Mapping of Th1-Cell Epitope Regions of *Mycobacterium tuberculosis* Protein MPT64 (Rv1980c) Using Synthetic Peptides and T-Cell Lines from *M. tuberculosis*-Infected Healthy Humans

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Key Words

Mycobacterium tuberculosis · MPT64 · Synthetic peptides · T-cell lines · Th1-cell reactivity

Abstract

Objective: To identify T helper 1 (Th1)-cell stimulating and HLA-promiscuous peptides of MPT64 (Rv1980c), a major secreted antigen of *Mycobacterium tuberculosis*. **Materials and Methods:** Peripheral blood mononuclear cells (PBMCs) were obtained from 35 healthy subjects and typed for HLA-DR molecules using genomic methods. To identify subjects infected with *M. tuberculosis*, PBMCs were tested in antigen-induced proliferation assays with whole cells and culture filtrate antigens of *M. tuberculosis*, *M. tuberculosis*-specific antigens ESAT-6 and CFP10, and MPT64. Culture filtrate-induced T-cell lines were established in vitro from 12 *M. tuberculosis*-infected and HLA-heterogeneous healthy subjects, and tested with 20 overlapping synthetic peptides covering the sequence of MPT64 in Th1-cell assays, i.e. antigen-induced proliferation and/or IFN- γ secretion. In addition, T-cell lines from three HLA-heterogeneous subjects were tested for cytotoxic activity against peptide-pulsed antigen-presenting cells. **Results:** PBMCs from 12 of 35 subjects responded to *M. tuberculosis*-specific antigens ESAT-6 and CFP10 as well as to MPT64, which suggested that they were infected

with *M. tuberculosis*. Ten of twelve T-cell lines established from these donors responded to MPT64, and nine T-cell lines responded to 1 or more of the peptides of MPT64 in antigen-induced proliferation assays. Furthermore, 18 of the 20 peptides of MPT64 were recognized by the T-cell lines in 1 or more assay systems, and at least 5 peptides were recognized by T-cell lines from HLA-DR-heterogeneous subjects. **Conclusion:** Th1-cell-reactive epitopes are scattered throughout the sequence of MPT64, and at least 5 of its peptides are presented to Th1-cells in a HLA-promiscuous manner.

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Introduction

Tuberculosis (TB) is among the top 10 causes of worldwide mortality, and has been declared a global emergency by the World Health Organization. In spite of worldwide efforts to control TB, about 9 million people develop active disease and 1.7 million people die of TB each year [1]. The impact of current efforts to reduce the global problem of TB is less than expected [1], and therefore improved diagnostic and therapeutic efforts need to be combined with additional preventive efforts, including the development of new vaccines [1].

Among the potential candidates to develop new anti-TB vaccines is MPT64, a major secreted antigen of *Mycobacterium tuberculosis* [2]. A number of studies performed in the past have shown that MPT64 is relevant to immune response in humans [2–5], and immunization of experimental animals with preparations containing MPT64 provided protection against challenge with *M. tuberculosis* [6]. Although these studies encourage the use of MPT64 as a new candidate vaccine against TB, this protein requires further characterization for promiscuous recognition by the immune system of *M. tuberculosis*-infected healthy individuals who did not progress to active disease, most probably due to the development of protective immunity.

Although the exact correlates of protective immunity in TB are not exactly defined, Th1-cells secreting IFN- γ are considered the major cells that mediate protection against TB [7–9]. In addition, antigen-specific cytotoxic cells may also be crucial for protection against TB [10]. Furthermore, Th1-cells recognize mycobacterial antigenic proteins mostly in association with highly polymorphic human leukocyte antigen (HLA) molecules, particularly HLA-DR [11, 12]; therefore, to be effective in HLA-heterogeneous humans, the antigens proposed as anti-TB vaccine candidates should be recognized by Th1-cells in a HLA-DR-nonrestricted (promiscuous) fashion [13–15]. Moreover, to avoid holes in the Th1-cell repertoire, an ideal candidate vaccine should have a large number of epitopes, which are scattered throughout the sequence and well separated from each other to allow appropriate epitope processing [16].

In the present study, HLA-heterogeneous healthy blood donors, previously infected with *M. tuberculosis*, were identified based on HLA typing and the reactivity of their peripheral blood mononuclear cells (PBMCs) to the antigens ESAT-6 and CFP10 in antigen-induced proliferation assays, which are considered *M. tuberculosis*-specific [17, 18]. Furthermore, antigen-specific T-cell lines were established from these subjects and tested for reactivity to MPT64 and its peptides in Th1-cell and cytotoxicity assays to confirm the HLA-promiscuous nature of MPT64 and to identify its HLA-promiscuous peptides.

Materials and Methods

Complex and Purified Antigens of M. tuberculosis

The complex mycobacterial antigens used in this study were irradiated whole-cell *M. tuberculosis* H37Rv [19] and *M. tuberculosis* culture filtrate (MTCF) enriched for secreted antigens [20]. The MTCF was kindly provided by Dr. P.J. Brennan (Colorado

State University, Colo., USA) through the repository of TB research materials, NIH contract No. AI-25147, USA. The mature MPT64 (lot 6523A2) was prepared from the mycobacterial culture filtrate, as previously described [4].

Overlapping Synthetic Peptides of MPT64, ESAT-6 and CFP10

Twenty synthetic peptides (20 mers overlapping neighboring peptides by 10 aa) spanning the sequence of mature MPT64 (fig. 1) were purchased from Thermo Hybaid, Ulm, Germany. These peptides, as well as the synthetic peptides covering the sequences of ESAT-6 and CFP10, were synthesized using fluorenylmethoxycarbonyl chemistry, as described previously [19]. The stock concentrations (5 mg/ml) of the peptides were prepared in normal saline (0.9%) by vigorous pipetting, and the working concentrations were prepared by further dilution in tissue culture medium RPMI-1640 (Life Technologies, Paisley, Scotland, UK), as previously described [21]. To represent full-length proteins, the peptides of ESAT-6 and CFP10 were pooled to test for Th1-cell reactivity, as described previously [19].

Isolation of PBMCs and HLA Typing

PBMCs were isolated from the buffy coats of 35 randomly selected healthy subjects donating blood at the Central Blood Bank, Kuwait, as described previously [22]. Informed consent was obtained from all the subjects included in the study, and the research protocol was approved by the Ethics Committee, Faculty of Medicine, Kuwait University, Kuwait.

PBMCs were HLA typed genomically by using sequence-specific primers for various HLA-DR alleles in PCR, as described previously [23]. In brief, a HLA-DR 'low-resolution' kit containing the primers to type for HLA-DR genotypes was purchased from Dynal (Oslo, Norway), and used in PCR as specified by the manufacturer. DNA amplifications were carried out in a Gene-Amp PCR system 2400 (Perkin-Elmer Cetus, Norwalk, Conn., USA), and the amplified products were analyzed by agarose gel electrophoresis, using standard procedures [24]. Serologically defined HLA-DR molecules were identified from the genotypes by following the guidelines provided by Dynal.

Antigen-Induced Proliferation of PBMCs

To determine the recognition of MPT64 and its peptides by Th1-cells, PBMCs from 35 healthy subjects were screened first for responses to *M. tuberculosis*, MTCF and peptide pools of ESAT-6, CFP10 and MPT64 in antigen-induced proliferation assays using standard procedures, as described previously [25, 26]. In brief, PBMCs (2×10^5 cells/well) suspended in 50 μ l complete tissue culture medium [RPMI 1640 + 10% human AB serum + penicillin (100 U/ml) + streptomycin (100 μ g/ml) + gentamicin (40 μ g/ml) + fungizone (2.5 μ g/ml)] were seeded into 96-well tissue culture plates (Nunc, Roskilde, Denmark). Each antigen was added to the wells in triplicate at a final concentration of 5 μ g/ml and control cultures lacked the antigen. The plates were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cultures were pulsed (4 h) on day 6 with 1 μ Ci of [³H]thymidine (Amersham Life Science, Little Chalfont, England) and harvested on filter mats with a Skatron harvester (Skatron Instruments, Oslo, Norway). The radioactivity incorporated was measured by liquid scintillation counting and expressed as counts per minute (cpm). Average cpm was calculated from triplicate cultures stimulated with each antigen. The results were presented as stimulation in-

Peptide No.	1 st aa	aa sequence	last aa
1	1	APKTYCEELKGTDTGQACQI	20
2	11	GTDGTGQACQIQMSDPAYNIN	30
3	21	QMSDPAYNINISLPSYYPDQ	40
4	31	ISLPSYYPDQKSLENYIAQT	50
5	41	KSLENYIAQTRDKFLSAATS	60
6	51	RDKFLSAATSSTPREAPYEL	70
7	61	STPREAPYELNITSATYQSA	80
8	71	NITSATYQSAIPPRGTQAVV	90
9	81	IIPRGTQAVVLKVYQNAGGT	100
10	91	LKVYQNAGGTHPTTTYKAFD	110
11	101	HPTTTYKAFDWDQAYRKPIIT	120
12	111	WDQAYRKPIITYDTLWQADTD	130
13	121	YDTLWQADTDPLPVVFPVQ	140
14	131	PLPVVFPVQGELSKQTGQQ	150
15	141	GELSKQTGQQVSIAPNAGLD	160
16	151	VSIAPNAGLDPVNYQNFAVT	170
17	161	PVNYQNFAVTNDGVIFFFNP	180
18	171	NDGVIFFFNPGELLPEAAGP	190
19	181	GELLPEAAGPTQVLVPRSAI	200
20	191	TQVLVPRSAIDSMILA	205

Fig. 1. Synthetic peptides (P1 to P20) covering the sequence of mature MPT64 antigen. The peptides overlap each other by 10 aa. The single-letter designations for amino acids are used.

dex (SI), which is defined as follows: SI = cpm in antigen-stimulated cultures/cpm in cultures without antigen. SIs of ≥ 2 were considered positive proliferations in response to antigenic stimulations [22].

Establishment and Testing of MTCF-Induced T-Cell Lines

MTCF-induced T-cell lines were established from PBMCs of MPT64-responding donors ($n = 12$) after stimulation with MTCF according to standard procedures [27]. The T-cell lines were tested for peptide-induced proliferation in the wells of 96-well tissue culture plates (Nunc, Roskilde, Denmark) in the presence of autologous and allogeneic HLA-typed APCs, as described previously [28]. In brief, adherent cells obtained from irradiated (24 Gy) PBMCs (seeded into the wells of 96-well plates at 1×10^5 cells/well) were used as APCs. The T-cell lines were added to the wells at a concentration of 5×10^4 cells/well. Peptides were added in triplicate at a final concentration of 5 μ g/ml, and the control wells lacked the peptides. The plates were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air. On day 3, the cultures were pulsed with radioactive thymidine, harvested on filter mats, and the amount of radioactivity incorporated was measured by liquid scintillation counting, as described above for PBMCs. The cpm values were used to calculate SI (as given above for PBMCs), and the cellular proliferation in response to a given antigen was considered positive with SI ≥ 2 [22].

IFN- γ Secretion by T-Cell Lines

The cultures with T-cell lines were set up as described above for proliferation assays and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After 3 days of incubation, the

culture supernatants were collected and assayed for IFN- γ concentrations using immunoassay kits (Coulter/Immunotech, Marseille, France) as specified by the manufacturer. The detection limit of the IFN- γ assay kit was 0.4 international units (IU)/ml. Secretion of IFN- γ in response to a given antigen was considered positive with E/C > 2 (E/C = IFN- γ concentration in cultures stimulated with antigen/IFN- γ concentration in cultures without antigen) and IFN- γ concentration ≥ 1.5 IU/ml [22].

Cytotoxicity Assay

Cytotoxic activity of T-cell lines against peptide-pulsed monocytes/macrophages was assessed by the neutral red release assay according to standard procedures [20, 28]. In brief, adherent monocytes/macrophages from 1×10^6 autologous irradiated PBMCs in 24-well Costar plates were pulsed with individual peptides. The T-cell lines were added at a concentration of 2×10^5 cells/well. After 7 days of incubation at 37°C, the wells were washed and the adherent macrophages were allowed to take up neutral red for 30 min. The dye taken up by macrophages was released by adding 0.5 ml of 0.05 M acetic acid in 50% ethanol, and quantitated by spectrophotometric measurement of optical density at 540 nm (OD540). The results, expressed as percent (%) cytotoxicity, were calculated from OD540 values according to the following formula: % cytotoxicity = [(OD540 control - OD540 experimental)/(OD540 control)] $\times 100$, where OD540 control = OD540 of cultures with adherent cells plus T-cells, and the OD540 experimental = OD540 of cultures with adherent cells plus T-cells plus MPT64. The % cytotoxicity values above 40% were considered significant [20].

Table 1. Antigen-induced proliferation (SI) of MTCF-induced T cell lines from 12 healthy donors to MPT64 and its peptides

Antigen/ peptide	HLA-DR molecules expressed by donors											
	4, 13, 52, 53	4, 53	3, 52	4, 5, 52, 53	3, 52	ND	6, 7, 52, 53	4, 13, 52, 53	ND	7, 53	3, 52	ND
MPT64	3.5	<i>14</i>	<i>2.5</i>	<i>8.8</i>	<i>3.4</i>	<i>18</i>	<i>30</i>	<i>329</i>	<i>15</i>	<i>14</i>	1.0	1.3
P1	1.1	1.0	1.3	1.5	1.4	0.9	1.7	0.8	0.9	1.0	1.2	1.0
P2	0.9	<i>3.0</i>	1.2	1.2	1.5	0.8	1.6	1.1	0.9	1.0	1.4	0.9
P3	1.7	1.1	1.0	<i>6.2</i>	<i>2.1</i>	1.0	<i>12</i>	1.0	<i>9.5</i>	1.0	1.6	1.0
P4	0.8	<i>4.0</i>	1.5	1.4	1.4	1.0	1.4	<i>330</i>	1.2	1.4	1.1	1.2
P5	1.0	1.3	1.1	1.2	1.5	1.0	1.8	0.9	1.0	1.0	0.9	1.0
P6	1.0	<i>15</i>	0.9	1.4	1.9	1.0	3.9	1.0	1.0	1.2	1.6	0.9
P7	1.0	1.7	1.0	1.3	<i>2.0</i>	0.9	2.7	1.0	0.9	0.9	1.6	0.9
P8	1.0	1.8	0.9	2.3	1.0	0.7	3.2	1.1	1.1	1.0	1.3	1.0
P9	2.5	1.2	1.1	9.8	<i>2.0</i>	0.8	8.2	1.8	<i>4.3</i>	0.9	1.7	1.2
P10	1.4	2.3	2.3	1.9	1.4	1.1	2.6	1.2	0.9	0.9	1.2	1.0
P11	1.3	1.5	1.5	<i>2.1</i>	1.4	1.0	3.5	1.0	1.0	1.0	1.2	1.1
P12	1.3	1.6	1.2	1.8	1.4	<i>6.0</i>	2.8	1.0	1.0	1.4	1.8	1.4
P13	1.7	1.4	1.5	<i>2.1</i>	1.6	1.2	3.6	1.0	1.0	1.0	1.5	1.1
P14	1.0	6.8	1.5	1.9	1.1	1.0	3.3	1.1	1.1	1.0	1.4	1.3
P15	1.2	5.9	1.5	1.8	1.6	1.2	2.6	0.8	0.9	1.2	1.7	1.2
P16	1.9	3.9	0.5	2.5	1.2	0.5	3.1	1.0	1.8	1.3	1.2	1.2
P17	1.4	1.8	1.3	2.5	1.0	0.9	4.0	0.9	2.0	0.9	1.1	1.0
P18	1.9	2.3	1.4	5.5	3.4	0.7	8.5	0.9	2.0	0.9	1.2	1.3
P19	1.9	2.5	1.8	6.5	0.9	0.7	6.7	0.9	1.9	0.9	1.5	1.3
P20	1.9	2.2	2.0	<i>11</i>	3.3	0.6	8.5	1.0	1.5	1.0	1.5	0.9

Positive responses (SI ≥ 2) are given in italics. ND = Not determined.

Results

Although PBMCs from all of the donors responded to *M. tuberculosis* and MTCF in antigen-induced proliferations assays, 14 donors responded to ESAT6 and/or CFP10; 12 of these 14 donors also responded to MPT64 (data not shown).

Testing of the MTCF-induced T-cell lines showed that all of the 12 T-cell lines responded to MT-CF (data not shown) and 10 of them responded to MPT64 (table 1). Both of the MPT64-nonresponding T-cell lines failed to respond to any of its peptides, whereas among the 10 MPT64 responders, the T-cell lines from 9 donors responded to 1 or more peptides (table 1). In addition, except for peptides P1 and P5, all other peptides of MPT64 induced the proliferation of 1–5 T-cell lines (table 1). HLA-DR typing of the subjects showed that the donors of T-cell lines responding to the peptides of MPT64 represented a heterogeneous group expressing DR3, DR4, DR5, DR6, DR7, DR52 and DR53 molecules

(table 1). Furthermore, the positive responses of T-cell lines from HLA-DR heterogeneous subjects to MPT64 and its peptides suggested that the full-length protein and at least 5 of its peptides (P3, P9, P18, P19 and P20) were presented to Th1-cells in HLA-promiscuous manner (table 1).

In addition to antigen-induced proliferation, T-cell lines from 5 and 3 HLA-DR heterogeneous donors were also tested for peptide-induced IFN- γ secretion (table 2), and cytotoxic activity against peptide-pulsed monocytes/macrophages (table 3), respectively. The results revealed that all of the tested T-cell lines recognized 1 or more peptides in these assays, and the epitopes recognized were scattered throughout the sequence of MPT64 (tables 2, 3). Furthermore, recognition of MPT64 in both assays (tables 2, 3), and of peptides P3, P9, P18, P19 and P20 in IFN- γ assays (table 2) by T-cell lines from HLA-heterogeneous subjects further suggested their promiscuous presentation to Th1-cells.

Table 2. Antigen-induced IFN- γ secretion (IU/ml) by MTCF-induced T-cell lines in response to MPT64 and its peptides

Antigen/peptide	HLA-DR molecules expressed by donors				
	4, 13, 52, 53	4, 53	3, 52	4, 5, 52, 53	3, 52
No antigen	13	1.1	19	48	1.3
MPT64	53	24	53	105	15
P1	12	0.6	18	57	1.9
P2	12	7.9	16	52	0.9
P3	41	1.0	48	98	4.3
P4	12	4.4	19	63	1.9
P5	17	0.6	17	52	1.3
P6	15	20	17	57	1.9
P7	13	1.8	17	57	5.4
P8	14	1.9	21	68	1.5
P9	48	0.4	45	98	2.1
P10	22	0.2	15	63	1.6
P11	22	0.2	18	68	1.6
P12	17	0.0	17	68	1.5
P13	41	0.2	17	77	0.9
P14	33	6.0	15	57	0.7
P15	30	4.0	19	68	1.1
P16	54	4.8	9.4	80	1.1
P17	17	0.5	20	74	0.5
P18	43	0.1	57	100	19
P19	35	1.1	37	100	1.0
P20	43	0.5	42	105	6.2

Positive IFN- γ responses (≥ 1.5 IU/ml with E/C > 2) are given in italics.

Table 3. Cytotoxicity activity (% cytotoxicity) of T-cell lines against monocytes/macrophages pulsed with the antigen MPT64 and its peptides

Antigen/peptide	HLA-DR molecules expressed by donors		
	4, 5, 52, 53	4, 53	4, 13, 52, 53
MPT64	81	82	84
P2	20	75	18
P3	59	19	17
P4	22	22	86
P6	30	70	22
P9	80	27	28
P14	21	64	18
P15	16	58	19
P18	69	25	23
P19	71	21	22
P20	79	22	25

Values showing significant cytotoxicity ($\geq 40\%$) are given in italics.

Discussion

MPT64 (Rv1980c), a 24-kDa protein of *M. tuberculosis*, is among the major secreted proteins of this pathogen [2]. It is hypothesized that actively secreted proteins of *M. tuberculosis* are the first ones to interact with the immune system, and, therefore, such proteins are important for activating immune response in individuals infected with *M. tuberculosis* [14]. This hypothesis may also be true for MPT64, because antibodies and T-cells reactive to this protein exist in TB patients [2–5]. It has also been previously shown that the T-cell responses to MPT64 are comparable to the responses induced by other secreted antigens, which have been suggested to be candidate vaccines against TB, i.e. Ag85B, MPT70 and MPT63, etc. [29]. These antigens have previously been characterized for HLA-promiscuous recognition using HLA-DR-typed PBMCs and T-cell lines from healthy subjects [17, 22, 23]. However, to our knowledge, such analysis for MPT64 has been performed for the first time in this study.

To identify the epitope regions of MPT64 recognized by Th1-cells, we used 20 peptides (overlapping with the neighboring peptides by 10 aa) covering the sequence of mature MPT64, i.e. aa 1–205 (fig. 1). Because most mycobacterial epitopes recognized by Th1-cells are sequences of ≤ 10 aa [14], the overlaps of 10 aa between the neighboring peptides used in this study were expected to minimize missing of the regions and epitopes required for Th1-cell recognition.

MPT64 is an antigen that is specific for *M. tuberculosis* complex [30], and therefore the selection of healthy donors suitable for reactivity to MPT64 required identification of subjects infected with *M. tuberculosis*. An approach that has been previously used for this purpose is to identify the subjects that respond to *M. tuberculosis* region of difference 1 antigens ESAT-6 and CFP10 in Th1-cell assays [22]. Because of the specificity of ESAT-6 and CFP10 to *M. tuberculosis* [18], subjects responding to these antigens are considered infected with *M. tuberculosis* [22]. On this basis, we identified 14 ESAT-6/CFP10-responding donors, among 35 tested, and PBMCs from 12 of the 14 donors also responded to MPT64 in Th1-cell assays. The testing of MTCF-induced T-cell lines showed that all of the 12 T-cell lines responded to MTCF (data not shown) and 10 T-cell lines responded to MPT64 in Th1-cell assays (table 1). The nonresponsiveness of the two T-cell lines could have been due to selective expansion of T-cells that are activated in response to other secreted antigens present in MTCF, as has previously been shown for MPB70 [20]. When tested with the individual peptides

of MPT64, 9 of the 10 MPT64-responding T-cell lines proliferated in response to 1 or more peptides (table 1). The selective responsiveness of MPT64-responding T-cell lines to its peptides suggests that the peptide epitopes recognized were not cryptic and were relevant to natural processing pathways. Moreover, these results further support the appropriateness of the overlapping peptides to represent the natural protein for Th1-cell recognition, because T-cell lines from 9 of the 10 (90%) donors, responding to the natural MPT64 protein, responded to the peptides as well (table 1). These data strengthen the previous observations showing that overlapping synthetic peptides could faithfully replace full-length proteins in Th1-cell assays [14, 15, 31, 32]; thus, avoiding the hazards and complexities involved in obtaining full-length proteins either by purification of the natural protein from the culture filtrates of live *M. tuberculosis* or expression and purification of recombinant proteins of *M. tuberculosis* expressed in heterologous hosts [4, 33–35].

When tested for Th1-cell responses using T-cell lines, 18 of 20 peptides induced positive responses with 1 or more T-cell lines in antigen-induced proliferation (table 1) and/or IFN- γ secretion assays (table 2), which shows that Th1-cell epitopes were scattered throughout the sequence of MPT64. Furthermore, MPT64 and at least 5 of its peptides (P3, P9, P18, P19 and P20) were recognized by T-cell lines from HLA heterogeneous donors in Th1-cell assays (tables 1, 2); and thus MPT64 as well as these peptides qualify to be HLA promiscuous for recognition by Th1-cells [22]. However, none of the peptides can replace the full-length protein as none of them

showed reactivity with all T-cell lines responding to full-length MPT64 in Th1-cell assays.

Three of the T-cell lines from HLA-heterogeneous donors were also tested for cytotoxic activity against monocytes/macrophages pulsed with peptides reactive in Th1-cell assays, and the results suggest that several epitopes were present in MPT64, which could mediate cytotoxic activity (table 3). This property of MPT64 also makes it suitable for use as a vaccine candidate because cytotoxic activity of mycobacterial antigen-specific Th1-cells has recently been shown to have protective effect against *M. tuberculosis* [10].

Conclusion

The study demonstrates the presence of a large number of Th1-cell epitopes in MPT64, which are relevant to natural processing pathways. The recognition of MPT64 and its peptides by Th1-cells from *M. tuberculosis*-infected and HLA-heterogeneous healthy subjects supports the notion that MPT64 deserves consideration for inclusion as a candidate vaccine, either by itself, or in a protein cocktail, to protect against human TB.

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