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dendritic cell-based functional validation
of MHC class I-restricted *Mycobacterium
tuberculosis* epitopesSmita K. Nair^{1*}, Georgia D. Tomaras^{1*}, Ana Paula Sales^{2†}, David Boczkowski¹, Cliburn Chan²,
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Emergence of drug-resistant strains of the pathogen *Mycobacterium tuberculosis* (Mtb) and the ineffectiveness of BCG in curtailing Mtb infection makes vaccine development for tuberculosis an important objective. Identifying immunogenic CD8+ T cell peptide epitopes is necessary for peptide-based vaccine strategies. We present a three-tiered strategy for identifying and validating immunogenic peptides: first, identify peptides that form stable complexes with class I MHC molecules; second, determine whether cytotoxic T lymphocytes (CTLs) raised against the whole protein antigen recognize and lyse target cells pulsed with peptides that passed step 1; third, determine whether peptides that passed step 2, when administered *in vivo* as a vaccine in HLA-A2 transgenic mice, elicit CTLs that lyse target cells expressing the whole protein antigen. Our innovative approach uses dendritic cells transfected with Mtb antigen-encoding mRNA to drive antigen expression. Using this strategy, we have identified five novel peptide epitopes from the Mtb proteins Apa, Mtb8.4 and Mtb19.

Based on the 2013 World Health Organization (WHO) report, *Mycobacterium tuberculosis* (Mtb) infection caused tuberculosis (TB) in 8.6 million people and 1.3 million deaths in 2012 and remains a major health concern in the developing world and among HIV-positive people. This situation is further exacerbated by the emergence of drug-resistant strains, multidrug-resistant (MDR), extensively drug-resistant (XDR) and totally drug-resistant strains^{1,2}. Patients infected with these drug-resistant Mtb strains require much longer treatment courses and have lower success rates compared to susceptible strains^{3,4}. No truly effective vaccine to prevent infectious TB exists^{5–8}. Bacillus Calmette-Guérin (BCG) is a live attenuated vaccine used in some parts of the world, but due to its limited efficacy it is not routinely utilized in the United States⁹. Although there have been numerous vaccine studies to date, there is not a consensus as to the best antigens to include in a potential subunit vaccine against TB^{5–8,10}.

A cellular CD8+ T cell immune response against Mtb requires that peptides derived from Mtb proteins be presented in the context of MHC class I molecules on Mtb-infected cells. These peptides are specifically recognized by CD8+ cytotoxic T lymphocytes (CTL) that lyse the infected cell. Current data in both humans and the murine model of TB infection indicate that CD8+ T lymphocyte responses are crucial to immunity against Mtb^{11–19}. The antigen-specific CD8+ T cells lyse Mtb-infected monocyte-derived macrophages and alveolar macrophages after recognition of mycobacterial antigens presented in the context of MHC class I molecules. Thus, identification of *M. tuberculosis* peptides that bind to class I alleles is critical for understanding the immune response to TB infection and for the development of effective vaccines.

To generate an effective peptide-based vaccine it is critical to identify peptides that not only stimulate a peptide-specific T cell response, but also represent biologically derived peptides that are presented by pathogen-infected cells^{20,21}. This would ensure that a peptide vaccine would stimulate a peptide-specific T cell response that will ultimately lyse the infected target cells. In a review²², Lauemoller *et al.* concluded that peptide binding to MHC is the single most selective event involved in antigen handling and the most important to predict. Since no peptide can become an epitope without first binding to MHC, a more detailed understanding of peptide binding to MHC molecules would facilitate the identification of potentially immunogenic T cell epitopes. Epitope mapping studies



that have utilized peptide binding prediction algorithms have already identified HLA-A*0201 restricted epitopes in the Mycobacterium proteins ESAT-6^{17,23}, CFP10, CFP21²⁴, 85B²⁵, 85A²⁶, 19 kDa lipoprotein, 16 kDa, 44 kDa, thymidylate synthase¹¹, RNA poly B-subunit (RpoB)¹¹, permease protein A-1¹¹, 38 kDa²⁷, 28 kDa²⁷ and other proteins^{28,29}.

For the results reported herein, we employed a high throughput epitope discovery technology available through Beckman Coulter, Inc. (BCI) named iTopiaTM that is optimized for use in identifying HLA class I associated epitopes. This technology does not rely on *in silico* algorithmic predictions, but instead provides for rapid experimental testing of all possible peptides and their *bona fide in vitro* binding characteristics to a broad group of the most common class I MHC alleles (A*0101, A*0201, A*2402, A*0301, A*1101, B*0702, B*0801, and B*1501). Our overall study objective toward a vaccine design for Mtb was to identify novel T cell epitopes capable of binding with high affinity and slow off-rates to HLA alleles and then to test the selected epitopes for immunogenicity. For this study, we examined three Mtb antigens (Apa, Mtb8.4 and Mtb19) all previously reported to be immunogenic^{30–36}, but that have not been finely mapped for potential T cell epitopes.

Apa, a 50–55-kDa protein (Rv1860 gene) of *M. tuberculosis*³³, has been shown to induce IFN- γ secretion from CD8+ T cells isolated from individuals with latent TB, as defined by a positive response to Purified Protein Derivative (PPD). In guinea pigs an Apa-specific vaccine consisting of DNA priming followed by a poxvirus recombinant booster injection resulted in protection against a virulent *M. tuberculosis* challenge³². Mtb8.4, a small protein present in the culture filtrate of *M. tuberculosis*, elicited Th1 responses in murine and human cells^{30,31}. Notably, class I responses against this protein were demonstrated in PBMCs from PPD-positive humans^{30,31,36}. Mtb8.4-specific T cells were detected in mice immunized with *Mycobacterium bovis*-BCG and in mice infected with *M. tuberculosis*³⁰. More importantly, mice were protected from challenge with virulent *M. tuberculosis* and also demonstrated a strong induction of CD8+ cytotoxic T lymphocyte (CTL) responses after immunization with either plasmid DNA encoding Mtb 8.4 or Mtb 8.4 recombinant protein³⁰. Finally, Mtb19 is a 19-kDa secreted lipoprotein that is another relevant target of cellular immunity to *M. tuberculosis* in the murine model of TB infection^{16,18}. Although targeting Apa, Mtb8.4 and Mtb19 holds promise, to date very few studies have systematically addressed identification and functional validation of HLA-binding epitopes in Apa, Mtb8.4³⁴ and Mtb19³⁵ proteins for any of the human alleles.

In this study we determined the immunogenicity of peptide epitopes that were selected based on optimal MHC class I binding

properties. We then evaluated whether the cytotoxic T cells specific for the Mtb antigen were capable of recognizing and lysing cells that expressed the peptide-HLA complex. These CTL were generated *in vitro* using autologous dendritic cells (DCs) transfected with RNA encoding the Mtb antigen^{37–40}. Mtb antigen-encoding RNA transfected DCs were used to induce Mtb antigen-specific CTL *in vitro*. In this study, we identified peptide epitopes by determining the ability of peptide-pulsed targets to be recognized and lysed by Mtb antigen-specific CTL. Peptides that were potential CTL epitopes, based on recognition and lysis of peptide-pulsed targets, were then used to stimulate antigen-specific T cells *in vivo* in HLA-A2 transgenic mice.

Results

Identifying peptide epitopes using iTopiaTM. We mapped and validated MHC class I-restricted T cell epitopes of Mtb antigens, using as our primary mapping tool iTopia^{TM41–43}, a Beckman Coulter, Inc. (BCI) technology. We analyzed overlapping 9-mer peptides (with 8 aa overlaps) that spanned the entire protein sequence of seven different *M. tuberculosis* antigens: CFP-10, ESAT6, Ag85A, Ag85B, Mtb19, Apa, and Mtb8.4. We identified potential peptide epitopes among these 9-mers via a sequence of screening steps, such that only peptides that passed a given step were moved forward to the subsequent step. We performed an initial screening for MHC class I binding to one of eight alleles (A*0101, A*0201, A*2402, A*0301, A*1101, B*0702, B*0801, and B*1501) using the iTopiaTM system. The binding measurements produced by iTopiaTM along with the affinity (ED₅₀) and off-rate (t_{1/2}) measurements of peptide binding to the MHC class I molecules, were used to calculate the peptide's cScore (described in Methods and Supplementary Methods). The set of peptides with cScores above a 0.1 threshold were identified as the 9-mers with highest potential for being peptide epitopes, and were moved into the functional validation phase of study. The cumulative result of peptide mapping for the 3 Mtb proteins of interest (Apa, Mtb8.4 and Mtb19) is provided in Table 1. The peptide mapping data for all the seven *M. tuberculosis* antigens: CFP-10, ESAT6, Ag85A, Ag85B, Apa, Mtb8.4 and Mtb19 is provided in Supplementary Table 1.

Generation of Mtb antigen-specific CTL responses using DCs transfected with RNA encoding Mtb antigen to stimulate autologous T cells *in vitro*. Although we identified a large number of potential epitopes that met the selection criteria (1396 9-mers for all seven TB proteins and 8 MHC alleles, see Supplementary Table 1), we focused on the functional validation of the epitopes in three of the lesser studied Mtb antigens, namely Apa, Mtb8.4 and Mtb19

Table 1 | Summary of *Mycobacterium tuberculosis* peptide mapping

# Peptides	Mtb Apa		Mtb 8.4		Mtb 19	
	317		102		151	
	C > 0.0	C > 0.1	C > 0.0	C > 0.1	C > 0.0	C > 0.1
A*0101	5	2	3	0	2	1
A*0201	157	54	69	26	55	17
A*2402	52	12	21	6	10	2
A*0301	13	4	3	1	6	3
A*1101	20	6	7	2	12	3
B*0702	51	14	15	3	8	1
B*0801	10	2	5	1	2	0
B*1501	31	6	31	10	13	3
Total Peptides	339	100	154	49	108	31
% Total Peptides/Target	13.4%	3.9%	18.9%	6.0%	8.9%	2.6%

The relative MHC binding, Affinity (ED₅₀), and Off-Rate (t_{1/2}) for each of the 8 alleles (A*0101, A*0201, A*2402, A*0301, A*1101, B*0702, B*0801, and B*1501) were measured for the peptides that passed the initial screen. The number of peptides tested and that received a cScore [C] greater than 0.0 and 0.1 for each of the 8 alleles are shown. % Total Peptides/Target is the number of identified epitopes divided by the total number of peptide-MHC targets tested (# peptides * 8 alleles) multiplied by 100.



(reducing the number of 9-mers to 570 for 8 MHC alleles, Table 1). Moreover, since HLA-A2 and HLA-B7 mice are available for functional validation, we further narrowed our analysis to HLA-A*0201- and HLA-B*0702-binding 9-mers from Mtb antigens, Apa, Mtb8.4 and Mtb19. Since our analysis was restricted to the top 20 peptides with cScore > 0.1, we analyzed a total of 75 peptides (57 HLA-A*0201- and 18 HLA-B*0702-binding peptides) using the validation strategy described in the results below.

To determine if peptides we identified are presented and recognized by antigen-specific CTL, our first task was to generate Mtb antigen-specific T cells *in vitro* that could then be used in subsequent experiments to functionally screen Mtb peptides. To this end, we have developed an *in vitro* system wherein we can routinely generate primary, antigen-specific cytotoxic T cells using DCs transfected with RNA encoding the full-length protein^{44,45} to stimulate autologous, naïve T cells *in vitro*^{37–40}. We, therefore, generated *in vitro* transcribed (IVT) RNA encoding Apa, Mtb8.4 and Mtb19. Figure 1 demonstrates that DCs transfected with Mtb antigen RNA stimulate primary, antigen-specific CTL *in vitro*. The specificity of the lysis is demonstrated by the fact that Mtb antigen-specific effectors lyse only the corresponding Mtb antigen-expressing targets and not the control targets (DCs transfected with human actin RNA). Mtb antigen-specific CTL generated using this DC-RNA T cell stimulation platform were used to functionally screen peptides from Table 1.

Identification of endogenously processed and presented MHC class I HLA-A*0201-restricted and MHC class I HLA-B*0702-restricted peptides. The antigen-specific CTL generated using the protocol described in Figure 1 were used to determine immunodominance among the peptide epitopes that were identified using our screening algorithm. We determined whether the HLA-A*0201-restricted CTL epitopes predicted using the binding, affinity and off-rate algorithm were indeed endogenously processed peptides. Thus, peptide-pulsed target cells were tested for recognition and lysis by antigen-specific CTL^{22,46}. The CTL assay also determines if the peptides with higher cScore are more efficiently recognized by Mtb antigen-specific T cells. CTL reactivity was determined by monitoring lysis of target cells, T2 cell line (TAP-deficient, HLA-A*0201+ cells) (Figure 2) or autologous DCs from HLA-A*0201+ donors (Figure 3) that were pulsed with peptides. Target cells pulsed with control peptides (depicted as C) were used as negative controls to determine the specificity of lysis. The peptides listed in Figures 2 and 3 are ranked in descending order from the highest to the lowest

cScore binders. The peptide ID number refers to the nomenclature of the peptide and represents the starting amino acid position within the protein. This analysis was repeated using cells derived from 3 additional HLA-A*0201+ donors to determine reproducibility in the immunodominance of the top 20 cScore-ranked peptide epitopes. Each experiment is tabulated in Supplementary Table 2: 2a (Apa), 2b (Mtb8.4) and 2c (Mtb19). The sequence of peptides that were consistently recognized by antigen-specific CTL and their cScore ranking is provided in Table 2.

To functionally validate HLA-B*0702-binding peptide epitopes, HLA-B*0702-restricted T cells specific to Mtb antigens were generated *in vitro* using cells from healthy HLA-B*0702+ donors. These HLA-B*0702-restricted T cells were used in CTL assays with autologous DCs pulsed with peptide as targets (Figure 4a). To further validate the HLA-B*0702-binding peptide epitopes, we used an alternate approach. HLA-B7 transgenic mice were immunized subcutaneously three times with autologous DCs transfected with Mtb antigen-encoding RNA. Antigen-specific T cells were restimulated *in vitro* with RNA-transfected DCs and used to validate HLA-B*0702-restricted CTL reactivity using autologous DCs pulsed with peptides as targets (Figure 4b). As shown in Figure 4 and Table 2, both approaches revealed the presence of two dominant epitopes, one from Mtb antigen Apa and the second from Mtb antigen 19. The analysis was restricted to peptides with cScore > 0.1, therefore the analysis included 14 peptides from Apa, 3 peptides from Mtb8.4 and 1 peptide from Mtb19. This experiment was repeated 4 times using cells from 2 donors and cells from HLA-B7 mice and each experiment is represented in the Supplementary Table 2d.

***In vivo* immunogenicity of peptide epitopes.** HLA-A*0201-restricted peptides (2 peptides selected for each Mtb protein, Table 2) that were consistently recognized in CTL analysis *in vitro* were analyzed for immunogenicity by measuring their ability to induce a T cell response *in vivo*. HLA-A2 mice were immunized intradermally as indicated in the Methods with 100 µg of peptide, emulsified 1:1 in IFA, for a total of 3 immunizations in conjunction with anti-CD40 antibody. Non-adherent splenocytes were restimulated *in vitro* using peptide-pulsed DCs. CTL reactivity of these epitope-specific T cells was determined by measuring lysis of Mtb antigen-encoding RNA-transfected DCs as shown in Figure 5. Data in Figure 5 demonstrates that 5 out of the 6 HLA-A*0201-restricted peptides that were selected based on binding (Table 1) and functional screening (Figures 2 & 3), when administered *in vivo* in HLA-A2

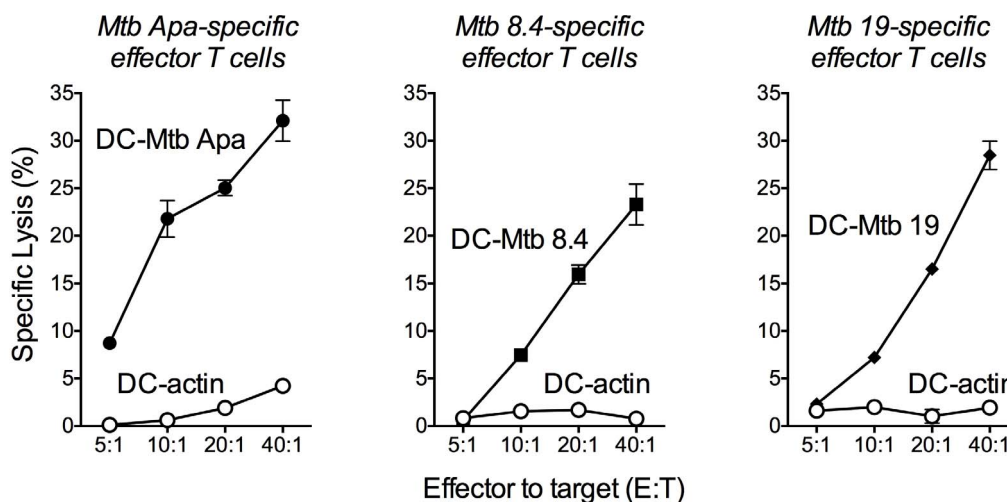


Figure 1 | Induction of Mtb antigen-specific CTL using Mtb antigen encoding RNA-transfected DCs to stimulate autologous T cells. RNA-transfected mature DCs were co-cultured with autologous negatively isolated CD3+ T cells as described in Methods. Following *in vitro* stimulation, T cells were harvested and assessed for their ability to lyse targets in a standard 4-hour europium-release assay using autologous DCs transfected with RNA as targets. Standard deviation reflects variation between triplicate wells. This experiment was done 2 times with similar results. E:T is effector to target ratio.

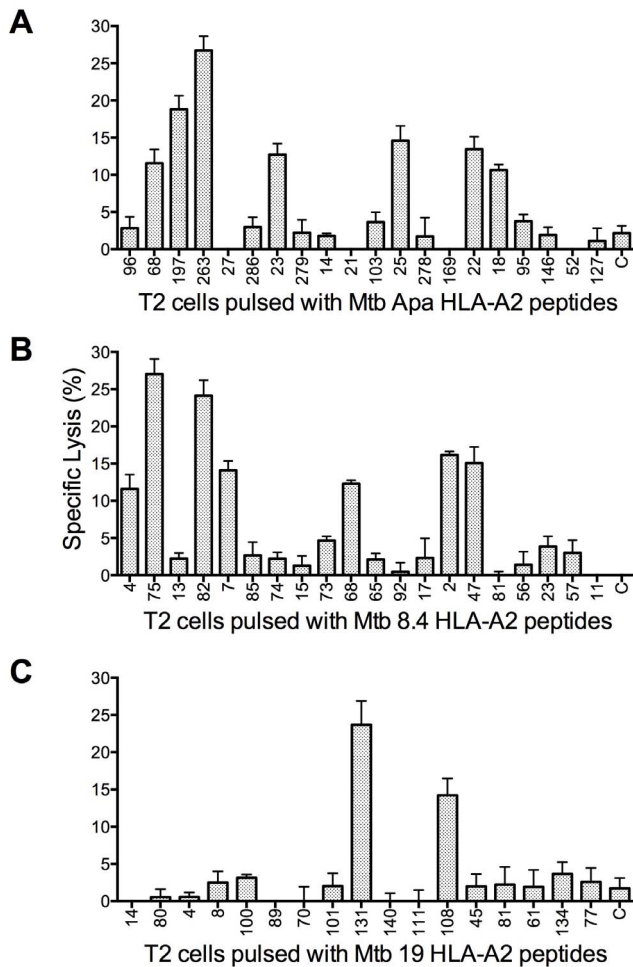


Figure 2 | Identification of HLA-A*0201-restricted epitopes endogenously processed and presented by Mtb antigen encoding RNA-transfected DCs using T2 cells as targets. Mtb antigen-specific T cells were generated as described in Methods. Europium-labeled T2 cells were incubated with the peptides (100 μ M) for 1 h prior to addition of T cells followed by a standard 4 h europium release assay. Negative control (C) is an HLA-A*0201 peptide with a low cScore. Standard deviation reflects variation between triplicate wells. This experiment was done 3 times and data from each experiment is shown in Supplementary Table 2a–c. (2A). Mtb Apa, (2B). Mtb8.4, (2C). Mtb19.

transgenic mice as a vaccine, elicit cytotoxic T cells that lyse target cells expressing the Mtb antigen.

Discussion

A number of methods have been proposed for identification of T cell epitopes based on the ability to predict or measure peptide binding to MHC class I molecules^{47–49}. Most algorithmic approaches are limited to assessment of the theoretical potential for epitope binding in common alleles and do not measure actual binding or address the potential significant contribution of immunogenic epitopes in other HLA alleles. The methods that do measure binding are not amenable to high throughput and, therefore, it is difficult to screen large numbers of peptides for many different MHC types. The iTopiaTM technology addresses these issues⁴³. Using a modified iTopiaTM algorithm to accurately assess binding, affinity and off-rate, we mapped and validated MHC class I-restricted T cell epitopes within selected target regions of lesser-studied Mtb antigens. Of note is the fact that the identified epitopes in Mtb have a proclivity for restriction by B*0702 and B*1501, as previously shown³⁴, in addition to A*0201 (Table 1 and Supplementary Table 1). This higher restriction by B alleles may

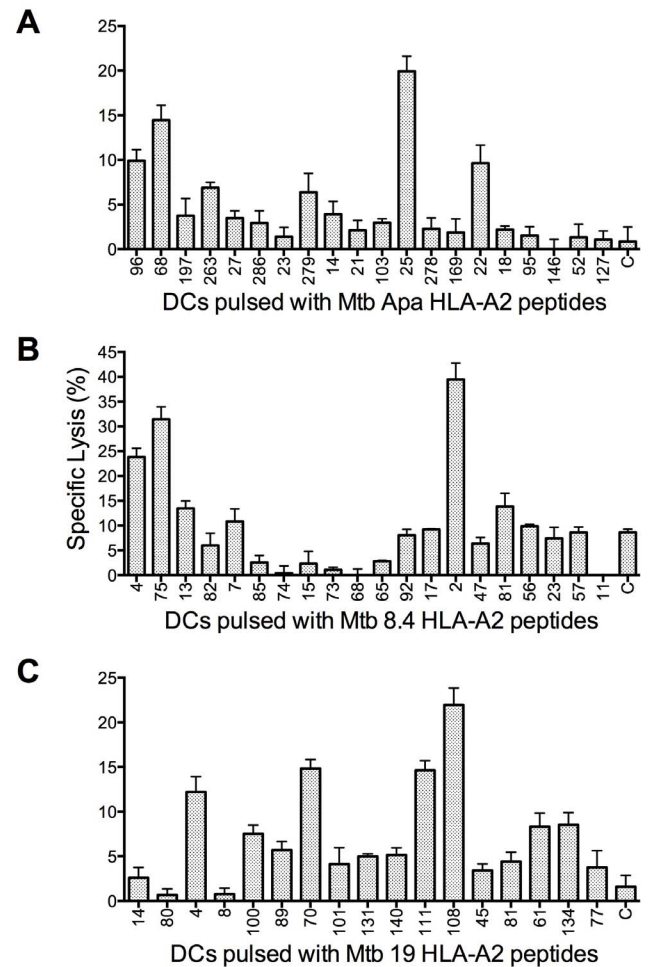


Figure 3 | Identification of HLA-A*0201-restricted epitopes endogenously processed and presented by Mtb antigen encoding RNA-transfected DCs using DCs as targets. Mtb antigen-specific T cells were generated as described in Methods. Europium-labeled DCs were incubated with the peptides (100 μ M) for 1 h prior to addition of T cells followed by a standard 4 h europium release assay. Negative control (C) is an HLA-A*0201 peptide with a low cScore. Standard deviation reflects variation between triplicate wells. This experiment was done 2 times and data from each experiment is shown in Supplementary Table 2a–c. (3A). Mtb Apa, (3B). Mtb8.4, (3C). Mtb19.

be suggestive of their potential immunogenicity in populations where HLA-B MHC alleles predominate.

To immunologically assess the function of these peptide epitopes, something that no algorithm is currently able to do, we generated Mtb antigen-specific T cells using DCs transfected with Mtb antigen-encoding RNA, technology that we originally established to stimulate tumor-specific immune responses *in vivo*^{37–40}. Use of antigen-encoding mRNA offers an important, and not fully appreciated, practical advantage over the use peptide or protein: ease of generation. Given a sequence, the corresponding mRNA can be generated in a simple process involving RT-PCR and transcription, with cloning of the cDNA intermediate into bacterial plasmid. Thus, the analysis of multiple antigenic targets is not hindered by the need to manufacture protein antigen or to identify class I and class II peptides corresponding to specific MHC alleles.

In this study, we identified functional peptide epitopes by determining the ability of peptide-pulsed targets to be recognized and lysed by Mtb antigen-specific CTL. Peptides that were potential CTL epitopes, based on recognition and lysis of peptide-pulsed targets, were then used to stimulate antigen-specific T cells *in vivo* in



Table 2 | Peptides recognized by Mtb antigen-specific cytotoxic T lymphocytes

PeptideID	Sequence	Rank	cScore	Experimental Results - % Lysis ± SD [p value compared to control]					
				Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	
Mtb Apa HLA-A2 peptides									
Apa-197	YMPYPTGTRI	3	0.580	21 ± 0.6 [0.002]	19 ± 2 [0.007]	18 ± 2 [0.02]	4 ± 2 [0.09]	11 ± 2 [0.2]	
Apa-263	WLGTAIINPV	4	0.522	29 ± 1 [0.0002]	27 ± 2 [0.003]	14 ± 0.6 [0.001]	7 ± 0.6 [0.04]	28 ± 2 [0.005]	
Apa-25	SASLVTAV	12	0.392	12 ± 0.3 [0.007]	15 ± 2 [0.01]	40 ± 4 [0.005]	20 ± 1.7 [0.01]	19 ± 1.5 [0.006]	
Mtb 8.4 HLA-A2 peptides									
Mtb8.4-4	SLTALSAGV	1	0.627	27 ± 2 [0.005]	12 ± 2 [0.002]	24 ± 1.8 [0.005]	11 ± 2 [0.006]	16 ± 0.6 [0.006]	
Mtb8.4-75	APPPQRAAM	2	0.572	25 ± 0.4 [0.001]	27 ± 2.5 [0.002]	32 ± 2.5 [0.005]	1 ± 1 [0.6]	21 ± 2 [0.01]	
Mtb8.4-82	AMAAQLGAV	4	0.447	28 ± 1 [0.0005]	24 ± 2 [0.002]	6 ± 2.5 [0.2]	1 ± 0.7 [0.5]	20 ± 1.5 [0.01]	
Mtb8.4-2	RLSLTALSA	14	0.261	11 ± 0.5 [0.07]	16 ± 0.5 [0.002]	40 ± 3 [0.005]	0.5 ± 0.3 [0.5]	16 ± 1.4 [0.001]	
Mtb 19 HLA-A2 peptides									
Mtb19-131	KITGTATGV	9	0.184	37 ± 3 [0.004]	24 ± 3 [0.01]	12 ± 2 [0.009]	5 ± 0.3 [0.06]	19 ± 3 [0.02]	
Mtb19-108	VTLGYTSGT	12	0.143	31 ± 1 [0.002]	14 ± 2.5 [0.02]	14 ± 1 [0.002]	22 ± 2 [0.004]	26 ± 3 [0.009]	
Mtb Apa HLA-B7 peptides									
Apa-286	LVAPPAPA	4	0.155	34 ± 3.4 [0.008]	28 ± 1.6 [0.002]	22 ± 2.4 [0.01]	35 ± 3 [0.002]		
Mtb 19 HLA-B7 peptides									
Mtb19-146	SPVNKSFEI	1	0.159	15 ± 0.7 [0.02]	9 ± 1.7 [0.04]	16 ± 1.8 [0.007]	15 ± 1.5 [0.007]		

Based on *in vitro* functional validation data, peptides highlighted in yellow were used in a subsequent *in vivo* immunogenicity experiment in HLA-A2 transgenic mice. Statistical significance comparing two groups was done using paired two-tailed Student's *t* test. A probability of less than 0.05 ($p < 0.05$) was considered statistically significant. Data in italics represents an insignificant *p* value as compared to control peptide. Standard deviation (SD) reflects variation between triplicate wells. A detailed overview of all peptides tested and data from each experiment is provided in Supplementary Table 2.

HLA transgenic mice. Our unique approach has identified Mtb peptide epitopes that are preferentially recognized by Mtb antigen-specific T cells. Notably, peptides with high binding scores were not necessarily the peptides recognized by Mtb-antigen-specific T cells, suggesting that detailed measurements of peptide-MHC binding (ED_{50} and $t_{1/2}$) as well as functional analysis are both important in identifying immunogenic peptide epitopes. In follow-up studies, we will conduct *ex vivo* validation of HLA A*0201 epitopes of TB peptides identified in this study, using PBMCs from patients infected with Mtb followed by *in vivo* studies to determine if HLA-A2 mice vaccinated with these epitopes are protected against a virulent Mtb challenge. Although broad applicability of peptide vaccines for global diseases may be limited because of MHC restrictions and low immunogenicity, these limitations can be resolved by making multiple epitope vaccines, including CD4 T cell epitopes, and with the use of adjuvants or chemical modifications to increase immunogenicity^{7,8}. Furthermore, this study is also relevant in the selection of antigenic targets (immunogenic pathogen proteins) that can be incorporated into vaccine strategies (vector- or protein-based) designed to induce protective CD8+ T cell responses^{5,8,12}.

It is also noteworthy that we did not see correlation between iTopia™ and the SYFPETHI and Bimas algorithms (peptide rankings compared in Supplementary Table 2), indicating that regardless of the algorithm used to identify MHC class I binding peptides, a functional validation should be performed to ultimately identify immunogenic T cell epitopes. The workflow for identification and validation of immunologically relevant peptides is shown in Supplementary Figure 1.

To summarize, the immunogenic peptide epitopes selected by binding, affinity and off-rate measurements followed by functional validation are listed below.

Mtb Apa: HLA-A*0201-binding peptides 25 (aa SASLVTAV), 197 (aa YMPYPTGTRI) and 263 (aa WLGTAIINPV) and HLA-B*0702-binding peptide 286 (aa LVAPPAPA) are potentially immunogenic epitopes. Positive lysis of T2 cell targets but not DC targets (refer to Supplementary Table 2) is presumably because T2 cells are artificially designed to exclusively present a high number of HLA-A*0201-binding epitopes thus increasing the density of these peptide epitopes on the cell surface. Moreover DCs express multiple HLA alleles and the individual alleles may differ in the levels of expression. We also noticed differences in peptide recognition between the different donors, although most donors were selected based on the expression of HLA-A*0201 and HLA-B*0702. In confirmatory *in vivo* immunogenicity assays, both peptides tested, peptides 25 and 263, induced immune responses in HLA-A2 transgenic mice (Figure 5).

Mtb 8.4: HLA-A*0201-binding peptides 4 (aa SLTALSAGV), 75 (aa APPPQRAAM), 82 (aa AMAAQLGAV) and 2 (aa RLSLTALSA) are potentially immunogenic epitopes. None of the 3 HLA-B*0702-restricted peptide epitopes that were selected using iTopia™ were immunogenic using our functional validation criteria. In confirmatory *in vivo* immunogenicity assays, both peptides tested, peptides 4 and 75, induced immune responses in HLA-A2 transgenic mice (Figure 5).

Mtb 19: HLA-A*0201-binding peptides 108 (aa VTLGYTSGT), 131 (aa KITGTATGV) and HLA-B*0702-binding peptide 146 (aa SPVNKSFEI) are potentially immunogenic epitopes. Although peptide 131 (aa KITGTATGV) was a strong candidate in functional assays using *in vitro* validation criteria, this peptide gave minimal CTL responses when used as an immunogen in HLA-A2 transgenic mice (Figure 5). Mtb 19 peptide 108 did induce a response *in vivo* in HLA-A2 transgenic mice (Figure 5).

Overall, we observed a weak correlation between the strength of peptide-MHC binding (as measured via iTopia™ as well as computed by prediction algorithms) and immunogenicity (as measured using functional lysis assays). This was also the case for two highly

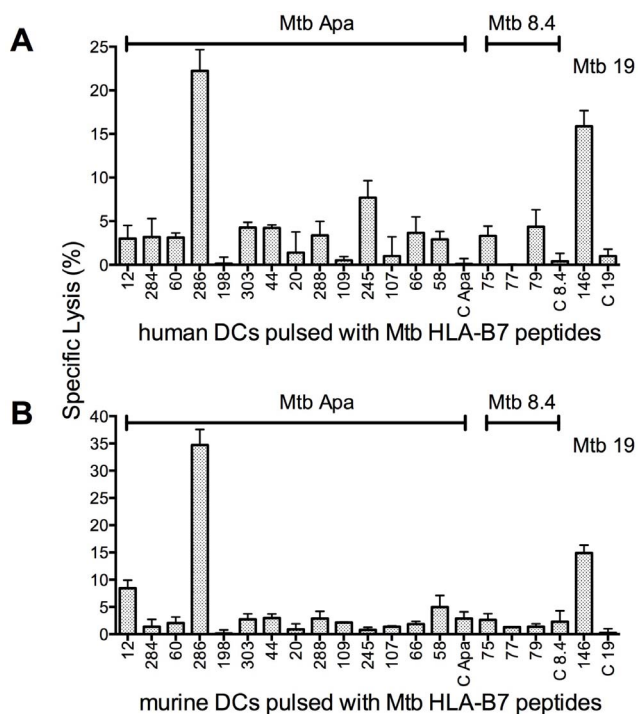


Figure 4 | Identification of HLA-B*0702-restricted epitopes for Mtb Apa, Mtb 8.4 and Mtb 19. (A). Human CD3⁺ T cells were stimulated with autologous DCs transfected with either Mtb Apa RNA or Mtb 8.4 RNA or Mtb 19 RNA. Stimulations were as described in methods. Autologous human DCs pulsed with B*0702-restricted peptides were used as targets. Europium-labeled DCs were incubated with peptides (100 μ M) for 1 h prior to addition of T cells followed by a standard 4 h europium-release assay. (B). HLA-B7 transgenic mice were immunized with murine DCs transfected with Mtb RNA 3 times at weekly interval. Splenocytes were harvested 8–9 days later and stimulated as described in methods. CTL assay was done after 5 days with murine DCs as targets. Europium-labeled DCs were incubated with the peptides (100 μ M) for 1 h prior to addition of T cells followed by a standard 4 h europium release assay. Standard deviation reflects variation between triplicate wells. This experiment was done 4 times and data from each experiment is shown in Supplementary Table 2d. Peptides analyzed were based on cScore 0.1 or above (14 peptides for Mtb Apa, 3 peptides for Mtb 8.4 and 1 peptide for Mtb 19). Negative controls (C Apa, C 8.4 and C 19) are HLA-B*0702 peptides with a low cScore.

immunogenic peptides derived from melanoma tumor antigen MART (EAAGIGILTV) and influenza matrix protein M1 (GILGFVFTLT). These peptides elicit immunodominant HLA-A*0201 restricted T cell responses *in vitro* but do not have the highest predicted binding to HLA-A*0201 when compared to other peptides derived from the protein (data not shown).

While minimal binding between peptide and MHC is a requirement for immunogenicity, it alone is not sufficient or predictive of immunogenicity. Hence, peptide-MHC binding assessment serves as a first screening step to eliminate primarily true negative peptides, that is, peptides that most likely will fail to elicit an effective CTL response because they fail to be presented by MHC class I molecules. This pre-screening step is essential in the process of identification of peptide epitopes because experimental assays to assess immunogenicity are both costly and time-consuming; as such, it is unrealistic to perform such assays for large numbers of peptides. For these reasons, iTopiaTM epitope discovery system (as possibly, *in silico* prediction algorithms) is a useful tool in the identification of peptide epitopes. In conclusion, in addition to identifying possible peptide epitopes for inclusion in peptide vaccine strategies, this immunological validation study may aid in the development of a more reliable algorithm for

prediction of immunogenic epitopes that elicit potent MHC class I-restricted CD8⁺ T cell responses.

Methods

Ethics statement. *Human.* Primary human cells used in these experiments were isolated from leukapheresis products that were obtained from human subjects following informed written consent using protocols approved by the Duke University Institutional Review Board.

Murine. In conducting the research described in this paper, the investigators adhered to the “Guide for the Care and Use of Laboratory Animals” as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities at the Duke vivarium are fully accredited by the American Association for Accreditation of Laboratory Animal Care, and all studies were conducted using protocols approved by the Duke University IACUC.

Peptide synthesis. All peptides (8 amino acids (aa), 9 aa and 10 aa) were synthesized at 80% or higher purity as assessed by HPLC and NMR profiling for each peptide (JPT Peptide Technologies). Peptides were dissolved in DMSO to generate 0.01 M stock solutions. The stock solutions were diluted in AIM-V to 0.001 M (10^{-3} M) and used in assays at 100 μ M.

MHC class I binding, affinity and off-rate assays. Eight different Human Leukocyte Antigen (HLA) alleles that cover 85–90% of the world population were assessed (A*0101, A*0201, A*2402, A*0301, A*1101, B*0702, B*0801, B*1501). MHC molecules were produced using recombinant technology by Beckman Coulter Inc. Peptide-MHC class I binding, affinity, and off-rate were measured using iTopiaTM epitope discovery system as follows. Solid phase MHC class I molecules were biotinylated and bound to avidin coated 96-well microtiter plates. Class I devoid of peptide is unstable, so the bound class I molecule included a placeholder peptide to maintain its integrity. The complexes were denatured to release placeholder peptide, and free β 2 microglobulin and peptide were washed away. The test peptide, additional β 2 microglobulin and antibody were added. The anti-HLA class I monoclonal antibody (mAb) only recognizes a properly folded class I molecule that contains peptide. To test affinity, peptides were incubated in decreasing concentrations (8 serial dilutions: 10^{-4} , 10^{-8} M) for 18 hours. Results are expressed as the concentration of peptide required to achieve 50% binding or half the complexes to form (ED_{50}). The entire plate was used to estimate common parameters of the affinity assay. To determine off-rate: peptide-MHC I complexes were allowed to form (18 hours) as in binding assay. Rate of dissociation from MHC was measured by loss of fluorescence over 8 different times (0, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0 hours) at 37°C. Results are expressed as the amount of time needed to achieve 50% dissociation ($t_{1/2}$) of peptide from MHC complex. Positive controls were included on every plate. Each positive control value was within 3STDEV of the mean for all targets as tracked throughout the project with Levey-Jennings plots (data not shown). Plates were read on a plate reader with a fluorescent read-out (M2, Molecular Devices). This information was used to derive the binding score (or cScore) for each peptide (Table 1, Supplementary Table 1 and Supplementary Methods).

Cloning of genes. Dr. Richard Frothingham (Duke University) provided chromosomal DNA from Mtb H37Rv (ATCC 25681, type strain). PCR primers were designed based on the genomic sequence (GenBank AL123456) to amplify the Mtb genes for Apa (GenBank CCP44626; synonyms: Rv1860, antigen MPT-32, ModD), Mtb8.4 (GenBank NP_215690; synonym: Rv1174c), and Mtb19 (Genbank CCP46590; synonyms: Rv3763, LpQH, 19 kDa lipoprotein). Full-length Mtb genes were then sub-cloned into the RNA expression plasmid. The RNA expression plasmid is a derivative of pcDNA3.1(–) (Invitrogen) containing a T7 RNA polymerase site 5' to the insertion site and a string of 64 adenosine residues 3' to the insert. *In vitro* RNA transcription from this plasmid produces RNA with poly-A tail 64 bases in length. Successful sub-cloning of each gene was confirmed by DNA sequencing of each plasmid (data not shown).

***In vitro* transcription of RNA.** The plasmids were digested with the restriction enzyme SpeI to linearize the DNA followed by *in vitro* transcription using the T7 mMessage Machine (Ambion). The *in vitro* transcribed (IVT) RNA was purified using a commercial kit (RNeasy, Qiagen), quantified by spectrophotometry, and analyzed by MOPS formaldehyde gel electrophoresis to confirm the synthesis of full-length RNA (data not shown)⁴⁴.

Generation of human dendritic cells (DCs). Peripheral blood mononuclear cells (PBMCs) were thawed, washed in PBS and resuspended at 2×10^8 cells in 30 ml AIM-V media (Invitrogen) in T-150 tissue culture flasks⁴⁵. Cells were incubated for 1 hour at 37°C, 5% CO₂ in a humidified incubator. The non-adherent cells were removed by rocking the flask from side to side to dislodge them. The adherent cells were replenished with 30 ml AIM-V media with 800 U/ml human GM-CSF and 500 U/ml human IL-4 then incubated at 37°C. DCs were harvested on day 6, by collecting all non-adherent cells, followed by cold PBS wash. DCs that were still adherent were dissociated with cell dissociation buffer (Invitrogen), 37°C for 20 min. The DCs were washed, counted and maintained on ice until use.

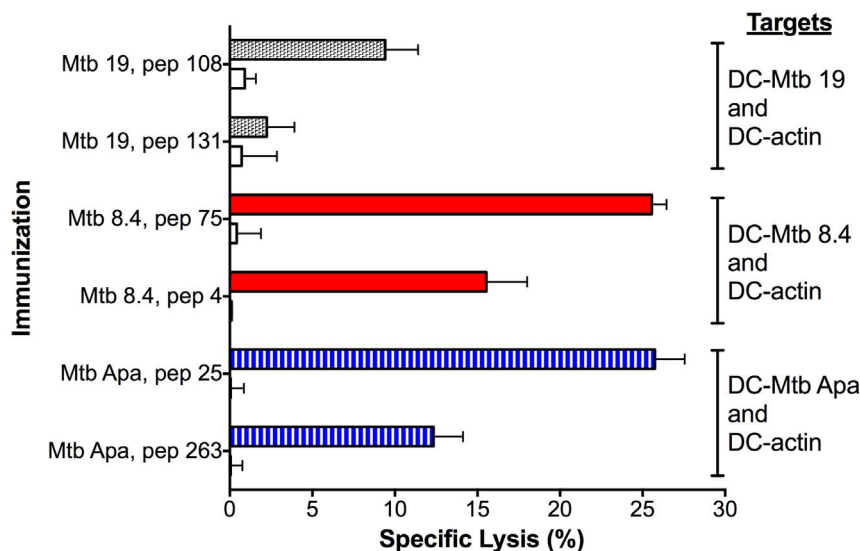


Figure 5 | *In vivo* immunogenicity of Mtb peptides in HLA-A2 transgenic mice. Mice were immunized intradermally 3 times at 1-week intervals with HLA-A*0201 peptides emulsified in IFA as described in detail in Methods. Peptides (2 representing each Mtb protein) were chosen based on *in vitro* assays shown in Figures 2 and 3 and Table 2. 10 days after the third immunization, spleens were harvested and non-adherent splenocytes were restimulated with autologous DCs pulsed with peptide and 50 U/ml IL-2. CTL assay was done 5 days later using the corresponding Mtb RNA-transfected DCs as targets. Actin RNA-transfected DCs were used as control targets. Standard deviation reflects variation between triplicate wells. E:T = 40:1.

Transfection of DCs with RNA encoding Mtb proteins. DCs were washed two times and gently resuspended in Opti-MEM at $2.5\text{--}3 \times 10^7/\text{ml}$. 100–200 μl were transferred to a 2-mm cuvette for electroporation using an Electro Square Porator ECM 830 from BTX® Harvard Apparatus, Inc (Holliston, MA). 3–4 μg RNA per 10^6 cells was added to the cuvette and mixed by pipetting up and down gently but thoroughly. The cells and RNA were pulsed at 300 Volts for 500 microseconds. The efficiency of RNA transfection was assessed using RNA encoding green fluorescent protein (GFP) followed by flow cytometric analysis (data not shown)⁵⁴. Transfected DCs were immediately transferred to plates containing media at 10^6 cells/ml and incubated in media containing the maturation cytokine cocktail at 37°C, 5% CO₂ for 8–15 hours. Maturation media is AIM-V containing GM-CSF (800 U/ml), IL-4 (500 U/ml), TNF- α (5 ng/ml), IL-1 β (5 ng/ml), IL-6 (150 ng/ml) and PGE₂ (1 $\mu\text{g}/\text{ml}$). All cytokines are obtained from Peprotech Inc. PGE₂ is purchased from Sigma.

Stimulation of T cells with RNA-transfected DCs *in vitro*. Human DCs transfected with antigen (or control)-encoding RNA were used to stimulate T cell responses *in vitro*. PBMCs were thawed, washed, resuspended in PBS and treated with DNase I (Sigma) at 200 U/ml for 20 min at 37°C. The EasySep T cell Enrichment Kit (StemCell Technologies, Vancouver, Canada) was used to negatively isolate CD3+ T cells from DNase I-treated PBMCs. The isolated CD3+ T cells were stimulated with RNA-transfected, matured DCs at a responder to stimulator ratio (R:S) of 10 : 1 in the presence of 25 ng/ml IL-7 at 37°C. Stimulations were done in RPMI 1640 with 10% FCS, 2 mM L-glutamine, 20 mM HEPES, 1 mM Na-pyruvate, 0.1 mM MEM non-essential amino acids, MEM amino acids (50 \times solution), 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 5×10^{-5} M β -mercaptoethanol (CTL stimulation medium). The responder T cell concentration was $2\text{--}2.5 \times 10^6$ cells/ml. IL-2 was added on day 3 at 50–100 U/ml. On day 7, the T cells were restimulated with RNA-electroporated DC at a R:S ratio of 10 : 1 in complete RPMI-10% FCS in the presence of 50–100 U/ml IL-2, with the T cells at $1\text{--}2 \times 10^6$ cells/ml in CTL stimulation medium. After 5–7 days the T cells were harvested, counted and used as effector cells in a europium-based CTL assay⁵⁰.

Target cells for CTL assays. T2 cells pulsed with peptides or autologous DCs pulsed with peptides or transfected with RNA were used as targets. T2 (ATCC® CRL-1992™) is an HLA-A*0201+ T-B lymphoblastoid hybrid cell line deficient of transporter associated with antigen processing (TAP) proteins and therefore can be successfully loaded with the candidate peptide epitopes⁵¹. DCs were transfected with RNA and incubated overnight in GM-CSF+IL-4 AIM-V media prior to europium labeling for use as target cells. 10^4 europium-labeled target cells in 100 μl of CTL medium (without penicillin-streptomycin) were incubated with 100 μM of peptide per well in 96-well V-bottom plates for 1 hour at 37°C. Effector cells were added to these peptide-pulsed, europium-labeled target cells for CTL assay at the indicated effector to target (E:T) ratio.

***In vitro* cytotoxicity assay.** 5–10 $\times 10^6$ target cells were labeled with europium for 20 min at 4°C using a standard published procedure⁵⁰. 10^4 europium-labeled targets and serial dilutions of effector cells at varying E:T ratio or at fixed E:T ratio as indicated in the figure legends were incubated in 200 μl of CTL medium (CTL stimulation medium with no penicillin-streptomycin) in 96-well V-bottom plates.

The plates were centrifuged at $500 \times g$ for 3 min and incubated at 37°C for 4 hours. 50 μl of the supernatant was harvested and added to 150 μl of enhancement solution (Perkin-Elmer) in 96-well flat-bottom plates and europium release was measured by time resolved fluorescence using the VICTOR3 Multilabel Counter (Perkin-Elmer).

Specific cytotoxic activity was determined using the formula: % specific release = [(experimental release - spontaneous release)/(total release - spontaneous release)] \times 100. Spontaneous release of the target cells was less than 25% of total release by detergent in all assays for the results of the assay to be valid. Spontaneous release of the target cells was determined by incubating target cells in medium without T cells. Non-specific lysis was determined by measuring the lysis of target cells loaded with control peptides (C). All assays were done in triplicates and repeated 2–3 times to confirm data.

Mice. 4–6 week old C57Bl/6 HLA-A2 transgenic mice (Stock #004191, Jackson Labs) and C57Bl/6 HLA-B7 transgenic mice (STOCK Tg(HLA-B*0702/H2-K^b)B7.xx $\beta 2^{\text{mml}}/\text{Ure}^{\text{c}}$ Tg($\beta 2\text{M}$)55Hpl, Taconic) were used. The HLA-A2 (HLA-A*0201) transgenic mice express $\alpha 1$, $\alpha 2$ domain of HLA-A*0201, $\alpha 3$ domain of H-2D^d and human $\beta 2\text{M}$ ($\beta 2$ microglobulin). The HLA-B7 (HLA-B*0702) transgenic mice express $\alpha 1$, $\alpha 2$ of HLA-B*0702, $\alpha 3$ domain of H-2K^b and human $\beta 2\text{M}$.

Generation of murine bone marrow precursor-derived DCs. Marrow from tibias and femurs of mice were harvested followed by treatment with ammonium chloride Tris buffer for 3 min at 37°C to deplete the red blood cells^{38,52}. The precursors were plated in RPMI-5% FCS with GM-CSF (15 ng/ml) and IL-4 (10 ng/ml) and supplemented with 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium-pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.1 mM non-essential amino acids and 5×10^{-5} M β -mercaptoethanol in 6-well plates. Cells were maintained at $10^6/\text{ml}$, 3 ml/well and incubated at 37°C and 5% CO₂. After 3 days the floating cells (mostly granulocytes) were removed and the adherent cells replenished with fresh GM-CSF and IL-4 containing medium. 4 days later the non-adherent DCs were harvested, washed, pulsed with peptide (100 μM) or electroporated with RNA as described above and matured for 5 hours at 37°C in GM-CSF+IL4 RPMI-5% FCS medium supplemented with LPS (E. coli 026:B6, 100 ng/ml, Sigma).

CTL induction *in vivo*. Naïve mice were immunized intradermally, 3 times at 12-day intervals. Each immunization consisted of an emulsion containing 100 μg of peptide, 1 : 1 with incomplete Freund's adjuvant in a total volume of 100 μl per injection. Mice were also injected with 50 μg of anti-CD40 (eBioscience) given subcutaneously on day 0 and day 1 of each immunization. Alternately, mice were immunized subcutaneously, at the base of each ear, 3 times at 7–10 day intervals with peptide-pulsed or RNA-transfected DCs ($2\text{--}3 \times 10^5$ DCs per mouse in 100 μl PBS, 50 μl per site).

Splenocytes were harvested 8–10 days after the third immunization and depleted of red blood cells with ammonium chloride Tris buffer. Splenocytes were adhered for 1 hour in tissue culture plates to deplete adherent cells. 10^7 non-adherent splenocytes were cultured with 2×10^5 stimulator cells (DCs pulsed with corresponding peptides used for immunization or Mtb RNA-transfected DCs) in 5 ml of RPMI with 10% FCS, 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.1 mM non-essential amino acids and 5×10^{-5} M



β-mercaptoethanol (complete RPMI-10% FCS) per well and 50 U/ml of IL-2 in a 6-well tissue culture plate at 37°C and 5% CO₂. Effectors were harvested 5 days later on Histopaque 1083 gradient (Sigma) for harvesting viable lymphocytes prior to use in a CTL assay (described above). DCs transfected with Mtb antigen-encoding RNA or pulsed with Mtb peptides were used as targets.

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Author contributions

Conceived and designed the experiments: S.N., G.T. and K.W. Performed the experiments: S.N., D.B., G.T., A.S., K.P. and Y.C. Analyzed the data: S.N., G.T., K.W., D.B., A.S., K.P. and Y.C. Contributed reagents/materials/analysis tools: S.N., G.T., K.W., A.S., C.C., J.D., T.K. and S.P. Wrote the manuscript: S.N., K.W., A.S., D.B., S.P. and G.T. All authors reviewed the manuscript and approved the final version of the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: S.N. and D.B. are co-inventors on a patent that describes the use of dendritic cells transfected with tumor antigen encoding RNA that has been licensed by Argos Therapeutics (Durham, NC) through Duke University. S.N. and D.B. have no financial interests in Argos Therapeutics and are not compensated by Argos Therapeutics.

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