FIGURE LEGENDS

Figure 1. Rab GTPases regulating phagosome maturation are released from *M. tuberculosis* phagosomes. Phagosome maturation is achieved through a series of interactions by Rab GTPases with the phagosome. Phagosomal acidification and recruitment of cathepsin D are regulated by Rab GTPases. *M. tuberculosis* modulates the localization of Rab GTPases (underlined) to arrest phagosome maturation, resulting in the inhibition of phagolysosome biogenesis.

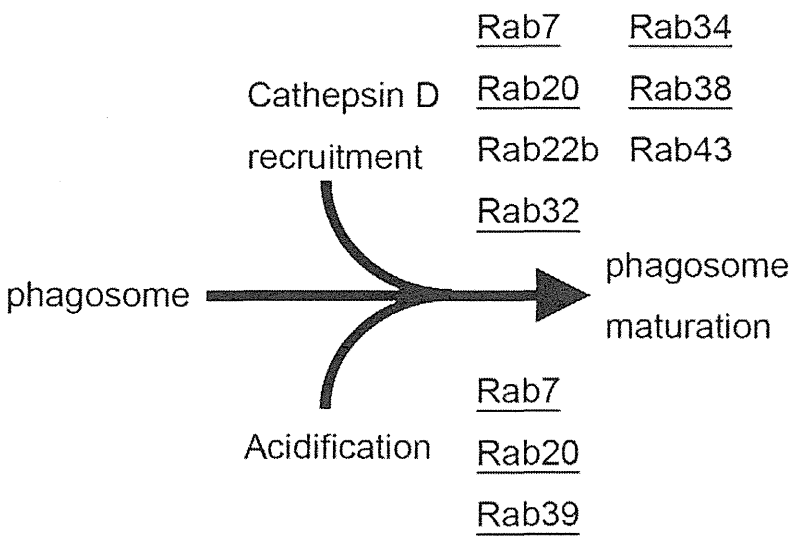


Figure 2. Electron micrographs of *M. tuberculosis*-containing phagosomes in Coronin-1a-knockdown macrophages. Control (A) or Coronin-1a-knockdown (B) macrophages were infected with *M. tuberculosis*, fixed, and subjected to thin-section electron microscopy. Autophagy-specific membrane structures surround the infected mycobacterial bacilli in Coronin-1a-knockdown macrophages.

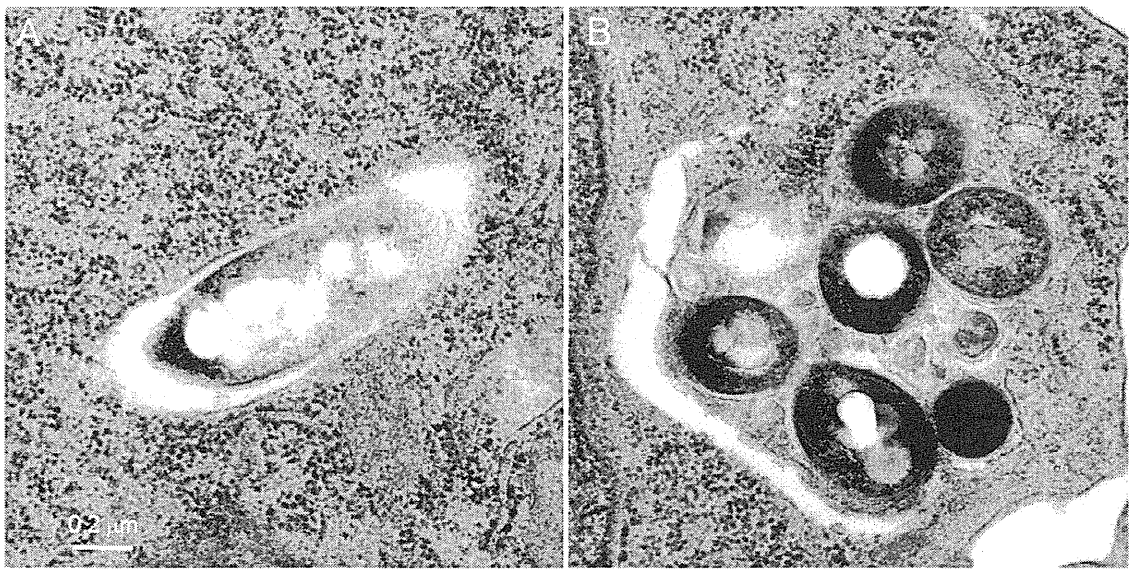
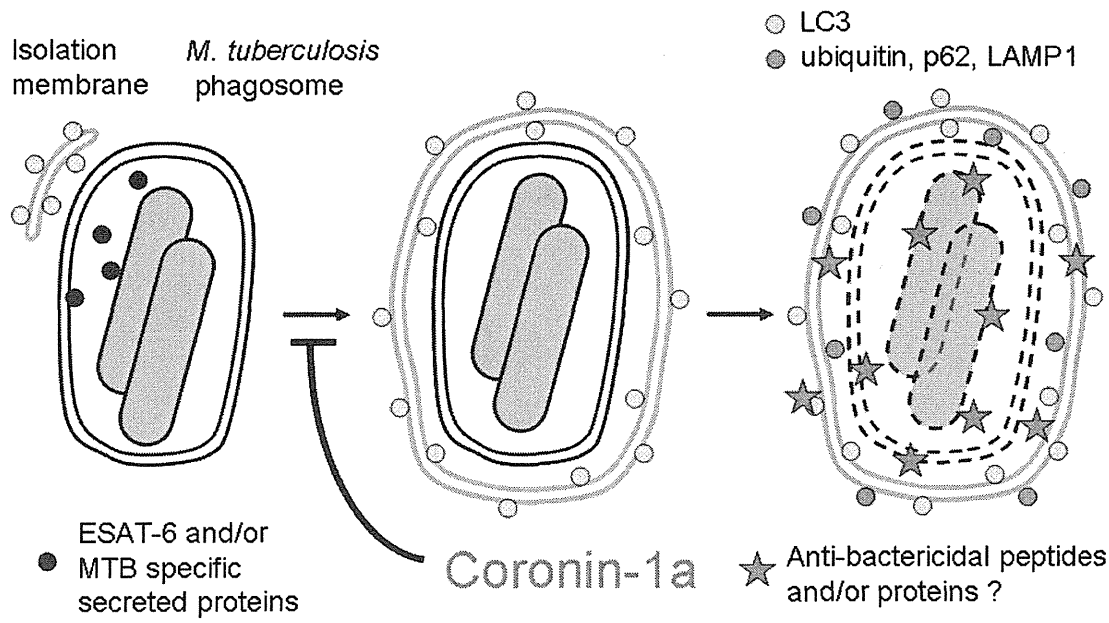


Figure 3. Coronin-1a inhibits autophagosome formation to *M. tuberculosis* in alveolar macrophages. *M. tuberculosis* phagocytosed by alveolar macrophages secretes ESAT-6 and/or *M. tuberculosis*-specific proteins that damage phagosomal membranes immediately after phagocytosis. These secreted proteins, or phagosomal membrane damage, act as signals for stimulation of autophagosome formation by *M. tuberculosis* via the p38 MAP kinase signaling pathway. *M. tuberculosis* is eliminated by anti-bacterial factors during autophagosome maturation and autophagolysosome biogenesis. Coronin-1a inhibits autophagosome formation and supports mycobacterial survival.



Chapter 22

Identification of T Cell Epitopes of *Mycobacterium tuberculosis* with Biolistic DNA Vaccination

Toshi Nagata and Yukio Koide

Abstract

Tuberculosis (TB) has been listed as one of the most prevalent and serious infectious diseases worldwide. The etiological pathogen of TB is *Mycobacterium tuberculosis* (Mtb), a facultative intracellular bacterium. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only approved vaccine against TB to date. BCG has been widely used, but the efficacy is questionable, especially in adult pulmonary TB. Therefore, more effective, safe and reliable TB vaccines have been urgently needed. T cell-mediated cellular immune response is a key immune response for effective protective immunity against TB. DNA vaccines using Mtb antigens have been studied as promising future TB vaccines. Most TB DNA vaccine studies so far reported used intramuscular or intradermal injection with needles, as these methods tend to induce a type 1 helper T lymphocyte (Th1)-type immune response that is critical for the protective immunity. We have been using DNA vaccines with gene gun bombardment for T cell epitope identification of various Mtb antigens. We show here our strategy to identify precise Mtb T cell epitopes using DNA vaccines with gene gun bombardment.

Key words: *Mycobacterium tuberculosis*, Codon usage, T cell epitope, Cytotoxic T lymphocyte, Type 1 helper T lymphocyte, Interferon-γ

1. Introduction

1.1. Tuberculosis and the Vaccine

According to the global burden of disease caused by tuberculosis (TB) in 2009, there were 9.4 million incident cases of TB with approximately one third of the world total population being infected (1). *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only approved attenuated live vaccine to date against TB (2, 3). Despite the fact that BCG is among the most widely used vaccines throughout the world, TB still poses a serious global health threat. Whereas BCG is believed to protect newborns and

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young children against early manifestations of TB, its efficacy against pulmonary TB in adults is still a subject of debate (4) and was reported to wane with time since vaccination (5). Variable levels of the protective efficacy ranging from 0 to 80% have been reported in different studies (2, 4). Moreover, the viable nature of BCG makes it partly unsafe in case of immunocompromised individuals. This highlights the need to develop more effective, safe and reliable vaccines against TB, and several TB vaccine candidates have now entered clinical trials (6).

The T cell-mediated immune response is critical for the development of resistance against mycobacterial infection (7, 8). It has been well established that major histocompatibility complex (MHC) class II-restricted $CD4^+$ type 1 helper T lymphocytes (Th1) are important mediators of host defense against TB. In addition, MHC class I-restricted $CD8^+$ cytotoxic T lymphocytes (CTL) have also been reported to be required for the optimum control of mycobacterial infection (9, 10).

1.2. Codon Usage

In DNA vaccines against pathogens such as bacteria, protozoa, and viruses, interspecies difference of codon usage is one of the major obstacles for the effective induction of specific immune responses. We evaluated the codon optimization effect on CTL induction using the DNA vaccine against *Listeria monocytogenes* and malaria parasite (11). Using mammalian culture cells, we analyzed the translation efficiency of several genes composed of different levels of optimization to mammalian cells, but encoding an identical CTL epitope, and showed that the codon optimization level of the genes is not precisely proportional to, but correlates well with the translation efficiency in mammalian cells. These results also correlated well with the induction level of specific CTL response in vivo (12). For evaluation of the codon optimization level, the relative synonymous codon usage (RSCU) value has been used (13). The RSCU values of codons used in *L. monocytogenes* showed the opposite relationship to the RSCU values of codons used in mice and humans, indicating that native codons frequently used in *L. monocytogenes* are rarely used in mice and humans (see Table 1, Note 1). However, such a relationship is not necessarily applicable in *Mycobacterium tuberculosis* (Mtb). The RSCU values of codons used at high frequency in Mtb genes are quite similar to those in mouse and human genes (see Table 1). The Mtb genome has a similar G+C content as mammalian genomes. Therefore, the effect of codon difference on expression efficiency of Mtb DNA vaccines would be minimal.

1.3. DNA Vaccination Against TB

Many papers on DNA vaccination against Mtb have been published since 1996 (14, 15). So far, a variety of Mtb antigen genes have been used for DNA vaccines, which include heat shock protein

Table 1
RSCU values of codons in genes derived from *L. monocytogenes*, *M. tuberculosis*, *Mus musculus*, and *Homo sapiens*

RSCU						RSCU					
		L. monocytogenes	*M. tuberculosis*	*Mus musculus*	*Homo sapiens*			*L. monocytogenes*	*M. tuberculosis*	*Mus musculus*	*Homo sapiens*
Phe	UUU	1.304	0.419	0.845	0.771	Ser	UCU	1.225	0.251	1.148	1.091
	UUC	0.696	1.581	1.155	1.130		UCC	0.624	1.260	1.334	1.364
Leu	UUA	2.420	0.101	0.365	0.409		UCA	1.153	0.410	0.825	0.851
	UUG	0.943	1.087	0.767	0.727		UCG	0.569	2.106	0.336	0.338
Leu	CUU	1.231	0.350	0.756	0.741	Pro	CCU	1.206	0.240	1.206	1.122
	CUC	0.285	1.084	1.215	1.215		CCC	0.229	1.175	1.229	1.334
	CUA	0.885	0.295	0.457	0.406		CCA	2.017	0.430	1.119	1.082
	CUG	0.236	3.083	2.441	2.502		CCG	0.548	2.155	0.446	0.462
Ile	AUU	1.736	0.463	0.996	1.030	Thr	ACU	1.259	0.278	0.970	0.910
	AUC	0.769	2.380	1.558	1.521		ACC	0.390	2.621	1.449	1.483
	AUA	0.495	0.157	0.447	0.449		ACA	1.691	0.353	1.137	1.055
Met	AUG	1.000	1.000	1.000	1.000		ACG	0.659	1.175	0.445	0.471
Val	GUU	1.468	0.382	0.562	0.685	Ala	GCU	1.427	0.337	1.155	1.049
	GUC	0.450	1.532	0.879	0.985		GCC	0.364	1.789	1.544	1.644
	GUA	1.392	0.229	0.386	0.425		GCA	1.513	0.398	0.884	0.876
	GUG	0.690	1.858	1.614	1.905		GCG	0.697	1.476	0.417	0.431
Tyr	UAU	1.401	0.460	0.822	0.840	Cys	UGU	1.384	0.512	0.915	0.860
	UAC	0.599	1.113	1.178	1.160		UGC	0.616	1.488	1.085	1.140

(continued)

Table 1
(continued)

RSCU						RSCU					
ter	UAA	–	–	–	–	ter	UGA	–	–	–	–
ter	UAG	–	–	–	–	Trp	UGG	1.000	1.000	1.000	1.000
His	CAU	1.380	0.578	0.786	0.795	Arg	CGU	2.094	0.695	0.531	0.510
	CAC	0.620	1.422	1.214	1.205		CGC	0.839	2.321	1.106	1.203
Gln	CAA	1.711	0.525	0.513	0.507		CGA	0.788	0.598	0.718	0.654
	CAG	0.289	1.475	1.486	1.493		CGG	0.487	2.004	1.118	1.243
Asn	AAU	1.365	0.408	0.835	0.892	Ser	AGU	1.474	0.389	0.887	0.743
	AAC	0.636	1.592	1.165	1.108		AGC	0.954	1.583	1.469	1.257
Lys	AAA	1.710	0.504	0.767	0.820	Arg	AGA	1.575	0.111	1.268	1.195
	AAG	0.290	1.496	1.233	1.180		AGG	0.218	0.272	1.259	1.195
Asp	GAU	1.483	0.546	0.878	0.904	Gly	GGU	1.475	0.774	0.697	0.646
	GAC	0.516	1.454	1.122	1.096		GGC	0.785	2.031	1.342	1.392
Glu	GAA	1.653	0.689	0.807	0.819		GGA	1.264	0.415	1.036	0.981
	GAG	0.347	1.310	1.193	1.181		GGG	0.477	0.785	0.925	0.980

(Hsp) 65, Hsp 70, Antigen (Ag) 85A, Ag85B, and ESAT6. DNA immunization with naked DNA has been shown to efficiently induce cellular as well as humoral immune responses. DNA vaccines in most of these reports used needle injection via intramuscular or intradermal routes, although some studies used gene gun (16). The DNA immunization with needle injection tends to raise predominant Th1 responses, which is indispensable for induction of the protective immunity. On the other hand, gene gun DNA immunization is apt to produce "mixed type" (Th1 and Th2; producing interferon (IFN)-γ) and interleukin (IL)-4) T cell responses, which is not necessarily adequate for induction of the protective immunity (17). The difference is considered to be mainly due to the difference in the amount of antigen produced from the plasmids (high amounts in needle injection and low amounts in gene gun bombardment). Therefore, DNA vaccination with gene gun will need additional factors such as adjuvants for eliciting protective immunity against Mtb.

We realized that DNA immunization with gene gun bombardment is an excellent method for identification of Mtb T cell epitopes, as it is highly reproducible and efficiently induces T cell responses, especially $CD8^+$ CTL (18). Identification of T cell epitopes in Mtb antigens is indispensable for accurate analysis of T cell responses against Mtb antigens by analyses with specific MHC tetramers or intracellular cytokine staining. A variety of T cell epitopes of Mtb antigens have been reported. Some of them are listed in Table 2. Huygen and colleagues have reported identification of T cell epitopes of Ag 85 family proteins (Ag85A, Ag85B, and Ag85C) (19, 20) using intramuscular DNA immunization. We have used gene gun DNA immunization method for identification of $CD8^+$ and $CD4^+$ T cell epitopes of Mtb antigens including MPT51 (21–23), MDP1 (24), and low-molecular-mass secretory antigens (CFP11, CFP17, and TB18.5) (25). After immunization, immune spleen cells were examined for their IFN-γ responses to overlapping peptides covering full-length proteins by measuring IFN-γ levels by enzyme-linked immunosorbent assay (ELISA) or by counting the numbers of IFN-γ-secreting cells by enzyme-linked immunospot assay (ELISPOT). We combined these methods with computer algorithms to predict T cell epitopes (Fig. 1). These programs are helpful for narrowing down the amino acid region of the bona fide T cell epitope. However, the algorithms are still not perfect for accurate identification of T cell epitopes at this time. A peptide that shows the highest score in these algorithms is not necessarily the best T cell epitope. Experimental validation is definitely necessary to determine actual T cell epitopes.

Table 2
T cell epitopes of Mtb antigens (examples)

Antigen	Epitope peptide	MHC restriction	Reactive T cells	References
Ag85A	p60–68 (9-mer)	K^d	Mouse CD8	(19)
	p144–152 (9-mer)	K^d	Mouse CD8	(19)
	p101–120 (20-mer)	E^d	Mouse CD4	(32)
	p241–260 (20-mer)	A^b	Mouse CD4	(32)
	p261–280 (20-mer)	A^b	Mouse CD4	(20)
Ag85B	p240–254 (15-mer)	A^b	Mouse CD4	(33,34)
	p262–279 (18-mer)	A^b	Mouse CD4	(20)
	p143–152 (10-mer)	A*0201	Human CD8	(35)
	p199–207 (9-mer)	A*0201	Human CD8	(35)
	p10–27 (18-mer)	DR3, 52, 53	Human CD4	(36)
	p19–36 (18-mer)	Promiscuous	Human CD4	(36)
	p91–108 (18-mer)	Promiscuous	Human CD4	(36)
MPT51	p24–32 (9-mer)	D^d	Mouse CD8	(21)
	p171–190 (20-mer)	A^b	Mouse CD4	(21)
	p53–62 (10-mer)	A*0201	Human CD8	(22)
	p191–202 (12-mer)	Promiscuous	Human CD4	(23)
Hsp65	p489–503 (15-mer)	A^d	Mouse CD4	(37)
	p369–377 (19-mer)	A*0201	Human CD8	(38)
	p3–13 (11-mer)	DR3	Human CD4	(39)
ESAT6	p1–20 (20-mer)	$H2^{b,d}$	Mouse CD4	(40)
	p51–70 (20-mer)	$H2^{a,k}$	Mouse CD4	(40)
	p72–95 (24-mer)	DR52, DQ2	Human CD4	(41)
CFP10	p32–39 (8-mer)	K^k	Mouse CD8	(42)
	p11–25 (15-mer)	A^k	Mouse CD4	(42)
MDP1	p23–31 (9-mer)	D^b	Mouse CD8	(24)
	p41–60 (20-mer)	A^d, E^k	Mouse CD4	(24)
	p111–130 (20-mer)	E^k	Mouse CD4	(24)
	p141–160 (20-mer)	E^k	Mouse CD4	(24)

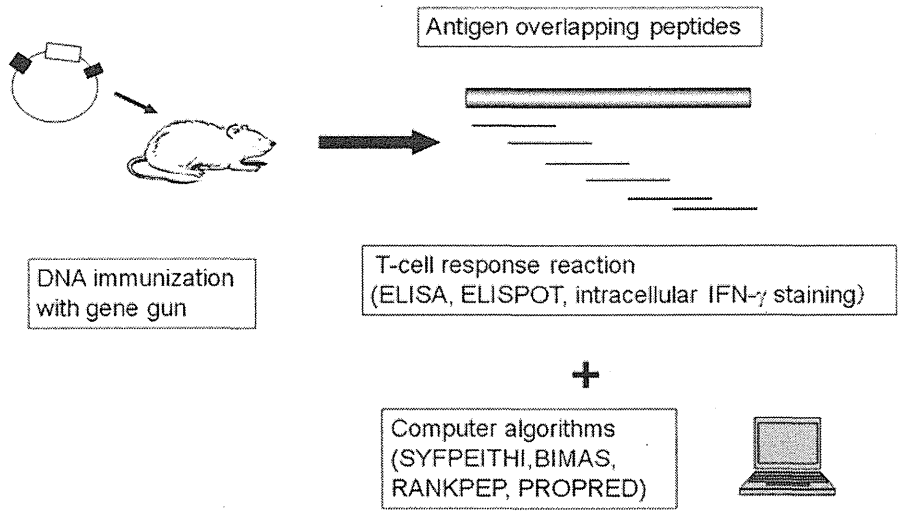

Fig. 1. Schematic diagram for identification of T cell epitopes with DNA immunization.

2. Materials

2.1. Preparation of Plasmid DNA

1. Mammalian expression plasmid such as pCI (Promega, Madison, WI, USA) (see Note 2).
2. Appropriate restriction enzymes.
3. Qiagen Plasmid Midi or Maxi Kit (Qiagen Sciences, MD, USA).

2.2. Preparation of DNA/Gold Cartridge

1. 1.5 mL Microfuge tube.
2. 1.0 μm Gold microcarrier (Bio-Rad Laboratories, Herculus, CA, USA).
3. Plasmid DNA solution in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). DNA concentration should be >1 μg/μL.
4. 0.05 M Spermidine (Sigma, St. Louis, MO, USA) in distilled water.
5. 1 M $CaCl_2$ in distilled water.
6. Small variable speed vortex mixer.
7. Microfuge.
8. Ultrasonic cleaner (e.g., Bransonic 1,210 J; Branson Ultrasonics, Danbury, CT, USA).
9. Fresh 100% (v/v) ethanol.
10. Polyvinylpyrrolidone (PVP) (Sigma): Prepare a stock solution of 20 mg/mL PVP in 100% (v/v) ethanol. Dilute this solution with 100% (v/v) ethanol to prepare PVP solution at 0.05 mg/mL (see Note 3). Prepare 3.5 mL of the dilute solution for

each 30-in. length of gold-coat tubing (25 in. to be coated) in the tubing prep station. Keep these solutions tightly capped, when not in use. Prepare the solution freshly.

11. 1.5 mL Disposable polypropylene tube with a screw cap.
12. Nitrogen pressure regulator.

2.3. Loading the DNA/Gold Suspension into Tubing Using the Tubing Prep Station

1. Tefzel tubing (Bio-Rad Laboratories).
2. Tubing prep station (Bio-Rad Laboratories).
3. Nitrogen tank with compressed nitrogen gas.
4. Flowmeter.
5. 10 mL Syringe.
6. Tubing cutter (Bio-Rad Laboratories).

2.4. In Vivo Delivery of DNA-Coated Particle to Epidermis with Gene Gun

1. Inbred mice such as BALB/c or C57BL/6 mice. Mice between 2 and 4 months of age were used for immunization.
2. Razor.
3. Commercial depilatory.
4. Helios gene gun (Bio-Rad Laboratories).
5. Cartridge holder (Bio-Rad Laboratories).
6. Compressed helium gas.
7. Helium pressure regulator.
8. 70% (v/v) Ethanol in a spray bottle.

2.5. Preparation of Immune Spleen Cells and the Immunological Assays

1. Diethyl ether.
2. Scissors and two tweezers.
3. Peptides: Synthesize peptides spanning the entire amino acid sequences of Mtb antigen proteins as approximately 20-mer peptides overlapping by ten residues. All peptides were dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mM and stored at –80°C until use.
4. Sterile Petri dishes (60×15 mm). Use bacteriological type, not tissue culture type for cells not to adhere to the dish bottom.
5. 5 mL Syringe.
6. Low-speed centrifuge for sedimenting cells.
7. Stainless metal mesh (wire size ca. 300 μm, pore size ca. 500 μm).
8. ACK lysis solution: 0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.2.
9. Sterile round-bottom 96-well plate for ELISA.
10. 75 mL Flask for MHC stabilization assay.
11. RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (RPMI/10% FCS).

12. CO_2 incubator.
13. 96-Well half-size microwell plate (e.g., EIA RIA Plate A/2; Costar, Cambridge, MA, USA) for ELISA.
14. 96-Well nitrocellulose-backed microwell plate (e.g., MultiScreen 96-well plates; Millipore, Billerica, MA, USA) for ELISPOT.
15. Microplate washer for ELISA (e.g., ImmunoWash 1575; Bio-Rad Laboratories).
16. Microplate reader for ELISA (e.g., IWAKI EZS-ABS Microplate Reader; IWAKI Asahi Techno Glass, Tokyo, Japan).
17. Dissecting microscope or an ELISPOT plate reader for ELISPOT.
18. Coating solution: 0.1 M sodium carbonate, pH 9.6, for ELISA and ELISPOT.
19. Washing buffer: PBS containing 0.05% Tween 20, for ELISA and ELISPOT.
20. FACS buffer: PBS supplemented with 1% FCS, for intracellular cytokine staining.
21. Blocking solution: 10% fetal calf serum or 1% bovine serum albumin in PBS. The blocking solution should be filtered to remove particulates before use. Commercially available reagents such as Blocking One (Nacalai Tesque, Kyoto, Japan) is also usable.
22. Blocking solution/Tween: Blocking solution containing 0.05% Tween 20.
23. Monoclonal antibodies (mAb): anti-murine IFN-γ antibody R4-6A2 as capture antibody and biotin-labeled anti-murine IFN-γ antibody XMG1.2 as detection antibody for ELISA; phycoerythrin (PE)-conjugated anti-IFN-γ antibody XMG1.2 as well as fluorescein isothiocyanate (FITC)-conjugated anti-CD8 and PerCP-Cy5.5-conjugated anti-CD4 antibodies for intracellular IFN-γ staining assay (all from BD Biosciences, Franklin Lakes, NJ, USA).
24. Streptavidin-conjugated horseradish peroxidase (SAv-HRP) (e.g., eBioscience, San Diego, CA, USA).
25. 3, 3′, 5, 5′-Tetramethylbenzidine (e.g., TMB No Hydrogen Peroxide One Component Substrate; BioFX Laboratories, Owings Mills, MD, USA, This solution is supplied as a ready to use solution.).
26. AEC (3-amino-9-ethyl-carbazole) Substrate Set (BD Biosciences).
27. BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences).

28. RMA-S cells or cells transfected with MHC class I gene (e.g., RMAS-K^d, EMAS-D^d, or RMAS-L^d cells for BALB/c mouse).
29. Special equipment: flow cytometry apparatus for intracellular cytokine staining and MHC stabilization assay.

3. Methods

3.1. Preparation of Plasmid DNA

1. Mammalian expression plasmid pCI was digested with appropriate restriction enzymes (see Note 2).
2. Ligate an Mtb antigen-containing DNA fragment to the digested pCI plasmid (see Note 4).
3. Prepare plasmid DNA with Qiagen Plasmid Midi or Maxi Kit according to QIAGEN plasmid purification handbook.

3.2. Preparation of DNA/Gold Cartridge

Prepare DNA/gold cartridge according to Helios gene gun system instruction manual. The method is described briefly below.

1. In a 1.5 mL microfuge tube, weigh out 25 mg gold particle (see Note 5).
2. To the measured gold, add 100 µL of 0.05 M spermidine.
3. Vortex the gold/spermidine mixture for a few seconds, then sonicate for 3–5 s using an ultrasonic cleaner to break up gold clumps.
4. To the gold/spermidine mixture, add the required volume of plasmid to achieve the desired DNA loading rate (DLR) (see Note 6).
5. Mix DNA, spermidine, and gold by vortexing approximately 5 s.
6. While vortexing the mixture at moderate rate on a variable speed vortexer, add 100 µL of 1 M $CaCl_2$ dropwise to the mixture slowly. The volume of 1 M $CaCl_2$ solution added should be equal to that of the spermidine in Step 3.
7. Allow the mixture to precipitate at room temperature for 10 min.
8. Most of the gold will now be in the pellet, but some may be on the sides of the tube. The supernatant should be relatively clear. Spin the DNA/gold solution in a microfuge approximately 15 s to pellet the gold particle. Remove the supernatant and discard.
9. Resuspend the pellet in the remaining supernatant by vortexing briefly. Wash the pellet three times with 1 mL of fresh 100% (v/v) ethanol each time. Spin approximately 5 s in a microfuge between each wash. Discard the supernatants.

10. After the final ethanol wash, resuspend the pellet in 200 μL of the ethanol solution containing 0.05 mg/mL PVP prepared freshly. Transfer this suspension to a 15 mL disposable polypropylene tube with a screw cap. Add 2.8 mL of the ethanol/PVP solution to the centrifuge tube for a 25-in. length of tubing.
11. The suspension is now ready for tube preparation. Alternatively, the DNA/gold particle suspensions can be stored for up to 2 months at –20°C. Prior to freezing, tighten the cap securely.

3.3. Loading the DNA/Gold Suspension into Tubing Using the Tubing Prep Station

Prepare DNA/gold-coating tubing according to Helios gene gun system instruction manual. The method is described briefly below.

1. Set up the Tubing prep station and connect to a nitrogen tank.
2. Prior to preparing cartridges, ensure that the tubing is completely dry by purging with nitrogen. Insert an uncut piece of tubing into the opening on the Tubing prep station.
3. Using the knob on the flowmeter, turn on the nitrogen and adjust the flow to 0.3–0.4 L/min (LPM). Allow nitrogen to flow through the tubing for at least 15 min immediately prior to using it.
4. Turn off the flow of nitrogen to the Tubing prep station using the knob on the flowmeter.
5. From the dried tubing cut about 30 in. (about 75 cm) length of tubing for each 3 mL of DNA/gold suspension.
6. Vortex the DNA/gold suspension in a 15 mL tube and, if necessary, sonicate briefly with an ultrasonic cleaner to achieve an even suspension of gold. Invert the tube several times to resuspend the gold. Immediately remove the cap and quickly draw the DNA/gold suspension into the tubing with 10 mL syringe.
7. Immediately bring the tubing to a horizontal position and slide the loaded tube, with 10 mL syringe attached, into the tubing prep station.
8. Allow the DNA/gold to settle for 3–5 min. Then, remove ethanol at the rate of 0.5–1.0 in. per sec using a syringe (this should require 30–45 s).
9. Detach the syringe from the tubing. Immediately turn the tubing 180° while in the groove.
10. Turn on the switch on the Tubing prep station to start rotating the Tubing prep station.
11. Allow the gold to smear in the tube for 20–30 s. Then, open the valve on the flowmeter to allow 0.35–0.4 LPM of nitrogen to dry the tubing, while it continues to rotate.
12. Continue drying the tubing while rotating for 3–5 min.

13. Turn off the motor on the Tubing prep station. Turn off the nitrogen by closing the valve on the flowmeter. Remove the tubing from the Tubing support cylinder.
14. Examine the coated tubing to verify that the DNA/gold is evenly distributed over the length of the tubing.
15. Using scissors, cut off and discard the ends of the tubing.
16. Use the Tubing cutter to cut the remaining tubing into 0.5-in. pieces.

3.4. In Vivo Delivery of DNA-Coated Particle to Epidermis with Gene Gun

The gold particle coated with plasmid DNA encoding Mtb antigens are injected into mice with the gene gun. The frequency of injections and the interval time between injections depends on the experiments and investigators (see Note 7).

1. Clip animal fur as closely as possible over the desired target area using a razor and brush fur off (see Notes 8 and 9). Spraying the clipping site with 70% (v/v) ethanol makes clipping easy.
2. Load cartridges into the cartridge holder and place it in gene gun.
3. Administer DNA-coated gold particles to the dermis with gene gun using helium discharge pressure of 350–400 psi (pounds per square inch; 350–400 psi is about 24.5–28 kg/cm^2).
4. Remove cartridge holder from gene gun.
5. Remove cartridge from cartridge holder.
6. Turn off the helium pressure to the system.
7. Turn the regulator value counterclockwise to de-pressurize the system.
8. Disconnect the helium gas and gene gun.

3.5. Preparation of Immune Spleen Cells and the In Vitro Stimulation with Mtb Antigen Peptides

Prepare spleen cells aseptically from DNA-immunized mice more than 2 weeks after the last immunization.

1. Sacrifice mice by cervical dislocation or inhalation of diethyl ether.
2. Make an incision in the back skin, grasp either side of the incision with tweezers and pull back the skin until spleen is observed through back membrane.
3. Make an incision over the spleen and transfer the spleen with tweezers to a 60 mm Petri dish containing about 5 mL of RPMI 1640 medium.
4. Put the spleen on sterilized metal mesh and crush it by the head of a 5 mL syringe to prepare a single cell suspension.
5. Transfer the cell suspension to a 15-mL tube.
6. Centrifuge the cell suspension at 400 $\times g$ for 5 min at 4°C.

7. Resuspend the cells with 3 mL of ACK lysis solution per spleen to lyse red blood cells.
8. Immediately add 10 mL of RPMI 1640 medium and centrifuge the cells at 400×*g* for 5 min at 4°C. Discard the medium by decantation. Repeat the washing step for three times. Check the cell pellet is whitish after this washing step. In addition, remove the cell debris during this step.
9. Transfer the immune spleen cells to 96-well plates at $1–2 \times 10^6$ cells/well and culture in RPMI/10% FCS in the presence of 7.5 μM of each antigen peptide at 37°C in a humidified 5% CO_2 incubator. Harvest the cell culture supernatants 24–72 h later and store them at −20°C until they are assayed by ELISA.

3.6. Quantification of IFN-γ by ELISA

IFN-γ amounts secreted by immune spleen cells are measured by ELISA. The commercially available ELISA kits such as Quantikine Immunoassay (R&D Systems, Minneapolis, MN, USA) are also usable.

1. Coat half-size 96-well plate with 30 μL/well of 2 μg/mL of capture antibody (anti-murine IFN-γantibody R4-6A2) in coating solution at 4°C overnight.
2. Wash with 100 μL/well of washing buffer three times manually or automatically with a microplate washer.
3. Block with 50 μL/well of blocking solution at 37°C for 2 h.
4. After washing with 100 μL/well of washing buffer three times, add the culture supernatants to the plate and incubate at 4°C overnight.
5. After washing with 100 μL/well of washing buffer three times, add 50 μL/well of 0.5 μg/mL of biotin-labeled anti-murine IFN-γ antibody XMG1.2 in blocking solution/tween to the plate and incubate for 1 h at room temperature.
6. After washing with 100 μL/well of washing buffer five times, add 50 μL/well of 0.1 μg/mL of SAv-HRP in blocking solution/tween to each well.
7. After washing, add 50 μL/well of 3, 3′, 5, 5′-tetramethylbenzidine and incubate for 30 min at room temperature.
8. Determine the absorbency at 450 nm with a microplate reader.

3.7. Detection of IFN-γ-Producing Cells by Enzyme-Linked ImmunoSpot Assay

Single cell suspensions are tested for antigen-specific IFN-γ secretion with ELISPOT. The commercially available ELISPOT kits such as BD ELISPOT kit (BD Biosciences) are also usable.

1. Coat nitrocellulose-backed 96-well plate with 50 μL/well of 2 μg/mL of capture antibody (anti-murine IFN-γ monoclonal antibody R4-6A2) in coating solution at 4°C overnight.

2. Wash with 100 µL/well of washing buffer three times manually or automatically with a microplate washer.
3. Block with 50 µL/well of RPMI 1640 medium with 10% FCS at 37°C for 2 h.
4. Stimulate 200 µL/well of the immune spleen cells at different densities (e.g., $1–2 \times 10^6$ cells/well) in RPMI/10%FCS medium with 7.5 µM of each peptide in each well.
5. Incubate the plates for 24 h at 37°C in a humidified 5% CO_2 incubator.
6. Aspirate cell suspension and wash wells two times with deionized water. Allow wells to soak for 3–5 min at each wash step.
7. Wash wells three times with 200 µL/well of washing buffer. Discard washing buffer.
8. Add 100 µL/well of 0.5 µg/mL of biotin-labeled detection antibody (anti-murine IFN-γ antibody XMG1.2) and incubate the plates for 2 h at room temperature.
9. Discard detection antibody solution. Wash wells three times with 200 µL/well of washing buffer.
10. After wash, add 100 µL/well of 0.1 µg/mL of SAv-HRP and incubate the plates for 1 h at room temperature.
11. Discard the SAv-HRP solution. Wash wells four times with 200 µL/well of washing buffer.
12. Add 100 µL/well of AEC substrate solution to detect bound SAv-HRP. Monitor spot development for 5–60 min. Do not let color overdevelop as this will lead to high background.
13. Stop substrate reaction by washing wells with deionized water.
14. Air-dry plates at room temperature for 2 h to overnight until it is completely dry.
15. Enumerate spots developed on the nitrocellulose filters manually under a dissecting microscope, or automatically using an ELISPOT plate reader.

3.8. Intracellular IFN-γ Staining Assay

1. Stimulate 200 µL/well of the immune spleen cells (1×10^7 cells/mL) in RPMI/10%FCS medium with 7.5 µM of each peptide in nitrocellulose-backed 96-well microwell plates.
2. Incubate the plates for 24–48 h at 37°C in a humidified 5% CO_2 incubator.
3. For the last 6–12 h of incubation, add GolgiStop (containing monensin) or GolgiPlug (containing brefeldin A) solution. GolgiStop solution: the final concentration should be 4 µL of GolgiStop for every 6 mL of cell culture. Prepare first 5 × GolgiStop solution in RPMI 1640 medium (4 µL GolgiStop solution in 1.2 mL RPMI 1640 medium) and add 50 µL of

5 × GolgiStop solution to each well (200 µL). GolgiPlug solution: the final concentration should be 1 µL of GolgiPlug for every 1 mL of cell culture. Prepare first 5 × GolgiPlug solution in RPMI 1640 medium (1 µL GolgiPlug solution in 0.2 mL RPMI 1640 medium) and add 50 µL of 5 × GolgiPlug solution to each well (200 µL).

4. Transfer the cells to 1.5 mL microfuge tubes and wash with 500 µL of FACS buffer.
5. Add 100 µL of FITC-conjugated anti-CD8 and PerCP-Cy5.5-conjugated anti-CD4 mAbs to the 1.5 mL microfuge tubes and incubate on ice for 30 min.
6. Wash twice with 500 µL of FACS buffer (see Note 10).
7. Perform intracellular IFN-γ staining with phycoerythrin (PE)-conjugated anti-IFN-γ mAb XMG1.2 using BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit according to the manufacturer's instruction.
8. Analyze the cells with a flow cytometer.

3.9. MHC Stabilization Assay

MHC stabilization assay was originally described in Ljunggren et al (26) (see Note 11).

1. Culture RMA-S cells or cells transfected with H2 class I gene (e.g., RMAS-K^d, EMAS-D^d, or RMAS-L^d cells for BALB/c mouse) in RPMI/10%FCS at 26°C overnight using 75-mL flask (see Note 12).
2. Transfer the cells to 96-well round-bottom microwell plates at 2×10^6 cells/well.
3. Incubate the plates for 1 h in the presence or absence of 5–50 µM of respective peptide at 26°C.
4. Incubate the plates at 37°C for 2 h in a humidified 5% CO_2 incubator.
5. Transfer the cells to 1.5 mL microfuge tubes and wash the cells with 500 µL of FACS buffer.
6. Stain cell-surface H2 class I molecules with 100 µL of FITC-conjugated mouse mAbs specific for each H2 class I molecule at appropriate concentrations in FACS buffer and incubate for 30 min on ice.
7. Wash twice the cells with 500 µL of FACS buffer and resuspend in 500 µL of FACS buffer.
8. Analyze the cells with a flow cytometer.
9. To allow comparison between multiple experiments and to reduce inter-experimental variations, the mean fluorescence intensity (MFI) values, which are direct measures of peptide binding, should be converted to percent maximal stabilization

values. The values are calculated using the following formula: (experimental MFI—control MFI)/(maximal MFI—control MFI)×100. Control MFI is obtained from cells incubated without peptide at 37°C, while the MFI of cells at 26°C is taken as maximal MFI.

3.10. Prediction of T Cell Epitopes by MHC Binding Peptide Prediction Algorithms

For the prediction of potential minimal murine T cell epitopes, which could bind to MHC class I molecules, the following MHC binding peptide prediction algorithms are used through their websites. These are: National Institutes of Health BioInformatics and Molecular Analysis Section (BIMAS) (http://bimas.dcrt.nig.gov/cgi-bin/molbio/ken_parker_comboform) (27), SYFPEITHI (http://www.syfpeithi.de/) (28), RANKPEP program (http://bio.dfci.harvard.edu/Tools/rankpep.html) (29), and Propred (http://www.imtech.res.in/raghava/propred/) (30).

4. Notes

1. Codon usage database of various species is available in internet (http://www.kazusa.or.jp/codon/).
2. pCI mammalian expression plasmid contains a human cytomegalovirus promoter/enhancer element that allows expression of the cloned Mtb gene in mammalian cells and high copy number in the cells. A variety of similar mammalian expression vectors are available and usable for TB DNA vaccine backbone.
3. It is better to optimize PVP concentration first: Prepare tubes with 0, 0.05, 0.1 mg/mL PVP. For most systems, 0.05 mg/mL PVP is a good starting point. PVP is a binding agent of DNA to the tube.
4. Addition of DNA encoding the signal sequence from mammalian genes such as that of interleukin-2 and tissue plasminogen activator to the 5′ of Mtb antigen gene induces secretion of Mtb antigens and may improve expression level of Mtb antigen.
5. Microcarrier loading quantity (MLQ) is the amount of microcarrier (gold) in each cartridge. For most systems, the MLQ of 0.5 mg gold per cartridge is a good starting point. A 25-in. length of tubing gives approximately 50 cartridges. So, the tubing will require 25 mg of gold resuspended in a volume of 3 mL of 100% (v/v) ethanol to give MLQ of 0.5 mg gold/cartridge.
6. DLR is the amount of DNA per mg gold. At MLQ of 0.5 mg gold/cartridge, DLR of 2 μg of plasmid DNA/mg gold results

in loading 1 μg of DNA/cartridge. For the MLQ, preparation of a 25-in. length of gold-coat tubing (25 mg of gold) requires 50 μg of plasmid DNA. For most systems, the DLR usually ranges between 1 and 2.5 μg of plasmid DNA/mg gold.

7. In general, the injection of 1–2 μg of plasmid DNA for 3–6 times with intervals of 1–3 weeks induce sufficient immune responses. We usually inject 1 μg of plasmid DNA four times with 1-week intervals.
8. Razor should be attached to the skin and moved along fur's strike. Otherwise, razor will damage the skin and give bleeding.
9. After clipping, a commercial depilatory can be used to completely remove the animal's fur. This treatment removed the stratum corneum from the skin, completely exposing the epidermis. Carefully rinse the skin with warm water following depilatory treatment. If the target site is wet or dirty, clean and dry with 70% (v/v) ethanol.
10. Before this step, treatment with antibodies specific for mouse FcγII/III receptors that block Fc receptors may be useful for reducing nonspecific staining by fluorescein-conjugated antibodies at the subsequent step.
11. Generally, RMA-S cells (26) and the cells transfected with H2 class I gene or T2 cells (31) and the cells transfected with human HLA class I gene are used for mouse or human system, respectively.
12. Instead of using CO_2 incubator at 26°C, the flask being tightly capped can be incubated at room temperature without CO_2 incubator.

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