tuberculosis (*Mtb*) (1). However, less than 10% of the infected individuals actually develop active diseases (approximately 9.4 million new incidences in 2008) (1), and *Mtb* infection remains latent with no overt clinical symptoms throughout the life in more than 90% of infected individuals. Such statistical evidence indicates the efficacy of naturally induced immune responses, at least in the resistant population. The risk of developing active TB is increased in immunocompromised individuals, especially in HIV-positive people (1). While infected immunocompetent individuals mount a cell-mediated immune response against *Mtb*, most of them do not achieve complete eradication of the microbe (2–5). Consequently, *Mtb* bacilli can persist within the human body, especially in the lung, for long periods of time in a metabolically inactive but reversible state known as dormancy (2–5). The major cause of adult pulmonary TB, the most prevalent form of the disease, is reactivation of such persistent *Mtb* bacilli (6), although the differences between resistant and susceptible populations to TB reactivation are not fully understood.

The current TB vaccine *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) was developed nearly 90 years ago and is the most widely used vaccine worldwide (7–10), although it has been reported to exhibit variable protective efficacy (11). This vaccine efficiently protects against severe disseminated TB (miliary TB) in newborns and young children, but fails to prevent adult pulmonary TB. Accordingly, BCG has virtually no effect on TB transmission in the adult population (12). The ineffectiveness of BCG to protect against the highly prevalent TB in adults has stimulated many efforts to establish more effective vaccine protocols. In general, these attempts can be divided into two categories, i.e., (i) subunit vaccines composed of one or a few antigens and (ii) recombinant viable vaccines superior to BCG (7, 8). Considering the complementary strength of both types of vaccines, a heterologous prime-boost regimen comprising a prime with a viable vaccine superior to BCG and a boost with a subunit vaccine seems to be the most promising combination (7, 8).

After *Mtb* enters the lungs by inhalation, the bacterial cell is engulfed by alveolar macrophages and probably dendritic cells (DCs); however, *Mtb* can escape from digestion by these phagocytic cells (13). Consequently, infected macrophages and DCs serve as mobile reservoirs that transport the bacteria, eventually to draining lymph nodes, where presentation of mycobacterial antigens to T cells occurs (2–5). As a regional immune response, activated antigen-specific T cells induce the formation of a granuloma around infected macrophages, primarily composed of monocyte-derived macrophages, $CD4^+$ T cells, and $CD8^+$ T cells (14). In the granuloma, surrounding T cells activate macrophages through the production of Th1 cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α (14), and IFN-γ augments the capacity to control *Mtb* in macrophages (15, 16). The granuloma can persist for years and deprive the arrested *Mtb* of oxygen and nutrients; therefore, the bacterial cells survive probably in a state of dormancy (17-20). Recent DNA microarray analysis revealed upregulation of the DosR regulon, which comprises 48 genes (including the heat shock protein (hsp) X/α-crystallin), in *Mtb* during its dormancy state (19, 20). Therefore, in this study, we assessed the immunogenicity of these antigens in DNA-vaccinated mice. We anticipate that our results can provide useful information not only for the establishment of novel vaccines to control TB reactivation but also for the development of biomarkers for new diagnosis of latent *Mtb* infection.

2. Materials and Methods

2.1. Preparation of DNAs

DNAs encoding the DosR regulon proteins (20) were amplified by PCR using genomic DNAs of *Mtb* H37Rv or BCG as templates. Primers were designed according to the genetic information obtained from the TubercuList (http://genolist.pasteur.fr/TubercuList/). Appropriate restriction sites were introduced at the 5′ and 3′ ends of the DNAs by PCR, and the PCR products were digested and ligated into the corresponding restriction sites of pCI (Promega, Madison, WI, USA) and pET-28b(+) (Novagen/Merck, Darmstadt, Germany) vectors for DNA vaccine and recombinant protein preparation, respectively. Plasmids were purified using commercially available Plasmid Purification Kits (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The nucleotide sequences were confirmed by automated DNA sequencing (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) using a dye primer cycle sequencing kit (Applied Biosystems).

2.2. Preparation of recombinant proteins

Escherichia coli BL21(DE3) competent cells (Novagen/Merck) were transformed with pET-28(+) vectors containing the DNAs for the DosR regulon proteins. Proteins were induced with isopropyl β-D(-)-

thiogalactopyranoside (IPTG, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Proteins were extracted from the bacterial cells with 7 M urea (Wako Pure Chemical Industries, Ltd.) and purified by Ni^{2+}-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) according to the manufacturer's instructions.

2.3. Mice and vaccination

Female BALB/c and C57BL/6 mice (8 and 12 weeks of age) were purchased from Japan SLC (Hamamatsu, Japan) and maintained in the animal facility of Hamamatsu University School of Medicine under specific pathogen-free conditions. All animal experiments were performed according to the Guidelines for Animal Experimentation issued by the Hamamatsu University School of Medicine. Mice were vaccinated with plasmids using a Helios gene gun system (Bio-Rad Laboratories, Hercules, CA, USA) three times at 2-week intervals as described previously (21).

2.4. Enzyme-linked immunosorbent assay (ELISA) for IFN-γ

Two weeks after the last vaccination, spleen cells from the vaccinated and non-vaccinated control mice were cultured in a 96-well plate (1×10^6 cells/0.2 ml/well) in the presence or absence of corresponding antigens for 2 days. IFN-γ concentration of the culture supernatants was determined by a sandwich ELISA as described previously (21).

2.5. Serum preparation and antibody titration

Two weeks after the last vaccination, sera from the vaccinated mice were prepared as described (22). Pre-vaccinated sera from the corresponding vaccinated mice were also prepared. Indirect ELISA to detect antigen-specific antibodies in the sera from mice (both pre- and post-vaccination) was conducted as described (23), with slight modifications, using peroxidase-labeled goat anti-mouse IgG+A+M (H+L) antibody (Zymed/Invitrogen, Carlsbad, CA, USA) and 3,3′,5,5′-tetramethylbenzidine (TMB) (BioFX Laboratories, Owings Mills, MD, USA).

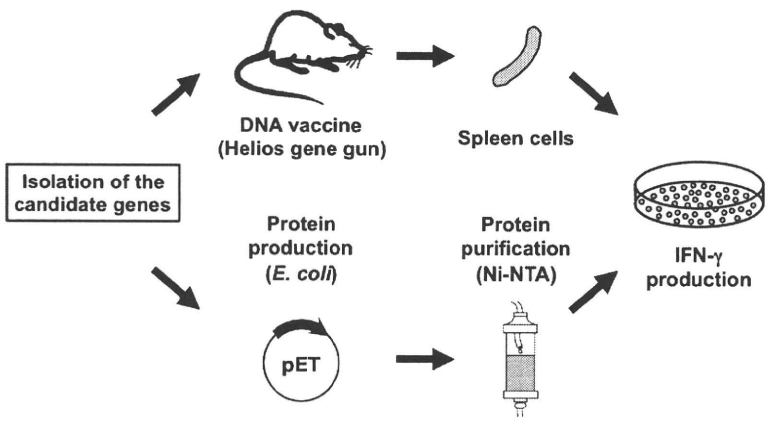

Fig. 1. Experimental design for the measurement of T-cell responses against latency-associated antigens. . DNAs for DosR regulon proteins were obtained by PCR using the genomic DNA of *Mtb* H37Rv or BCG as templates and were cloned into pCI and pET-28b(+) vectors for DNA vaccine and recombinant protein preparation, respectively. By using a Helios gene gun, BALB/c and C57BL/6 mice were vaccinated three times at 2-week intervals with the plasmids encoding antigen DNA. Two weeks after the last vaccination, spleen cells from the vaccinated and non-vaccinated mice were stimulated with corresponding antigen proteins prepared by the bacterial pET system, and the production of IFN-γ was tested by ELISA.

3. Results and Discussion

T cell responses against DosR regulon-encoded antigens were measured as illustrated in Fig. 1. The results of these experiments are shown in Fig. 2 and Table 1. Of the 32 DosR regulon-encoded antigens tested, 12 induced antigen-specific T-cell responses in vaccinated BALB/c mice and 9 induced responses in C57BL/6 mice. Of these antigens that induced responses, 5 antigens (Rv1998c, Rv2031c, Rv2032, Rv2623, and Rv3132c) induced T-cell responses in both mice strains, although the intensity of their responses varied. The antigen Rv0079 also showed a strong immunogenicity in a single BALB/c mouse and in 2 C57BL/6 mice, but failed to induce a T-cell response in the BALB/c mouse. In addition, Rv2628 induced strong T-cell responses in BALB/c mice only. In contrast, Rv2624c induced intermediate T-cell responses in C57BL/6 mice, while its immunogenicity was relatively low in BALB/c mice.

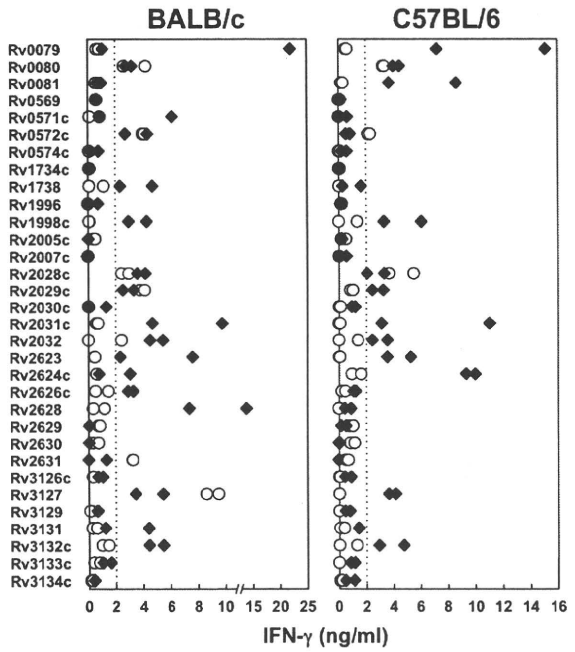

Fig. 2. T-cell responses against DosR regulon proteins. By using a Helios gene gun, BALB/c or C57BL/6 mice were vaccinated with the plasmids encoding the corresponding antigen DNA. Spleen cells (1×10^5 cells) from the vaccinated mice were cultured for 48 h in the presence of corresponding antigens, and the production of IFN-γ was then tested by a sandwich ELISA. Closed diamonds and open circles represent the individual results of immunized and naïve mice, respectively. Dotted vertical lines represent the thresholds between positive and negative.

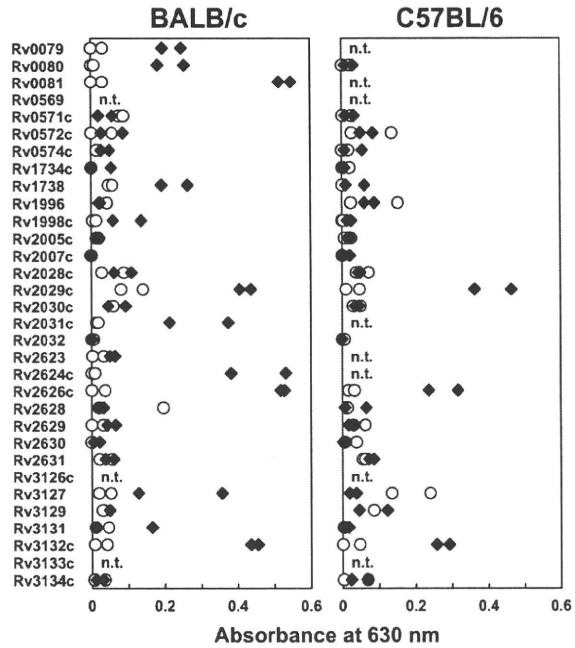

Fig. 3. Antibody production against DosR regulon proteins. Sera from mice (both pre- and post-vaccination) were diluted 100 times, and the titers for antigen-specific antibody were measured by ELISA. Closed diamonds and open circles represent the individual results of vaccinated and pre-vaccinated mice, respectively. n.t.: not tested.

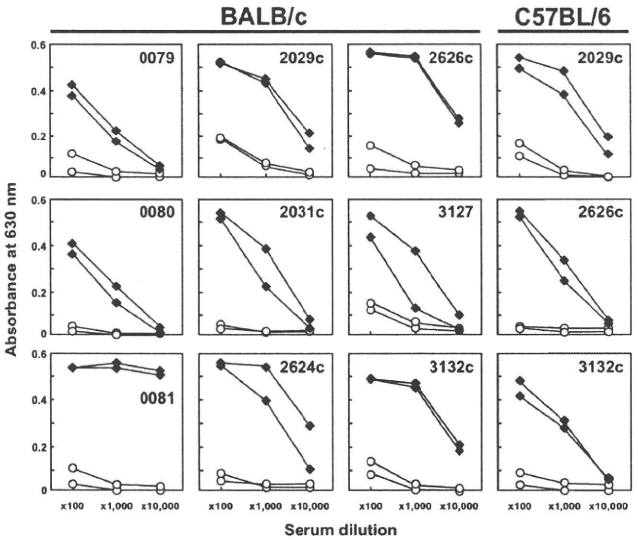

Fig. 4. Titration analysis of sera with high titers for antigen-specific antibody. Sera from mice (both pre- and post-vaccination) were serially diluted, and the titers for antigen-specific antibody were measured by ELISA. Closed diamonds and open circles represent the individual results of vaccinated and pre-vaccinated mice, respectively.

Table 1. Summary of the present study and comparison with a previous study

Antigen	AA[1]	Name/Despcription[2]	Present study								Roupie *et al.*[3]			
			IFN-γ production[4]				Ab secretion[5]				IFN-γ[6]		Ab	
			BALB/c		C57BL/6		BALB/c		C57BL/6		BALB	B6	BALB	B6
Rv0079	273	Hypothetical protein	+++	-	+++	++	++	+	n.t.[7]		n.t.	n.t.	n.t.	n.t.
Rv0080	152	Conserved hypothetical protein	-	-	-	-	++	+	-	-	n.t.	n.t.	n.t.	n.t.
Rv0081	114	Probable transcriptional regulatory protein	-	-	++	+	+++	+++	n.t.		n.t.	n.t.	n.t.	n.t.
Rv0569	88	Conserved hypothetical protein	-	-	-	-	n.t.		n.t.		n.t.	n.t.	n.t.	n.t.
Rv0571c	443	Conserved hypothetical protein	++	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv0572c	113	Hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv0574c	380	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv1733c	210	Probable conserved transmembrane protein	n.t.		n.t.		n.t.		n.t.		VL	VL	+	+
Rv1734c	80	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv1738	94	Conserved hypothetical protein	+	+	-	-	++	+	-	-	VL	VL	-	-
Rv1996	317	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv1998c	258	Conserved hypothetical protein	++	+	+	+	+	+	-	-	n.t.	n.t.	n.t.	n.t.
Rv2005c	295	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv2007c	114	FdxA: probable ferredoxin	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv2028c	279	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv2029c	339	PfkB: probable phosphofructokinase/phosphohexokinase	-	-	-	-	+++	++	+++	++	VL	VL	+	+
Rv2030c	681	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv2031c	144	HspX/Acr: heat shock protein/α-crystallin homolog	++	+	+++	+	++	++	n.t.		HIGH	INT	+	+
Rv2032	331	Acg: conserved hypothetical protein	+	+	+	+	-	-	-	-	INT	LOW	-	-
Rv2623	297	Tb31.7: conserved hypothetical protein	++	+	++	+	-	-	n.t.		n.t.	n.t.	n.t.	n.t.
Rv2624c	272	Conserved hypothetical protein	+	-	++	++	+++	++	n.t.		n.t.	n.t.	n.t.	n.t.
Rv2626c	143	Conserved hypothetical protein	+	+	-	-	+++	+++	++	++	HIGH	HIGH	+	+
Rv2627c	413	Conserved hypothetical protein	n.t.		n.t.		n.t.		n.t.		LOW	INT	-	-
Rv2628	120	Hypothetical protein	+++	++	-	-	-	-	-	-	INT	INT	-	+
Rv2629	374	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv2630	179	Hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv2631	432	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv3126c	104	Hypothetical protein	-	-	-	-	n.t.		n.t.		n.t.	n.t.	n.t.	n.t.
Rv3127	344	Conserved hypothetical protein	-	-	+	+	++	+	-	-	n.t.	n.t.	n.t.	n.t.
Rv3129	110	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv3131	332	Conserved hypothetical protein	+	-	-	-	+	-	-	-	n.t.	n.t.	n.t.	n.t.
Rv3132c	578	DevS: two component sensor histidine kinase	+	+	+	+	+++	+++	++	++	n.t.	n.t.	n.t.	n.t.
Rv3133c	217	DevR: two component transcriptional regulatory protein	-	-	-	-	n.t.		n.t.		n.t.	n.t.	n.t.	n.t.
Rv3134c	268	Conserved hypothetical protein	-	-	-	-	-	-	-	-	n.t.	n.t.	n.t.	n.t.

[1] Number of amino acids.

[2] Referred to the TubercuList World-Wide Web Server (http://genolist.pasteur.fr/TubercuList/).

[3] Infect. Immun. 75: 941-949, 2007. Only the results for DNA vaccinated mice are shown.

[4] Each symbol represents a result of an individual mouse. -: IFN-γ concentration <2 ng/ml, +: 2-5 ng/ml, ++: 5-10 ng/ml, +++: >10 ng/ml.

[5] Each symbol represents a result of an individual mouse. -: ABS at 630 nm <0.1, +: 0.1-0.2, ++: 0.2-0.4, +++: >0.4.

[6] HIGH: >800 pg/ml, INT (intermediate): 600-800 pg/ml, LOW: 250-600 pg/ml, VL (very low): <250 pg/ml (according to the authors' description).

[7] n.t.: not tested.

Antibody production against DosR regulon-encoded antigens in DNA-vaccinated mice was also examined, although some results have yet to be obtained. As shown in Fig. 3 and Table 1, at least 12 DosR regulon-encoded antigens induced antigen-specific antibody production in vaccinated BALB/c and 3 antigens induced antibody production in C57BL/6 mice. Of the antigens that produced antibody, 3 antigens (Rv2029c, Rv2626c, and Rv3132c) induced strong antibody production in both mice strains (Figs. 3 and 4). Rv0081 and Rv2624c also induced strong antibody production in BALB/c mice, while their effects in C57BL/6 mice have not been tested.

Table 1 summarizes the present study results and shows the results of a previous report for comparison (24). Both studies showed that Rv2031c (hsp X/α-crystallin) effectively induced antigen-specific cellular and humoral immune responses in both mice strains, although we have not assessed the humoral response in C57BL/6 mice. In addition to Rv2031c, our present study also showed that Rv3132c (desV) induced antigen-specific cellular and humoral immune responses in both mice strains, although its immunogenicity in T-cell responses seemed relatively low.

Rv2032 also showed a similar immunogenicity between the two studies, i.e., only T-cell responses were induced in both BALB/c and C57BL/6 mice. On the other hand, while Roupie *et al.* showed high and intermediate IFN-γ production by spleen cells from vaccinated C57BL/6 mice in response to Rv2626c and Rv2628, respectively, we did not detect T-cell responses against these antigens in the same strain. The discordance may stem from the differences in DNA-vaccine constructs, vaccination formula, and/or assay conditions, but the actual cause(s) has not been clarified. Some differences were also observed across studies in the results for the antigens showing low immunogenicity.

IFN-γ-secreting $CD4^+$ T cells, also known as Th1 cells, are important mediators of TB protection (15, 16). Attempts to induce TB antigen-specific Th1 cells have been the dominant theme of most TB vaccine development studies (7, 8). In addition, accumulating evidence shows that $CD8^+$ cytotoxic T lymphocytes (CTLs) also contribute to disease resistance (2–5). Therefore, identification of highly immunogenic antigens recognized by T cells is a particularly important step in the development of TB vaccines. However, since T-cell responses are induced in an MHC-restricted manner (25, 26), T-cell antigens found in murine systems are not necessarily applicable for human vaccines. In addition, HLA heterogeneity should also be considered during vaccine development. Recent studies revealed that the immunogenicity of latency-associated antigens varies not only between individuals of European and African descent but also among African people (27, 28). MHC class II binding motifs, however, are not as tightly restricted as those of MHC class I (29). Promiscuous peptides binding to different MHC class II molecules have been reported (30). Of note, some promiscuous peptides are associated with both mouse and human MHC class II molecules (31, 32). We are currently attempting to identify T-cell epitopes on the antigens found immunogenic in this study that are shared by both mouse and human MHC class II molecules.

In addition to the establishment of vaccine protocols and therapeutic regimens against TB, another important issue in TB management is the development of biomarkers applicable for monitoring clinical trials and for making more rapid and accurate diagnoses and prognoses of the outcome of infections and drug treatments (33, 34). For TB diagnosis, a tuberculin skin test (TST) is still used today, although this test has many major disadvantages, including poor specificity (false-positive tests caused by prior vaccination or previous exposure to environmental mycobacteria) and poor sensitivity (false-negative tests in children and immunocompromised individuals) (34). Newly established IFN-γ release assays using specific antigens of ESAT-6 and CFP-10, such as the QuantiFERON-TB Gold test (Cellestis Limited, Carnegie, Victoria, Australia) and the T-SPOT.TB test (Oxford Immunotech Limited, Abingdon, UK), can differentiate infection from BCG vaccination, but these methods still have some limitations (35).

We are hopeful that the immune responses against latency-associated antigens observed in this study can ultimately be used as biomarkers for the diagnosis of latent infection of *Mtb*. To this end, we have initiated a few human studies to measure the immunogenicity of latency-associated antigens. Since antibody-based assays are generally easier and cheaper than T cell-based assays, the former may be particularly suitable for the measurement of biomarkers, especially in developing countries. In this regard, we have found 3 strong B-cell antigens (Rv2029c, Rv2626c, and Rv3132c) that are commonly immunogenic in both strains tested. Rv0079, Rv0080, Rv1738, Rv2626c, and Rv3127 potentially have similar characteristics. Therefore, it would be interesting to verify whether the antibody production against these antigens in humans can be used as the biomarkers for the diagnosis of latent infections and/or active diseases. Such a study is currently in progress.

In conclusion, in this study we assessed the immunogenicity of latency-associated antigens in DNA-vaccinated mice and found that of 32 antigens, 12 and 9 antigens induced antigen-specific T-cell responses in vaccinated BALB/c and C57BL/6 mice, respectively. In addition, we found that at least 12 antigens induced antigen-specific antibody production in vaccinated BALB/c and 3 induced production in C57BL/6 mice. Our next aims are to identify the epitopes on these T-cell antigens and to assess the immune responses against them by using human blood samples.

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References

1. **World Health Organization (WHO).** 2009. 2009 Tuberculosis Facts Update.
2. **Flynn, J.L., and J. Chan.** 2001. Immunology of tuberculosis. Annu. Rev. Immunol. **19:**93-129.
3. **North, R.J., and Y.J. Jung.** 2004. Immunity to tuberculosis. Annu. Rev. Immunol. **22:**599-623.
4. **Kaufmann, S.H.E., and A.J. McMichael.** 2005. Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. Nat. Med. **11:**S33-44.
5. **Cooper, A.M.** 2009. Cell-mediated immune responses in tuberculosis. Annu. Rev. Immunol. **27:**393-422.
6. **Hunter, R.L., C. Jagannath, and J.K. Actor.** 2007. Pathology of postprimary tuberculosis in humans and mice: contradiction of long-held beliefs. Tuberculosis **87:**267-278.
7. **Kaufmann, S.H.E.** 2005. Recent findings in immunology give tuberculosis vaccines a new boost. Trends Immunol. **26:**660-667.
8. **Skeiky, Y.A., and J.C. Sadoff.** 2006. Advances in tuberculosis vaccine strategies. Nat. Rev. Microbiol. **4:**469-476.
9. **Oettinger, T., M. Jorgensen, A. Ladefoged, K. Haslov, and P. Andersen.** 1999. Development of the Mycobacterium bovis BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. Tuber. Lung Dis. **79:**243-250.
10. **Andersen, P.** 2007. Tuberculosis vaccines - an update. Nat. Rev. Microbiol. **5:**484-487.
11. **Colditz, G.A., T.F. Brewer, C.S. Berkey, M.E. Wilson, E. Burdick, H.V. Fineberg, and F. Mosteller.** 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA **271:**698-702.
12. **Corbett, E.L., C.J. Watt, N. Walker, D. Maher, B.G. Williams, M.C. Raviglione, and C. Dye.** 2003. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch. Intern. Med. **163:**1009-1021.
13. **Pieters, J.** 2008. Mycobacterium tuberculosis and the macrophage: maintaining a balance. Cell Host Microbe **3:**399-407.
14. **Ulrichs, T., and S.H.E. Kaufmann.** 2006. New insights into the function of granulomas in human tuberculosis. J. Pathol. **208:**261-269.
15. **Cooper, A.M., D.K. Dalton, T.A. Stewart, J.P. Griffin, D.G. Russell, and I.M. Orme.** 1993. Disseminated tuberculosis in interferon γ gene-disrupted mice. J. Exp. Med. **178:**2243-2247.
16. **Flynn, J.L., J. Chan, K.J. Triebold, D.K. Dalton, T.A. Stewart, and B.R. Bloom.** 1993. An essential role for interferon γ in resistance to Mycobacterium tuberculosis infection. J. Exp. Med. **178:**2249-2254.
17. **Chan, J., and J. Flynn.** 2004. The immunological aspects of latency in tuberculosis. Clin. Immunol. **110:**2-12.
18. **Boon, C., and T. Dick.** 2002. Mycobacterium bovis BCG response regulator essential for hypoxic dormancy. J. Bacteriol. **184:**6760-6767.
19. **Sherman, D.R., M. Voskuil, D. Schnappinger, R. Liao, M.I. Harrell, and G.K. Schoolnik.** 2001. Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding α-crystallin. Proc. Natl. Acad. Sci. U.S.A. **98:**7534-7539.
20. **Voskuil, M.I., D. Schnappinger, K.C. Visconti, M.I. Harrell, G.M. Dolganov, D.R. Sherman, and G.K. Schoolnik.** 2003. Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. J. Exp. Med. **198:**705-713.
21. **Suzuki, M., T. Aoshi, T. Nagata, and Y. Koide.** 2004. Identification of murine H2-D^d- and H2-A^b-restricted T-cell epitopes on a novel protective antigen, MPT51, of Mycobacterium tuberculosis. Infect. Immun. **72:**3829-3837.
22. **Harlow, E., and D. Lane.** 1988. Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. **Margulies, D.H.** 2005. Enzyme-Linked Immunosorbent Assays. Short Protocols in Immunology. John Wiley & Sons, Inc., Hoboken, NJ.

24. **Roupie, V., M. Romano, L. Zhang, H. Korf, M.Y. Lin, K.L. Franken, T.H. Ottenhoff, M.R. Klein, and K. Huygen.** 2007. Immunogenicity of eight dormancy regulon-encoded proteins of Mycobacterium tuberculosis in DNA-vaccinated and tuberculosis-infected mice. Infect. Immun. **75:**941-949.
25. **Zinkernagel, R.M., and P.C. Doherty.** 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. Adv. Immunol. **27:**51-177.
26. **Germain, R.N.** 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell **76:**287-299.
27. **Leyten, E.M., M.Y. Lin, K.L. Franken, A.H. Friggen, C. Prins, K.E. van Meijgaarden, M.I. Voskuil, K. Weldingh, P. Andersen, G.K. Schoolnik, S.M. Arend, T.H. Ottenhoff, and M.R. Klein.** 2006. Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. Microbes Infect. **8:**2052-2060.
28. **Black, G.F., B.A. Thiel, M.O. Ota, S.K. Parida, R. Adegbola, W.H. Boom, H.M. Dockrell, K.L. Franken, A.H. Friggen, P.C. Hill, M.R. Klein, M.K. Lalor, H. Mayanja, G. Schoolnik, K. Stanley, K. Weldingh, S.H. Kaufmann, G. Walzl, and T.H. Ottenhoff.** 2009. Immunogenicity of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in three high-burden populations in Africa. Clin. Vaccine Immunol. **16:**1203-1212.
29. **Margulies, D.H., K. Natarajan, K., J. Rossjohn, and McCluskey.** Major histocompatibility complex (MHC) molecules: structure, function, and genetics. Fundamental Immunology, 6th ed. Lippincott Williams & Wilkins Publishers, Philadelphia, PA.
30. **Sinigaglia, F., and J. Hammer.** 1994. Defining rules for the peptide-MHC class II interaction. Curr. Opin. Immunol. **6:**52-56.
31. **Sinigaglia, F., M. Guttinger, J. Kilgus, D.M. Doran, H. Matile, H. Etlinger, A. Trzeciak, D. Gillessen, and J.R. Pink.** 1988. A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules. Nature **336:**778-780.
32. **Wang, L.X., T. Nagata, K. Tsujimura, M. Uchijima, S. Seto, and Y. Koide.** 2010. Identification of HLA-DR4-restricted T-cell epitope on MPT51 protein, a major secreted protein derived from Mycobacterium tuberculosis using MPT51 overlapping peptides screening and DNA vaccination. Vaccine **28:**2026-2031.
33. **Kaufmann, S.H.E., and S.K. Parida.** 2008. Tuberculosis in Africa: learning from pathogenesis for biomarker identification. Cell Host Microbe **4:**219-228.
34. **Parida, S.K., and S.H.E. Kaufmann.** 2010. The quest for biomarkers in tuberculosis. Drug Discov. Today **15:**148-157.
35. **Mazurek, M., J. Jereb, A. Vernon, P. LoBue, S. Goldberg, and K. Castro.** 2010. Updated guidelines for using Interferon Gamma Release Assays to detect Mycobacterium tuberculosis infection - United States, 2010. MMWR Recomm. Rep. **59:**1-25.

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Review Article

Induction of Specific CD8$^+$ T Cells against Intracellular Bacteria by CD8$^+$ T-Cell-Oriented Immunization Approaches

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For protection against intracellular bacteria such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, the cellular arm of adaptive immunity is necessary. A variety of immunization methods have been evaluated and are reported to induce specific CD8$^+$ T cells against intracellular bacterial infection. Modified BCG vaccines have been examined to enhance CD8$^+$ T-cell responses. Naked DNA vaccination is a promising strategy to induce CD8$^+$ T cells. In addition to this strategy, live attenuated intracellular bacteria such as *Shigella*, *Salmonella*, and *Listeria* have been utilized as carriers of DNA vaccines in animal models. Vaccination with dendritic cells pulsed with antigenic peptides or the cells introduced antigen genes by virus vectors such as retroviruses is also a powerful strategy. Furthermore, vaccination with recombinant lentivirus has been attempted to induce specific CD8$^+$ T cells. Combinations of these strategies (prime-boost immunization) have been studied for the efficient induction of intracellular bacteria-specific CD8$^+$ T cells.

1. Introduction

The types of effective immune responses against infectious diseases depend on the location of the pathogens responsible. Generally extracellular pathogens are vulnerable to antibody-mediated effector mechanisms. On the other hand, protection against intracellular pathogens depends on the induction of specific cell-mediated immunity [1, 2]. Induction of effective resistance to infection depends on vaccines with the capacity of eliciting certain effectors.

In this review, we focused on strategies for the induction of CD8$^+$ T cells against intracellular bacterial infections. CD8$^+$ cytotoxic T-lymphocytes (CTL) are the main effectors against bacteria, such as *Rickettsia* or *Listeria monocytogenes*, located in the cytoplasm of host cells, while CD4$^+$ type 1 helper T (Th1) cells play a pivotal role in the protection against infections caused by intracellular bacteria, such as *Mycobacterium* or *Salmonella*, located in vacuolar compartments. Many reports have indicated that in addition to Th1 cells, CD8$^+$ CTL are also important for protection against these bacteria. After CD8$^+$ CTL are antigen-presented, they directly kill the infected cells with an oriented release of granules like perforin and granzymes, as well as by granule-independent mechanisms (reviewed in [3]). Therefore, the induction of the bacteria-specific CD8$^+$ T cells at an appropriate timing and magnitude is a key factor for protection against infections. Attenuated vaccines have been used for the induction of cellular immunity including that of CD8$^+$ T cells. However, a variety of immunization methods have been recently reported to effectively induce specific CD8$^+$ T cells. We reviewed methods to induce CD8$^+$ T cells specific for intracellular pathogenic bacteria with emphasis on our efforts to induce CD8$^+$ T cells specific for *Mycobacterium tuberculosis* and *L. monocytogenes*.

2. Intracellular Bacteria

Several bacteria have evolved in their mechanisms that allow them to survive in the host cells. These bacteria are considered intracellular based on their localization within the host cells, and are further categorized based on several criteria: bacteria such as *Chlamydia* and *Rickettsia* that cannot survive

Table 1: Intracellular bacteria.

Intracytosolic bacteria
Listeria monocytogenes Shigella flexneri**
Rickettsia prowaseki
Intravascular bacteria
Mycobacterium spp. (*M. tuberculosis, M. bovis, M. avium*)
Nocardia asteroides
Legionella pneumophila, Chlamydia trachomatis
Intralysosomal bacteria
Salmonella enterica Typhimurium*, *Salmonella enterica* Typhi
Yersinia enterocolitica, Coxiella burnetii*

Asterisks indicate bacteria utilized as carrier for DNA vaccine.

outside host cells are called obligate intracellular bacteria. On the other hand, facultative intracellular bacteria such as *Salmonella*, *Mycobacteria*, *Shigella*, and *Listeria* can survive outside of host cells.

Intracellular bacteria are also divided into three different groups depending on their intracellular niche (Table 1, reviewed in [4]) as follows: (1) cytoplasmic bacteria, which exit the phagosome and reside in the host cell cytoplasm: (2) intravascular bacteria, which persist in nonacidic vacuoles that have little interaction with the endosomal system of the host cells: and (3) intralysosomal bacteria, which persist in acidic, hydrolytic compartments that interact with the endosomal network of host cells.

(1) *Cytoplasmic Bacteria. L. monocytogenes* is a typical cytoplasmic, gram-positive, facultative intracellular bacterium. The *L. monocytogenes* infection system in mice has been studied and regarded as an excellent model system for intracellular bacterial infections [2, 5]. This bacterium has been known to induce major histocompatibility complex (MHC) class I-restricted $CD8^+$ T-cell responses in addition to MHC class II-restricted $CD4^+$ T-cell responses since it is capable of escaping from the phagocytic vesicles into the cytoplasm of the host cells with the help of listeriolysin O (LLO; Hly), thereby introducing the bacterial proteins into the MHC class I antigen processing pathway. Both $CD8^+$ CTL and $CD4^+$ Th1 have been shown to be amplified in listerial infections and to play a critical role in protective immunity.

(2) *Intravascular Bacteria.* Bacteria such as *Mycobacteria* and *Nocardia* have been shown to block the normal maturation steps of phagosomes of host cells. In other words, these bacteria inhibit the phagosome-lysosome fusion after being phagocytosed. Vacuoles containing *Mycobacteria* do not acidify below pH 6.2 to 6.5 and exhibit paucity of the vacuolar proton ATPase, which is responsible for the acidification of endosomal and lysosomal compartments. For protection against *M. tuberculosis* infection, both $CD4^+$ T cells and $CD8^+$ T cells have been shown to be critical [1, 2].

(3) *Intralysosomal Bacteria. Salmonella* has been reported to belong to the intralysosomal bacteria. More than 2000 serotypes of *Salmonella* have been described. DNA homology analysis revealed that the genus consists of two species: *S. enterica* and *S. bongori*. *S. enterica* is further subdivided into six subgroups both phenotypically and genetically. Bacteria that belong to *S. enterica* are often briefly designated as *S.* Typhimurium and *S.* Enteritidis based on their serotypes for convenience sake. *Salmonella* penetrates through M cells into the Peyer's patches where they are phagocytosed by the underlying macrophages. The precise nature of *Salmonella*-containing vacuoles is controversial and is dependent on investigators and the types of the cells infected by *Salmonella*, but, the vacuoles inhabited by *Salmonella* have characteristics consistent with those that are late endosomal or lysosomal in nature. *Salmonella* vacuoles in macrophages are subject to be acidified by the fusion with lysosomes. Although the acidification of these vacuoles is partially reduced in those containing live *Salmonella*, the pH of the vacuoles containing live bacteria is still relatively acidic. *Yersinia* also belongs to this category. After passing through M cells, they are engulfed by macrophages and carried to the mesenteric lymph nodes where replication occurs.

$CD8^+$ T cells have been considered to be critical for protection against these intracellular bacterial infections, especially, intracytosolic and intravascular bacterial infections. Intracellular bacteria themselves have been used as attenuated bacterial vaccines and also as carriers of DNA vaccines.

3. Antigen Processing and Presentation Required for CTL Induction

Both CTL and helper T cells have the same T-cell receptor molecules on their surfaces, apart from CD8 and CD4 molecules on their surfaces, respectively. Antigens (antigenic peptides) in association with MHC class I molecules on the surface of antigen-presenting cells (APC) are presented to $CD8^+$ CTL. Furthermore, antigens in association with MHC class II molecules are presented to $CD4^+$ Th cells. Therefore, the efficient induction of CTL and Th cells requires efficient presentation of antigenic molecules via MHC class I and II antigen processing and presentation pathways, respectively (reviewed in [6]).

MHC class I molecules have been shown to be expressed in almost all somatic cells except for neurons and germ cells. In order to prime $CD8^+$ CTL, antigenic peptides must be presented on MHC class I molecules on the surface of professional APC that possess special accessory molecules. In general, proteins located in the cytoplasm of APC (endogenous antigens) are processed with the proteasome complex and selected peptides go into the endoplasmic reticulum (ER) through transporters associated with antigen processing (TAP) molecules. Antigenic peptides of 8 to 10 amino acid residues bind to the groove of MHC class I molecules in the ER after which they travel to the cell surface and are presented to $CD8^+$ T cells (reviewed in [7]).

A new type of antigen presentation pathway to induce $CD8^+$ T cells against intravesicular bacteria such as *M. tuberculosis* has been proposed [8, 9]. Intracellular bacteria induce apoptosis in infected macrophages after they are

phagocytosed. The formed apoptotic vesicles are captured by dendritic cells (DC) and induce $CD8^+$ T cells. This cross-priming pathway explains how intravesicular bacteria induce $CD8^+$ T cells.

4. Attenuated Bacteria Vaccination

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is the only approved vaccine to date against tuberculosis (TB) and the most widely distributed attenuated bacterial vaccine [10, 11]. Despite 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 newborn and young children against early manifestations of TB, its efficacy against pulmonary TB in adults is still a subject of debate [12] and was reported to wane with time since vaccination [13]. Moreover, the viable nature of BCG makes it partly unsafe in case of immunocompromised individuals. This highlights the need to develop a more effective, safe, and reliable vaccine against TB [14]. One of weak points of BCG vaccination is that the vaccination cannot induce strong $CD8^+$ T-cell responses. In contrast to *M. tuberculosis*, growth of BCG is not affected in mice lacking β2-microglobulin [15, 16]. The weak $CD8^+$ T-cell responses by BCG vaccination may be caused by weak invasiveness of BCG compared with *M. tuberculosis*. In order to enhance $CD8^+$ T-cell responses by BCG, Kaufmann's group reported recombinant BCG strain harboring listeriolysin O (LLO; LLO) derived from *L. monocytogenes* [17]. LLO is a pore-forming sulfhydryl-activated cytolysin and is essential for the release of *L. monocytogenes* from phagosomal vacuoles into the cytoplasm of host cells. Although recombinanthly^+ BCG strain expressing LLO did not egress into the cytoplasmic compartment of host cells, it improved MHC class I presentation of cophagocytosed ovalbumin as compared with wild-type BCG strain. Further, they developed *urease C*-deficient hly^+ BCG [18]. Urease C deficiency inhibits to increase an intraphagosomal pH and facilitates LLO activity. They found that LLO promotes antigen translocation into the cytoplasm and enhance not only $CD8^+$ T-cell responses via cross-priming mechanisms, but also the apoptosis of infected macrophages. One of other well-studied recombinant BCG is BCG overexpressing antigen 85B (Ag85B) gene (rBCG30) [19]. Ag85B is one of the major secreted proteins in *Mycobacteria* and the over-expressing BCG elicits more effective protective immunity against *M. tuberculosis* challenge.

In addition to recombinant BCG, a variety of live attenuated *M. tuberculosis* strains have been reported. Several strategies are being pursued to develop the attenuated strains, which include auxotrophs (strains that are able to grow only in supplementation of particular nutrients) and mutants that have deletions in virulence genes such as RD1 (region of deletion-1) genes.

Recombinant attenuated *Salmonella* strains have also been examined for vaccines against *M. tuberculosis*. Wang et al. [20] reported that orogastrical immunization of BALB/c mine by a live attenuated *Salmonella* Typhimurium strain harboring the *M. tuberculosis* ESAT6 (early secreted antigenic target 6-kDa protein)-Ag85B fusion gene or

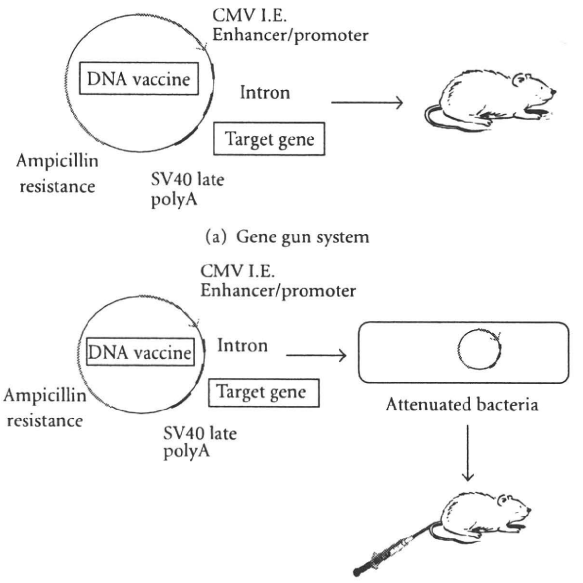

FIGURE 1: DNA vaccination system. (a) Naked DNA vaccination. Eukaryotic expression plasmids that have a strong enhancer/promoter such as cytomegalovirus immediate-early promoter/enhancer are used as DNA vaccines. Naked DNA vaccination carried out by needle injection (intramuscular, subcutaneous), gene gun bombardment, or topical application to skin. (b) Live attenuated bacteria carrier DNA vaccination. Live attenuated intracellular bacteria, such as *Listeria*, *Shigella*, or *Salmonella*, are used as carriers of DNA vaccines. They were given orally or parenterally (needle injection).

combination of this vaccine with BCG vaccine induced strong Ag85B-specific mucosal humoral and cellular immune responses including effector $CD8^+$ T-cell responses and exerted high protective efficacy in mice against *M. tuberculosis* challenge. Cross-priming mechanisms may occur for the $CD8^+$ T-cell responses by these recombinant *Salmonella* vaccines.

5. DNA Vaccination

DNA vaccination is a method by which target antigen genes are directly introduced into host cells. The vaccination strategy is categorized into two groups (Figure 1). One is the so-called naked DNA vaccination. Eukaryotic expression plasmids encoding target antigen genes are used for this strategy. Immunization methods for the naked DNA vaccines are intramuscular injections, gene gun bombardment of DNA-coated gold particles into the epidermis, intradermal DNA immunization [21], and topical application of DNA vaccines [22]. The other is carrier-mediated DNA vaccination. Liposomes, microparticle encapsulation, and attenuated bacteria have been examined as carriers of DNA (reviewed in [23, 24]). Here we briefly review naked DNA vaccination and attenuated bacteria carrier DNA vaccination for the induction of $CD8^+$ T cells specific for intracellular bacterial infections.

5.1. Naked DNA Vaccination. Genetic immunization with naked DNA has been shown to efficiently induce cellular as well as humoral immune responses. The reason why this method produces an efficient induction of immune responses is due to the fact that it involves efficient antigen presentation through DC [25, 26]. It is of particular interest that the amount of DNA required by gene gun DNA immunization is 100 to 1000 times less DNA than that by muscle DNA inoculation to generate equivalent antibody responses [27]. Muscle DNA immunization raises predominant Th1 responses, while gene gun DNA immunization is apt to produce type 2 helper T (Th2) responses [28]. This difference is considered to be mainly due to the difference in (1) the amount of antigen produced from the plasmids and (2) the amount of CpG motif present in plasmid DNA vaccines. In addition, gene gun DNA immunization has brought about highly reproducible and reliable results in antibody production and the induction of specific $CD8^+$ CTL and interferon-γ (IFN-γ) production from immune splenocytes [29].

Codon usage is a problem for the effective induction of specific immune responses by DNA vaccination against pathogenic bacteria. We constructed a plasmid DNA vaccine harboring a wild-type DNA sequence of a dominant CTL epitope of *L. monocytogenes* derived from LLO 91–99 (GYKDGNEYI). We then attempted to immunize mice with the DNA vaccine by intramuscular injection. However, this vaccine could not clearly induce LLO 91–99-specific CTL in BALB/c mice [30]. A reason for the induction failure may be the difference in codon usage between mammalian cells and *L. monocytogenes*. The *L. monocytogenes* genome is highly A+T-rich. In contrast, the mammalian genome is G+C-rich. This difference may affect the efficiency of *L. monocytogenes* gene expression in mammalian cells. To address this difference, we examined a DNA vaccine using the LLO 91–99 gene whose codons were optimized to those of the mammalian cells. The codon-optimized DNA vaccine gave an excellent specific $CD8^+$ CTL induction by intramuscular immunization [30]. We further evaluated the codon optimization effect on CTL induction using the DNA vaccine [31]. In this previously performed study, 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 derived from *L. monocytogenes* (LLO 91–99) 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 [31].

Several studies have been performed on the efficient induction of CTL of a particular specificity. We have demonstrated that the minigene DNA vaccine encoding only a dominant CTL epitope of *L. monocytogenes* (LLO 91–99) was effective for inducing CTL in vivo by gene gun DNA immunization [30]. This result suggests that the DNA vaccine plasmids are directly taken up by APC, which present target peptides to T cells by DNA immunization. Injection of a single CTL epitope minigene DNA generates a single CTL epitope peptide, which is supposed to enter the ER with the help of TAP molecules. However, Cho et al. [32] suggested that cross-priming is a predominant mechanism for inducing $CD8^+$ T cell responses in gene gun DNA immunization. Some CTL epitopes have been modified to have greater immunogenic capacity by substituting several amino acid residues (epitope enhancement) [33].

As one particular approach for the efficient induction of a $CD8^+$ T-cell subset, Wolkers et al. [34] showed that the carboxy-terminal fusion of a CTL epitope to a carrier protein of foreign origin efficiently induced $CD8^+$ CTL. They constructed DNA vaccines by encoding a carboxy-terminal fusion of CTL epitopes (NP 366–374 derived from influenza virus or E7 49–57 derived from human papilloma virus) into green fluorescent protein (GFP) and showed that the DNA vaccines induced a much larger clonal size of antigen-specific $CD8^+$ CTL by intramuscular immunization compared to the clonal size induced by these epitope minigene DNA vaccines. The purpose of the GFP fusion strategy was to provide $CD4^+$ T-cell help by recognizing $CD4^+$ T-cell epitopes in GFP protein. Maecker et al. [35] also showed that CTL induction by both intramuscular and intradermal DNA administration is dependent upon the generation of $CD4^+$ T-cell help via the class II MHC-dependent pathway. Our results showed that CTL minigene DNA vaccination by gene gun DNA immunization induced specific CTL without any $CD4^+$ T-cell help [36]. We speculate that the method of naked DNA immunization (needle injection or gene gun injection) determines requirement for $CD4^+$ T-cell help.

Several studies have attempted to produce multimerized CTL epitope DNA vaccines (polyepitope DNA vaccines). This vaccine was first evaluated by Whitton et al. [37]. They generated a recombinant vaccinia virus system for the expression of CTL-epitope minigenes tandemly fused in a "string-of-beads" manner and showed that this vaccine can induce $CD8^+$ CTL specific for each different epitope and protect vaccinated animals against infections. Subsequently, Thomson et al. [38] constructed a DNA vaccine plasmid containing 10 contiguous minimal CTL epitopes, which were restricted by five MHC alleles derived from five viruses (influenza virus, adenovirus, murine cytomegalovirus, Sendai virus, and lymphocytic choriomeningitis virus), a murine malaria parasite (*Plasmodium berghei*), and a tumor model antigen (ovalbumin). They administered in mice with the plasmid by intramuscular injection or gene gun-mediated intradermal injection and showed that the DNA vaccination successfully induced each epitope-specific CTL activity. Results of our single CTL-epitope DNA vaccine showed that a single dominant CTL epitope is sufficient for the induction of protective immunity [39], suggesting that selecting the most effective CTL epitope for each pathogen is critical for the efficacy of DNA vaccines.

Although some reports have suggested that the flanking sequences of a CTL epitope are important for the precise processing of the CTL epitope in vivo and that some CTL epitopes will interfere with other epitope function (Del Val et al. [40]), a majority of reports have shown that immunization with multimerized CTL epitope DNA without any spacer successfully induces CTL specific for each CTL epitope.

However, some reports (e.g., Velders et al. [41]) suggested the importance of defined flanking sequences around epitopes and the addition of ubiquitin. Ishioka et al. [42] evaluated minigene DNA vaccines encoding multiple HLA-restricted CTL epitopes employing HLA class I-transgenic mice. Such studies are useful as pilot experiments to evaluate DNA vaccines before attempting human studies.

We have used this naked DNA vaccination for identifying immunodominant $CD8^+$ T-cell epitopes of *M. tuberculosis* antigens [43–45]. We have used gene gun DNA immunization because it is highly reproducible and efficiently induces $CD8^+$ T cells [28]. After immunization, immune spleen cells were examined for their responses to overlapping peptides covering full-length proteins by measuring IFN-γ levels by enzyme-linked immunosorbent assay or by counting the numbers of IFN-γ-secreting cells by enzyme-linked immunospot assay. We combined these methods with computer algorithms, such as BIMAS [46] and SYFPEITHI [47], to predict T-cell epitopes. These programs were helpful for reducing 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 $CD8^+$ T-cell epitopes.

5.2. Live Attenuated Bacteria Carrier DNA Vaccination. Live attenuated bacteria, particularly intracellular bacteria, have been examined as carriers of DNA vaccines [48]. Advantages of these vaccination systems include (1) possible mucosal route of immunization, (2) propensity to infect APC, (3) relative ease of genetic manipulation, (4) adjuvant effects of carrier bacteria, (5) possible amplification of DNA vaccine plasmids in vivo, and (6) simplicity of handling and stocking. *Salmonella*, *Listeria*, and *Shigella* have been mainly examined for this purpose.

5.2.1. Shigella as a Carrier of DNA Vaccines. The first reported DNA vaccine-carrying bacterium was *Shigella*. Sizemore et al. [49, 50] showed that the strain *S. flexneri* 2a 15D harboring a plasmid expressing the *lacZ* reporter gene, which is controlled by an in vivo-induced promoter, elicited modest antibody and cellular immune responses against the reporter protein. The *Shigella* strain 15D (a derivative of the wild-type *S. flexneri* 2a strain 2457T) harbors a deletion mutation in the *asd* gene encoding aspartate β-semialdehyde dehydrogenase, an essential enzyme required for synthesizing the bacterial cell wall constituent diaminopimelic acid (DAP). The strain 15D retains invasiveness for mammalian cells but cannot survive in the absence of DAP supplementation in vivo. The use of an invasive yet nonreplicating attenuated vector such as 15D may be suitable for delivering plasmid DNA vaccines to mucosal lymphoid tissues. This study was supported by experiments in mice intranasally immunized with the strain 15D expressing measles virus envelope protein or nucleoprotein (NP) by Fennelly et al. [51]. They showed that mice vaccinated with the strain 15D harboring plasmid vectors encoding different measles virus antigens induced a vigorous antigen-specific response against measles virus. They observed the production of measles virus protein-specific $CD8^+$ T cells and IFN-γ responses, as well as modest production of specific serum antibodies.

5.2.2. Salmonella as a Carrier of DNA Vaccines. An attenuated *Salmonella* strain widely used is the *S.* Typhimurium *aroA* strain [52], which interferes with the biosynthesis of aromatic amino acids. Darji et al. [53] reported that orally administered attenuated *S.* Typhimurium *aroA* carrying plasmids containing the coding sequence of β-galactosidase (β-gal) of *Escherichia coli*, or truncated forms of ActA or LLO of *L. monocytogenes* driven by eukaryotic promoters induce efficient humoral and cellular immune responses. Immunization of *Salmonella* carrying a LLO-encoding expression plasmid elicited protective immunity against a lethal dose of *L. monocytogenes* challenge. As *Salmonella* is reported to induce apoptosis when it enters macrophages, bystander DC may capture the DNA vaccine plasmid through the phagocytosis of *Salmonella*-infected apoptotic cells [54]. In addition to oral *Salmonella* DNA vaccine administration, the nasal route of administration has also been examined. Darji et al. [55] compared the oral and nasal administration of *Salmonella* harboring a eukaryotic expression plasmid encoding β-gal. They showed that both routes could induce systemic T-cell responses but nasal administration was clearly inferior to oral administration. This may be due to the lower number of bacteria that could be applied nasally.

Several investigators have improved the *Salmonella* carrier by introducing genes conferring invasiveness. Introduction of the LLO gene of *L. monocytogenes* into the *S.* Typhimurium Δ*aroA* strain resulted in enhanced plasmid delivery [56].

5.2.3. Listeria as a Carrier of DNA Vaccines. The ability of *L. monocytogenes* to enter the host cytoplasm after phagocytosis and deliver plasmid DNA directly into the cytoplasm makes it an attractive DNA delivery platform for inducing cellular immune responses.

Hense et al. [57] evaluated *Listeria* as a vehicle for gene transfer using a variety of cell lines. They observed gene transfer into host cells after treating cells infected with plasmid-carrying *Listeria* with tetracycline, a bacteriostatic antibiotic. They speculated that the metabolic block by tetracycline treatment makes these bacteria susceptible to cellular defense mechanisms and induces release of plasmid into the host cell cytoplasm. They reported that bacterial properties required for the delivery of eukaryotic expression plasmids were strictly dependent on the ability of the bacteria to both invade eukaryotic cells and egress from the vacuole into the cytoplasm of the infected host cells. Dietrich et al. [58] reported on the DNA vaccination system of an attenuated self-destructing *L. monocytogenes* strain by demonstrating the feasibility of the system in the cell culture system using a deletion mutant of *L. monocytogenes* Δ2 that lacks the entire lecithinase operon including the virulence-associated genes *actA*, *mpl*, and *plcB* [59]. This strain can infect macrophages and replicate in the cytoplasm but cannot spread to adjacent

cells. This attenuated mutant was introduced with a plasmid containing a gene for the lysis protein PLY118 of the listerial bacteriophage A118. PLY118 expression was controlled by the *actA* promoter, which is active when *L. monocytogenes* is in the host cell cytoplasm. Thus, this *L. monocytogenes* mutant escapes from the phagosome and then lyses when the PLY118 gene is expressed in the cytoplasm. Autolysis of the *L. monocytogenes* mutant apparently releases the plasmid DNA into the host cell cytoplasm, allowing the expression of the transgene in the host cells. We applied this system for DNA vaccines against *M. tuberculosis* by constructing self-destructing attenuated *L. monocytogenes* Δ2 strains carrying eukaryotic expression plasmids for the mycobacterial antigen 85 complex (Ag85A and Ag85B) and MPT51 [60]. Intravenous immunization of BALB/c mice by these *Listeria*-carrying DNA vaccines elicited significant protective responses against virulent *M. tuberculosis*.

However, these plasmids are lost from the carrier *Listeria* in vivo [61]. Pilgrim et al. [62] modified the *Listeria* system in order to stabilize the plasmid in the *L. monocytogenes* carrier strain. They constructed an *L. monocytogenes* strain that has the chromosomal deletion region compassing the *trpS* gene (encoding tryptophanyl-tRNA synthetase) and also the *actA* gene. Since the *trpS* gene is essential for bacterial viability, the *trpS*-deleted *Listeria* can maintain itself only in the presence of plasmids carrying the *trpS* gene. They constructed DNA vaccine plasmids containing the *trpS* gene in addition to the listerial autolysis cassette consisting of the lysis gene of phage A118 (ply118) under the control of the *actA* promoter, which is activated only in the cytoplasm of infected mammalian host cells. They reported no plasmid loss for more than 50 generations of *Listeria*. This new *Listeria*-carrying DNA vaccine allows cell-to-cell spread, which was much more efficient in DNA delivery than the nonspreading counterparts like the Δ2 listerial strain.

6. Dendritic Cell Vaccination

DC are the most powerful APC that initiate the primary immune response. They capture pathogens and apoptotic cells at the portal of entry sites in the body and then they migrate to regional lymphoid organs where they present antigens to naive T cells [63]. DC have a distinct ability to prime naive T cells. Therefore, DC-based vaccines have been powerful for tumors and infectious diseases.

DC vaccines have been examined for efficacy as vaccines against infectious diseases as well as cancer. There are several strategies for using DC as vaccines against intracellular bacteria, including ex vivo pulses with bacteria or bacterial antigens or the transfer of genes encoding antigens to DC. McShane et al. [64] showed that for immunization with DC pulsed with $CD4^+$- or $CD8^+$ T-cell epitope peptides in the *M. tuberculosis* antigen 85A (Ag85A), copresentation of both epitope peptides on the same DC was required for protection. Badovinac et al. [65] showed that vaccination with LLO 91–99 peptide-coated DC generated $CD8^+$ T cells with the phenotype and function of memory cells in a short time (4–6 days after immunization) and that the early memory $CD8^+$ T cells underwent vigorous secondary expansion in response to a variety of booster immunizations leading to elevated numbers of effector and memory T cells and enhanced protective immunity against *Listeria* challenge infection.

Retroviral transduction is advantageous for long-term antigen presentation in vivo because the transgene integrates into the chromosome leading to gene expression throughout the life of the cell and its progeny. In our previous study [66], we showed that DC vaccination retrovirally transduced with a minimal CTL epitope derived from *L. monocytogenes* successfully induced the specific CTL and protective immunity against lethal listerial challenge. We also found that the retrovirally transduced DC vaccine was more effective than a CTL epitope peptide-pulsed DC vaccine or a minigene DNA vaccine for eliciting protective immunity. We also evaluated retrovirally transduced DC vaccination with the *M. tuberculosis*-derived Ag85A gene [67]. The results indicated that DC vaccination successfully induced specific cellular immunity, including immune responses of $CD4^+$ T cells and $CD8^+$ CTL, as well as specific antibody responses. In the system, the de novo synthesized Ag85A proteins in the Ag85A gene-transduced DC are processed via the MHC class I pathway to induce specific $CD8^+$ T cells. Specific $CD4^+$ T-cell responses to the proteins may also be evoked through the uptake of the secreted proteins by APC or direct antigen presentation by Ag85A gene-transduced DC.

These results showed that DC vaccination efficiently induces $CD8^+$ CTL, which have the capacity to protect vaccinated animals from pathogenic bacteria. DC vaccination would not be feasible for preventing acute infectious diseases because it is laborious and costly; however, DC vaccination could be a promising strategy against serious chronic infectious diseases.

7. Recombinant Virus Vaccination

Recombinant viral vector systems for gene therapy have been developed and their efficacy has been examined in gene delivery to DC and in direct immunization. Adenoviral vectors have been shown to deliver antigen genes to DC. For example, Wang et al. [68] reported that single intranasal immunization of BALB/c mice with Ag85A recombinant human adenovirus (type 5; Ad5) induced Ag85A-specific $CD8^+$ and $CD4^+$ T-cell responses and also provided protection against intranasal inhalation of *M. tuberculosis*. However, preexisting immunity against viral proteins expressed by the vector prevents effective immunization [69]. Therefore, immunogenicity of the rare adenovirus serotype 35 (Ad35) combined with Ad5 fiber knob (Ad35k5) were examined, and Ad5 fiber knob was found to be important for the immunogenicity in mice and Rhesus monkeys [70].

Retroviral vectors based on murine leukemia virus have been employed to express antigens in vivo. Splenic DC were found to contain injected proviral DNA and were able to efficiently present antigens to T cells [71], but the retroviral vectors only infect dividing cells and do not infect nondividing cells including DC. Therefore, antigen expression and succeeding immune responses would be limiting.

Lentiviral vectors have been shown to efficiently transduce a variety of nondividing cells [72, 73]. In addition, lentiviral vectors pseudotyped with minimal filovirus envelopes have been reported to increase gene transfer in murine lungs [74]. Third-generation self-inactivating lentiviral vectors have been excellent viral vectors because of their advanced safety profile and the presumed absence of preexisting antivector immunity, allowing in vivo adminstraion.

We showed that third-generation lentivirus vectors, which express the *M. tuberculosis* MPT51 antigen, efficiently induced cell-mediated immunity against pulmonary tuberculosis with intratracheal instillation [75]. We also showed that a single intratracheal MPT51 lentivirus administration was effective for inducing antigen-specific $CD8^+$ T-cell responses in the lung. Esslinger et al. [76] showed that lentiviral vector injection into the footpad of mice was capable of transducing regional DC which appeared in the draining lymph nodes and in the spleen. They showed that in vivo administration of lentivector was superior to the transfer of transduced DC or peptide/adjuvant vaccination in terms of both amplitude and longevity of the resultant CTL response. These results confirm the effectiveness of the lentiviral vector system for mucosal T cell-based vaccination.

8. Improvement in Immunization Regimen: Prime-Boost Immunization

Evaluation of vaccination has indicated that the repeated injection of the same vaccine has a limitation in terms of its overall immunological effects. Especially, DNA immunization has been reported to induce considerably strong immunological responses in the rodents, but not in the primates including human [77].

Instead of the repeated injection of the same vaccine, the heterologous prime-boost regimen including DNA vaccination, which is primed with naked DNA vaccination and boosted with recombinant viral vectors such as vaccinia virus and adenovirus, has been shown to evoke superior levels of immunity to DNA vaccine or recombinant virus alone [78]. The relatively low-level but persistent expression of immunogenic proteins in vivo by naked DNA vaccines has been suggested to be important for priming immunological responses and inducing enhanced cellular immunity [78]. Interestingly, Eo et al. [79] reported that mucosal immunological responses were optimal when animals were primed with the recombinant vaccinia virus vector and boosted with a naked DNA vaccine, which is an opposite approach compared to the regimen for systemic immunological responses. Strong immunization by recombinant virus vaccines may be necessary to give enough priming effects in the mucous membrane.

A variety of prime-boost regimens have been examined for *M. tuberculosis* infection [80]. Many investigators examined the regimens in which priming with DNA vaccines and boosting with other immunization strategies. Tanghe et al. [81] immunized C57BL/6 mice first with Ag85A DNA vaccine and then with recombinant Ag85A proteins and showed that this regimen induced stronger specific IFN-γ responses and better protective immunity against *M. tuberculosis* i. v. challenge compared to Ag85A DNA vaccine alone. Similarly, ESAT6 protein boosting immunization increased immune responses by ESAT6 DNA vaccines [82]. McShane et al. [83] showed that priming immunization with ESAT6 and MPT63 DNA vaccines and boosting with modified vaccinia virus Ankàra (MVA) harboring Ag85A gene (MVA85A) induced protective immunity against *M. tuberculosis* infection compared to BCG immunization in mice. Feng et al. [84] showed that priming with Ag85B DNA vaccine and boosting with BCG vaccine strengthened protective immunity against *M. tuberculosis* induced by BCG vaccine alone in mice. Skinner et al. [85] also reported that priming with ESAT6 and Ag85A DNA vaccines and boosting with BCG vaccine enhanced specific IFN-γ production from immune splenocytes compared with that by the DNA vaccine or BCG vaccine alone in mice. Furthermore, Romano et al. [86] showed that immunization of BALB/c mice with Ag85A DNA vaccine first and boosting with BCG vaccine induced stronger protective immunity against *M. tuberculosis* challenge than that by Ag85A DNA vaccine alone. These results demonstrated that DNA vaccine priming and BCG vaccine boosting enhanced immune responses induced by BCG vaccine alone.

The regimens in which BCG vaccine was used as a priming vaccine also have been tried. As the BCG vaccine has been injected to people all over the world, this regimen seems to be reasonable. Priming with BCG vaccine and intranasal boosting with MVA85A in mice enhanced Ag85A-specific $CD4^+$ and $CD8^+$ T-cell responses and strengthened protective immunity against aerosol *M. tuberculosis* challenge infection in mice [87]. This regimen was reported in humans. McShane et al. [88] reported that in volunteers who had been vaccinated 0.5–38 years previously with BCG, vaccination with MVA85A induced substantially higher levels of antigen-specific IFN-γ-secreting T cells and that at 24 weeks after vaccination, these levels were 5–30 times greater than in vaccinees administered a single BCG vaccination. Santosuosso et al. [89] reported that intranasal immunization of mice with recombinant Ag85A-expressing adenovirus after subcutaneous BCG immunization augmented Ag85A-specific $CD4^+$ and $CD8^+$ T-cell responses in the lung and protective immunity against intratracheal *M. tuberculosis* infection.

WHO have showed a list of TB vaccine candidates (TB vaccine pipeline: http://www.stoptb.org/retooling/). These TB vaccine strategies are based on the prime-boost regimens and the vaccine candidates are categorized into three vaccine groups, namely, (1) priming vaccines, (2) boosting vaccines, and (3) therapeutic vaccines after *M. tuberculosis* infection. Reports on the prime-boost regimens by these TB vaccine candidates have been publishing. Tchilian et al. [90] reported that priming with $\Delta ureC\,hly^+$ BCG and boosting with MVA85A induced protective immunity against *M. tuberculosis* infection in mice. The protective effects were much higher in $\Delta ureC\,hly^+$ BCG vaccination than that in parental BCG vaccination. MVA85A boost immunization enhanced Ag85A-specific T-cell responses, but did not affect bacterial numbers in the lung after *M. tuberculosis* aerosol infection.

9. Conclusions

A variety of immunization methods have been evaluated and reported to induce specific $CD8^+$ T cells against intracellular bacterial infections. Modified BCG vaccines have been examined to enhance $CD8^+$ T-cell responses. Naked DNA vaccination is a promising strategy to induce $CD8^+$ T cells. This method has a variety of advantages over conventional attenuated bacterial vaccination, including a relatively easy design and construction using recombinant DNA technology, relatively low cost, high stability, and safety. In addition to the naked DNA vaccination strategy, live attenuated intracellular bacteria such as *Shigella*, *Salmonella*, and *Listeria* have also been utilized as carriers of DNA vaccines in animal models. Furthermore, a strong and immediate induction of specific $CD8^+$ T cells is expected with DC vaccination. DC are the most potent antigen-presenting cells in the body. Immunization of DC pulsed with antigenic peptides or introduced with antigen genes by viral vectors such as retroviruses is a powerful strategy. Recombinant virus vaccination, especially recombinant lentivirus vaccination, has also been utilized to induce specific $CD8^+$ T cells. These strategies and combinations of different strategies (prime-boost immunization) have been examined for the efficient induction of intracellular bacteria-specific $CD8^+$ T cells.

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References

[1] J. Hess, U. Schaible, B. Raupach, and S. H. E. Kaufmann, "Exploiting the immune system: toward new vaccines against intracellular bacteria," *Advances in Immunology*, vol. 75, pp. 1–88, 2000.

[2] S. H. E. Kaufmann, "Immunity to intracellular bacteria," in *Fundamental Immunity*, W. E. Paul, Ed., pp. 1229–1261, Lippincott Williams & Wilkins, Philadelphia, Pa, USA, 5th edition, 2003.

[3] R. C. Bleackley, "Cytotoxic T lymphocytes," in *Fundamental Immunology*, W. E. Paul, Ed., pp. 1079–1095, Lippincott Williams & Wikins, Philadelphia, Pa, USA, 6th edition, 2008.

[4] D. G. Russell, "Where to stay inside the cell: a homesteader's guide to intracellular parasitism," in *Cellular Microbiology*, P. Cossart, P. Boquet, S. Normark, and R. Rappuoli, Eds., pp. 227–253, ASM Press, Washington, DC, USA, 2nd edition, 2005.

[5] P. Cossart and J. Mengaud, "*Listeria monocytogenes*. A model system for the molecular study of intracellular parasitism," *Molecular Biology and Medicine*, vol. 6, pp. 463–474, 1989.

[6] L. Delamarre and I. Mellman, "Cell biology of antigen processing and presentation," in *Fundamental Immunology*, W. E. Paul, Ed., pp. 614–630, Lippincott Williams & Wilkins, Philadelphia, Pa, USA, 6th edition, 2008.

[7] E. Pamer and P. Cresswell, "Mechanisms of MHC class I-restricted antigen processing," *Annual Review of Immunology*, vol. 16, pp. 323–358, 1998.

[8] F. Winau, S. H. E. Kaufmann, and U. E. Schaible, "Apoptosis paves the detour path for CD8 T cell activation against intracellular bacteria," *Cellular Microbiology*, vol. 6, no. 7, pp. 599–607, 2004.

[9] F. Winau, S. Weber, S. Sad, et al., "Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis," *Immunity*, vol. 24, no. 1, pp. 105–117, 2006.

[10] P. Andersen and T. M. Doherty, "The success and failure of BCG—implications for a novel tuberculosis vaccine," *Nature Reviews Microbiology*, vol. 3, no. 8, pp. 656–662, 2005.

[11] B. R. Bloom and P. E. M. Fine, "The BCG experience: implications for future vaccines against tuberculosis," in *Tuberculosis: Pathogenesis, Protection, and Control*, B. R. Bloom, Ed., pp. 531–557, ASM Press, Washington, DC, USA, 1994.

[12] P. E. M. Fine, "Variation in protection by BCG: implications of and for heterologous immunity," *Lancet*, vol. 346, no. 8986, pp. 1339–1345, 1995.

[13] J. A. C. Sterne, L. C. Rodrigues, and I. N. Guedes, "Does the efficacy of BCG decline with time since vaccination?" *International Journal of Tuberculosis and Lung Disease*, vol. 2, no. 3, pp. 200–207, 1998.

[14] S. H. E. Kaufmann, S. Baumann, and A. N. Eddine, "Exploiting immunology and molecular genetics for rational vaccine design against tuberculosis," *International Journal of Tuberculosis and Lung Disease*, vol. 10, no. 10, pp. 1068–1079, 2006.

[15] J. L. Flynn, M. M. Goldstein, K. J. Triebold, B. Koller, and B. R. Bloom, "Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 24, pp. 12013–12017, 1992.

[16] C. H. Ladel, S. Daugelat, and S. H. E. Kaufmann, "Immune response to *Mycobacterium bovis* bacille Calmette—Guérin infection in major histocompatibility complex class I- and II deficient knock-out mice: contribution of CD4 and CD8 T cells to acquired resistance," *European Journal of Immunology*, vol. 25, no. 2, pp. 377–384, 1995.

[17] J. Hess, D. Miko, A. Catic, V. Lehmensiek, D. G. Russell, and S. H. E. Kaufmann, "*Mycobacterium bovis* bacille Calmette—Guérin strains secreting listeriolysin of *Listeria monocytogenes*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 9, pp. 5299–5304, 1998.

[18] L. Grode, P. Seiler, S. Baumann, et al., "Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette—Guérin mutants that secrete listeriolysin," *The Journal of Clinical Investigation*, vol. 115, no. 9, pp. 2472–2479, 2005.

[19] M. A. Horwitz, G. Harth, B. J. Dillon, and S. Masleša-Galić, "Recombinant bacillus Calmette—Guérin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity againts tuberculosis than conventional BCG vaccines in a highly susceptible animal model," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13853–13858, 2000.

[20] Q.-l. Wang, Q. Pan, Y. Ma, et al., "An attenuated Salmonella-vectored vaccine elicits protective immunity against *Mycobacterium tuberculosis*," *Vaccine*, vol. 27, no. 48, pp. 6712–6722, 2009.

[21] E. Raz, D. A. Carson, S. E. Parker, et al., "Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 20, pp. 9519–9523, 1994.

[22] H. Fan, Q. Lin, G. R. Morrissey, and P. A. Khavari, "Immunization via hair follicles by topical application of naked DNA to normal skin," *Nature Biotechnology*, vol. 17, no. 9, pp. 870–872, 1999.

[23] S. Gurunathan, D. M. Klinman, and R. A. Seder, "DNA vaccines: immunology, application, and optimization," *Annual Review of Immunology*, vol. 18, pp. 927–974, 2000.

[24] M. A. Kutzler and D. B. Weiner, "DNA vaccines: ready for prime time?" *Nature Reviews Genetics*, vol. 9, no. 10, pp. 776–788, 2008.

[25] O. Akbari, N. Panjwani, S. Garcia, R. Tascon, D. Lowrie, and B. Stockinger, "DNA vaccination: transfection and activation of dendritic cells as key events for immunity," *Journal of Experimental Medicine*, vol. 189, no. 1, pp. 169–177, 1999.

[26] A. Porgador, K. R. Irvine, A. Iwasaki, B. H. Barber, N. P. Restifo, and R. N. Germain, "Predominant role for directly transfected dendritic cells in antigen presentation to $CD8^+$ T cells after gene gun immunization," *Journal of Experimental Medicine*, vol. 188, no. 6, pp. 1075–1082, 1998.

[27] T. M. Pertmer, T. R. Roberts, and J. R. Haynes, "Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery," *Journal of Virology*, vol. 70, no. 9, pp. 6119–6125, 1996.

[28] A. Yoshida, T. Nagata, M. Uchijima, T. Higashi, and Y. Koide, "Advantage of gene gun-mediated over intramuscular inoculation of plasmid DNA vaccine in reproducible induction of specific immune responses," *Vaccine*, vol. 18, no. 17, pp. 1725–1729, 2000.

[29] D. M. Feltquate, S. Heaney, R. G. Webster, and H. L. Robinson, "Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization," *Journal of Immunology*, vol. 158, no. 5, pp. 2278–2284, 1997.

[30] M. Uchijima, A. Yoshida, T. Nagata, and Y. Koide, "Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium," *Journal of Immunology*, vol. 161, no. 10, pp. 5594–5599, 1998.

[31] T. Nagata, M. Uchijima, A. Yoshida, M. Kawashima, and Y. Koide, "Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms," *Biochemical and Biophysical Research Communications*, vol. 261, no. 2, pp. 445–451, 1999.

[32] J. H. Cho, J. W. Youn, and Y. C. Sung, "Cross-priming as a predominant mechanism for inducing $CD8^+$ T cell responses in gene gun DNA immunization," *Journal of Immunology*, vol. 167, no. 10, pp. 5549–5557, 2001.

[33] J. A. Birzofsky, "Epitope selection and design of synthetic vaccines," *Annals of the New York Academy of Sciences*, vol. 690, pp. 256–264, 1993.

[34] M. C. Wolkers, M. Toebes, M. Okabe, J. B. A. G. Haanen, and T. N. M. Schumacher, "Optimizing the efficacy of epitope-directed DNA vaccination," *Journal of Immunology*, vol. 168, no. 10, pp. 4998–5004, 2002.

[35] H. T. Maecker, D. T. Umetsu, R. H. DeKruyff, and S. Levy, "Cytotoxic T cell responses to DNA vaccination: dependence on antigen presentation via class II MHC," *Journal of Immunology*, vol. 161, no. 12, pp. 6532–6536, 1998.

[36] A. Yoshida, T. Nagata, M. Uchijima, and Y. Koide, "Protective CTL response is induced in the absence of $CD4^+$ T cells and IFN-γ by gene gun DNA vaccination with a minigene encoding a CTL epitope of *Listeria monocytogenes*," *Vaccine*, vol. 19, pp. 4297–4306, 2001.

[37] J. L. Whitton, N. Sheng, M. B. A. Oldstone, and T. A. Mckee, "A "string-of-beads" vaccine, comprising linked minigenes, confers protection from lethal-dose virus challenge," *Journal of Virology*, vol. 67, no. 1, pp. 348–352, 1993.

[38] S. A. Thomson, M. A. Sherritt, J. Medveczky, et al., "Delivery of multiple CD8 cytotoxic T cell epitopes by DNA vaccination," *The Journal of Immunology*, vol. 160, pp. 1717–1723, 1998.

[39] T. Yamada, H. Uchiyama, T. Nagata, et al., "Protective cytotoxic T lymphocyte responses induced by DNA immunization against immunodominant and subdominant epitopes of *Listeria monocytogenes* are noncompetitive," *Infection and Immunity*, vol. 69, no. 5, pp. 3427–3430, 2001.

[40] M. Del Val, H. J. Schlicht, T. Ruppert, M. J. Reddehase, and U. H. Koszinowski, "Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein," *Cell*, vol. 66, no. 6, pp. 1145–1153, 1991.

[41] M. P. Velders, S. Weijzen, G. L. Eiben, et al., "Defined flanking spacers and enhanced proteolysis is essential for eradication of established tumors by an epitope string DNA vaccine," *The Journal of Immunology*, vol. 166, pp. 5366–5373, 2001.

[42] G. Y. Ishioka, J. Fikes, G. Hermanson, et al., "Utilization of MHC class I transgenic mice for development of minigene DNA vaccines encoding multiple HLA-restricted CTL epitopes," *The Journal of Immunology*, vol. 162, pp. 3915–3925, 1999.

[43] T. Aoshi, M. Suzuki, M. Uchijima, T. Nagata, and Y. Koide, "Expression mapping using a retroviral vector for $CD8^+$ T cell epitopes: definition of a *Mycobacterium tuberculosis* peptide presented by H2-D^d," *Journal of Immunological Methods*, vol. 298, no. 1-2, pp. 21–34, 2005.

[44] T. Aoshi, T. Nagata, M. Suzuki, et al., "Identification of an HLA-A*0201-restricted T-cell epitope on the MPT51 protein, a major secreted protein derived from *Mycobacterium tuberculosis*, by MPT51 overlapping peptide screening," *Infection and Immunity*, vol. 76, no. 4, pp. 1565–1571, 2008.

[45] M. Suzuki, T. Aoshi, T. Nagata, and Y. Koide, "Identification of murine H2-D^d- and H2-A^b-restricted T-cell epitopes on a novel protective antigen, MPT51, of *Mycobacterium tuberculosis*," *Infection and Immunity*, vol. 72, no. 7, pp. 3829–3837, 2004.

[46] K. C. Parker, M. A. Bednarek, and J. E. Coligan, "Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains," *Journal of Immunology*, vol. 152, no. 1, pp. 163–175, 1994.

[47] H.-G. Rammensee, J. Bachmann, N. P. N. Emmerich, O. A. Bachor, and S. Stevanović, "SYFPEITHI: database for MHC ligands and peptide motifs," *Immunogenetics*, vol. 50, no. 3-4, pp. 213–219, 1999.

[48] G. Dietrich, I. Gentschev, J. Hess, J. B. Ulmer, S. H. E. Kaufmann, and W. Goebel, "Delivery of DNA vaccines by attenuated intracellular bacteria," *Immunology Today*, vol. 20, no. 6, pp. 251–253, 1999.

[49] D. R. Sizemore, A. A. Branstrom, and J. C. Sadoff, "Attenuated *Shigella* as a DNA delivery vehicle for DNA-mediated immunization," *Science*, vol. 270, pp. 299–302, 1995.

[50] D. R. Sizemore, A. A. Branstrom, and J. C. Sadoff, "Attenuated bacteria as a DNA delivery vehicle for DNA-mediated immunization," *Vaccine*, vol. 15, no. 8, pp. 804–807, 1997.

[51] G. J. Fennelly, S. A. Khan, M. A. Abadi, T. Fabian Wild, and B. R. Bloom, "Mucosal DNA vaccine immunization against measles with a highly attenuated *Shigella flexneri* vector," *Journal of Immunology*, vol. 162, no. 3, pp. 1603–1610, 1999.

[52] S. K. Hoiseth and B. A. D. Stocker, "Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines," *Nature*, vol. 291, no. 5812, pp. 238–239, 1981.

[53] A. Darji, C. A. Guzmán, B. Gerstel, et al., "Oral somatic transgene vaccination using attenuated S. typhimurium," *Cell*, vol. 91, no. 6, pp. 765–775, 1997.

[54] M. L. Albert, B. Sauter, and N. Bhardwaj, "Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLS," *Nature*, vol. 392, no. 6671, pp. 86–89, 1998.

[55] A. Darji, S. zur Lage, A. I. Garbe, T. Chakraborty, and S. Weiss, "Oral delivery of DNA vaccines using attenuated *Salmonella typhimurium* as carrier," *FEMS Immunology and Medical Mirobiology*, vol. 27, pp. 341–349, 2000.

[56] C. Grillot-Courvalin, S. Goussard, and P. Courvalin, "Wild-type intracellular bacteria deliver DNA into mammalian cells," *Cellular Microbiology*, vol. 4, no. 3, pp. 177–186, 2002.

[57] M. Hense, E. Domann, S. Krusch, et al., "Eukaryotic expression plasmid transfer from the intracellular bacterium *Listeria monocytogenes* to host cells," *Cellular Microbiology*, vol. 3, no. 9, pp. 599–609, 2001.

[58] G. Dietrich, A. Bubert, I. Gentischev, et al., "Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide *Listeria monocytogenes*," *Nature Biotechnology*, vol. 16, pp. 181–185, 1998.

[59] N. Hauf, W. Goebel, F. Fiedler, Z. Sokolovic, and M. Kuhn, "*Listeria monocytogenes* infectoin of $P388D_1$ macrophages results in a biphasic NF-κB (RelA/p50) activation induced by lipoteichoic acid and bacterial phospholipases and mediated by IκBα and IκBβ degradation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 17, pp. 9394–9399, 1997.

[60] K. Miki, T. Nagata, T. Tanaka, et al., "Induction of protective cellular immunity against *Mycobacterium tuberculosis* by recombinant attenuated self-destructing *Listeria monocytogenes* strains harboring eukaryotic expression plasmids for antigen 85 complex and MPB/MPT51," *Infection and Immunity*, vol. 72, no. 4, pp. 2014–2021, 2004.

[61] S. Spreng, G. Dietrich, S. Niewiesk, V. ter Meulen, I. Gentschev, and W. Goebel, "Novel bacterial systems for the delivery of recombinant protein of DNA," *FEMS Immunology and Medical Microbiology*, vol. 27, pp. 299–304, 2000.

[62] S. Pilgrim, J. Stritzker, C. Schoen, et al., "Bactofection of mammalian cells by *Listeria monocytogenes*: improvement and mechanism of DNA delivery," *Gene Therapy*, vol. 10, no. 24, pp. 2036–2045, 2003.

[63] J. Banchereau and R. M. Steinman, "Dendritic cells and the control of immunity," *Nature*, vol. 392, pp. 245–252, 1998.

[64] H. McShane, S. Behboudi, N. Goonetilleke, R. Brookes, and A. V. S. Hill, "Protective immunity against *Mycobacterium tuberculosis* induced by dendritic cells pulsed with both $CD8^+$- and $CD4^+$-T-cell epitopes from antigen 85A," *Infection and Immunity*, vol. 70, no. 3, pp. 1623–1626, 2002.

[65] V. P. Badovinac, K. A. N. Messingham, A. Jabbari, J. S. Haring, and J. T. Harty, "Accelerated $CD8^+$ T-cell memory and prime-boost response after dendritic-cell vaccination," *Nature Medicine*, vol. 11, no. 7, pp. 748–756, 2005.

[66] Y. Nakamura, T. Suda, T. Nagata, et al., "Induction of protective immunity to Listeria monocytogenes with dendritic cells retrovirally transduced with a cytotoxic T lymphocyte epitope minigene," *Infection and Immunity*, vol. 71, no. 4, pp. 1748–1754, 2003.

[67] H. Nakano, T. Nagata, T. Suda, et al., "Immunization with dendritic cells retrovirally transduced with mycobacterial antigen 85A gene elicits the specific cellular immunity including cytotoxic T-lymphocyte activity specific to an epitope on antigen 85A," *Vaccine*, vol. 24, no. 12, pp. 2110–2119, 2006.

[68] J. Wang, L. Thorson, R. W. Stokes, et al., "Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis," *Journal of Immunology*, vol. 173, no. 10, pp. 6357–6365, 2004.

[69] P. Brossart, A. W. Goldrath, E. A. Butz, S. Martin, and M. J. Bevan, "Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL," *Journal of Immunology*, vol. 158, no. 7, pp. 3270–3276, 1997.

[70] A. Nanda, D. M. Lynch, J. Goudsmit, et al., "Immunogenicity of recombinant fiber-chimeric adenovirus serotype 35 vector-based vaccines in mice and Rhesus monkeys," *Journal of Virology*, vol. 79, no. 22, pp. 14161–14168, 2005.

[71] E. S. Song, V. Lee, C. D. Surh, et al., "Antigen presentation in retroviral vector-mediated gene transfer in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 5, pp. 1943–1948, 1997.

[72] L. Naldini, U. Blömer, P. Gallay, et al., "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector," *Science*, vol. 272, no. 5259, pp. 263–267, 1996.

[73] L. Yang, H. Yang, K. Rideout, et al., "Engineered lentivector targeting of dendritic cells for in vivo immunization," *Nature Biotechnology*, vol. 26, no. 3, pp. 326–334, 2008.

[74] M. F. Medina, G. P. Kobinger, J. Rux, et al., "Lentifiral vectors pseudotyped with minimal filovirus envelopes increased gene transfer in murine lung," *Molecular Therapy*, vol. 8, no. 5, pp. 777–789, 2003.

[75] D. Hashimoto, T. Nagata, M. Uchijima, et al., "Intratracheal administration of third-generation lentivirus vector encoding MPT51 from *Mycobacterium tuberculosis* induces specific $CD8^+$ T-cell responses in the lung," *Vaccine*, vol. 26, no. 40, pp. 5095–5100, 2008.

[76] C. Esslinger, L. Chapatte, D. Finke, I. Miconnet, P. Guillaume, and H. R. MacDonald, "In vivo administration of a lentiviral vaccine targets DCs and induces efficient $CD8^+$ T cell responses," *Journal of Clinical Investigation*, vol. 111, no. 11, pp. 1673–1681, 2003.

[77] S. Li, M. Rodrigues, D. Rodriguez, et al., "Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces $CD8^+$ T-cell-mediated protective immunity against malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 11, pp. 5214–5218, 1993.

[78] I. A. Ramshaw and A. J. Ramsay, "The prime-boost strategy: exciting prospects for improved vaccination," *Immunology Today*, vol. 21, no. 4, pp. 163–165, 2000.

[79] S. K. Eo, M. Gierynska, A. A. Kamar, and B. T. Rouse, "Prime-boost immunization with DNA vaccine: mucosal route of administration changes the rules," *Journal of Immunology*, vol. 166, no. 9, pp. 5473–5479, 2001.

[80] H. McShane and A. Hill, "Prime-boost immunisation strategies for tuberculosis," *Microbes and Infection*, vol. 7, no. 5-6, pp. 962–967, 2005.

[81] A. Tanghe, S. D'Souza, V. Rosseels, et al., "Improved immunogenicity and protective efficacy of a tuberculosis DNA vaccine encoding Ag85by protein boosting," *Infection and Immunity*, vol. 69, no. 5, pp. 3041–3047, 2001.

[82] Q.-M. Wang, S.-H. Sun, Z.-L. Hu, M. Yin, C.-J. Xiao, and J.-C. Zhang, "Improved immunogenicity of a tuberculosis DNA vaccine encoding ESAT6 by DNA priming and protein boosting," *Vaccine*, vol. 22, no. 27-28, pp. 3622–3627, 2004.

[83] H. McShane, R. Brookes, S. C. Gilbert, and A. V. S. Hill, "Enhanced immunogenicity of $CD4^+$ T-cell responses and protective efficacy of a DNA-modified vaccinia virus Ankara prime-boost vaccination regimen for murine tuberculosis," *Infection and Immunity*, vol. 69, no. 2, pp. 681–686, 2001.

[84] C. G. Feng, U. Palendira, C. Demangel, J. M. Spratt, A. S. Malin, and W. J. Britton, "Priming by DNA immunization augments protective efficacy of *Mycobacterium bovis* bacille Calmette-Guérin against tuberculosis," *Infection and Immunity*, vol. 69, no. 6, pp. 4174–4176, 2001.

[85] M. A. Skinner, A. J. Ramsay, G. S. Buchan, et al., "A DNA prime-live vaccine boost strategy in mice can augment IFN-γ responses to mycobacterial antigens but does not increase the protective efficacy of two attenuated strains of *Mycobacterium bovis* against bovine tuberculosis," *Immunology*, vol. 108, no. 4, pp. 548–555, 2003.

[86] M. Romano, S. D'Souza, P.-Y. Adnet, et al., "Priming but not boosting with plasmid DNA encoding mycolyl-transferase Ag85A from *Mycobacterium tuberculosis* increases the survival time of *Mycobacterium bovis* BCG vaccinated mice against low dose intravenous challenge with *M. tuberculosis* H37Rv," *Vaccine*, vol. 24, no. 16, pp. 3353–3364, 2006.

[87] N. P. Goonetilleke, H. McShane, C. M. Hannan, R. J. Anderson, R. H. Brookes, and A. V. S. Hill, "Enhanced immunogenicity and protective efficacy against *Mycobacterium tuberculosis* of bacille Calmette-Guérin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara," *Journal of Immunology*, vol. 171, no. 3, pp. 1602–1609, 2003.

[88] H. McShane, A. A. Pathan, C. R. Sander, et al., "Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans," *Nature Medicine*, vol. 10, no. 11, pp. 1240–1244, 2004.

[89] M. Santosuosso, S. McCormick, X. Zhang, A. Zganiacz, and Z. Xing, "Intranasal boosting with an adenovirus-vectored vaccine markedly enhances protection by parenteral *Mycobacterium bovis* BCG immunization against pulmonary tuberculosis," *Infection and Immunity*, vol. 74, no. 8, pp. 4634–4643, 2006.

[90] E. Z. Tchilian, C. Desel, E. K. Forbes, et al., "Immunogenicity and protective efficacy of prime-boost regimens with recombinant Δ*ureC hly*$^+$ *Mycobacterium bovis* BCG and modified vaccinia virus Ankara expressing *M. tuberculosis* antigen 85A against murine tuberculosis," *Infection and Immunity*, vol. 77, no. 2, pp. 622–631, 2009.

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Identification of murine T-cell epitopes on low-molecular-mass secretory proteins (CFP11, CFP17, and TB18.5) of *Mycobacterium tuberculosis*

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ABSTRACT

The low-molecular-mass secretory proteins of *Mycobacterium tuberculosis* have been shown to be major T-cell antigens during infection with the pathogenic bacterium. In this study, we determined murine T-cell epitopes on three low-molecular-mass proteins, CFP11 (Rv2433c), CFP17 (Rv1827), and TB18.5 (Rv0164) using DNA immunization of inbred mice. We analyzed interferon-γ production from immune splenocytes in response to overlapping peptides covering these proteins. We identified two CD8+ T-cell epitopes on CFP11 and CFP17, one in BALB/c mice and the other in C57BL/6 mice, respectively. On TB18.5, we identified a CD8+ T-cell epitope in BALB/c mice and a CD4+ T-cell epitope in C57BL/6 mice. With the aid of computer algorithms, we could identify the minimal CD8+ T-cell epitopes. These T-cell epitopes are feasible for analysis of the role of antigen-specific T cells during *M. tuberculosis* infection.

1. Introduction

According to the latest tuberculosis (TB) global burden estimates, there were 9.27 million incident cases of TB in 2007 with approximately one third of the total world population being infected [1]. To date, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only approved vaccine against TB [2,3]. Despite the fact that BCG is among the most widely used vaccines throughout the world, TB still poses a serious threat to global health. Whereas BCG is believed to protect newborn and young children against early manifestations of TB, its efficacy against pulmonary TB in adults is still a subject of debate [4], and has been reported to wane with time since vaccination [5]. Variable levels of 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 cases of immunocompromised individuals. This highlights a need to develop a more effective, safe and reliable vaccine against TB [6].

A 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+ T cells are important mediators of host defense against TB. In addition, MHC class I-restricted CD8+ T cells have also been reported to be required for the optimum control of mycobacterial infection [9,10].

An essential step towards the development of a new vaccine against TB is gaining more information on the antigenic architecture of *Mycobacterium tuberculosis* to identify T-cell epitopes responsible for eliciting protective immune responses [6,11]. Determination of the complete genome sequence of *M. tuberculosis* facilitated this step considerably [12]. The observation that only immunization with live *M. tuberculosis* can induce effective protective T-cell responses [13] has drawn great attention to the study of proteins that are actively secreted into the culture medium by the replicating organism due to their potential immunogenic role.

M. tuberculosis culture fluid proteins (CFPs) have been shown to be of particular relevance as protective T-cell antigens [14–16]. Especially, relatively low-molecular-mass polypeptides (less than 20 kDa) in CFPs have been reported to be major antigens that evoke T-cell responses [17,18]. ESAT-6 and CFP10 proteins are widely used these days in whole blood interferon (IFN)-γ release assays for TB diagnosis [19]. CFP17 was purified using two-dimensional electrophoresis and identified as a T-cell antigen using the mouse system reported by Weldingh and co-workers [20,21]. In their study, CFP17 was demonstrated as one of the most potent inducers of IFN-γ release among the investigated CFPs in *M. tuberculosis*-immune mice. TB18.5 was reported in a study by Lim et al. [22], using peripheral blood mononuclear cells from healthy tuberculin reactors, to be a T-cell-stimulating antigen in humans. In that study, CFP17 and TB18.5 were described as MTSP14 and MTSP17, respectively. CFP11, CFP17, and TB18.5 were identified as human immunodominant T-cell antigens in a study by Sable et al. [23]

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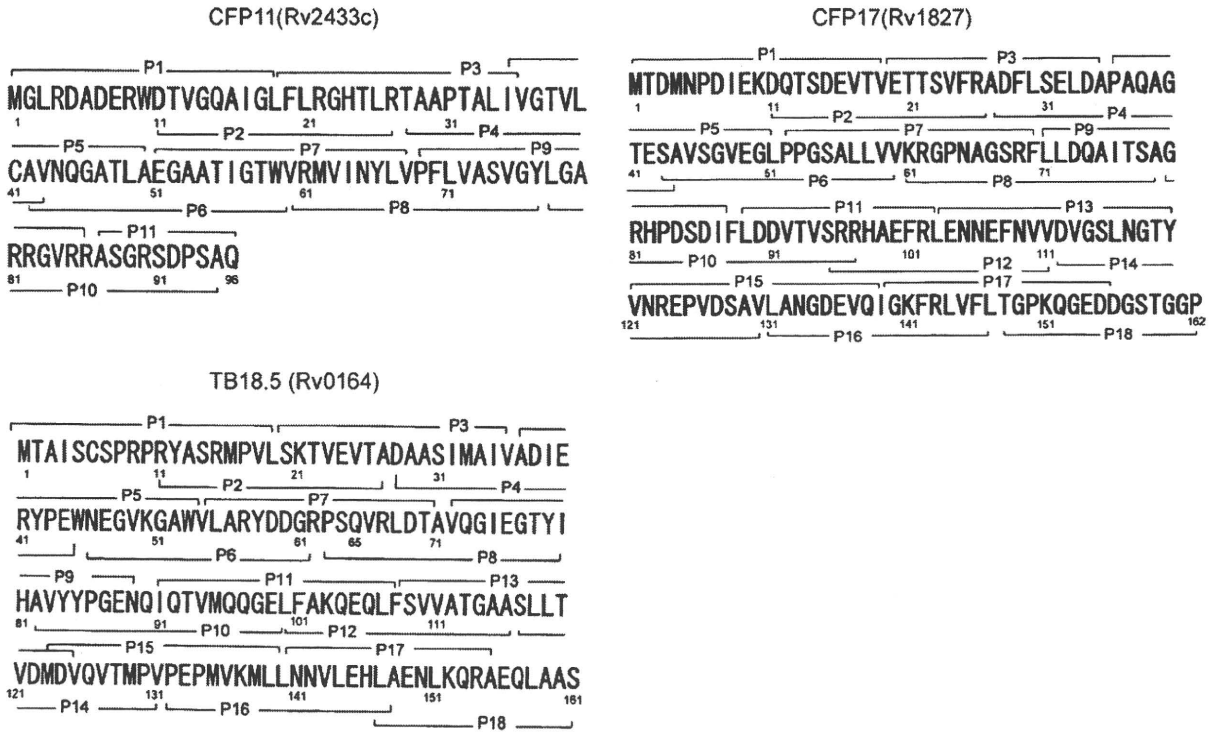

Fig. 1. Schematic representation of overlapping synthetic peptides of CFP11, CFP17, and TB18.5 proteins of *M. tuberculosis*. All peptides covering entire CFP11 (Rv2433c), CFP17 (Rv1827), and TB18.5 (Rv0164) proteins of *M. tuberculosis* were synthesized as approximately 20-mer molecules overlapping by approximately 10 amino acids.

using human peripheral blood and pleural fluid mononuclear cells. In that study, CFP11, which was described as one of the newly identified T-cell antigens, was reported to induce marked increase in serum IgG levels as well as IFN-γ production and lymphocyte proliferation. In the same study, TB18.5 was in the top 10 out of 104 polypeptides that induced prominent T-cell responses based on IFN-γ production and lymphocyte proliferation. These proteins were purified from the culture filtrate of *M. tuberculosis*, but they were also found in the cell wall fraction following two-dimensional electrophoresis analysis [24]. The structure of CFP11 has been elucidated by de novo methods [25].

DNA immunization with gene gun bombardment is a reliable method to induce reproducible T-cell responses [26], and has been used for the identification of T-cell epitopes of *M. tuberculosis* antigens including antigen (Ag) 85 family proteins (Ag85A, Ag85B, and Ag85C) [27,28] and MPT51 [29,30]. Here, we identified murine T-cell epitopes on three low-molecular-mass secretory proteins, CFP11 (Rv2433c), CFP17 (Rv1827), and TB18.5 (Rv0164) using gene gun immunization of inbred mice with plasmid DNA, restimulation with overlapping synthetic peptides spanning the entire amino acid (aa) sequences, and MHC binding peptide prediction algorithms for the prediction of minimal T-cell epitopes.

2. Materials and methods

2.1. Animals

BALB/c and C57BL/6 mice (Japan SLC; Hamamatsu, Japan) were maintained in the Animal Facility at Hamamatsu University School of Medicine. Mice between 2 and 4 months of age were used for immunization. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Plasmid construction

DNAs encoding CFP11, CFP17, and TB18.5 were amplified from *M. tuberculosis* H37Rv genome by PCR. Primers used for PCR are as follows: 5′-CGAGAATTCACCATGGGCCTGCGCGACG-3′ and 5′-ATAGTTTAGCGGCCGCACCGTCATTGTGCTGATGG-3′ for CFP11, 5′-CGACTCGAGCACCATGACGGACATGAACCCGG-3′ and 5′-ATAGTTTAGCGGCCGCTCACGGGCCCCC-3′ for CFP17, and 5′-CGAGAATTCACCATGACGGCAATCTCGTGC-3′ and 5′-ATAGTTTAGCGGCCGCTTAGCTGGCCGCGAC-3′ for TB18.5 (the underlined portions indicate restriction enzyme recognition sequences.) The PCR fragments were inserted between EcoRI and Not I sites for CFP11 and TB18.5, and between Xho I and Not I for CFP17, located downstream of cytomegalovirus immediate-early enhancer/promoter region of eukaryotic expression plasmid, pCI (Promega, Madison, WI, USA), resulting in pCI-CFP11, pCI-CFP17, and pCI-TB18.5, respectively. The integrity of the nucleotide sequence was validated by automated DNA sequencing with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) using a Dye Primer Cycle Sequencing kit (Applied Biosystems). The aa sequences of CFP11, CFP17, and TB18.5 are identical in *M. tuberculosis* and *M. bovis* (Fig. 1). CFP11 aa sequences are NP_216949 (NCBI reference number) for *M. tuberculosis*, NP_856106 (NCBI) for *M. bovis*; CFP17 aa sequences are CAB01474 (GenBank reference number) for *M. tuberculosis*, CAD94561 (GenBank) for *M. bovis*; and TB18.5 aa sequences are YP_177617 (NCBI) for *M. tuberculosis*, CAD93033 (GenBank) for *M. bovis*.

2.3. Immunization

Helios gene gun system (Bio-Rad Laboratories, Hercules, CA, USA) was used for DNA immunization. Preparation of a DNA-coated gold particle cartridge was performed according to the manufacturer's instructions. Finally, 0.5 mg of gold particles was coated with