	20.18	YP_177968.1	93
gi|224,990,769|ref.|YP_002645456.1|	integral membrane protein	SPISGVGILVV	24.43	NP_216911.1	99
gi|224,988,961|ref.|YP_002643648.1|	hypothetical protein JTY_0586	TLLIVGGRD	26.1	NP_215085.1	99
gi|224,989,290|ref.|YP_002643977.1|	type II citrate synthase	TLVENFLR	27.99	NP_215411.1	99
gi|224,988,713|ref.|YP_002643400.1|	hypothetical protein JTY_0335	TLYFAGQGF	29.75	NP_214840.1	99
gi|224,989,399|ref.|YP_002644086.1|	hypothetical protein JTY_1027	VAEIAFTV	33.45	NP_215513.1	100
gi|224,991,644|ref.|YP_002646333.1|	F420–0-gamma-glutamyl ligase	VALAVRGLGSCWIGS	25.69	NP_217779.1	100
gi|224,989,068|ref.|YP_002643755.1|	enoyl-CoA hydratase	VAVLWGAGGTFCAGAD	21.01	YP_177745.1	100
gi|224,991,227|ref.|YP_002645916.1|	mycothione reductase	VIAAGSRPVIP	21.14	YP_177910.1	100
gi|224,992,237|ref.|YP_002646927.1|	polyketide synthase associated protein	WFTGQIPVT	30.6	NP_218341.1	100
gi|224,988,668|ref.|YP_002643355.1|	PPE family protein	WGPPPGEDP	22.03	YP_177709.1	99

the aligned pairs were 100 % identical. The lowest identity among proteins that generated MHC bound peptides was 98 % (Tables 2 & 3).

3.4. Identification of immunodominant BCG peptides

We investigated whether T cells from BCG immunized mice could recognize BCG derived peptides as a preliminary step to establishing the immunological relevance of the peptides. By using the IFN-γ ELISPOT assay, we examined the 94 BCG-derived peptides for immune responses after immunizing mice with live BCG. For BCG vaccination of mice, we initially adhered to the commonly accepted dose of 1×10^6 viable CFUs of BCG for subcutaneous vaccination. However, our ELISPOT assay failed to demonstrate significant IFN-γ responses to BCG peptides, regardless of whether mice were vaccinated once or twice. Therefore, we conducted subsequent experiments using a 25-fold higher dose of BCG (2.5×10^7 CFUs). This elevated dose did not induce adverse effects on mouse skin. Our results revealed that the prime and boost vaccination strategy with 2.5×10^7 BCG was effective in eliciting significant IFN-γ responses to BCG peptides, unlike the single vaccination approach. We acknowledge that the standard dose of 1×10^6 CFUs may be appropriate for inducing protective immunity in vivo in vaccination/challenge studies with an interval between BCG vaccination and Mtb challenge of eight to ten weeks, as described before [23]. However, our experimental design involved a general vaccine protocol with a shorter interval of four weeks between vaccinations and evaluation occurring two weeks after the final vaccination. Our findings suggest that the utilization of a higher dose of BCG and repeated vaccination was crucial for inducing significant immune responses, particularly in the context of the ELISPOT assay utilized in our study.

Splenocytes from BCG-immunized mice were stimulated in vitro with the specific MHC-bound peptides, or control peptides, and the number of IFN-γ secreting cells was calculated. Among the 94 BCG derived MHC bound peptides tested, 10 showed positive response (Fig. 4A). Seven of these 10 peptides originated from MHC class II and 3 originated from MHC class I. The top 4 positive peptides (B7, B8, B11 and F12) were derived from 3 BCG source proteins [B7 & B8 – Cell surface lipoprotein Mpt83 (JTY 2890); F12 – Protease IV (JTY 0744); and B11 – Integral membrane protein (JTY 0631)]. All three BCG source proteins are 100 % identical to Mtb proteins Rv2873, Rv0724 and Rv0615, respectively. Rv2873 (Mpt83) protein is a mycobacterial lipoprotein found on the surface of Mtb. The nine peptide source proteins in BCG and Mtb that correspond to various antigens and their functions, and potential relevance to vaccines are listed in Fig. 4B.

4. Discussion

Over the past fifteen years, our group has used immunoproteomics to identify T cell antigens from *C. trachomatis* and *S. enterica* [10–16]. *Chlamydia* T cell antigens identified by immunoproteomics were successfully used in murine models as T cell protein-based subunit vaccines against *Chlamydia* infection [13–16]. Four MHC class-II binding *Salmonella* peptides identified via our immunoproteomic approach stimulated significantly higher level of IFN-γ production by CD4 T cells from the spleens of mice with persistent *Salmonella* infection [11]. One of these peptides (LpdA), derived from the *Salmonella* dihydrolipoamide dehydrogenase protein, was used to rigorously define the CD4 T cell maturation pathway [17,18].

In our approach, peptides originating from pathogens and presented

Table 3
MHC class II-bound BCG peptides and source proteins identified using immunoproteomics (ELISPOT positives are in bold).

Protein ID	BCG T cell antigens	MHC class II-bound peptides	Ion Score	Mtb homolog	Identity
gi|224,989,069|ref.| YP_002643756.1|	transmembrane transport protein	AAALTLGP	21.14	NP_215190.1	99
gi|224,992,121|ref.| YP_002646810.1|	aspartokinase	AAVGVNI	22.98	NP_218226.1	100
gi|224,989,006|ref| YP_002643693.1|	**integral membrane protein**	**AGIAAGALTLAAWG**	**27.37**	**NP_215129.1**	**100**
gi|224,991,939|ref.| YP_002646628.1|	acetyl-CoA acetyltransferase	AGSVGGSTGVVAASLVQS	29.88	NP_218040.1	100
gi|224,990,964|ref.| YP_002645651.1|	lipoprotein	AGYVDIANIECTAGQ	22.34	NP_217101.1	99
gi|224,990,770|ref.| YP_002645457.1|	PE-PGRS family protein	AINAPTLALLGRPLI	21.4	YP_177878.1	99
gi|224,988,979|ref.| YP_002643666.1|	MCE-family protein	AVAQAPDGLGGKFGESIVN	21.99	YP_177740.1	99
gi|224,988,520|ref.| YP_002643207.1|	transcriptional regulatory protein	AVEAYVTHIES	20.39	NP_214649.1	100
gi|224,989,829|ref.| YP_002644516.1|	hypothetical protein JTY_1460	AVSRPSYRQCVQASAQT	21.26	NP_215940.1	100
gi|224,990,048|ref.| YP_002644735.1|	chalcone synthase	AVVAVGDRRAE	30.18	NP_216181.1	100
gi|224,990,194|ref.| YP_002644881.1|	PPE family protein	DITAQLSQLIS	20.85	YP_177844.1	99
gi|224,989,041|ref.| YP_002643728.1|	alpha-mannosidase	DLMVALVRAGV	21.08	NP_215162.1	99
gi|224,989,056|ref.| YP_002643743.1|	arylsulfatase	DVGFGASSAFGGPCRTSTAEL	23.51	NP_215177.1	99
gi|224,988,790|ref.| YP_002643477.1|	membrane bound polyketide synthase	EVSWEALEHAGIIPE	21.03	NP_214919.1	100
gi|224,991,801|ref.| YP_002646490.1|	hypothetical protein JTY_3449	GFGRFSTFDFPLLSSGKTS	25.72	NP_217895.1	99
gi|224,988,510|ref.| YP_002643197.1|	PE-PGRS family protein	GGDGGQGGAGRGL	23.9	WP_141766561.1	100
gi|224,990,034|ref| YP_002644721.1|	**PE-PGRS family protein**	**GGTGGANSGSLFASPGGT**	**28.47**	**YP_177826.1**	**99**
gi|224,989,963|ref.| YP_002644650.1|	inv protein	GLAAADPATRPV	22.05	NP_216082.1	100
gi|224,990,006|ref.| YP_002644693.1|	hypothetical protein JTY_1637	IFRGVATTVSAISDPM	23.71	NP_216140.1	99
gi|224,991,315|ref| YP_002646004.1|	**acyl-CoA synthetase**	**ILTTSSAVDDVVQH**	**22.15**	**NP_217457.1**	**100**
gi|224,991,247|ref| YP_002645936.1|	**cell surface lipoprotein**	**ILTYHVIAGQASPSRIDG**	**61.89**	**NP_217389.1**	**100**
gi|224,988,740|ref.| YP_002643427.1|	PPE family protein	LAANYTITIERPAAF	20.8	YP_177721.2	99
gi|224,989,547|ref.| YP_002644234.1|	enoyl-CoA hydratase	LACDFILASDSAFFMLANTK	20.11	NP_215657.1	100
gi|224,991,153|ref| YP_002645842.1|	**alanine rich oxidoreductase**	**LADDIAAARA**	**20.31**	**NP_217297.1**	**100**
gi|224,990,541|ref.| YP_002645228.1|	S-adenosyl-methyltransferase	LAIGGRIAVLAYQSL	20.72	NP_216681.4	100
gi|224,990,914|ref.| YP_002645601.1|	transmembrane protein	LFAIGGVVWGVMDGRADAKASPDPD	27.07	NP_217052.1	100
gi|224,992,076|ref.| YP_002646765.1|	periplasmic dipeptide-binding lipoprotein	LMSYDAVGKPSLEVAQSIESA	23.09	NP_218183.1	100
gi|224,992,146|ref.| YP_002646835.1|	hypothetical protein JTY_3796	NMAPLTLAGV	31.67	NP_218251.1	100
gi|224,991,535|ref.| YP_002646224.1|	PPE family protein	QGAAAAAMAAAAAPYAGWLA	21.44	YP_177937.1	71
gi|224,991,292|ref.| YP_002645981.1|	nitrogen regulatory protein P-II	QKGHTEVYR	21.17	NP_217435.1	100
gi|224,988,498|ref.| YP_002643185.1|	gdp-mannose 4,6-dehydratase	SIATFRPDAVFHLAAQSYP	20.51	NP_214626.1	100
gi|224,991,247|ref| YP_002645936.1|	**cell surface lipoprotein**	**SILTYHVIAGQASPSRIDG**	**25.61**	**NP_217389.1**	**100**
gi|224,990,212|ref.| YP_002644899.1|	hypothetical protein JTY_1844	SVRVGVDTWVVGVPGSL	21.66	NP_216341.1	100
gi|224,989,830|ref.| YP_002644517.1|	hypothetical protein JTY_1461	TGAPLGLDR	24.81	NP_215941.1	99
gi|224,992,092|ref.| YP_002646781.1|	bifunctional membrane-associated penicillin-binding protein 1A/1B	VAGLDVDAAR	37.82	YP_178005.1	100
gi|224,988,571|ref.| YP_002643258.1|	beta-glucosidase	VASANANTVVVL	28.85	NP_214700.1	99

(*continued on next page*)

Table 3 (*continued*)

Protein ID	BCG T cell antigens	MHC class II-bound peptides	Ion Score	Mtb homolog	Identity
gi|224,989,118|ref|YP_002643805.1|	**protease IV**	**VLRVDSPGGS**	**23.97**	**NP_215238.1**	**100**
gi|224,989,815|ref.|YP_002644502.1|	aminoglycosides/tetracycline-transport integral membrane protein	WFLIALPIGAVTGGW	20.04	NP_215926.1	100
gi|224,991,464|ref.|YP_002646153.1|	hypothetical protein JTY_3107	WLRADIADPL	21.23	NP_217603.1	99

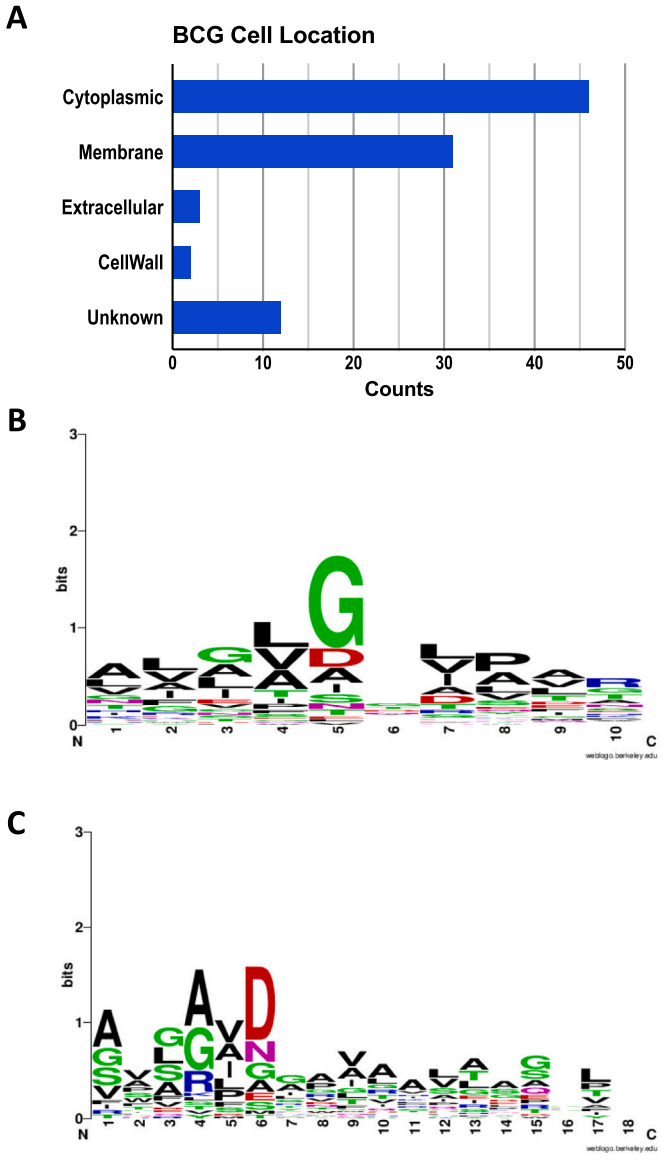
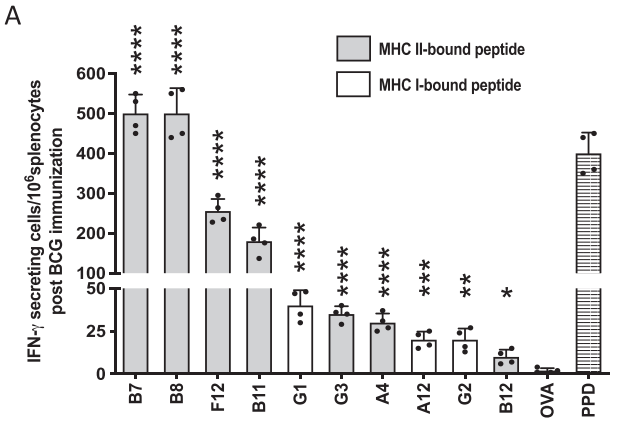


Fig. 3. Characteristics of MHC-bound BCG peptides. Cellular location of BCG-derived MHC-bound peptide source proteins (A); Logo plots of MHC class I peptides (B) and MHC class II peptides (C) from BCG-infected DCs created with the WebLogo program.



B

Peptide	Source Protein in BCG & Mtb	Functions & Potential Relevance to Vaccines
B7 & B8	JTY_2890, Rv2873 (Surface-exposed lipoprotein Mpt83)	Immunogenic and involved in the host-pathogen interaction.
F12	JTY_0744, Rv0724 (Protease IV)	Play roles in bacterial metabolism and virulence.
B11	JTY0631, Rv0615 (Integral Membrane Protein)	Involved in transport and membrane integrity.
G1	JTY_3586, Rv3521 (Hypothetical Protein)	Uncharacterized protein
G3	JTY_1665, Rv1651c (PE-PGRS Family Protein)	Involved in immune evasion, play roles in antigenic variation and immune system interactions
A4	JTY_2793, Rv2781c (Alanine-Rich Oxidoreductase)	Involved in metabolic processes, can be crucial for bacterial survival and virulence.
A12	JTY_3269, Rv3244c (Lipoprotein LpqB)	Involved in cell wall processes, often immunogenic.
G2	JTY_2831, Rv2818c (Hypothetical Protein)	Uncharacterized protein
B12	JTY_2958, Rv2941 (Acyl-CoA Synthetase)	Involved in fatty acid metabolism, essential for bacterial growth and survival.

Fig. 4. Identification of immunodominant BCG peptides. (A) Peptide-specific IFN-γ responses in the BCG vaccinated mice assessed by ELISPOT assay. Splenocytes pooled from five vaccinated mice were stimulated in vitro for 20 h with 94 individual BCG peptides at 2 μg/ml respectively. Purified protein derivative (PPD) was used as a positive control and an irrelevant ovalbumin (OVA) peptide was used as a negative control. The results reflected the average of 4 replicates from two independent experiments and were expressed as the means ± SD. No background was subtracted from these responses, as the background (medium only) yielded extremely low counts (≤ 3 spots). The data of ten positive peptides (listed above and highlighted in bold font in Tables 2 & 3) which elicited significant IFN-γ secreting cells (≥ 10 spots per well) were presented. The remaining 84 BCG peptides (in regular font in Tables 2 & 3) that did not induce significant IFN-γ secreting cells (falling below the cut off of <10 spots per well) were not depicted in this figure. *, **, *** and **** indicate *P* values of <0.05, <0.01, <0.001 and < 0.0001 respectively, in comparison to results for the negative control, OVA peptide. (B) The nine peptide source proteins in BCG and Mtb, and their functions and potential relevance to vaccines.

by MHC molecules are extracted directly from infected BM-DCs and detected using tandem mass spectrometry. This method enables the utilization of genomic data under physiological conditions to map out the entire T-cell immunoproteome of the organism. Although the present study did not involve the evaluation of the BCG peptides in vivo, our previous studies on *Chlamydia* demonstrated that the *C. muridarum* MHC class II peptides identified via immunoproteomics were recognized by *Chlamydia*-specific CD4 T cells harvested from immune mice and adoptive transfer of dendritic cells pulsed ex vivo with the peptides provided protection against intranasal and genital tract *Chlamydia* infection in mice [10]. Using immunoproteomics, we identified 27 *C. muridarum* CD4 T cell antigens, representing about 3 % of the *Chlamydia* proteome. After excluding *Chlamydia* proteins with significant sequence homology to human and other bacterial proteins, we tested 13

antigens in vivo and found that 11 conferred significant protection against *Chlamydia* genital challenge in mice [16].

We also found that the peptides originally identified in C57BL/6 and their source proteins are readily recognized by T cells from C57BL/6 mice. Source proteins of the peptides, but not peptides, were also recognized by *Chlamydia*-specific T cells from Balb/c and C3H/HeN mice that are of different genetic background [15]. Our previous study further revealed that T cell epitopes at distinct sequences within the identified proteins are presented by specific MHC haplotypes, indicating that the proteins (antigens) identified via immunoproteomics are immunologically recognized across genetically different MHC background suggesting they are immunodominant [14]. In addition, multiple-component vaccines (cocktail subunit vaccines) are likely to cover a broader range of epitopes for T cell recognition among different MHC genetic backgrounds, providing protection in humans.

Since a peptide identified via immunoproteomics is specific to an MHC haplotype, it is unlikely that a peptide identified in mice is also reactive in humans. However, our immunoproteomics studies in mice suggest that a peptide source protein contains multiple epitopes for different MHC backgrounds. Thus, it is likely that a peptide source protein identified via immunoproteomics in mice also contains human epitopes that can bind to HLA haplotypes. The value of the immunoproteomics approach used in this study is to discover immunodominant peptide source proteins that have potential as subunit vaccines, inducing immunity to pathogens such as TB and *Chlamydia*, which are known to mainly depend on cell-mediated immunity. It would be ideal to identify epitopes directly from human DCs infected with a specific pathogen. However, the requirement for a high number of DCs, ranging from five to ten billion for each experiment, and the diverse HLA profiles of individuals make this technique currently unfeasible with human DCs.

The IFN-γ ELISPOT assay is an important tool in the immunoproteomics approach to screen pathogen-derived peptides and efficiently discover immunodominant antigens. In this study, about 10 % of the peptides were recognized as positive in the IFN-γ ELISPOT assay. This is likely because peptides bound to MHC molecules on APCs, such as DCs, need to be recognized by cognate T cells via TCR-peptide/MHC complex binding to trigger T cell activation. The lack of cognate T cells in the host T cell reservoir will result in no reactivity. Moreover, only a subset of the activated T cells generates IFN-γ responses, which are essential for protective immunity against TB. Those peptides that activate T cells to generate non-IFN-γ responses, which may induce pathological reactions, would not be selected.

BCG share about 98 % of its genome with Mtb. The source proteins of all 10 positive peptides identified in the current study are 99–100 % identical to Mtb. However, BCG vaccine strains lack several regions and specific genes that are present in virulent Mtb strains, particularly those involved in virulence, immune evasion, and certain metabolic pathways. These deletions are primarily responsible for the attenuation of BCG, making it non-virulent compared to Mtb. For example, BCG Tokyo 172 strain has 3984 genes with a deletion of 189 genes compared to Mtb H37Rv strain that has 4173 genes. Therefore, further investigations using virulent Mtb instead of BCG vaccine strain via this immunoproteomic approach to discover Mtb-specific immunodominant antigens would be crucial. The identified Mtb-specific antigens could be used as subunit vaccines that are co-administered with BCG for prime or boost to enhance and broaden the immune responses.

Strategies to improve TB vaccination mainly involve one of two approaches: live attenuated vaccines, such as the optimization of the current BCG vaccine, or the development of novel vaccines, such as subunit, vectored, and mRNA vaccines [24]. The selection of BCG-shared or Mtb-specific antigens included in TB vaccines is fundamental to the success of these approaches. A review by Singh et al. [25] summarized current efforts to improve the efficacy of BCG through the development of recombinant strains including rBCG expressing immunodominant antigens, mammalian cytokines, listeriolysin, perfringolysin, and the ESX-1 variants. The review also introduced various BCG prime-boost vaccination regimens in both animal models and human studies. For example, in an NHP model, a multistage vaccine containing Ag85B, ESAT-6, and Rv2660c antigens with the adjuvant IC31 as a boost to BCG delayed and reduced clinical disease after Mtb challenge, and also prevented the development of latent TB infection (LTBI) [26].

The immunoproteomic approach used in this study provides an innovative platform to discover new and immunodominant antigens for the evaluation of vaccine candidates against TB. Currently there are 17 Mtb vaccine candidates in clinical development and 7 are subunit booster vaccine for BCG [27]. A novel subunit vaccine that recently entered late-stage development (Phase 2a) to prevent pulmonary TB is the M72/AS01 vaccine [28]. M72/AS01 is composed of two Mtb antigens (a fusion recombinant protein of Mtb32A and Mtb39A antigens) and the GSK proprietary adjuvant system AS01 (liposome based MPL-A and QS21). This vaccine, administered in adulthood is intended to boost BCG immunity administered in childhood. The vaccine efficacy in a recent randomized, double blinded clinical trial at month 36 to prevent pulmonary TB was 49.7 % [29].

The current study demonstrates, for the first time, the isolation of BCG derived peptides from class I and class II MHC molecules of professional antigen-presenting cells using MS/MS analysis. 94 BCG-derived MHC-bound peptides were discovered, 55 of which were presented by MHC class I molecules and 39 by MHC class II molecules from BCG-infected DCs. Mice that were infected with BCG were able to recognize a subset of these peptides (Fig. 4A). In particular B7 and B8 peptides derived from Mpt83 protein were most antigenic and warrant further immunological study.

Mpt83, a mycobacterial lipoprotein found on the surface of Mtb was the top T cell antigen identified in this study because of its high antigenicity. Mpt83 was previously shown to be recognized by IFN-γ secreting T cells from patients with active TB and stimulated protective immunity to Mtb in mice when immunized with MPL-A/DDA adjuvant [30]. Because of the results found in our study, we speculate that the addition of the Mpt83 T cell antigen could substantially increase the efficacy of the M72/AS01 vaccine in new clinical trials of subunit vaccines to boost BCG immunity and prevent reactivation of latent TB.

CRediT authorship contribution statement

Karuna P. Karunakaran: Writing – original draft, Project administration, Investigation, Funding acquisition, Conceptualization. **Hong Yu:** Writing – review & editing, Investigation, Formal analysis. **Xiaozhou Jiang:** Methodology, Investigation. **Queenie W.T. Chan:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Lynette Sigola:** Writing – review & editing, Supervision, Formal analysis. **Leonard A. Millis:** Writing – review & editing, Formal analysis, Data curation. **Jiaqi Chen:** Formal analysis, Data curation. **Patrick Tang:** Writing – review & editing, Methodology, Conceptualization. **Leonard J. Foster:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Robert C. Brunham:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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