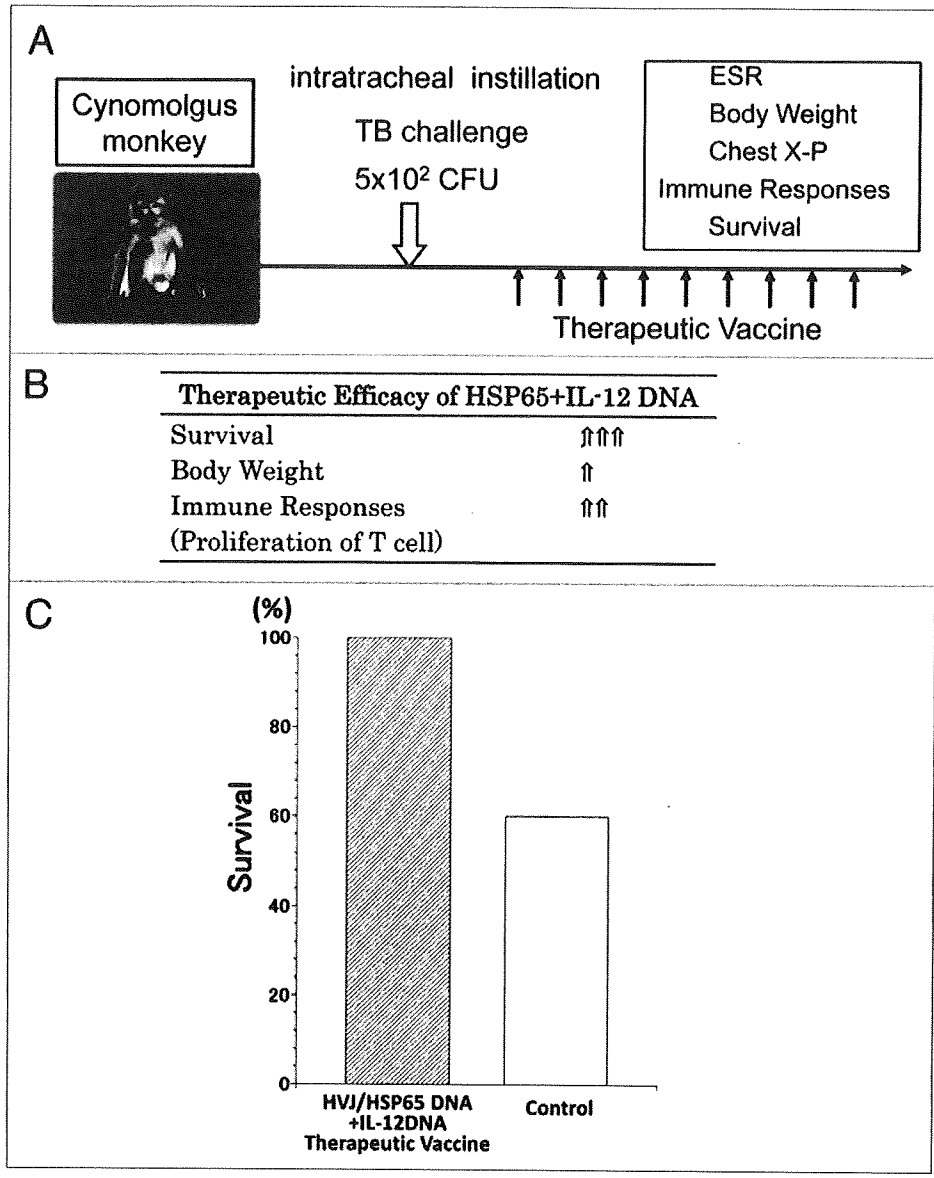

Figure 4. (A) Therapeutic effect of HVJ-Envelope/HSP65DNA + IL-12DNA vaccine on TB-infected cynomolgus monkeys. (B) Therapeutic efficacy of HSP65 + IL-12DNA. (C) Therapeutic Efficacy (survival) of HSP65 + IL-12 DNA vaccine 130 days after TB infection.

CTL and Granulysin Vaccine

The increasing threat posed by drug-resistant strain of *M. tuberculosis* is to a reappraisal of the possibility of treating TB by immunotherapy (**Fig. 2**). Rook et al. analyze 6 strategies that have been shown to be therapeutic in animal models of TB and identify a common pathway underlying the activity of the superficially different immunotherapeutic protocols.[29] 6 strategies are (1) DNA vaccine encoding hsp65 from *M. leprae*, (2) heat-killed *M. vaccae*, (3) fragmented, lipid-depleted *M. tuberculosis* delivered in liposomes (RUTI), (4) HE2000, (5) inhibition of IL-4, (6) inhibition of TGFβ. This pathway involves enhanced induction of $CD8^+$ CTLs and downregulation of interleukin-4 and pathways. This unifying analysis strengthens the rationale for future trials of immunotherapy in humans and points to surrogate markers that could be studied in such trials.[29]

There is increasing evidence for the importance of cytotoxic cells.[72] Their role in immunity to TB in mice has been revealed by knocking out genes involved in presentation via major histocompatibility complex (MHC) class I (e.g., transporter associated with antigen processing-1, CD8, β2m and MHC class I heavy chain), by cell-transfer experiments, and by depletion of $CD8^+$ CTLs with antibodies.[73] $CD8^+$ CTLs play a major role in the control of the latent TB.[74] N-acetyl muramyl dipeptide (MDP: N-acetylmuramyl-L-alanyl-D-isoglutamine mycobacterial peptidoglycan(PGN)) presented adjuvant activity, augmented the CTL differentiation against TB as well as cancer cells, and activated innate immunity via the host sensor NOD2.[75,76]

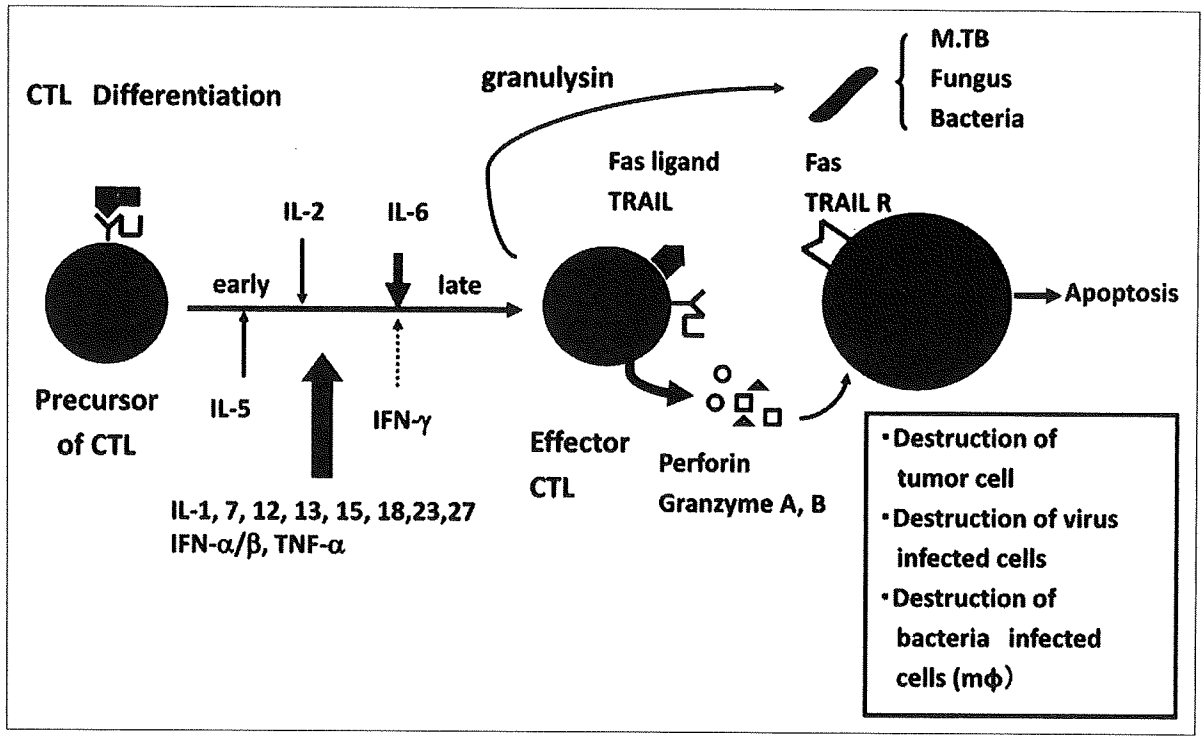

Figure 5. Induction of Cytotoxic T Cells and Killing Mechanism.

Table 3. Induction of decrease in TB number in vivo and CTL differentiation by 15K granulysin and 9K granulysin

Function / Two Kinds of Granulysin	Decrease in TB number	Induction of CTL against TB	Proliferation of T cells against TB	IFNγ production	Granulysin expression in CD8⁺T: Patients with MDR-TB	Granulysin expression in CD8⁺T: Patients with Drag-sensitive TB
15K Granulysin	++ (strong augmentation)	++	++	++	⇓⇓	⇓
9K Granulysin	++	+ (augmentation)	+	++	N.D	N.D

(++ ; strong augmentation, + ; augmentation); (⇓⇓ ; strong suppression, ⇓ ; suppression).

CD8⁺ cells and other CTLs might be even more important in humans, in whom they have additional effector modalities not present in the mouse, such as granulysin, which can kill *M. tuberculosis*,[77] and lymphocyte subsets that recognize antigens presented by HLA-E or by group1 CD1 molecules (CD1a, CD1b, etc.,) in addition to MHC class 1.[73,78-81]

It has been suggested that the granulysin has the function of in vitro cytotoxic activity against *M. tuberculosis* outside the macrophage cells, and contributes the in vitro reduction of *M. tuberculosis* in the macrophage in the presence of perforin.[71] However, the precise role of granulysin in the in vivo defense for the tuberculosis infection has not been elucidated yet.

CTL play an important role for the protection against TB[1,2] (Fig. 5). The granulysin protein expression as well as IL-6, IFNγ

Table 4. Therapeutic efficacy against tuberculosis by 15K granulysin transgenic mice and 9K granulysin transgenic mice

Tg mouse	CFU of TB (log) (*p < 0.05)
15K Granulysin Tg mouse	5.3 ± 0.1*
wild type C57BL/6 mouse	5.9 ± 0.2
9K Granulysin Tg mouse	5.8 ± 0.4*
wild type C57BL/6 mouse	6.7 ± 0.2
Secreted 9K Granulysin Tg mouse	5.7 ± 0.6*
wild type C57BL/6 mouse	6.7 ± 0.2

(CFU, Colony Forming Unit); (*significant ($p < 0.05$) by Student's Test).

Table 5. Preclinical and/or phase I, II clinical trial vaccines for tuberculosis

	Vaccine Type
(A) Priming, Pre-Exposure	
(1) Phase I: 2008	
(a) **rBCG30**	**recombinant 85B BCG**
(b) **rBCG30ΔureC:Hly (VPM1002)**	**recombinant listeriolysin BCG**
(c) AERAS-407	recombinant perfringiolysin
(d) rBCG30ARMF, rBCG Mtb B30, rBCG h IFNγ	recombinant 85B BCG
(e) Nas L3/Htk BCG	nasal vaccine/heat killed whole BCG
(f) $mc^{2}6220$, $mc^{2}6221$, $mc^{2}6222$, $mc^{2}6231$	non-replicating, *M. Tuberculosis* strain (Δlys A Δpan CD)
(g) $mc^{2}5059$	replicating pro-apoptotic *M. bovis* BCG (ΔnuoG)
(2) Phase I 2009 or Later	
(a) HBHA (heparin-binding haemagglutinin)	methylated 21-KDA protein
(b) Attenuated Live Vaccine based on Phop	attenuated TB (virulence gene phop inactivation)
(c) paBCG (pro-apoptotic BCG)	decrease in anti-apoptotic enzyme activity

and IL-2 activities in the culture supernatants of PBL from patients with MDR-TB and patients with Diabetes Mellitus (DM)-TB were evaluated since IL-6, IFNγ and IL-2 act as cytotoxic T cell differentiation factor.[59,82] All these activities were very low in MDR-TB and DM-TB in comparison with healthy volunteers.

Two major protein products, 15 kDa (15K) granulysin and 9 kDa (9K) granulysin, are detected in CTL and NK cells. Granulysin exhibits potent cytotoxic activity against a broad panel of microbial targets, including bacteria, fungi and parasites. Granulysin is present in human $CD8^{+}$ (and some $CD4^{+}$) CTLs, NK cells, NK T Cells and γ/δ T cells. It is a member of the saposin-like protein family, colocalizes with perforin and granzymes in the cytolytic granules of human CTL and NK cells[82-85] (**Table 3**).

We found that 15K granulysin was secreted from CD8 positive CTL, and 15K granulysin could enter human macrophages and killed *M. tuberculosis* in the cytoplasm of macrophages.[82]

Expression of 15K granulysin protein and mRNA in CD8 positive T cells in the patients with drug sensitive TB were lower than that in the healthy volunteers.[86] Expression of 15K granulysin protein in CD8 positive T cells in the patients with multi-drug resistant tuberculosis (MDR-TB) was significantly lower than that in the patients with drug-sensitive TB (**Table 3**). 15K granulysin production stimulated with PHA-P, ConA, alloantigens and PPD antigens was suppressed significantly in the supernatants of PBL from MDR-TB patients.[82] Furthermore, we established 15K granulysin transgenic mice and 9K granulysin transgenic mice (**Table 4**). It was demonstrated first that 15K granulysin transgenic mice as well as 9K granulysin transgenic mice exerted in vivo anti-TB effect, decrease in the number of TB and in vivo induction of cytotoxic T cells against TB, proliferation of T cells against TB, and augmentation of cytokine production. As shown in **Table 4**, in vivo anti-TB effect of 15K granulysin transgenic mouse was observed. CFUs of *M. tuberculosis* in the lungs, 4 weeks after TB injection, were decreased in 15K granulysin transgenic mice, compared to wild type mice. Furthermore, CFUs of TB in the lungs of 9K granulysin transgenic mice, were also decreased compared to wild type mice (**Table 4**). These findings demonstrate for the first time an in vivo effect of 15K granulysin and 9K granulysin against TB infection. Thus, granulysin vaccine therapy might provide a weapon against MDR-TB and XDR-TB (extremely drug resistant TB).[78]

Anti-TNF therapy reduced the expression in lymphocytes of perforin and granulysin, 2 components of the T cell-mediated antimicrobial response to intercellular pathogens. Specifically, *M. tuberculosis*-reactive $CD8^{+}CCR7\text{-}CD45RA^{+}$ effector memory T cell (T_{EMRA} cells) expressed the highest levels of granulysin, lysed *M. tuberculosis*, and infected macrophages and mediated an antimicrobial activity against intracellular *M. tuberculosis*. Furthermore, T_{EMRA} cells expressed cell surface TNF and bound the anti-TNF therapeutic infliximad in vitro, making them susceptible to complement-mediated lysis. Immune therapy with anti-TNF was associated with reduced numbers of $CD8^{+}T_{EMRA}$ cells and decreased anrimicrobial activity against *M. tuberculosis*.[53,87]

Long-term memory T cell immunity for tuberculosis vaccines. Standard BCG vaccinations (intradermal) protect infants from severe TB meningitis and milliary TB, but provide highly variable protection against pulmonary TB later in life (adult). Therefore, an important issue for new TB vaccination strategies attempting to do better than BCG is the ability to induce long-term memory immunity that is protective against primary-disease progression and reactivation of latent TB. Memory T cells express CD44, CD45RO and IL-7 receptor etc. Two subpopulations (central memory T cells and effector memory T cells) of memory T cells are identified.[88,89] Some cytokine, such as IL-7 and IL-15 enhance memory T cell responses and their induction by vaccines or their exogenous addition to vaccines might be useful in optimizing long-term memory responses.[90]

HSP65 DNA + IL-12 vaccine showed significant prophylactic efficacy on TB infection even when the interval between BCG priming and HSP65 + IL-12 DNA vaccine booster was very long in a monkey model, suggesting that this vaccine may augment the memory T cell differentiation and survival against TB.

Other Vaccines

Recombinant BCG vaccine. Recombinant BCG strains overexpressing specific mycobacterial antigens or engineered to escape from the phagosome, live, attenuated vectors expressing

Table 6. Preclinical and/or phase I, II clinical trial vaccines for tuberculosis

	Vaccine Type
(B) BOOSTING, PRE-Exposure	
(1) Phase I: 2008	
(a) MVA85A	**MVA virus expressingAg85A DNA**
(b) M72	**Mtb32 + Mtb29 fusion protein**
(c) AERAS-402	**Replication-incompetent adenovirus 35 vector expressing *M. Tuberculosis* antigens Ag85A, Ag85B, and TB 10.4.DNA**
(d) SSI Hybrid-1	**fusion protein (Ag85B-ESAT-6)**
(e) SSI HyVac4/AERAS-404	fusion protein (Ag85B-TB10.4)
(f) AERAS-405	Shigella-delivered recombinant double-stranded RNA nucleocapsid (Ag85A, 85B, Rv3407, latency antigen)
(g) r30	recombinant Ag85B protein
(h) Nas L3/Htk BCG	
(2) Phase I: 2009 or Later	
(a) Hsp C™ TB Vaccine	Heat shock protein antigen complexes (Hsp Cs)
(b) HBHA (heparin-binding haemagglutinin)	
(c) NasL3/AM85B conjugate	Nasal vaccine/Man capped Arabinomannan oligosaccharide
(d) PPI, PP2, PP3	BCG boosting
(e) AC_2SGL Diacylated Sulfoglycolipids	AC_2 SGL Mycobacterial lipids
(f) HVJ-liposome/Hsp65 DNA + IL-12 DNA	**HSP65 DNA from H37Rv TB HVJ-liposome vector**
(g) HVJ-envelope/HSP65 DNA+IL-12 DNA	**HSP65 DNA from H37Rv TB HVJ-envelope vector**
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mycobacterial antigens have been tested. Tuberculosis is the leading cause of death in AIDS patient, yet the current tuberculosis vaccine, BCG is contraindicated for immunocompromised individuals, including human immunodeficiency virus (HIV)-positive person, although Tullius et al. reported that a replication-limited recombinant BCG (30-kDa) vaccine designed for HIV-positive person is safer and more efficacious than BCG using SCID mice[91] (Table 5).

Intranasal, intratracheal or oral vaccine. Several new TB-vaccine candidates have been evaluated for their protective efficacy in animal models using the mucosal route of immunization. The adjuvants and delivery systems are crucially important in such vaccines.[22,60,92,93]

Today, nearly 200 new "laboratory bench" vaccine candidates have been developed by different research groups.[94] They include live attenuated vaccines,[60] recombinant virus-restored vaccines, recombinant bacteria-vectored vaccines (including BCG vector),[60] DNA vaccines and subunit vaccines including fusion proteins (Tables 5 and 6).

Antigen specific $CD4^+$ and $CD8^+$ T cells were elicited by intranasal immunization of mice with a vaccinia virus-based vaccine or an adenoviral-based vaccine expressing Ag85A.

A vaccine of Act DNA-deleted Listeria monocytogenes including Ag85A, Ag85B or MPT51 DNA was used for oral vaccine which augmented intestinal mucosal immunity. This vaccine exerted prophylactic efficacy on TB infection in mice.[60]

However, so far no mucosal TB vaccine candidate has reached clinical trial.

MPT51DNA vaccine, Lipocalin2 DNA vaccine and SLPI (secretory leukocyte protease inhibitory protein) DNA vaccine. T. Aoshi et al. identified an HLA-A*0201-restricted $CD8^+$T cell epitope on MPT51 by using a strategy that included HLA-A*0201 transgenic mice and gene gun immunization with expression plasmid DNA encoding MPT51.[60,95] They found HLA-A*0201-restricted $CD8^+$CTL which may play a pivotal role in protection against *M. tuberculosis* infection. Takeda K et al. reported lipocalin 2 and SLPI produced from macrophages and lung alveolar epithelial cells stimulated by TLR have killing activity against TB in vitro.[96,97] Lipocalin 2 (-/-) mice and SLPI (-/-) mice were very sensitive to TB infection.[96,97] Therefore, we are now constructing novel stronger therapeutic vaccine containing MPT51 + Lipocalin 2 + SLPI + HSP65 + IL-12 DNA vaccine in the collaboration with Koide and Takeda.

Clinical Trial

MTB72f. The MTB72f vaccine is a fusion molecule consisting of two antigens that are strong targets for T helper 1 (TH1) cells in PPD-positive individuals. Rv1196 (MTB32) is inserted into the middle of the serine protease Rv0125 (MTB39), which is thus present as two fragments. MTB72F in the A502A adjuvant formulation has recently completed two Phase I trials in healthy PPD-negative adults in the USA and Belgium. The vaccine was well tolerated and safe, and could induce both antigen-specific humoral and cell-mediated immune responses[98-100] (Table 6).

MVA85A. MVA85A is a modified vaccine virus Ankara (MVA) strain expressing antigen 85A, another member of the Ag85 family of protective antigens. In phase I studies in humans, MVA85A was found to be safe and well tolerated, and this vaccine has induced strong immune responses, particularly in previously BCG-vaccinated individuals.[61]

Boosting BCG vaccination with MVA85A downregulates the immunoregulatory cytokine TGFβ1 (**Table 6**).[101] MVA85A induces cellular immune responses in UK volunteers.[102] The safety and immunogenicity of MVA85A in West Africa support its accelerated development as a booster vaccine for tuberculosis. T cell responses were better maintained in BCG-naive Gambian than BCG-naive UK vaccine. $CD4^+$T cells responses were predominantly stimulated. $CD8^+$T cell responses were observed in subjects who were HLA B-35,[102] (**Table 6**).

AERAS-402 DNA. This DNA vaccine is intended for use as a boosting vaccine in BCG-primed individuals. The vaccine is a serotype 35 adenovirus which is incapable of replicating and contains DNA that expresses a fusion protein created from three *M. tuberculosis* antigens: 85A, 85B and TB 10.4.

HSP65 DNA + IL-12DNA. We reported the activities of a network of Asian investigators involved in the development of therapeutic vaccines for use in patients infected with MDR-TB, and described a network of hospitals within Japan which could be linked in the clinical testing of new TB vaccines[1,2,103] (**Table 6**).

Several new TB vaccines were reported[104-108] and some of them were currently in Phase I human testing. This requires a small study in healthy, PPD-negative individuals (usually adults) in the country in which the vaccine was developed. Additional Phase I trials may be conducted in PPD^+ individual, children, infants, or other groups for which the vaccine may be indicated ultimately. The critical issues which impact upon the design of TB vaccine field trials have been reviewed recently.[109] The determination of safety and immunogenicity are prerequisites for any new TB vaccine to go forward into Phase III (efficacy) trials.

Phase I and II vaccine trials are relatively small and inexpensive, however, Phase III trials of new TB vaccines will be large, complicated, costly endeavors requiring international private/public partnership and a long planning process. The complexities of evaluating new TB vaccines during the product development phase have been analyzed recently.[110] McMurray discussed the role of the Aeras Global TB Vaccine Foundation in the movement of TB vaccines from the bench through clinical testing to the bedside.[23] True protective efficacy can only be measured in phase III trials. Because of the absence of accurate methods to measure infection rates, especially when BCG or related vaccine are given, the long latency of *M. tuberculosis* infection and delayed reactivation disease, efficacy trials need large sample sizes (at least 10,000) with long-term follow-up (5 years). New diagnostic tests capable of distinguishing between immunity induced by BCG vaccination and *M. tuberculosis* infection (i.e., Quantiferon test) might allow for an infection and point to be studied in future vaccine trials.[111]

Conclusions

Several kinds of vaccines against TB were developed by the progress of method for genes, immunity and animal models. Among the vaccine candidates shown in **Tables 5 and 6** (WHO STOP TB partnership 2008), the results of MVA (Modified vaccinia Ankara) Ag85A, HVJ-Envelope/HSP65DNA + IL-12DNA, Recombinant BCG (listeriolysin), Recombinant BCG (Ag85A), Mtb72f fusion protein, ESAT6/Ag85A fusion protein vaccines might provide a significant rationale in for moving these vaccines into clinical application. It will furthermore be a high priority for the clinical development programs to evaluate the current vaccines for post-exposure vaccine which prevents reactivation of TB in the large proportion of the global population latently infected with TB.

References

1. Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, Nagasawa T, et al. Novel prophylactic and therapeutic vaccine against tuberculosis. Vaccine 2009; 27:3267-70.
2. Yoshida S, Tanaka T, Kita Y, Kuwayama S, Kanamaru N, Muraki Y, et al. DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation. Vaccine 2006; 24:1191-204.
3. Flynn JL, Chan J. Immunology of tuberculosis. Annu Rev Immunol 2001; 19:93-129.
4. Hess J, Schaible U, Raupach B, Kaufmann SH. Exploiting the immune system: toward new vaccines against intracellular bacteria. Adv Immunol 2000; 75:1-88.
5. Geluk A, van Meijgaarden KE, Franken KL, Drijfhout JW, D'Souza S, Necker A, et al. Identification of major epitopes of *Mycobacterium tuberculosis* AG85B that are recognized by HLA-A*0201-restricted $CD8^+$ T cells in HLA-transgenic mice and humans. J Immunol 2000; 165:6463-71.
6. Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Andersen P, et al. Human cytolytic and interferon gamma-secreting $CD8^+$ T lymphocytes specific for *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 1998; 95:270-5.
7. Wong P, Pamer EG. CD8 T cell responses to infectious pathogens. Annu Rev Immunol 2003; 21:29-70.
8. McShane H, Behboudi S, Goonetilleke N, Brookes R, Hill AV. Protective immunity against *Mycobacterium tuberculosis* induced by dendritic cells pulsed with both CD8(+)- and CD4(+)-T-cell epitopes from antigen 85A. Infect Immun 2002; 70:1623-6.
9. Srivastava IK, Liu MA. Gene vaccines. Ann Intern Med 2003; 138:550-9.
10. Okada M, Kishimoto T, editors. The potential application and limitation of cytokine/growth factor manipulation in cancer therapy. In "Cell proliferation in Cancer: Regulatory Mechanisms of Neoplastic Cell Growth" (Ed.,) Pusztai L, Lewis C, Yap E: Oxford University Press 1996; 218-44.
11. Roy MJ, Wu MS, Barr LJ, Fuller JT, Tussey LG, Speller S, et al. Induction of antigen-specific $CD8^+$ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 2000; 19:764-78.
12. Le TP, Coonan KM, Hedstrom RC, Charoenvit Y, Sedegah M, Epstein JE, et al. Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. Vaccine 2000; 18:1893-901.
13. Moore AC, Hill AV. Progress in DNA-based heterologous prime-boost immunization strategies for malaria. Immunol Rev 2004; 199:126-43.
14. Boyer JD, Chattergoon MA, Ugen KE, Shah A, Bennett M, Cohen A, et al. Enhancement of cellular immune response in HIV-1 seropositive individuals: A DNA-based trial. Clin Immunol 1999; 90:100-7.
15. Huygen K. DNA vaccines: application to tuberculosis. Int J Tuberc Lung Dis 1998; 2:971-8.
16. Lowrie DB. DNA vaccines against tuberculosis. Curr Opin Mol Ther 1999; 1:30-3.
17. Orme IM. The search for new vaccines against tuberculosis. J Leukoc Biol 2001; 70:1-10.
18. Doherty TM, Andersen P. Tuberculosis vaccine development. Curr Opin Pulm Med 2002; 8:183-7.
19. McMurray DM. Guinea pig model of tuberculosis, 113-134. In Bloom BR (ed), Tuberculosis: pathogenesis, protection and control. ASM Press, Washington, DC 1994.

20. Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, Nagasawa T, et al. Evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA + IL-12 DNA) against tuberculosis using the cynomologus monkey model of TB. Vaccine 2007; 25:2990-3.
21. Kita Y, Tanaka T, Yoshida S, Ohara N, Kaneda Y, Kuwayama S, et al. Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model. Vaccine 2005; 23:2132-5.
22. Hoft D. Tuberculosis vaccine development: goals, immunological design and evaluation. Lancet 2008; 372:164-75.
23. Gupta UD, Katoch VM, McMurray DN. Current status of TB vaccines. Vaccine 2007; 25:3742-51.
24. Tanaka F, Abe M, Akiyoshi T, Nomura T, Sugimachi K, Kishimoto T, et al. The anti-human tumor effect and generation of human cytotoxic T cells in SCID mice given human peripheral blood lymphocytes by the in vivo transfer of the Interleukin-6 gene using adenovirus vector. Cancer Res 1997; 57:1335-43.
25. Dranoff G, Mulligan RC. Gene transfer as cancer therapy. Adv Immunol 1995; 58:417-54.
26. Pardoll DM. Paracrine cytokine adjuvants in cancer immunotherapy. Annu Rev Immunol 1995; 13:399-415.
27. Okada M, Takemoto Y, Okuno Y, Hashimoto S, Yoshida S, Fukunaga Y, et al. The development of vaccines against SARS corona virus in mice and SCID-PBL/hu mice. Vaccine 2005; 23:2269-72.
28. Okada M, Okuno Y, Hashimoto S, Kita Y, Kanamaru N, Nishida Y, et al. Development of vaccines and passive immunotherapy against SARS corona virus using SCID-PBL/hu mouse models. Vaccine 2007; 25:3038-40.
29. Rook GAW, Lowrie DB, Hernandez-Pando R. Immunotherapeutics for tuberculosis in experimental animals: Is there a common pathway activated by effective protocols. J Infectious Diseases 2007; 196:191-8.
30. Mitsuyama M, McMurray DN. Tuberculosis: vaccine and drug development. Tuberculosis 2007; 87:10-3.
31. Andersen P. Tuberculosis vaccines—an update. Nature Reviews Microbiology 2007; 5:484-7.
32. Huygen K, Content J, Denis O, Montgomery DL, Yawman AM, Deck RR, et al. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. Nat Med 1996; 2:893-8.
33. Ulmer JB, Liu MA, Montgomery DL, Yawman AM, Deck RR, DeWitt CM, et al. Expression and immunogenicity of *Mycobacterium tuberculosis* antigen 85 by DNA vaccination. Vaccine 1997; 15:792-4.
34. Baldwin SL, D'Souza C, Roberts AD, Kelly BP, Frank AA, Lui MA, et al. Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. Infect Immun 1998; 66:2951-9.
35. Kamath AT, Feng CG, Macdonald M, Briscoe H, Britton WJ. Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. Infect Immun 1999; 67:1702-7.
36. Skeiky YA, Ovendale PJ, Jen S, Alderson MR, Dillon DC, Smith S, et al. T cell expression cloning of a *Mycobacterium tuberculosis* gene encoding a protective antigen associated with the early control of infection. J Immunol 2000; 165:7140-9.
37. Dillon DC, Alderson MR, Day CH, Lewinsohn DM, Coler R, Bement T, et al. Molecular characterization and human T-cell responses to a member of a novel *Mycobacterium tuberculosis* mtb39 gene family. Infect Immun 1999; 67:2941-50.
38. Morris S, Kelley C, Howard A, Li Z, Collins F. The immunogenicity of single and combination DNA vaccines against tuberculosis. Vaccine 2000; 18:2155-63.
39. Lowrie DB, Tascon RE, Bonato VL, Lima VM, Faccioli LH, Stavropoulos E, et al. Therapy of tuberculosis in mice by DNA vaccination. Nature 1999; 400:269-71.
40. Tanghe A, Lefèvre P, Denis O, D'Souza S, Braibant M, Lozes E, et al. Immunogenicity and protective efficacy of tuberculosis DNA vaccines encoding putative phosphate transport receptors. J Immunol 1999; 162:1113-9.
41. Brandt L, Elhay M, Rosenkrands I, Lindblad EB, Andersen P. ESAT-6 subunit vaccination against *Mycobacterium tuberculosis*. Infect Immun 2000; 68:791-5.
42. Fonseca DP, Benaissa-Trouw B, van Engelen M, Kraaijeveld CA, Snippe H, Verheul AF. Induction of cell-mediated immunity against *Mycobacterium tuberculosis* using DNA vaccines encoding cytotoxic and helper T-cell epitopes of the 38-kilodalton protein. Infect Immun 2001; 69:4839-45.
43. Delogu G, Brennan MJ. Comparative immune response to PE and PE_PGRS antigens of *Mycobacterium tuberculosis*. Infect Immun 2001; 69:5606-11.
44. Khera A, Singh R, Shakila H, Rao V, Dhar N, Narayanan PR, et al. Elicitation of efficient, protective immune responses by using DNA vaccines against tuberculosis. Vaccine 2005; 23:5655-65.
45. Delogu G, Li A, Repique C, Collins F, Morris SL. DNA vaccine combinations expressing either tissue plasminogen activator signal sequence fusion proteins or ubiquitin-conjugated antigens induce sustained protective immunity in a mouse model of pulmonary tuberculosis. Infect Immun 2002; 70:292-302.
46. Derrick SC, Yang AL, Morris SL. A polyvalent DNA vaccine expressing an ESAT6-Ag85B fusion protein protects mice against a primary infection with *Mycobacterium tuberculosis* and boosts BCG-induced protective immunity. Vaccine 2004; 23:780-8.
47. Tanghe A, Denis O, Lambrecht B, Motte V, van den Berg T, Huygen K. Tuberculosis DNA vaccine encoding Ag85A is immunogenic and protective when administered by intramuscular needle injection but not by epidermal gene gun bombardment. Infect Immun 2000; 68:3854-60.
48. Franco LH, Wowk PF, Silva CL, Trombone AP, Coelho-Castelo AA, Oliver C, et al. A DNA vaccine against tuberculosis based on the 65 kDa heat-shock protein differentially activates human macrophages and dendritic cells. Genet Vaccines Ther 2008; 6:3.
49. Gurunathan S, Klinman DM, Sender RA. DNA vaccines: immunology, application and optimization. Annu Rev Immunol 2000; 18:927-74.
50. Laddy DJ, Weiner DB. From plasmids to protection: a review of DNA vaccines against infectious diseases. Int Rev Immunol 2006; 25:99-123.
51. Ishii KJ, Akira S. Innate immune recognition of, and regulation by DNA. Trends Immunol 2006; 27:525-32.
52. Zelenay S, Elias F, Flo J. Immunostimulatory effects of plasmid DNA and synthetic oligodeoxynucleotides. Eur J Immuol 2003; 33:1382-92.
53. Hacker H, Vabulas RM, Takeuchi O, Hoshino K, Akira S, Wagner H. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. J Exp Med 2000; 192:595-600.
54. Takeshita F, Ishii KJ, Ueda A, Ishigatsubo Y, Klinman DM. Positive and negative regulatory elements contribute to CpG oligonucleotide-mediated regulation of human IL-6 gene expression. Eur J Immunol 2000; 30:108-16.
55. Takeshita F, Klinman DM. CpG ODN-mediated regulation of IL-12 p40 transcription. Eur J Immunol 2000; 30:1967-76.
56. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel Toll/IL-I receptor domain-containg adapter that preferentially activates the IFNbeta promoter in the Toll-like receptor signaling. J Immunol 2002; 169:6668-72.
57. Rosada RS, Torre LG, Frantz FG, Trombone A PF, Zarate Blades CR, Fonseca DM, et al. Protection against tuberculosis by a single intranasal administration of DNA-hsp65 vaccine complexed with cationic liposomes. BMC Immunology 2008; 9:38.
58. Ulmer JB, Wahren B, Liu MA. Gene-based vaccines: recent technical and clinical advances. Trends in molecular medicine 2006; 12:216-72.
59. Okada M, Tanaka T, Yoshida S, Kita Y, Kanamaru N, Hashimoto S, et al. Novel vaccination (HVJ-liposome/HSP65 + IL-12 DNA and recombinant 72f BCG) against tuberculosis using cynomolgus monkey. US-Japan Cooperative Medical Science Program. Fortieth Tuberculosis and Leprosy Research Conference (Seattle USA) 2005; 46-56.
60. Miki K, Nagato T, Tanaka T, Kim Y-H, Uchijima M, Ohara M, et al. Induction of protective cellular immunity against *M. tuberculosis* by attenuated self-destructing Listeria monocytogenes strains harboring eukaryotic plasmid for antigen 85 complex and MPB/MPT51. Infect Immun 2004; 72:2014-21.
61. McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosters BCG-primed and naturally acquired antimycobacterial immunity in humans. Nature Med 2008; 10:1240-4.
62. Dietrich J, Billeskov R, Doherty TM, Andersen P. Synergistic effect of bacillus Calmette Guerin and a tuberculosis subunit vaccine in cationic liposomes: increased immunogenicity and protection. J Immunol 2007; 178:3721-30.
63. Mata-Hara V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant Monophosphory Lipid A as a TRIF-biased agonist of TLR4. Science 2007; 316:1628-32.
64. Walsh GP, Tan EV, dela Cruz EC, Abalos RM, Villahermosa LG, Young LJ, et al. The *Philippine cynomolgus* monkey provides a new nonhuman primate model of tuberculosis that resembles human disease. Nat Med 1996; 2:430-6.
65. Saeki Y, Matsumoto N, Nakano Y, Mori M, Awai K, Kaneda Y. Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for in vitro and in vivo gene transfer. Hum Gene Ther 1997; 8:2133-41.
66. Okada M, Kitahara M, Kishimoto S, Matsuda T, Hirano T, Kishimoto T. IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells. J Immunol 1988; 141:1543-9.
67. Okada M, Klimpel GR, Kuppers RC, Henney CS. The differentiation of cytotoxic T cells in vitro I. Amplifying factor(s) in the primary response is Lyt 1 + cell dependent. J Immunol 1979; 122:2527-33.
68. Okada M, Kishimoto T, Igarashi T, Teranishi T, Yamamura Y. LPS- or 8Br-cyclic AMP-induced production of T cell-activating factor(s) in macrophage tumor cell line J774.1. J Immunol 1978; 120:1097-101.
69. Okada M, Yoshimura N, Kaieda T, Yamamura Y, Kishimoto T. Establishment and characterization of human T hybrid cells secreting immunoregulatory molecules. Proc Natl Acad Sci USA 1981; 78:7717-21.
70. Okada M, Sakaguchi N, Yoshimura N, Hara H, Shimizu K, Yoshida N, et al. B cell growth factors and B cell differentiation factor from human T hybridomas. Two distinct kinds of B cell growth factor and their synergism in B cell proliferation. J Exp Med 1983; 157:583-90.
71. Skeiky YA, Alderson MR, Ovendale PJ, Guderian JA, Brandt L, Dillon DC, et al. Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. J Immunol 2004; 172:7618-28.
72. Boom WH. New TB vaccine: is there a requirement for CD8+ T cells? J Clin Invest 2007; 117:2092-4.

73. Woodworth JS, Behar SM. *Mycobacterium tuberculosis*-specific CD8+ T cell and their role in immunity. Crit Rev Immunol 2006; 26:317-52.
74. van Pinxteren LA, Cassidy JP, Smedegaard BH, Agger EM, Andersen P. Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. Eur J Immunol 2000; 30:3689-98.
75. Igarashi T, Okada M, Azuma I, Yamamura Y. Adjuvant activity of synthetic N-acetyl-muramyl-L-alanyl-D-isoglutamine and related compounds on cell-mediated cytotoxicity in syngeneic mice. Cell Immunol 1977; 34:270-8.
76. Coulombe F, Dirangahi M, Veyrier F, Leseleuc L, Gleason JL, Yang Y, et al. Increased NOD2-mediated recognition of N-glycolyl muramyl dipeptide. J Exp Med 2009; 206:1709-16.
77. Stegelmann F, Bastian M, Swoboda K, Bhat R, Kiessler V, Krensky AM, et al. Coordinate expression of CC chemokine ligand 5, granulysin and perforin in CD8+ T cells provides a host defense mechanism against *Mycobacterium tuberculosis*. J Immunol 2005; 175:7474-83.
78. Wilkinson RJ, Zhu X, Wilkinson KA, Lalvani A, Ivanyi J, Pasvol G, et al. 38,000 MW antigen-specific major histocompatibility complex class I restricted interferon-γ secreting CD8+ T cell in healthy contacts of tuberculosis. Immunology 1998; 95:585-90.
79. Lewinsohn DM, Zhu L, Madison VJ, Dillon DC, Fling SP, Reed SG, et al. Classically restricted human CD8+ T lymphocytes derived from *Mycobacterium tuberculosis*-infected cells: definition of antigenic specificity. J Immunol 2001; 166:439-46.
80. Carraza C, Juarez E, Torres M, Ellner JJ, Sada E, Schwander SK. *Mycobacterium tuberculosis* growth control by lung macrophages and CD8 cells from patient contacts. Am J Respir Crit Care Med 2006; 174:238-45.
81. Tully G, Kortsik C, Höhn H, Zehbe I, Hitzler WE, Neukirch C, et al. Highly focused T cell responses in latent human pulmonary *Mycobacterium tuberculosis* infection. J Immunol 2005; 174:2174-84.
82. Kita Y, Kanamaru N, Hashimoto S, Nishida Y, Nakatani H, Takao K, et al. The study of Nobel vaccination using granulysin transgenic mice. Sixth World Congress on Vaccines, Immunization and Immunotherapy 2008; 39.
83. Pena SV, Hanson DA, Carr BA, Goralski TJ, Krensky M. Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. J Immunol 1997; 158:2680-8.
84. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediatd by granulysin. Science 1998; 282:121-5.
85. Huang LP, Lyu SC, Clayberger C, Krensky AM. Granulysin-mediated tumor rejection in transgenic mice. J Immunol 2007; 178:77-84.
86. Okada M, Tanaka T, Inoue Y, Yoshida S, Ohara N, Yamada T, et al. New (DNA-, recombinant BCG- and subunit-) vacctination against tuberculosis and cytotoxic activity. US-Japan Cooperatie Medical Science Program. Thirty-six Tuberculosis and Leprosy Research Conference (New Orleans USA) 2001; 127-33.
87. Bruns H, Meinken C, Schanenberg P, Harter G, Kern P, Modlin RL, et al. Anti-TNF immunotherapy reduces CD8+ cell-mediated antimicrobial activity against *Mycobacterium tuberculosis* in humans. J Clin Invest 2009; 119:1167-77.
88. Campbell JJ, Murphy KE, Kunkel EJ, Brightling CE, Soler D, Shen Z, et al. CCR7 expression and memory T cell diversity in humans. J Immunol 2001; 166:877-84.
89. Geglnat J, Sallusto F, Lanzavecchua A. Cytokine-driven proliferation and differentiation of human naïve, central memory and effector memory CD4(+) T cells. J Exp Med 2001; 194:1711-9.
90. Schuns KS, Lefrancois L. Cytokine control of memory T cell development and survival. Nat Rev Immunol 2003; 3:269-79.
91. Tullius MV, Harth G, Maslesa-Galic S, Dillon BJ, Harwitz MA. A replication-limited recombinant *Mycobacterium bovis* BCG vaccine against tuberculosis designed for human immunodeficiency virus-positive persons is safer and more efficacious than BCG: Infection and Immunity 2008; 76:5200-14.
92. Kaneda Y. Applications of Hamagglatinating Virus of Japan in therapeutic delivery systems. Expert Opin Drug Deliv 2008; 5:221-33.
93. Yoshida A, Inagawa H, Kohchi C, Hino M, Ito F, Tomoda K, et al. Selective delivery of rifampicin incorporated into poly (DL-lactic-co-glycolic) acid microspheres after phagocytotic uptake by alveolar macrophages, and the killing effect against intracellular *Mycobacterium bovis* Calmette-Guette-Guerin. Antimicrobial Agents and Chemotherapy (in press).
94. Hanekom WA, Dockrell HM, Ottenhoff THM, Doherty TM, Fletcher H, McShane H, et al. Immunological outcomes of new tuberculosis vaccine trials: WHO panel recommendation. PLos Medicine 2008; 5:145.
95. Aoshi T, Nagato T, Suzuki M, Uchijima M, Hashimoto D, Rafiei A, et al. Identification of an HLA-A0201-restricted T-cell epitope on the MPT51 protein, a major secreted protein derived from *Mycobacterium tuberculosis*, by MPT51 overlapping peptide screening. Infect and Immunity 2008; 7:1565-91.
96. Saiga H, Nishimura J, Kuwata H, Okuyama M, Matsumoto S, Sato S, et al. Lipocalin 2-dependent inhibition of mycobacterial growth in alveolar epithelium. J Immunol 2008; 81:521-7.
97. Nishimura J, Saiga H, Sato S, Okuyama M, Kayama H, Kuwata H, et al. Potent antimycobacterial activity of mouse secretory leukocyte protease inhibitor. J Immunol 2008; 180:4032-9.
98. Skeiky YA, Lodes MJ, Guderian JA, Mohamath R, Bement T, Alderson MR, et al. Cloning expression and immunological evaluation of two putative secreted serine protease antigens of *Mycobacterium tuberculosis*. Infect Immun 1999; 67:3998-4007.
99. Reed S, Alderson MR, Dalemans W, Lobet Y, Skeiky YA. Prospects for a better vaccine against tuberculosis. Tuberculosis 2003; 83:213-9.
100. Hussey G. Prospects for new tuberculosis vaccines. SAMJ 2007; 97:1001-2.
101. Fletcher HA, Pathan AA, Berthoud TK, Dunachie SJ, Whela KT, Alder NC, et al. Boosting BCG vaccination with MVA85A domn-regulates the immunoregulatory cytokine TGFβ1. Vaccine 2008; 26:5264-75.
102. Brooks RH, Hill PC, Owiafe PK, Ibanga HB, Jeffries DJ, Donkor SA, et al. Safety and immunogenicity of the candidate tuberculosis vaccine MVA85A in west Africa. Plos one 2008; 3:2921.
103. Okada M. Japanese/Asian efforts on TB vaccine development. 10th International Conference on Emerging Infection Diseased in the pacific Rim 2005; 23-4.
104. Kaufmann SH. Recent finding in immunology give tuberculosis vaccines a new booster. Trends Immunol 2005; 26:660-7.
105. Horwitz MA, Harth G. A new vaccine against tuberculosis affords grater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. Infect Immun 2003; 71:1672-9.
106. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser Eddine A, et al. Increased vaccine against tuberculosis of recombinant *Mycobacterium bovis* bacilli Camett-Guerin mutants that secrete listeriolysin. J Clin Invest 2005; 115:2472-9.
107. Winau F, Weber S, Sad S, de Diego J, Hoops SL, Breiden B, et al. Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. Immunity 2006; 24:105-17.
108. Dietrich J, Aagaard C, Leah R, Olsen AW, Stryhn A, Doherty TM, et al. Exchanging ESAT6 with TB10.4 in an Ag85B fusion molecule-based tuberculosis polyprotein vaccine: efficient protection and ESAT6-based sensitive monitoring of vaccine efficacy. J Immunol 2005; 174:6332-9.
109. Ibanga HB, Brookes RH, Hill PC, Owaife PK, Fletcher HA, Leinhardt C, et al. Early clinical trials with a new tuberculosis vaccine, MVA85A, in tuberculosis-endemic countries: issues in study design. Lancet Infect Dis 2006; 6:522-8.
110. Sadoff JC, Hone D. The role of "go no-go" decisions in TB vaccine development. Microbes Infect 2005; 7:899-904.
111. Mori T, Sakatani M, Yamagishi F, Takashima T, Kawabe Y, Nagao K, et al. Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. Am J Respir Crit Care Med 2004; 170:59-64.

A NOVEL THERAPEUTIC AND PROPHYLACTIC VACCINE (HVJ-ENVELOPE/HSP65 DNA+IL-12 DNA) AGAINST TUBERCULOSIS USING THE CYNOMOLGUS MONKEY MODEL

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[Abstract]

We have developed a novel tuberculosis (TB) vaccine; a combination of the DNA vaccines expressing mycobacterial heat shock protein 65 (HSP65) and interleukin 12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-envelope and –liposome (HSP65 + IL-12/HVJ). An IL-12 expression vector (IL-12DNA) encoding single-chain IL-12 proteins comprised of p40 and p35 subunits were constructed. This vaccine provided remarkable protective efficacy in mouse and guinea pig models compared to the BCG vaccine on the basis of C.F.U of number of TB, survival, an induction of the CD8 positive CTL activity and improvement of the histopathological tuberculosis lesions. This vaccine provided therapeutic efficacy against multi-drug resistant TB (MDR-TB) and extremely drug resistant TB (XDR-TB) (prolongation of survival time and the decrease in the number of TB in the lung) as well as protective efficacy in murine models. Furthermore, we extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis. This novel vaccine provided a higher level of the protective efficacy than BCG based upon the assessment of mortality, the ESR, body weight, chest X-ray findings and immune responses. All monkeys in the control group (saline) died within 8 months, while 50% of monkeys in the HSP65+hIL-12/HVJ group survived more than 14 months post-infection (the termination period of the experiment). Furthermore, the combination of HSP65 + IL-12/HVJ and BCG by the priming-booster method showed a synergistic effect in the TB-infected cynomolgus monkey (100% survival). In contrast, 33% of monkeys from BCG Tokyo alone group were alive (33% survival). Furthermore, this vaccine exerted therapeutic efficacy (survived and immune responses) in the TB-infected monkeys. These data indicate that our novel DNA vaccine might be useful against Mycobacterium tuberculosis including XDR-TB and MDR-TB for human therapeutic clinical trials.

[Introduction]

Tuberculosis is a major global threat to human health, with about 2 million people dying every year from M.tuberculosis (TB) infections. The only tuberculosis vaccine currently available is an attenuated strain of M.bovis BCG (BCG), although its efficacy against adult TB disease remains controversial. Furthermore, multi-drug resistant tuberculosis (MDR-TB) and extremely drug resistant TB (XDR-TB) are becoming big problems in the world. In such circumstances, the development of therapeutic vaccine against TB as well as prophylactic vaccine against TB is required. Therefore, we have recently developed a novel TB vaccine, a DNA vaccine expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-envelope (liposome) (HSP65+IL-12/HVJ). In order to explore the preclinical use of tuberculosis DNA vaccine combinations of IL-12 DNA with Hsp65 DNA, we choose the viral-based hybrid antigen delivery system hemagglutinating virus of Japan (HVJ)-liposome because this delivery system results in a high transfection efficacy, repeated gene transfection without reduction of gene transfer efficiency *in vivo*, and no apparent toxicity. The vaccine was 100 fold more efficient than BCG in the mouse model on the basis of the elimination of M. tuberculosis mediated by the induction of CTL [1]. A nonhuman primate model of TB will provide critical information for vaccine development. In fact, in the previous study we evaluated the protective efficacy of HSP65+IL-12/HVJ in the cynomolgus monkey model, which is an excellent model of human tuberculosis [2,3]. Furthermore, we observed the synergistic effect of the HSP65 + IL-12/HVJ and BCG using a priming-booster method in the TB-infected cynomolgus monkeys. The combination of the two vaccines showed a very strong prophylactic efficacy against *M. tuberculosis* (100% survival) as we have seen previously in the murine model of TB [4]. In the present study, we evaluated therapeutic effect and prophylactic effect of this vaccine on the MDR-TB infection and XDR- TB infection in murine and monkey models.

[Materials and Methods]

DNA vaccines encoding *M.tuberculosis* HSP65 and human IL-12 were encapsulated into HVJ-Envelope or HVJ-liposomes [5].

At 5 and 10 weeks after intravenous or aerosol intratracheal challenge of *M.Tuberculosis* H37RV, the number of CFU in the lungs, spleen, and liver were counted and therapeutic efficacy of HVJ-Envelope DNA vaccines was evaluated [1].

Cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial Research Center. The animals were vaccinated nine times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65 + hIL-12/HVJ: 400ug i.m.), one week after the challenge with the *M.tuberculosis* Erdman strain (5×10^2) by intratracheal instillation. Immune responses and survival were examined as described in our previous studies [2,4].

[Results]

The protective efficacy of the HSP65+IL-12 /HVJ and BCG using the priming-booster method in the TB infected cynomolgus monkeys was very strong. All four monkeys from the group of BCG-priming and the DNA vaccine (HVJ-liposome/HSP65+IL-12 DNA vaccine) booster were alive more than 12 months post-infection (Fig,1). In contrast, only 2 monkeys out of 6 from the BCG Tokyo group were alive (33% survival). 50% of the monkeys from the saline control group and DNA vaccine-priming and the BCG Tokyo vaccine booster group, respectively, were alive more than 12 months in the study. In addition, both HSP65+hIL-12/HVJ improved ESR and chest X-ray findings. IL-2 and IFN-γ production were augmented in the group vaccinated with HSP65+hIL-12/HVJ (data not shown). Furthermore, proliferation of PBL was strongly enhanced. Taken together, these results clearly demonstrate that BCG priming and the HSP65+hIL-12/HVJ booster could provide extremely strong protective efficacy against M. tuberculosis in the cynomolgus monkey model.

Fig.2A shows the results of immune responses of cynomolgus monkey at 11 weeks after challenge of *M.tuberculosis* Erdman strain (5×10^2) by intratracheal instillation. The proliferation of PBL in therapeutic vaccination of monkeys in the group with HVJ-Env/HSP65 DNA +IL-12 DNA was augmented. This vaccine also improved the survival of monkeys, compared to the saline (control) group, during the period between 0 weeks and 19 weeks after TB challenge(Fig.2B).

[Discussion]

The HSP65+hIL-12/HVJ vaccine exerted a significant therapeutic effect against TB, as indicated by: (1) extension of survival of mice infected with XDR-TB, (2) augmentation of immune responses, in a cynomologus monkey model which closely mimics human TB disease. It is important to evaluate the survival of monkey [2,3,4]. During the period between 0 weeks and 19 weeks after TB challenge, increase in the survival rate of the monkeys treated with this vaccine were observed, compared to the control monkeys treated with saline [6]. Thus, our results with the HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine in the murine therapeutic model and cynomologus monkey therapeutic model should provide a significant rationale for moving this vaccine into clinical trial. Furthermore, we have established chronic TB disease model using mouse infected with TB in the aerosol chamber (data not shown). By using this model, therapeutic efficacy of this vaccine was also observed.

In the present study, it was demonstrated that BCG vaccine priming and HSP65+h IL-12/HVJ booster could provide extremely strong (100% survival) efficacy against M.Tuberculosis compared to BCG alone (33% survival) in the cynomologus monkey model. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data on the HVJ-envelope DNA vaccine in anticipation of a Phase I clinical trial.

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[References]

[1] Yoshida S, Tanaka T, Kita Y, Kuwayama S, Kanamaru N, Muraki Y, et al. DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation. Vaccine 2006;24:1191-1204.

[2] Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, Nagasawa T, et al. Evaluation of a novel vaccine(HVJ-liposome/ HSP65 DNA+IL-12 DNA) against tuberculosis using the cynomologus monkey model of TB. Vaccine 2007; 25(16):2990-3

[3] Walsh GP, Tan EV, dela Cruz EC, Abalos RM, Villahermosa LG, Young LJ, et al. The Philippine cynomolgus monkey provides a new nonhuman primate model of tuberculosis that resembles human disease. Nat Med 1996;2(4):430-6.

[4] Kita Y, Tanaka T, Yoshida S, Ohara N, Kaneda Y, Kuwayama S, et al. Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model. Vaccine 2005;23:2132-2135.

[5] Saeki Y, Matsumoto N, Nakano Y, Mori M, Awai K, Kaneda Y. Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus). Hum Gene Ther. 1997;8(17):2133-41.

[6] Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, et al. Novel prophylactic and therapeutic vaccine against Tuberculosis. Vaccine. (in press)

Fig.1

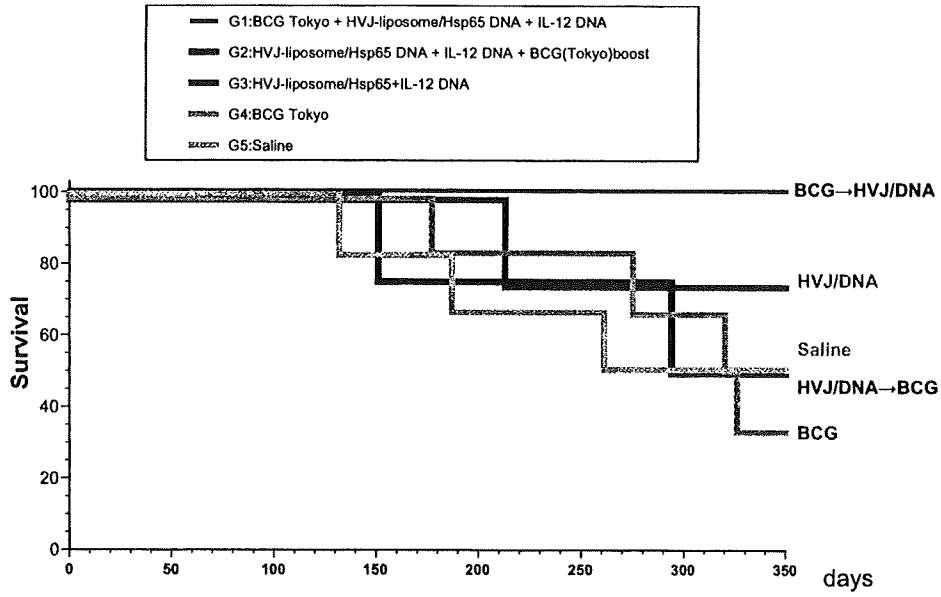


Fig.2A

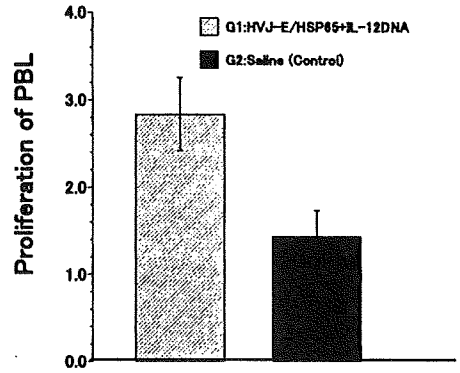
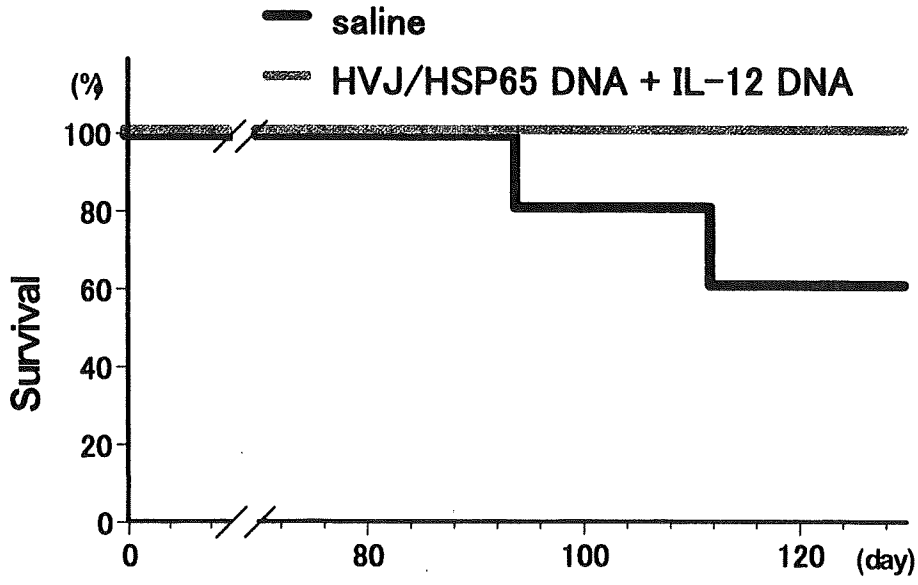


Fig.2B



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A Novel Therapeutic and Prophylactic Vaccine (HVJ-Envelope / Hsp65 DNA + IL-12 DNA) against Tuberculosis Using the Cynomolgus Monkey Model

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Abstract

We have developed a novel tuberculosis (TB) vaccine; a combination of the DNA vaccines expressing mycobacterial heat shock protein 65 (HSP65) and interleukin 12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-envelope and –liposome (HSP65 + IL-12/HVJ). An IL-12 expression vector (IL-12DNA) encoding single-chain IL-12 proteins comprised of p40 and p35 subunits were constructed. This vaccine provided remarkable protective efficacy in mouse and guinea pig models compared to the BCG vaccine on the basis of C.F.U of number of TB, survival, an induction of the CD8 positive CTL activity and improvement of the histopathological tuberculosis lesions. This vaccine also provided therapeutic efficacy against multi-drug resistant TB (MDR-TB) and extremely drug resistant TB (XDR-TB) (prolongation of survival time and the decrease in the number of TB in the lung) in murine models. Furthermore, we extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis. This novel vaccine provided a higher level of the protective efficacy than BCG based upon the assessment of mortality, the ESR, body weight, chest X-ray findings and immune responses. All monkeys in the control group (saline) died within 8 months, while 50% of monkeys in the HSP65+hIL-12/HVJ group survived more than 14 months post-infection (the termination period of the experiment). Furthermore, the BCG priming and HSP65 + IL-12/HVJ vaccine (booster) by the priming-booster method showed a synergistic effect in the TB-infected cynomolgus monkey (100% survival). In contrast, 33% of monkeys from BCG Tokyo alone group were alive (33% survival). Furthermore, this vaccine exerted therapeutic efficacy (100% survival) and augmentation of immune responses in the TB-infected monkeys. These data indicate that our novel DNA vaccine might be useful against *Mycobacterium tuberculosis* including XDR-TB and MDR-TB for human therapeutic clinical trials.

Keywords HSP65□IL-12DNA vaccine□ Tuberculosis□Monkey□Therapeutic vaccine

1. Introduction

Tuberculosis is a major global threat to human health, with about 2 million people dying every year from *Mycobacterium tuberculosis* (TB) infection. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG (BCG), although its efficacy against adult TB disease remains controversial. Furthermore, multi-drug resistant tuberculosis (MDR-TB) and extremely drug resistant TB (XDR-TB) are becoming big problems in the world. In such circumstances, the development of therapeutic vaccine against TB as well as prophylactic vaccine against TB is required. Therefore, we have recently developed a novel TB vaccine, a DNA vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ). This vaccine was 100 fold more efficient than BCG in the murine model

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on the basis of the elimination of *M. tuberculosis* mediated by the induction of CTL [1,2]. Futhermore the HSP65 + IL-12/HVJ vaccine using HVJ-envelope was 10,000 fold more efficient than BCG in the murine TB-prophylactic model. A nonhuman primate model of TB will provide information for vaccine development. In fact, in the previous study we evaluated the protective efficacy of HSP65 + IL-12/HVJ in the cynomolgus monkey model, which is an excellent model of human tuberculosis [1,4]. In the present study, we observed the synergistic effect of the HSP65 + IL-12/HVJ and BCG using a priming-booster method in the TB-infected cynomolgus monkeys. The combination of the two vaccines showed a very strong prophylactic efficacy against *M. tuberculosis* (100% survival) as we have seen previously in the murine model of TB [2,5]. Moreover, we evaluated therapeutic effect of this vaccine on the MDR-TB infection and XDR- TB infection in murine and monkey models, indicating that the vaccine exerts therapeutic efficacy against TB, MDR-TB and XDR-TB.

2. Method for the evaluation of the efficacy of vaccines on the *M.tuberculosis*-infected mice

DNA vaccines encoding *M.tuberculosis* HSP65 and human IL-12 were encapsulated into HVJ-Envelope or HVJ-liposomes [6]. CTL activity was assessed by $^{51}C_r$-release assay[1,7]. At 5 and 10 weeks after intravenous challenge of *M.tuberculosis* H37RV, the number of CFU in the lungs, spleen, and liver were counted and therapeutic efficacy of HVJ-Envelope DNA vaccines was evaluated [1]. Therapeutic efficacy was also evaluated by chronic TB infection model of mice using aerosol challenge of TB (15CFU/mouse: Madison aerosol exposure chamber, University of Wisconsin).

3. Method for the evaluation of the efficacy of the vaccine on the *M.tuberculosis*-infected monkeys

Cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial Research Center. The animals were vaccinated nine times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65 + hIL-12/HVJ: 400ug i.m.), one week after the challenge with the *M.tuberculosis* Erdman strain (5×10^2) by intratracheal instillation. Immune responses and survival were examined as described in our previous studies [2,5].

4. Results and Discussion

(a) Prophylactic efficacy

All 4monkeys in the control group (saline) died within 8 months, while 50% (2monkeys out of 4) of monkeys in the HSP65+hIL-12/HVJ group survived more than 14 months post-infection (the termination period of the experiment)(data not shown).

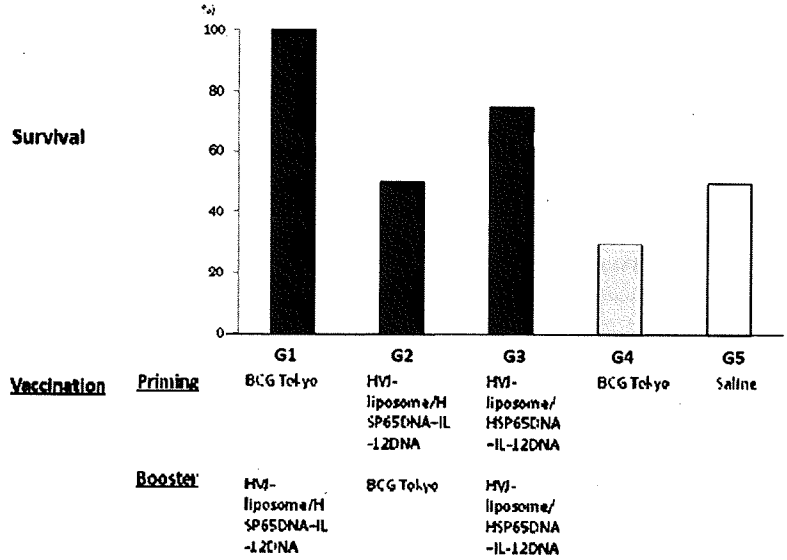

Fig 1. Protective efficacy (survival) of HSP65+IL-12/HVJ and BCG using priming- booster method against TB challenged cynomolgus monkey 350 days after TB using cynomolgus monkey models.

Table 1. Efficacy of HSP65 + IL-12 DNA Vaccine in Monkey

Efficacy of HSP65 + IL-12 DNA in Monkeys	
Survival	. . .
Chest X-p (improvement)	.
Erythrocyte Sedimentation Rate (improvement)	. .
Body Weight	.
Immune Responses	
IFN-γ	. .
IL-6	. .
IL-2	. .
Proliferation of T cell	.

Furthermore, using 32 monkeys, the protective efficacy of the HSP65+IL-12 /HVJ and BCG using the priming-booster method in the TB infected cynomolgus monkeys was very strong. All four monkeys from the group of BCG-priming and the DNA vaccine (HVJ-liposome/HSP65+IL-12 DNA vaccine) booster were alive more than 12 months post-infection (Fig.1). In contrast, only 2 monkeys out of 6 from the BCG Tokyo group were alive (33% survival). 50% of the monkeys from the saline control group and DNA vaccine-priming and the BCG Tokyo vaccine booster group, respectively, were alive more than 12 months in the study. In addition, HSP65+hIL-12/HVJ improved both ESR and chest X-ray findings. IL-2 and IL-6 production were augmented in the group vaccinated with BCG vaccine-priming and the DNA vaccine-booster (Table1). Furthermore, proliferation of PBL was strongly enhanced. Taken together, these results clearly demonstrate that BCG priming and the HSP65+hIL-12/HVJ booster could provide extremely strong protective efficacy against *M.tuberculosis* in the cynomolgus monkey model.

(b)Therapeutic efficacy

The survival of vaccinated mice after XDR-TB (extremely drug resistant TB) was investigated. All mice in the control group died of TB within 160 days after XDR-TB infection. In contrast, mice treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA prolonged the survival periods significantly by statistical analysis($p<0.05$). (data not shown) It was demonstrated that this vaccine had a therapeutic activity against XDR-TB (Table 2A).

At 5 and 10 weeks after intravenous challenge of MDR-TB, the CFU of TB in the lungs, spleen, and liver were counted and therapeutic efficacy of HVJ-Envelope DNA vaccine was evaluated.

HVJ-Envelope/HSP65 DNA +IL-12 DNA vaccine treatment significantly reduced the bacterial loads of MDR-TB as compared to saline control group($P<0.05$) (Table2).

Therapeutic efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA was also observed, using in vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice constructed with human PBL (SCID-PBL/hu)[8,9]. Therapeutic vaccination with HVJ-Envelope/HSP65 DNA+IL-12 DNA group resulted in significantly therapeutic activity even in SCID-PBL/hu mice which exerted human T cell immune responses(Table 2A).

Table 2A. The Development of Novel Vaccines for M.tuberculosis using animal model

Vaccine	mouse	guinea pig	monkey	SCID-PBL/hu	human
HVJ-Envelope/ Hsp65 DNA + IL-12 DNA	prophylactic Effect 10,000 fold effective than BCG	effective	effective	effective	Plan (phase. , .)
	Therapeutic Effect	plan	effective		
	Therapeutic Effect against MDR-TB XDR-TB	plan	plan		
HVJ-liposome/ Hsp65 DNA + IL-12 DNA	Prophylactic Effect 100 fold effective than BCG	effective	effective (100% survival)		

Vaccine	mouse	guinea pig	monkey	SCID-PBL/hu	human
HVJ-Envelope/ Hsp65 DNA + IL-12 DNA + Ag85B DNA + Ag85A DNA	plan	plan	Therapeutic Effect	plan	
15Kgranulysin recombinant 15K granulysin	Therapeutic Effect		plan		
15K granulysin DNA	Therapeutic Effect		plan		

Furthermore, the therapeutic activity of this vaccine was evaluated in a nonhuman primate model infected with *M.tuberculosis*.

Immune responses of cynomolgus monkey at 11 weeks after challenge of *M.tuberculosis* Erdman strain by intratracheal instillation were augmented. The proliferation of PBL in therapeutic vaccination of monkeys in the group with HVJ-Envelope/HSP65 DNA +IL-12 DNA was augmented (data not shown). This vaccine also improved the survival of monkeys, compared to the saline (control) group, after TB challenge(Fig.2). All five monkeys from the group of HVJ-Envelope/HSP65DNA+IL-12DNA vaccine were alive (100% survival). In contrast, 3 monkeys out of 5 from the saline control group were alive (60% survival). These results demonstrate that HVJ-Envelope/HSP65DNA+ IL-12DNA vaccine could provide strong therapeutic efficacy against TB, MDR-TB or XDR-TB in the cynomolgus monkey models as well as murine models

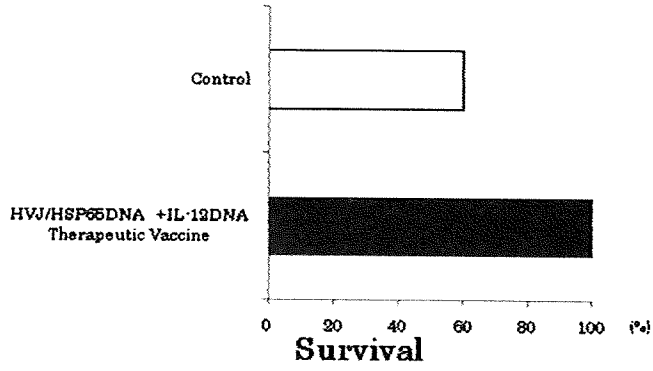

Fig 2. Therapeutic efficacy (survival) of HVJ-Envelope/HSP65DNA+IL-12DNA vaccine 130 days after TB infection using cynomolgus monkey models.

(c) Discussion

The HSP65+hIL-12/HVJ vaccine exerted a significant prophylactic effect against TB, as indicated by: 1) extension of survival for over a year; 2) improvement of ESR and chest X-ray findings; 3) increase in the body weight; 4) augmentation of immune responses, in a cynomolgus monkey model which closely mimics human TB disease. It is very important to evaluate the long survival period in a monkey model, as human TB is a chronic infection disease. Furthermore, the decrease in the body weight of TB patients is usually accompanied by a progression of the disease. [10]

DNA vaccine are a relatively new approach to immunization for infectious diseases.[1,2,5,11-14]

Prophylactic and therapeutic DNA vaccines were established by using several kinds of vectors such as (1) HVJ-liposome, (2) HVJ-envelope, (3) adenovirus vector, (4) adeno-associated virus vector (AAV), (5) lenti-virus vector.[1,2,9]

We have developed a hemagglutinating virus of Japan envelope (HVJ-Envelope) using inactivated Sendai virus, as a nonviral vector for drug delivery.[15-17] It can deliver very efficiently DNA, siRNA, proteins and anti-cancer drugs into cells both in vitro and in vivo[15,18,19]. Therefore, HVJ-Envelope was used as an efficient and safe vector for DNA vaccine against TB in the present study.

In the guinea pig model, HSP65+gpIL-12/HVJ provided better protection against the pulmonary pathology caused by pulmonary infection with TB than BCG vaccination (data not shown). In the present study, it was demonstrated that BCG vaccine priming and HSP65+h IL-12/HVJ booster could provide extremely strong (100% survival) efficacy against *M.tuberculosis* compared to BCG alone (33% survival) in the cynomologus monkey model. In Japan and other countries, the BCG vaccine is inoculated into human infants (0~6months after birth). Therefore, BCG priming in infants and HSP65+h IL-12/HVJ boosters for adults (including junior high school students, high school students and old persons) may be required for the significant improvement of clinical protective efficacy against TB.

Furthermore, the HSP65+hIL-12/HVJ vaccine exerted a significant therapeutic effect against TB, as indicated by: (1) extension of survival of mice infected with XDR-TB, (2) decrease in the CFU of TB in lungs, liver and spleen of mice infected with MDR-TB as well as drug-sensitive TB(H37RV), (3) decrease in the CFU of TB in these organs of mice challenged with TB in the in vivo humanized immune model of SCID-PBL/hu, (4) augmentation of immune responses, in a cynomologus monkey model which closely mimics human TB disease. It is important to evaluate the survival of monkey [7,8]. Increases in the survival rate of the monkeys treated with this vaccine were observed, compared to the control monkeys treated with saline. Increase in the survival rate of the monkeys treated with HVJ-Envelope/HSP65DNA+IL-12DNA+Ag85B DNA+Ag85A DNA was also strongly observed in the therapeutic models of monkeys(Table 2B). In the recent study, it is demonstrated that granulysin vaccine shows therapeutic efficacy against TB in mice(Table 2B). Therefore, the combination of these therapeutic vaccines might be useful in the future.

MDR-TB and XDR-TB are becoming big problems in the world. About 500,000 new patients with MDR-TB are shown every year. However, the effective drugs against MDR-TB are few.

The HVJ-Envelope/HSP65DNA+IL-12DNA vaccine exerted the therapeutic activity even against XDR-TB, which is resistant to RFP, INH, SM, EB, KM, EVM, TH, PAS, LVFX, PZA and only sensitive to CS. Thus, our results with the HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine in the murine therapeutic model and cynomolgus monkey therapeutic model should provide a significant rationale for moving this vaccine into clinical trial. Furthermore, we have established chronic TB disease model using mouse infected with TB in the aerosol chamber (data not shown). By using this model, therapeutic efficacy of this vaccine was also observed.

Thus, we are taking advantage of the availability of multiple animal models to accumulate essential data on the HVJ-envelope DNA vaccine in anticipation of a phase I clinical trial.

5. Acknowledgements

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6. References

1. **Yoshida S, Tanaka T, Kita Y, et al.** DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation. Vaccine 2006, 24 1191-1204.
2. **Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, Nagasawa T, et al.** Evaluation of a novel vaccine(HVJ-liposome/ HSP65 DNA+IL-12 DNA) against tuberculosis using the cynomologus monkey model of TB. Vaccine 2007; 25(16):2990-3
3. **Okada M, Kita Y.** Tuberculosis vaccine development ; The development of novel (preclinical) DNA vaccine. Human Vaccines 6:3,1-12 2010
4. **Walsh GP, Tan EV, dela Cruz EC, Abalos RM, Villahermosa LG, Young LJ, et al.** The Philippine cynomolgus monkey provides a new nonhuman primate model of tuberculosis that resembles human disease. Nat Med 1996;2(4):430-6.
5. **Kita Y, Tanaka T, Yoshida S, Ohara N, Kaneda Y, Kuwayama S, et al.** Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model. Vaccine 2005;23:2132-2135.
6. **Saeki Y, Matsumoto N, Nakano Y, Mori M, Awai K, Kaneda Y.** Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus). Hum Gene Ther. 1997;8(17):2133-41.
7. **Okada M, Yoshimura N, Kaieda T, Yamamura Y, Kishimoto T.** Establishment and characterization of human T hybrid cells secreting immunoregulatory molecules. Proc Natl Acad Sci U S A. 1981; 78(12):7717-21.
8. **Oada M, Okuno Y, Hashimoto S, Kita Y, Kanamaru N, Nishida Y, et al.** Development of vaccines and passive immunotherapy against SARS corona virus using SCID-PBL/hu mouse models. Vaccine 2007;25:3038-3040.
9. **Tanaka F, Abe M, Akiyoshi T, Nomura T, Sugimachi K, Kishimoto T, et al.** The anti-human tumor effect and generation of human cytotoxic T cells in SCID mice given human peripheral blood lymphocytes by the in vivo transfer of the Interleukin-6 gene using adenovirus vector. Cancer Res 1997;57(7):1335-43.
10. **Flynn JL, Chan J.** Immunology of tuberculosis. Annu Rev Immunol. 2001; 19:93-129.
11. **Huygen K.** DNA vaccines: application to tuberculosis. Int J Tuberc Lung Dis 1998;2(12):971-8.
12. **Lowrie DB.** DNA vaccines against tuberculosis. Curr Opin Mol Ther 1999;1(1):30-3.
13. **Hoft D.** Tuberculosis vaccine development:goals, immunological design, and evaluation. Lancet 2008;372:164-75.

14. **Gupta UD, Katoch VM, McMurray DN.** Current status of TB vaccines. Vaccine. 2007;25:3742-51.
15. **Kaneda Y, Nakajima T, Nishikawa T, Yamamoto S, Ikegami H, Suzuki N et al.** Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. Mol Ther 2002; 6: 219–226.
16. **Kaneda Y.** New vector innovation for drug delivery: development of fusigenic non-viral particles. Curr Drug Targets 2003; 4: 599–602.
17. **Kaneda Y, Yamamoto S, Nakajima T.** Development of HVJ envelope vector and its application to gene therapy. Adv Genet 2005; 53: 307–332.
18. **Ito M, Yamamoto S, Nimura K, Hiraoka K, Tamai K, Kaneda Y.** Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin. J Gene Med 2005; 7: 1044–1052.
19. **Mima H, Yamamoto S, Ito M, Tomoshige R, Tabata Y, Tamai K et al.** Targeted chemotherapy against intraperitoneally disseminated colon carcinoma using a cationized gelatin-conjugated HVJ envelope vector. Mol Cancer Ther 2006; 5: 1021–1028.

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Novel prophylactic and therapeutic vaccine against tuberculosis

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ABSTRACT

We have developed a novel tuberculosis (TB) vaccine; a combination of the DNA vaccines expressing mycobacterial heat shock protein 65 (HSP65) and interleukin 12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-envelope and -liposome (HSP65 + IL-12/HVJ). This vaccine provided therapeutic efficacy as well as remarkable protective efficacy via $CD8^+$ T and $CD4^+$ T cells in murine models compared with the saline controls, on the basis of CFU of number of multi-drug resistant TB (MDR-TB), and survival of extremely drug resistant TB (XDR-TB) challenged mice. Furthermore, we extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis. This vaccine exerted therapeutic efficacy (survival and immune responses) in the TB-infected monkeys. These data indicate that our novel DNA vaccine might be useful against *Mycobacterium tuberculosis* including XDR-TB and MDR-TB for human therapeutic clinical trials.

1. Introduction

Tuberculosis is a major global threat to human health, with about 2 million people dying every year from *Mycobacterium tuberculosis* (TB) infection. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG (BCG), although its efficacy against adult TB disease remains controversial. Furthermore, multi-drug resistant tuberculosis (MDR-TB) and extremely drug resistant TB (XDR-TB) are becoming big problems in the world. In such circumstances, the development of therapeutic vaccine against TB as well as prophylactic vaccine against TB is required. Therefore, we have recently developed a novel TB vaccine, a DNA vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ). This vaccine was 100-fold more efficient than BCG in the murine model on the basis of the elimination of *M. tuberculosis* mediated by the induction of CTL [1,2]. A nonhuman primate model of TB will provide information for vaccine development. In fact, in the previous study we evaluated the protective efficacy of HSP65 + IL-12/HVJ in the cynomolgus monkey model, which is an excellent model of human tuberculosis [1,3]. Furthermore, we observed the synergistic effect of the HSP65 + IL-12/HVJ and BCG using a priming-booster method in the TB-infected cynomolgus monkeys. The combination of the two vaccines showed a very strong prophylactic efficacy against *M. tuberculosis* (100% survival) as we have seen previously in the murine model of TB [4]. In the present study, we evaluated therapeutic effect and prophylactic effect of this vaccine on the MDR-TB infection and XDR-TB infection in murine and monkey models.

2. Materials and methods

DNA vaccines encoding *M. tuberculosis* HSP65 and human IL-12 were encapsulated into HVJ-envelope or HVJ-liposomes [5]. CTL activity was assessed by ^{51}Cr-release [1,6].

At 5 and 10 weeks after intravenous challenge of *M. tuberculosis* H37RV, the number of CFU in the lungs, spleen, and liver were counted and therapeutic efficacy of HVJ-envelope DNA vaccines was evaluated [1]. Therapeutic efficacy was also evaluated by chronic TB infection model of mice using aerosol challenge of TB

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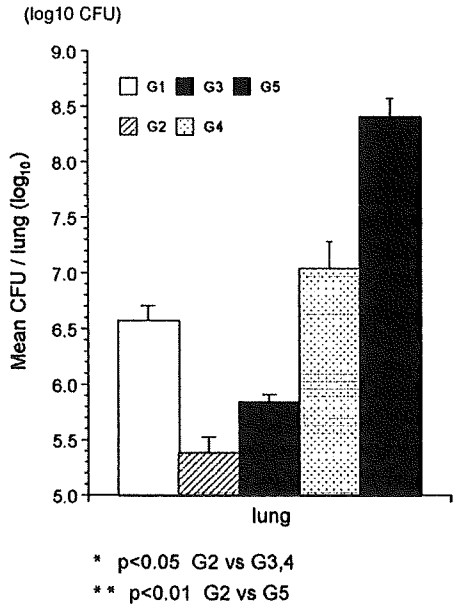

Fig. 1. The *in vivo* necessity of CD8 positive T cells and CD4 positive T cells for prophylactic efficacy of the HVJ-envelope/HSP65 DNA + IL-12 DNA vaccine. Anti-CD8 antibody and/or anti-CD4 antibody were injected i.p. every 5 days after the challenge of TB. BCG was used as a priming vaccine and this DNA vaccine was immunized 2 times (HVJ-envelope/HSP65 DNA 50 μg + IL-12 DNA 50 μg) as booster vaccine. 4 weeks after last immunization, 5×10^5 H37RV were challenged i.v. into mice. G1: without vaccine (□). G2: vaccine (▨). G3: vaccine + anti-CD8 antibody (■). G4: vaccine + anti-CD4 antibody (▩). G5: vaccine + anti-CD8 antibody + anti-CD4 antibody (■) (G2–G3: $P < 0.05$) (G2–G4: $P < 0.05$) (G2–G5: $P < 0.01$).

(15 CFU/mouse: Madison aerosol exposure chamber, University of Wisconsin). 5 weeks after aerosol infection of TB, the vaccine was administered to mice 6 times in 3 weeks.

Cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial Research Center. The animals were vaccinated 9 times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65 + hIL-12/HVJ: 400 μg i.m.), 1 week after the challenge with the *M. tuberculosis* Erdman

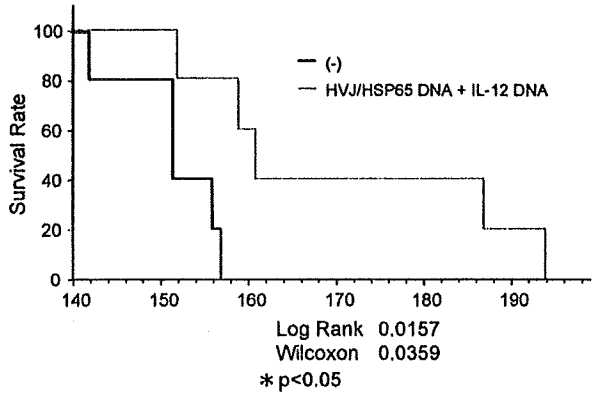

Fig. 2. Therapeutic efficacy of HVJ-envelope/HSP65 DNA + IL-12 DNA vaccine on the extremely drug resistant *Mycobacterium tuberculosis* (XDR-TB). The survival of DBA/1 mice treated with HVJ-envelope/HSP65 DNA (50 μg) + IL-12 DNA vaccine (50 μg) 3 times after 5×10^5 XDR-TB, injection i.v. XDR-TB is resistant to RFP, INH, SM, EB, KM, EVM, TH, PAS, LEFX and PZA. XDR-TB is only sensitive to CS. Survival rate of mice treated with HVJ-envelope/HSP65 DNA + IL-12 DNA (■). Survival rate of control mice without treatment (■). Kaplan–Meier's method (log rank test and Wilcoxon) was used to compare the survival of each group (G1–G2: log rank 0.0157 Wilcoxon 0.0359).

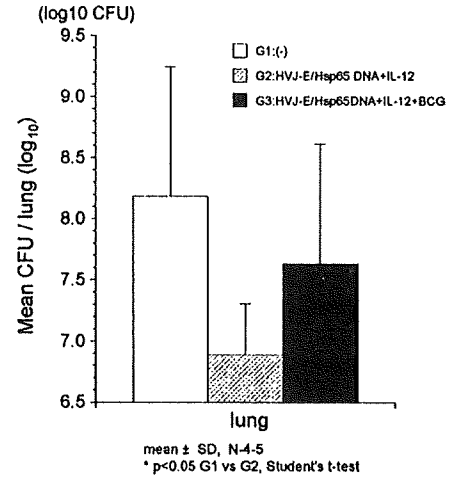

Fig. 3. Therapeutic efficacy of HVJ-envelope/HSP65 DNA + IL-12 DNA vaccine on MDR-TB TNF R gene disrupted DBA/1 mice were treated with HVJ-envelope/HSP65 DNA + IL-12 DNA vaccine 3 times after 5×10^5 MDR-TB injection i.v. CFU of MDR-TB in the lungs of mice, 4 weeks after MDR-TB injection, were assessed as described in Section 2. G1: (−) (□). G2: treated with HVJ-envelope/HSP65 DNA + IL-12 DNA (▨). G3: treated with HVJ-envelope/HSP65 DNA + IL-12 DNA and BCG (■). Student's *t*-test was used to compare the CFU of TB of each group (G1–G2: $P < 0.05$).

strain (5×10^2) by intratracheal instillation. Immune responses and survival were examined as described in our previous studies [2,4].

3. Results

The purpose of this study was to elucidate the therapeutic efficacy of a TB vaccine we have developed in a murine and nonhuman primate TB model [1,3].

The *in vivo* necessity of CD8 positive T cells as well as CD4 positive T cells to exert the prophylactic efficacy of the HVJ-

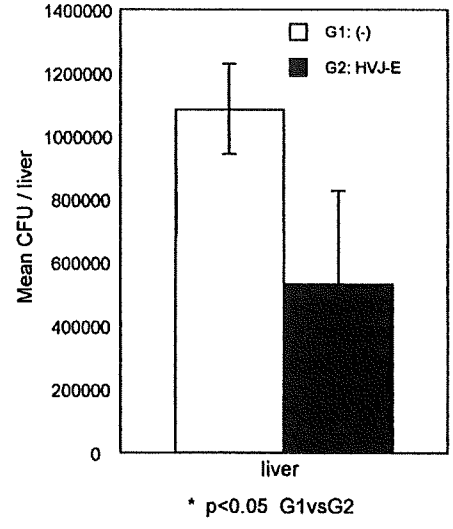

Fig. 4. Therapeutic efficacy of HVJ-envelope/HSP65 DNA + IL-12 DNA, using *in vivo* humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice (SCID-PBL/hu). Groups of animals were treated with 3 times with HVJ-envelope/HSP65 DNA + IL-12 DNA (50 μg i.m.) 10 days after the third vaccination, mice were sacrificed and CFU of TB in the liver of mice were accessed as described in Section 2 (1, 2). 1×10^7 PBL from a healthy human volunteer were injected i.p. into IL-2 receptor γ-chain disrupted NOD-SCID mice. 21 days after injection of PBL, mice were challenges with 5×10^5 H37RV i.v. and then treated with the vaccine. G1: (−) control (□). G2: treated with HVJ-envelope/HSP65 DNA + IL-12 DNA (■). Student's *t*-test was used to compare the CFU of TB of each group (G1–G2: $P < 0.05$).