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Tuberculosis vaccine development
The development of novel (preclinical) DNA vaccine

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Keywords: MDR-TB therapy, monkey, CTL, recombinant DNA

A third of the world's population is infected with *Mycobacterium tuberculosis* and 2 million people die from tuberculosis every year. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG, although its efficacy against adult tuberculosis disease remains controversial. Furthermore multi-drug resistant tuberculosis is becoming big problems in the world. Therefore, the development of novel therapeutic vaccine as well as novel prophylactic vaccine against tuberculosis is required.

This review provides a summary of novel vaccines (especially DNA vaccines) in preclinical stage using mouse, guinea pig and monkey models. In several promising novel vaccines, the studies were extended to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis. The review also provides recent advances of the precise studies of induction of immunity including CD8 positive cytotoxic T cells and effector molecules such as granulysin by these vaccines, against multi-drug resistant tuberculosis and extremely drug resistant tuberculosis.

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 was not effective. 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 as well as prophylactic vaccine against TB is required. TB vaccines were classified into four main vaccines (1) DNA vaccines, (2) recombinant BCG vaccines (3) subunit vaccines and (4) attenuated TB vaccines.

It is well established that protective immunity to *M. tuberculosis* depends on both $CD4^+$ and $CD8^+$ T cells.[1-8] Because DNA vaccination results in the generation of cellular immune responses, including those of a Th-1-type response and protection in animal models of infectious diseases.[9,10] In fact, several human clinical trials have recently been initiated to test the efficacy of DNA vaccines against emerging and re-emerging infectious diseases including hepatitis B,[11] malaria[12,13] and HIV infections.[14] DNA vaccination has also shown potential for the development of tuberculosis vaccines in the mouse model.[15-18] However, in a guinea pig model, which is arguably one of the most biologically relevant systems available for studying human pulmonary tuberculosis, DNA vaccines has not been proven more efficacious than BCG.[19] The efficacy of any experimental tuberculosis vaccine remains to be evaluated in human clinical trials and, thus, a vaccine against tuberculosis is still anxiously awaited.

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Vaccines for TB (DNA Vaccines)

DNA vaccines are a relatively new approach to immunization for infectious diseases.[1,2,15,16,20-23] Many gene therapies, including DNA vaccines, have been used for the treatment of several kinds of cancers for more than twenty years via the activation of $CD8^+$ cytotoxic T cells.[24-26] Plasmids containing genes have also been used to induce protection against a variety of bacterial, viral (such as SARS corona virus), protozoal and helminth infection in animal models.[27,28] DNA vaccines by the use of several kinds of vectors including HVJ-envelope, HVJ-liposome, adenovirus vector and AAV vector were established.

DNA vaccines can induce strong cellular immunity against TB and can invoke both CD4 and CD8 T-cell responses.[2,29] The first data showing significant protection against *M. tuberculosis* came from naked DNA immunization in mice. Several mycobacterial antigens delivered as naked DNA have elicited protection against *M. tuberculosis*. Hsp65 antigens (derived from H37Rv *M. tuberculosis*)-, Ag 85B-, Ag 85A-, M.tb8.4-, M.tb41-, PPT39-, MPT51-, MPT63-, MPT64-, MPT83-, ESAT-6-, Pst-3- and the 38 kDA lipoprotein-DNA vaccines were studied (**Table 1**).[1,20,30-44]

Ag85A antigen has been used to prime immune responses followed by homologous or heterologous boosting vaccines in mice. Protection as defined by a reduction in the numbers of bacteria recovered from the lungs was observed. Antigen Ag85B, an abundant 30 kDa secreted protein of *M. tuberculosis*, has shown protection in mice as well as in guinea pigs against TB.[34] Similarly DNA vaccines encoding secreted proteins Ag85B and MPT64 have also been reported to protect mice from *M. tuberculosis* H37Rv challenge by prompting a Th1 response. The effectiveness of DNA vaccines can be increased by codelivery of multiple DNA plasmids or chimeric DNA vaccines.[45-47] Derrick et al. used a DNA vaccine cocktail consisting of Ag85B, ESAT.

Table 1. Mycobacterial antigens and cytokines delivered as DNA vaccine

DNA Vaccines
HSP65
IL-12
Ag 85A
Ag 85B
MPT51
HSP70
ESAT-6
IL-6 + IL-6 Receptor + gp130
γIFN
Mtb8.4
Mtb41
Mtb39
MPT63
MPT64
Psts-3
38 kDa lipoprotein
MPT12
IL-15
IL-23
IL-27

KatG, MPT8.4, MPT12, MPT63, MPT64 and MPT83 to reduce the bacterial burden in the lungs of normal mice as well as mice lacking CD4 T cells after aerosol challenge.

DNA vaccines are safe, cheap, stable and effective for inducing cellular immunity (helper T cell, cytotoxic T cell, macrophages) and humoral immunity in preclinical models of infectious diseases.[48-50] These vaccines are also able to activate innate immune responses. They interact with Toll-like receptor 9 (TLR 9: the pattern recognition receptor) through unmethylated CpG oligodeoxynucleotides (CpG ODNs9),[51,52] resulting in the upregulation of cytokines gene expression through MyD88 molecule.[53-56]

DNA vaccines have been in general proved to be safe and well tolerated in preclinical and clinical studies.[57,58] The naked plasmid DNA molecules have not caused any adverse effects on the biochemical and hematological blood values and have caused neither detectable organ pathology nor systemic toxicity. Furthermore, there has been no evidence of autoimmunity, development of anti-nuclear or anti-DNA antibodies, or plasmid DNA integration into chromosomes.

TB Vaccine Strategies

Pre-infection vaccination strategy. From a public health perspective, delivering a vaccine prior to mycobacterial infection and soon after birth makes most sense.

Booster vaccination strategy (prophylactic). A second option would be to use a new TB vaccine as a booster sometime after neonatal BCG vaccination.

Table 2. Vectors for DNA vaccines against tuberculosis

Types	Characteristics
(1) HVJ-Envelope	Very Good Expression (GMP Level)
(2) HVJ-liposome	Good Expression
(3) Adenovirus	Good Expression. Transient
(4) Adeno Assosiated Virus (AAV) Vector	AAV 2/5 Good Expression. Long Term
(5) Lentivirus	Non-proliferating cell
(6) Liposome	safety
(7) Sendai virus Vector	Good Expression
(8) Gene Gun	safety
(9) Vaccinia Virus (Attenuated: MVA)	
(10) BCG (recombinant BCG)	
(11) Attenuated Listeria	

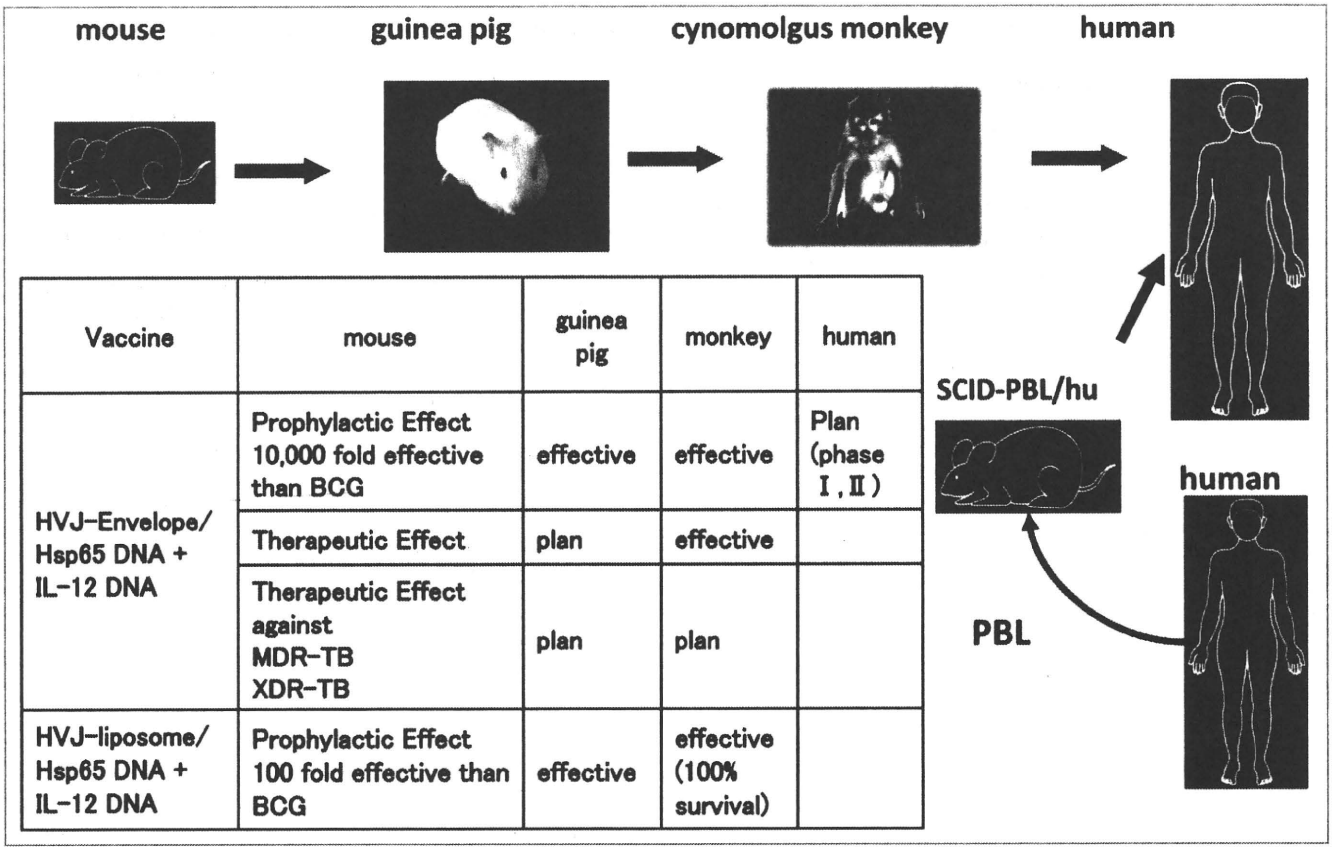
Post-infection vaccine strategy. A third option is to prevent disease by enhancing or boosting immunity in persons already infected a post-infection vaccine strategy. This approach is attractive because more than 2 billion persons worldwide are already infected and therefore at risk of progression to disease.

Therapeutic vaccine. A fourth option would be to use a vaccine as an adjunct to anti-TB treatment, to shorten therapy or reduce the risk of relapse, a therapeutic vaccine. This may be particularly relevant in situations where multi-drug resistant TB cases are common.

Vector and Adjuvant

Prophylactic and therapeutic DNA vaccines were established by using several kinds of vectors such as HVJ-liposome, HVJ-envelope, adenovirus vector, adeno-associated virus vector (AAV), lenti-virus vector, vaccinia-virus vector, poliovirus vector, BCG, attenuate Listeria[1,2,20,24,59-61] (**Table 2**).

MPL + QS21 + Squallene (AS101), or cationic liposome dimethyl dioctadecyl ammonium bromide (DDA) is promising adjuvant for TB vaccines.[62] Lipopolysaccharide (LPS), a component of bacterial cell walls, is driven by the adaptor proteins myeloid differentiation factor 88 (MyD88) and Toll-interleukin 1 receptor domain-containing adapter inducing interferon-β (TRIF), which together mediate signaling by the endotoxin receptor Toll-like receptor 4 (TLR4). Monophosphoryl lipid A (MPLA) is a low-toxicity derivative of LPS with useful immunostimulatory properties, which is nearing regulatory approval for use as a human vaccine adjuvant. Mata-Havo et al. reported that, in mice, the low toxicity of MPLA's adjuvant function is associated with a bias toward TRIF signaling which is likely caused by the active suppression, rather than passive loss, of proinflammatory activity of this LPS derivative. This finding



Vaccine	mouse	guinea pig	monkey	human
HVJ-Envelope/ Hsp65 DNA + IL-12 DNA	Prophylactic Effect 10,000 fold effective than BCG	effective	effective	Plan (phase I, II)
	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)	

Figure 1. The development of novel vaccines for *M. tuberculosis* using animal models.

may have important implication for the development of future vaccine adjuvants.[63]

Animal Model

Murine models are usually used for the first screening of novel TB vaccines, because (1) genetic background of mice is homogeneous, (2) only small spaces are required for the experiments (3) we can use TB-resistant C57BL/6 mice and TB sensitive BALB/C mice.[1,2]

Guinea pig models are also used for the evaluation of efficacy of novel TB vaccines. Guinea pig is sensitive to TB and makes granulomatous TB lesions in the lungs.[19]

Cynomolgus monkey model is the best animal TB model as reported by Walsh and E.V. Tan in Leonard Wood Memorial Institute. TB infection in the cynomolgus monkeys is very similar to human TB disease[1,20,21,64] (**Fig. 1**). Monkeys make caseous necrosis in TB granulomas in the lungs as human make caseous necrosis.

HVJ-Envelope/HSP65 DNA + IL-12 DNA Vaccine

DNA vaccines against TB using murine models. *Prophylactic DNA vaccines.* We investigated the immunogenicity and protective efficacy of DNA vaccine combinations expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) using gene gun bombardment and the hemagglutinating virus of Japan (HVJ)-liposome method.[65] A mouse IL-12 expression vector (mIL-12 DNA) encoding single-chain IL-12 proteins comprised of p40 and p35 subunits were constructed. In a mouse model, a single gene gun vaccination with the combination of Hsp65 DNA and mIL-12 DNA provided a remarkably high degree of protection against challenge with virulent *M. tuberculosis*; bacterial numbers were 100-fold lower in the lungs compared to BCG-vaccinated mice. To explore the clinical use of the DNA vaccines, we evaluated HVJ-liposome encapsulated Hsp65 DNA and mIL-12DNA (Hsp65 + mIL-12/HVJ). The HVJ-liposome method improved the protective efficacy of the Hsp65 DNA vaccine compared to gene gun vaccination. Hsp65 + mIL-12/HVJ induced $CD8^+$ cytotoxic T lymphocyte activity against Hsp65 antigen.[66-70] Most importantly, Hsp65 + mIL-12/HVJ vaccination resulted in a greater degree of protection than that evoked by BCG. This protective efficacy was associated with the emergence of IFNγ-secreting T cells and activation of proliferative T cells and cytokines (IFNγ and IL-2) production upon stimulation with Hsp65 and antigens from *M. tuberculosis* (**Fig. 2**). These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to BCG vaccine.[2]

The in vivo necessity of CD8 positive T cells as well as CD4 positive T cells to exert the prophylactic efficacy of the HVJ-envelope/HSP65 DNA + IL-12 DNA vaccine was demonstrated in mice.

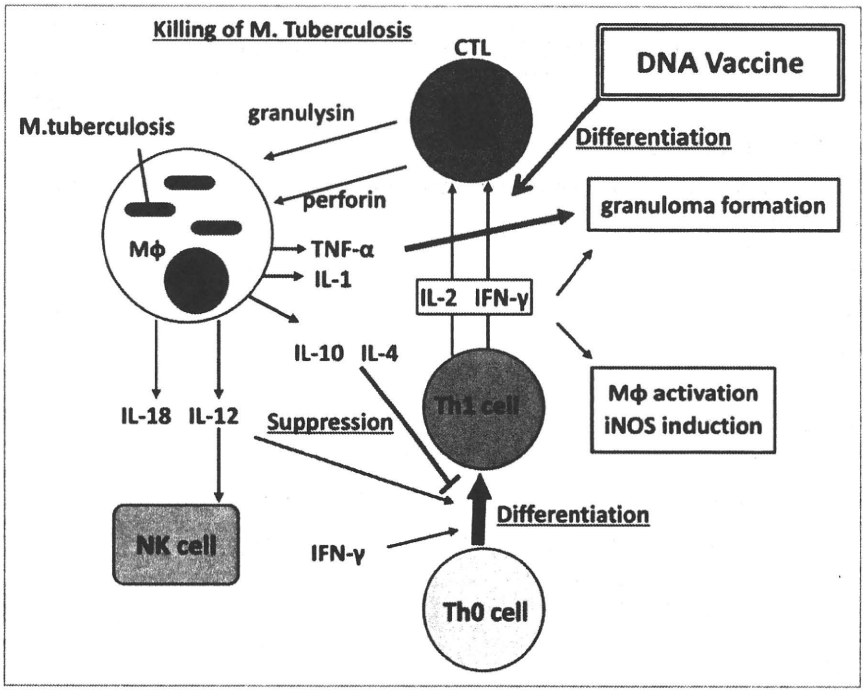


Figure 2. Induction of CTL and prophylactic effect by DNA vaccines against *Mycobacterium tuberculosis*.

Furthermore, by using BCG priming-DNA vaccine booster method, *M. tuberculosis* numbers in the lungs of DNA vaccinated mice were 10,000 (ten thousand) lower compared to BCG alone vaccinated mice (**Fig. 1**).

Therapeutic DNA vaccines. 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.

Figure 1 shows the therapeutic efficacy of HVJ-Envelope/Hsp65DNA + IL-12DNA vaccine against XDR-TB (extremely drug resistant TB).[1] Mice treated with this DNA vaccine prolonged the survival periods significantly by statistical analysis. The 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.

This DNA 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 (**Fig. 1**).

Furthermore, we have established chronic TB disease model using mouse infected with TB in the aerosol chamber (data not shown).[1] By using this model, therapeutic efficacy of this vaccine was also observed.

DNA vaccines against TB using guinea pig models. 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) (**Fig. 1**).

DNA vaccines against TB using cynomolgus monkey models. *Prophylactic DNA vaccines.* We have developed two novel TB vaccines: HSP65 + IL-12/HVJ and a recombinant BCG harboring the 72f fusion gene (72f rBCG).[21,71] We extended our studies to a cynomolgus monkey model,[64] which is currently the best animal model of human tuberculosis, to evaluate the HSP65 + IL-12/HVJ and 72f rBCG vaccines. Vaccination with HSP65 + IL-12/HVJ as well as 72f rBCG vaccines provided better protective efficacy as assessed by the Erythrocyte Sedimentation Rate, chest X-ray findings and immune responses than BCG. Most importantly, HSP65 + IL-12/HVJ resulted in an increased survival for over a year. This is the first report of successful DNA vaccination and recombinant BCG vaccination against *M. tuberculosis* in the monkey model which closely mimics human TB disease[21] (**Fig. 3**).

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 with TB is usually accompanied by progress of TB disease.

Furthermore, 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. 3**).[20] In contrast, only 2 monkeys out of 6 from the BCG Tokyo alone group were alive (33% survival).[20] 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. Furthermore, IFNγ production and proliferation of PBL from monkeys vaccinated with these vaccines were strongly enhanced. Taken together, these results clearly demonstrated that

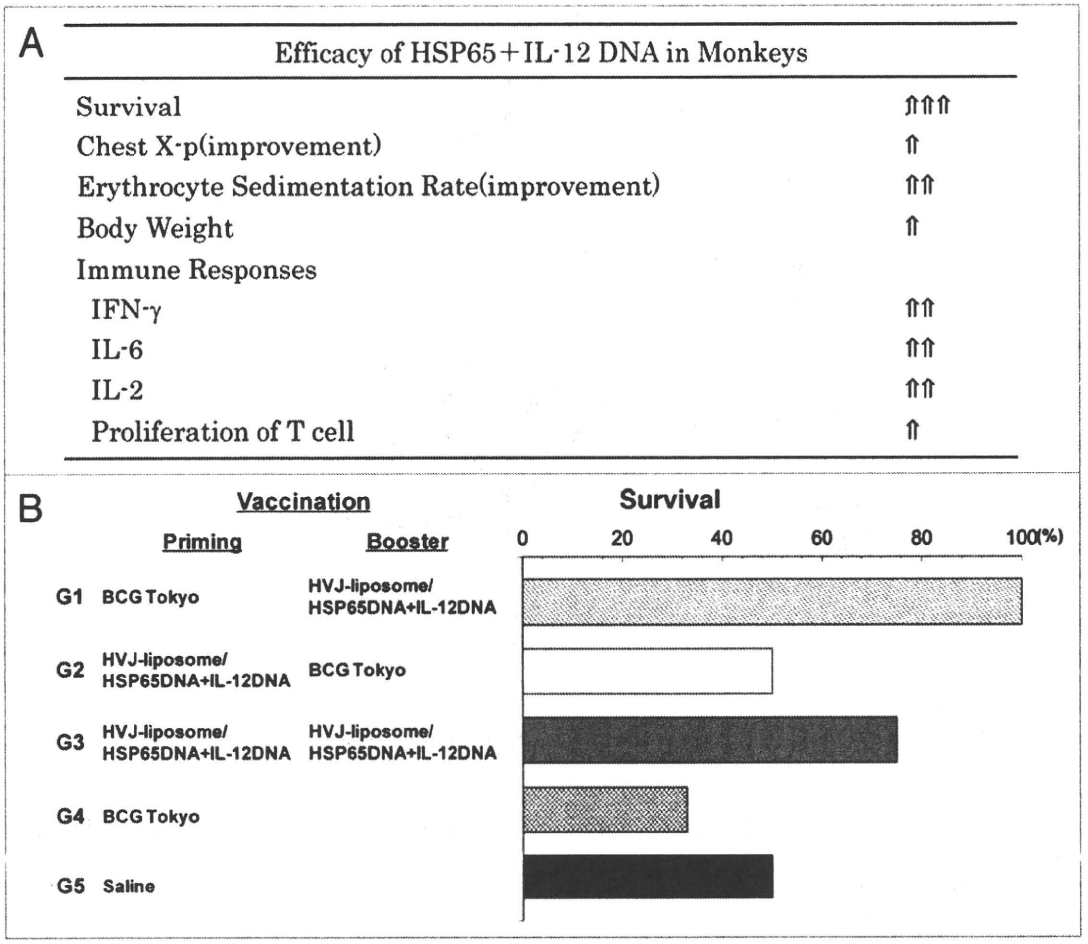


Figure 3. (A) Efficacy of HSP65 + IL-12 DNA in monkeys. (B) Protective efficacy (survival) of HSP65 + IL-12/HVJ and BCG using priming-booster method against TB challenged cynomolgus monkey 350 days after TB infection.

BCG priming and the HSP65 + hIL-12/HVJ booster could provide extremely strong protective efficacy against *M. tuberculosis* in the cynomolgus monkey model.[20]

In Japan and other countries, the BCG vaccine is inoculated into human infants (0–6 months after birth). Therefore, BCG priming in infants and HSP65 + hIL-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.

Our results with the HSP65 + hIL-12/HVJ vaccine in the cynomolgus monkey model should provide a significant rationale for moving this vaccine into clinical trials. In fact, the 72f fusion protein vaccine entered Phase I testing after its evaluation in cynomolgus monkeys in Leonard Wood Memorial[64] by Reed and Skeiky.

Therapeutic DNA vaccines. Furthermore, the therapeutic activity of this vaccine was evaluated in a nonhuman primate model infected with *M. tuberculosis*.[1]

Figure 4 shows the results of immune responses of cynomolgus monkey at 11 weeks after challenge of *M. tuberculosis* Erdman strain (5 x 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 (**Fig.** 4B). This vaccine also improved the survival of monkeys, compared to the saline (control) group after TB challenge (**Fig.** 4C).[1]

This vaccine exerted a significant therapeutic effect against TB, as indicated by augmentation of survival and immune responses, in a cynomolgus monkey model. It is important to evaluate the survival of monkey.[20,21]

Thus, our results with this DNA vaccine in the murine therapeutic model and cynomolgus monkey therapeutic model should provide a significant rationale for moving this vaccine into clinical trial.

Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig and monkey) (**Fig.** 1) to accumulate essential data on the HVJ-envelope DNA vaccine in anticipation of a phase I clinical trial.

DNA vaccines against TB using SCID-PBL/hu model. 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).[21,24,59] This DNA vaccine resulted in significantly therapeutic activity even in SCID-PBL/hu mice which exerted human T-cell immune responses (**Fig.** 1).

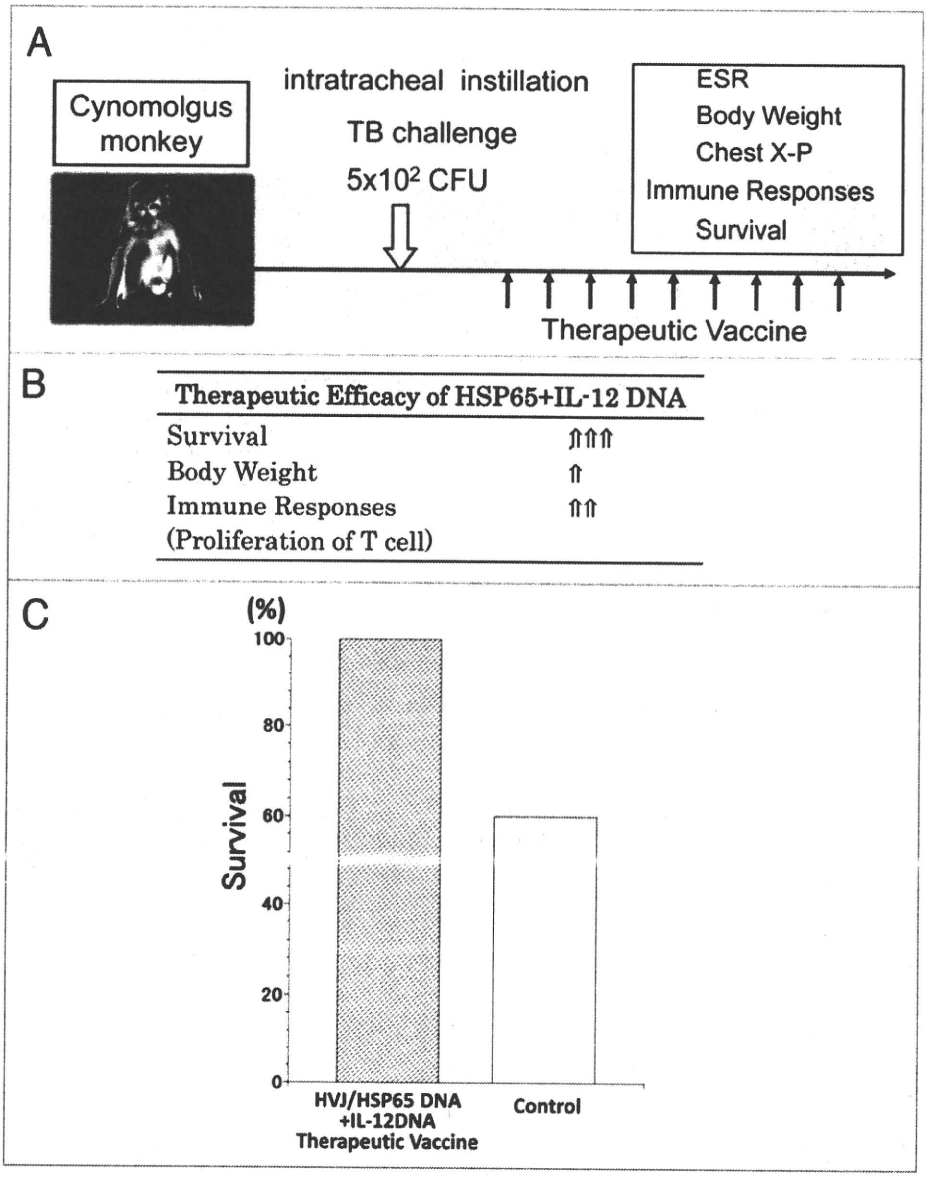


Figure 4. (A) Therapeutic effect of HVJ-Envelope/HSP65DNA + IL-12DNA vaccine on TB-infected cynomolgus monkeys. (B) Therapeutic efficacy of HSP65 + IL-12DNA. (C) Therapeutic efficacy (survival) of HSP65 + IL-12 DNA vaccine 130 days after TB infection.

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 six 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] Six 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]

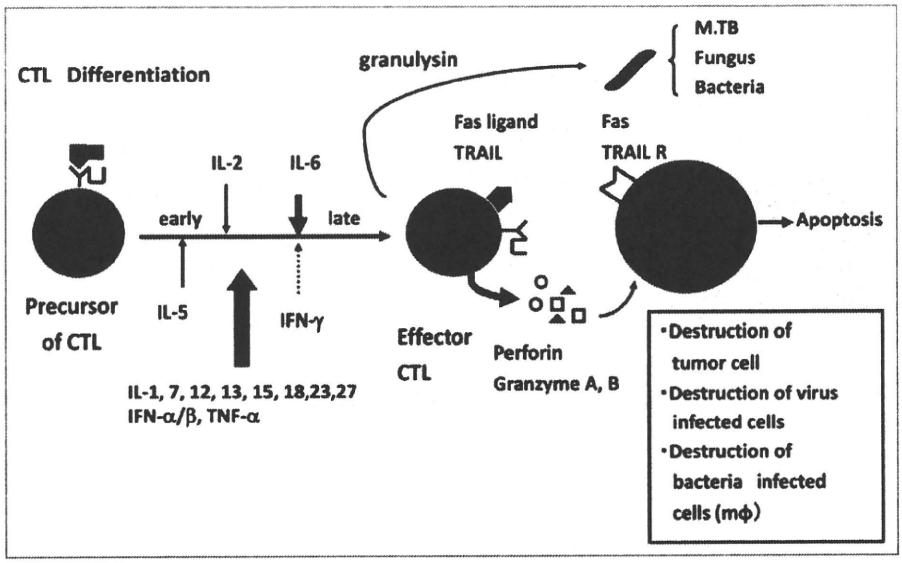


Figure 5. Induction of cytotoxic T cells and killing mechanism.

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

Function					Granulysin expression in CD8⁺T	
Two Kinds of Granulysin	**Decrease in TB number**	**Induction of CTL against TB**	**Proliferation of T cells against TB**	**IFNγ production**	**Patients with MDR-TB**	**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 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]

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

A) Priming, Pre-Exposure	
(1) Phase I: 2008	Vaccine Type
(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

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, two 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 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]

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

(B) BOOSTING, PRE-Exposure	
(1) Phase I: 2008	Vaccine Type
(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) PP1, 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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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. 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 response 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.

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Development of therapeutic and prophylactic vaccine against tuberculosis using monkey and transgenic mice models

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Key words: monkey, prime-boost method, HVJ-envelope/HSP65DNA + IL-12DNA, *Mycobacterium tuberculosis*, vaccine, 15 KDa granulyin, granulysin transgenic mouse, patients with tuberculosis, prophylactic efficacy, SCID-PBL/hu

Abbreviations: HVJ, hemagglutinating virus of Japan; Tg, transgenic; MDR-TB, multi-drug resistant tuberculosis; 15 K granulysin, 15 kilodalton granulysin; 9 K granulysin, 9 kilodalton granulysin; PBL, peripheral blood lymphocyte; ESR, erythrocyte sedimentation rate

Purpose: BCG is not efficacious against *M. tuberculosis* (TB) in adult. Therefore, novel TB vaccines were established by using three kinds of animal models (cynomolgus monkey model which is the best animal model of human TB, IL-2R knock out SCID mice as a human immune model and granulysin transgenic mouse).

Methods and Results: DNA vaccine expressing TB Hsp65 and IL-12 was delivered by the hemagglutinating virus of Japan (HVJ)-envelope. The BCG prime followed by Hsp65 + IL-12/HVJ vaccine boost showed a synergistic effect in the TB-infected cynomolgus monkey (100% survival). In contrast, 33% of monkeys were alive in BCG alone group. Furthermore, the prolongation of survival period of the monkey was observed by the combination of BCG and DNA vaccine even when the boost was performed after long-term period (4 month) from prime. This combination also improved the erythrocyte sedimentation rate (ESR), increased the body weight and augmented the proliferation of PBL and IL-12 production at higher levels than BCG alone or saline. Furthermore, this vaccine exerted therapeutic efficacy in IL-2R knock out SCID-PBL/hu mice, which were transplanted with human T cells. Granulysin is an important defensive molecule expressed by human T cells and NK cells and has a cytolytic activity against microbes including *Mycobacterium tuberculosis* (TB) and tumors. Expression of 15 kD (15 K) granulysin protein and mRNA in CD8 positive T cells in the patients infected with drug sensitive (TB) or multi-drug resistant M. tuber*culosis (MDR-TB)* were lower than that in the healthy volunteers, suggesting that granulysin treatment might improve the tuberculous disease in human. Therefore, we established two kinds of granulysin transgenic mice (15 K granulysin transgenic mice and 9 K granulysin transgenic mice). It was demonstrated that 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB effect, including the decrease of the number of TB and augmentation of the CTL activity. These are the first findings which demonstrate in vivo effects of 15 K granulysin and 9 K granulysin against TB infection. Moreover, DNA vaccine expressing 15 K granulysin showed a therapeutic activity against TB in mice.

Conclusion: These data indicate that monkey, IL-2R gene-knock out SCID-PBL/hu and granulysin transgenic mice models provide useful tools for the development of novel vaccines (HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine and granulysin vaccine) against TB.

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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 as well as prophylactic vaccines against TB is required.

Cynomolgus monkey model is the best animal TB model as reported by Walsh and Tan.[1] TB infection in the cynomolgus monkey is very similar to human TB disease.[1-3] In the present study, the long term prime-boost period prophylactic efficacy of

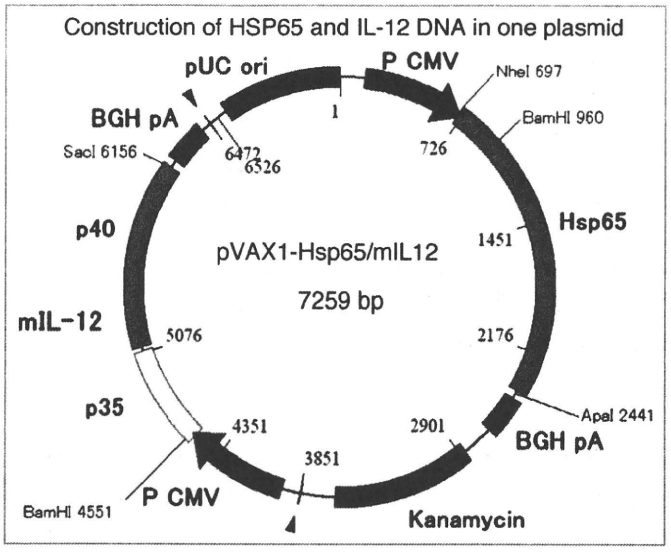

Figure 1. Construction of DNA vaccine for clinical trial. HVJ-Envelope/HSP65DNA + IL-12DNA vaccine was constructed for GMP-level-vaccine which contains two kinds of DNA in one plasmid vector (pVAX1) for clinical prophylactic trial.

vaccine was investigated using monkey models. In vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice constructed with human PBL (SCID-PBL/hu) provide a useful tool for investigating human immune responses activated by vaccine.[4,5] Transgenic mice which contain the components of vaccine also provide a lot of information about novel TB vaccines. Therefore, using cynomolgus monkey model, SCID-PBL/hu mice model and transgenic mice model, we have developed several kinds of novel vaccines against TB.

Granulysin, a member of the saposin-like protein family, colocalizes with perforin and granzymes in the cytolytic granules of human CTL and NK cells, has cytotoxic activity against intracellular pathogens in infected cells in the presence of perforin and has a cytotoxic effect against tumor cells.[6-8] The granulysin is expressed in human CD8 positive cytotoxic T cells and NK cells. 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.[7] However, the precise role of granulysin in the in vivo defense for the tuberculosis infection has not been elucidated yet. Therefore, we have established 15 K and 9 K granulysin transgenic mice to elucidate in vivo role of granulysin and to develop novel vaccines against the infection of *M. tuberculosis*. These 15 K granulysin transgenic mice and 9 K granulysin transgenic mice showed in vivo anti TB effect. This is the first demonstration of an in vivo action of granulysin for TB using granulysin transgenic mice. We have also developed novel TB vaccine of HVJ-Envelope/HSP65 DNA + IL-12 DNA.[3,9,10] Therefore, these findings suggest that granulysin or granulysin DNA may be useful as a TB vaccine, in the combination of other DNA vaccine.

Results

Cynomolgus monkey model. The prophylactic efficacy of HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine against TB was

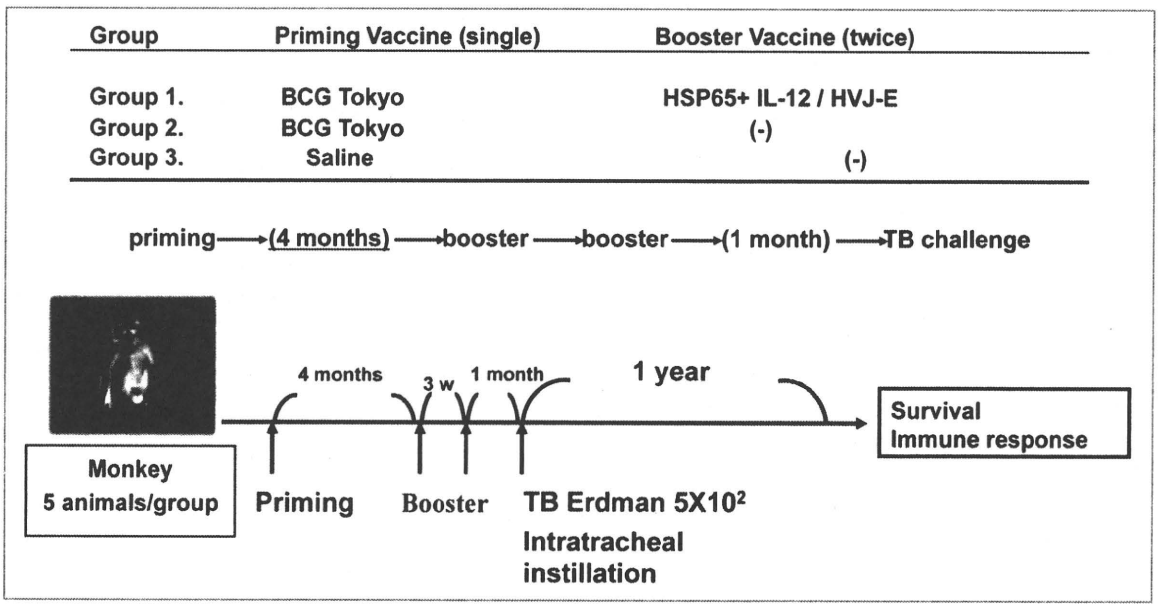

Figure 2. Evaluation of prophylactic efficacy of HVJ-Envelop/HSP65DNA + IL-12DNA vaccine on the infection of cynomolgus monkeys. Protective efficacy of HSP65 + IL-12/HVJ and BCG using prime-boost method against TB challenged cynomolgus monkeys. Group of animals were vaccinated three times (every 3 weeks) with (1°) BCG Tokyo, (2°) HSP65 + IL-12/HVJ, (3°) HSP65 + IL-12/HVJ = G_1. BCG prime-HVJ/DNA boost group; (1°) BCG, (2°) saline, (3°) saline = G_2. G_2 group animals were vaccinated with BCG once; (1°) saline, (2°) saline, (3°) saline = G_3. 4 month after the prime BCG vaccine, 2° vaccine was immunized. 3 weeks after the 2° vaccine, 3° vaccine was treated. One month after the third vaccination, monkeys were challenged with the *M. tuberculosis* (5 x 10^2 CFU) by intratracheal instillation. Prophylactic efficacy was evaluated by survival periods, erythrocyte sedimentation rate (ESR), body weight, chest X-rays, immune responses and DTH reaction against PPD for 16 months.

investigated using GMP-level-vaccine which contains two kinds of DNA in one plasmid vector for clinical trial (**Fig. 1**).

Long-term interval model (4 months) between prime and boost vaccinations was used in this study prior to intratracheal instillation of the challenge dose (**Fig. 2**).

In Group1 (BCG prime—DNA vaccine boost) monkeys, the regimen of vaccines improved ESR, compared to the regimen of Group3 (saline control group) or that of Group2 (BCG alone control group) (**Fig. 3**).

This vaccination method (BCG prime—DNA vaccine boost) also increased the body weight of 4 TB-infected monkeys out of 5 in Group1 as shown in **Figure 4**. In contrast, 2 monkeys in Group3 (saline) or 2 monkeys in Group2 (BCG alone) showed the decrease in body weight after the infection of TB.

The proliferation of PBL from monkeys in the base-line period was almost same among these G_1, G_2 and G_3 groups. However, proliferation of PBL from monkeys in G_1 group, (BCG prime-DNA vaccine boost group), was higher than those in G_2 (BCG alone group) and G_3 (saline control group) at 4 weeks after third vaccinations (G_1–G_3: $p < 0.05$) (**Fig. 5**).

Furthermore, IFNγ production from PBL in G_1 group (BCG prime-DNA vaccine boost group) was higher than those in G_2 (BCG alone group) and G_3 (saline control group) (data not shown).

By using long-term prime-boost method and vector containing two kinds of genes in one plasmid, the most reproducible and prophylactic efficacy based on the prolongation of survival was observed in Group1 monkeys (BCG prime-DNA boost, **Fig. 6**). The combination of BCG prime and DNA vaccine boost improved the survival (100% at 230 days and 80% at 360 days after TB challenge, respectively). In contrast, BCG vaccine alone in G_2 group monkeys showed 60% survival at 355 days and 40% survival at 360 days. The treatment of saline (G_3) showed 50% survival at 360 days.

Thus, even using the experimental model of long-term interval (4 months interval) between prime period and boost period, we could observe the prophylactic efficacy of this BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine in monkeys.

Transgenic mice model. The granulysin expression in the $CD3^+CD8^+CD4^-$ PBL-T cells of the patients with drug sensitive TB and MDR-TB was significantly lower than that of normal volunteer (data not shown).

We also analyzed the 15 K granulysin in the culture supernatants of PBL from patients with MDR-TB and healthy volunteer. The amounts of 15 K granulysin were measured after the stimulation with PPD, Hsp65 protein and killed TB H37Ra antigen. The production of 15 K granulysin was suppressed in the culture supernatants of PBL from patients with MDR-TB, compared to that from healthy volunteer (data not shown). Thus, it was suggested that granulysin treatment might improve the tuberculosis disease in human.

Therefore, to elucidate the in vivo mechanism of granulysin, we have established granulysin transgenic mice. We established eleven distinct transgenic mice including 15 K granulysin transgenic mice and 9 K granulysin transgenic mice. We confirmed the expression of mRNAs and proteins of 15 K granulysin and

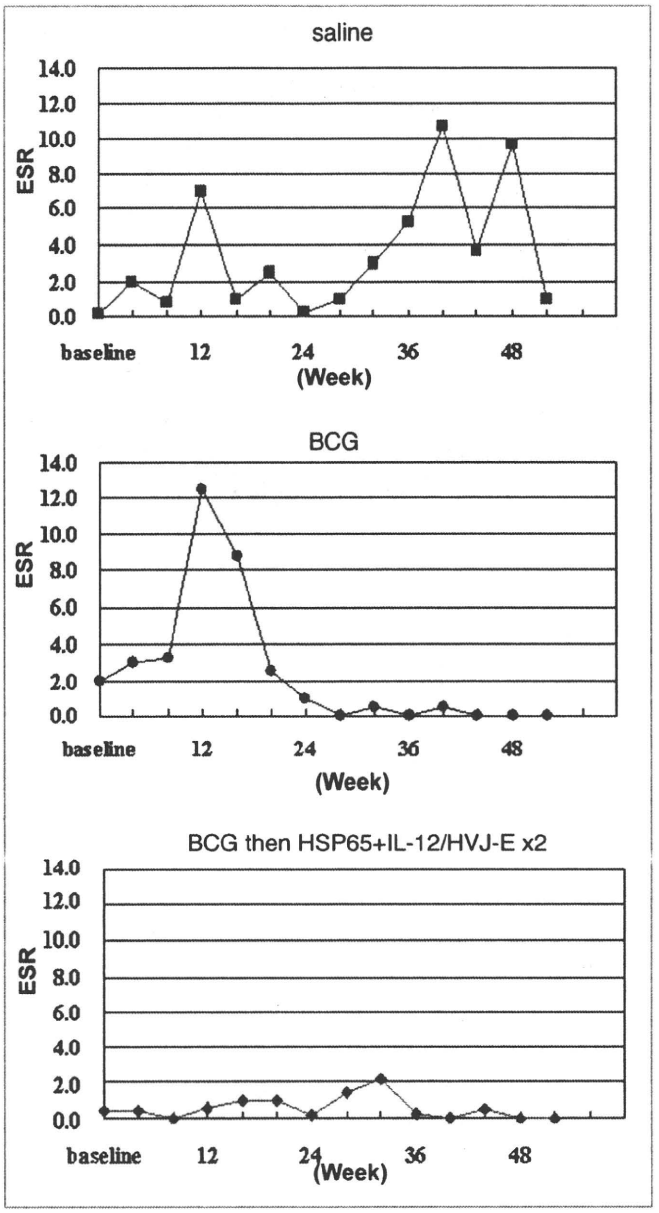

Figure 3. Improvement of erythrocyte sedimentation rate (ESR) in the cynomolgus monkeys immunized with BCG prime-HVJ-envelope/HSP65DNA + IL-12DNA boost vaccine. Cynomolgus monkeys were immunized and challenged as described in Figure 2. ESR of all monkeys was evaluated every month and mean values of ESR of 5 monkeys were shown.

9 K granulysin in established transgenic mice, respectively (data not shown). 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB effects, in vivo induction of cytotoxic T cells specific for TB, proliferation of T cells after the stimulation with TB antigens and augmentation of cytokine production.

As shown in **Figure 7**, in vivo anti-TB efficacy of 15 K granulysin transgenic mouse was observed.

CFU of *M. tuberculosis* was decreased at 4 weeks after the intravenous injection of 5×10^5 TB in the lungs of 15 K granulysin transgenic mice compared to those of wild type mice and

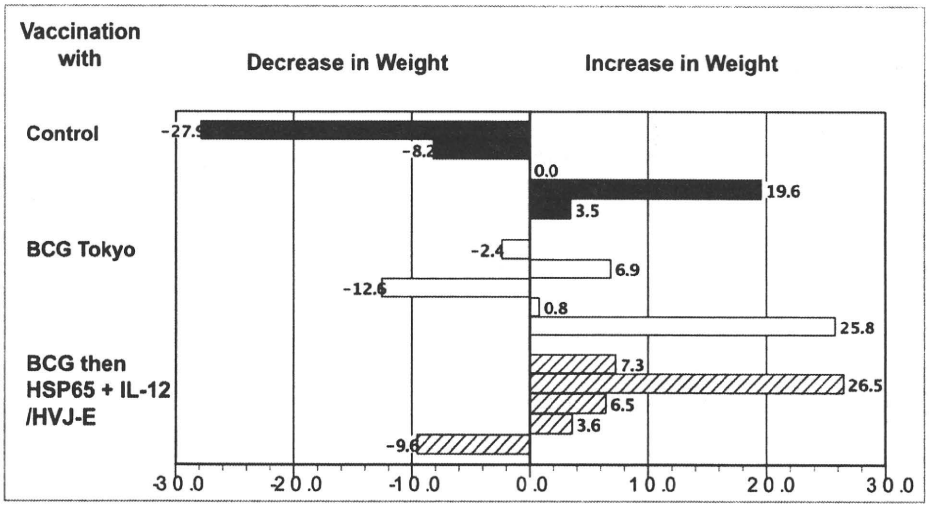

Figure 4. The increase in the body weight of monkeys vaccinated with HVJ-Envelope/Hsp65 DNA + IL-12 DNA. Monkeys vaccinated with BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine boost were challenged as described in Figure 2. Body weight of all monkeys was evaluated every month and values of body weight of monkeys at 16 weeks after TB challenge were shown.

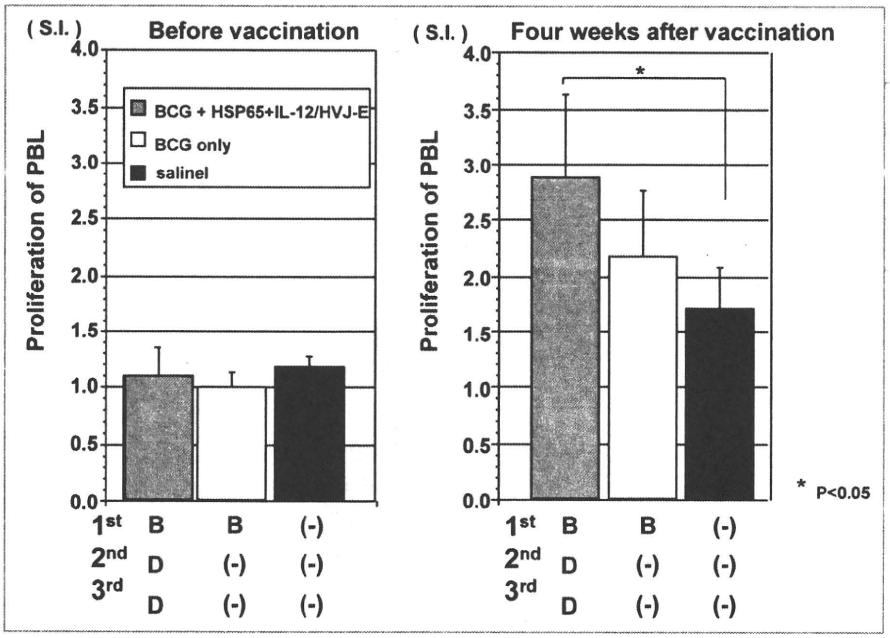

Figure 5. Augmentation of the proliferation of PBL in the monkeys immunized with BCG prime—HVJ-Envelope/Hsp65 DNA + IL-12 DNA boost vaccine. The proliferation of PBL (base line: before vaccination) from monkeys and PBL from monkeys vaccinated and challenged as described in Figure 2 were shown. Stimulation Index (S.I) of the ^{3}H-TdR uptake of monkey PBL at 11 weeks after TB challenge were shown. Student's t test were used to compare T cell proliferation between groups (p-value: G_1–G_3 $p < 0.05$).

normal C57BL/6 mice ($p < 0.05$). Furthermore, CFU of TB in the lungs of 9 K granulysin transgenic #1 mice and 9 K granulysin transgenic #17 mice were also decreased at 4 weeks after TB injection compared to that of wild type mice ($p < 0.05$) (Fig. 8). Thus, 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB efficacy and decreased the number of TB in the lungs.

SCID-PBL/hu model. We have very important and interesting SCID-PBL/hu models capable of analyzing in vivo human T cell immune responses and evaluating the efficacy of novel vaccines against TB, as reported first in Cancer Research 1997.

We used IL-2 receptor γ-chain gene knock out SCID-PBL/hu mice to analyze human immune responses.

Now, the therapeutic effects of HSP65 + IL-12 DNA vaccine in G_3 group (50 μg i.m.) on TB infection is observed in this IL-2 receptor γ-chain gene disrupted SCID-PBL/hu-model ($p < 0.05$) (**Table 1**). Human CTL activity against TB was associated with the efficacy of TB vaccine (data not shown).

Taken together, using cynomolgus monkey model, SCID-PBL/hu mice model and granulysin transgenic mice model, we have developed two kinds of novel vaccines, HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine and granulysin vaccine, against TB.

Discussion

In the present study, using cynomolgus monkey model, SCID-PBL/hu mice model and granulysin transgenic mice model, we have developed two kinds of novel vaccines, HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine and granulysin vaccine, against TB.

Most importantly, we demonstrated that even when the boost was performed after long-term period (4 month) from prime, the prolongation of monkey survival was observed by the combination of BCG and this HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine in the present study. This combination improved the erythrocyte sedimentation rate (ESR), increased the body weight and augmented the proliferation of PBL and IFNγ production, more than BCG alone or saline.

In the mouse system, by using BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine boost method, the number of *M. Tuberculosis* in the lungs of DNA-vaccinated mice were 10,000 (ten thousand) times lower compared to BCG alone vaccinated mice in our study (data not shown).

In parallel with the protective effect of HVJ-Envelope/DNA vaccines + BCG vaccine using prime-boost method on bacterial loads, histopathological analysis shows that mice vaccinated with

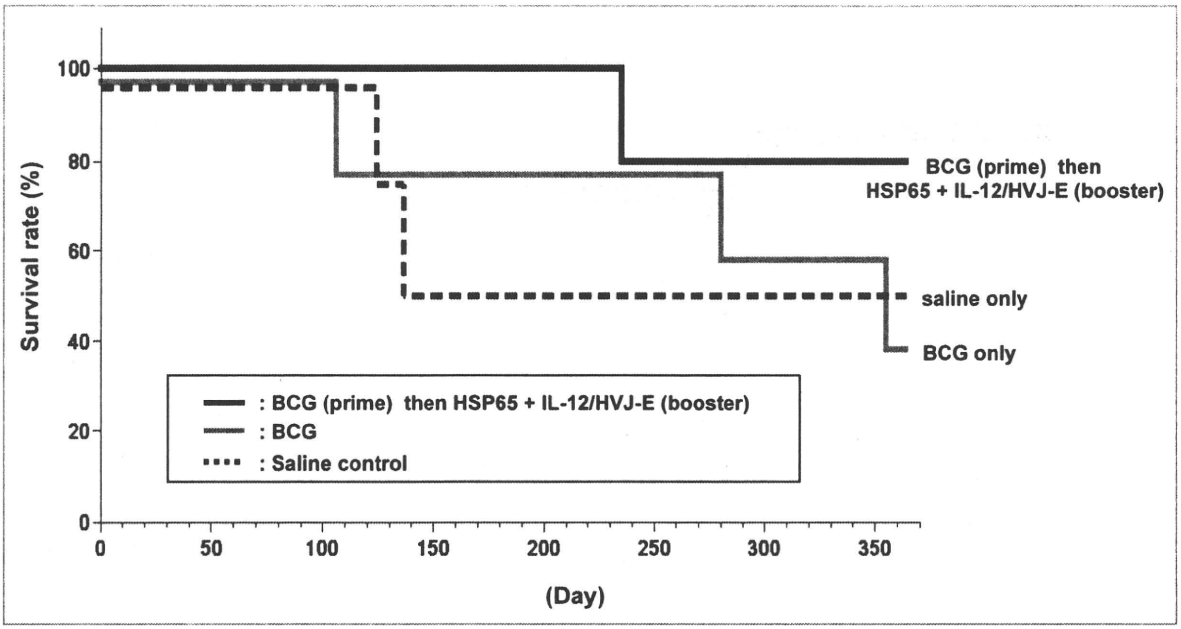

Figure 6. Protective efficacy of Hsp65 + IL-12/HVJ and BCG using prime—boost method against TB challenged cynomolgus monkeys. Group of animals were vaccinated three times as described in Figure 2. Group of animals were vaccinated with (1°) BCG Tokyo, (2°) Hsp65 + IL-12/HVJ, (3°) Hsp65 + IL-12/HVJ = G_1(—). BCG prime-HVJ/DNA boost group; (1°) BCG, (2°) saline, (3°) = G_2(– –). G_2 group animals were vaccinated with BCG once; (1°) saline, (2°) saline, (3°) saline = G_3(– – –). One month after the third vaccination, monkeys were challenged with the *M. tuberculosis*.

this BCG prime-HVJ-Envelope/HSP65 DNA + IL-12 DNA boost had fewer and smaller lesions in the lungs and significantly less lung granuloma than the naïve mice and mice vaccinated with BCG alone (data not shown).

We extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis, to evaluate the HSP65 + IL-12/HVJ.[1,2] Vaccination with BCG prime-HSP65 + IL-12/HVJ boost provided better protective efficacy as assessed by the Erythrocyte Sedimentation Rate, chest X-ray findings and immune responses than BCG alone. Importantly, HSP65 + IL-12/HVJ resulted in an increased survival for over a year. This was the first report of successful DNA vaccination against *M. Tuberculosis* in the monkey model which closely mimics human TB disease.[3]

Furthermore, the protective efficacy of the HSP65 + IL-12/HVJ and BCG using the prime-boost method in the TB-infected cynomolgus monkeys was very strong. All four monkeys from the group of BCG-prime and the DNA vaccine (HVJ-liposome/HSP65 + IL-12 DNA vaccine) boost were alive more than 12 months post-infection.[3] In contrast, only 2 monkeys out of 6 from the BCG Tokyo alone group were alive (33% survival).

Prime-boost method was reported in the study of MVA85A vaccine, which is a modified vaccine virus Ankara (MVA) strain expressing antigen 85A. In phase I studies in humans, this vaccine has induced high immune responses in previously BCG-vaccinated individuals.[14] Boosting of BCG vaccination with MVA85A downregulates the immunoregulatory cytokine TGFβ.[15] Aeras-402 DNA (DNA that expressed 85A, 85B and TB10.4) vaccine using adenovirus vector is intended for use as a boosting vaccine in BCG-primed individuals.[16] Several vaccines use a prime-boost strategy to enhance the immune responses.[17]

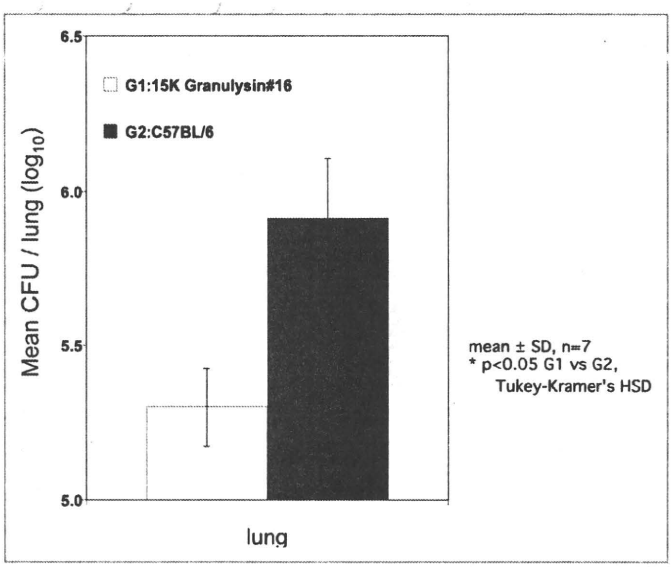

Figure 7. In vivo inhibition of the growth of *M. tuberculosis* in the 15 K granulysin transgenic mice. (In vivo anti-TB effect of 15 K granulysin transgenic mouse). Seven 15 K granulysin #16 transgenic mice and seven wild type C57BL/6 mice were injected with 5 x 10^5 H37RV *M. tuberculosis* i.v. 4 weeks after the challenge of *M. tuberculosis*, mice were sacrificed. CFU of *M. tuberculosis* in the lungs of these mice were assessed described in Material and Methods. CFU of 15 K granulysin #16 transgenic mice (□). CFU of control wild C57BL/6 mice (■). Student's t-test was used ($p < 0.05$).

In Japan and other countries, the BCG vaccine is inoculated into human infants (0–6 months after birth). Therefore, BCG prime in infants and HSP65 + hIL-12/HVJ boosts for adults

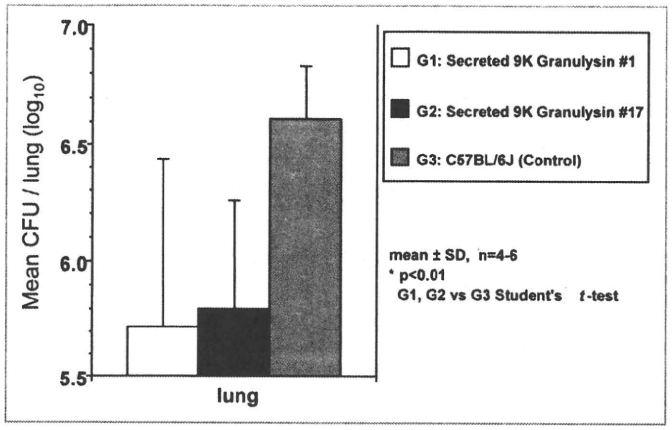

Figure 8. In vivo inhibition of the growth of *M. tuberculosis* in the 9 K granulysin transgenic mice. (In vivo anti-TB effect of 9 K granulysin transgenic mouse). Five 9 K granulysin #1 transgenic mice, five 9 K granulysin #7 mice and five wild type C57BL/6 mice were injected with 5 x 10^5 H37RV *M. tuberculosis* i.v. and 4 weeks after the challenge of *M. tuberculosis*, mice were sacrificed. CFU of *M. tuberculosis* in the lungs of these mice were assessed described in Material and Methods. CFU of 9 K granulysin #1 transgenic mice G_1 (□). CFU of 9 K granulysin #17 transgenic mice G_2 (■). CFU of control wild type C57BL/6 mice G_3 (■). Student's t-test was used to compare the CFU of each group(G_1–G_3: $p < 0.01$ G_2–G_3: $p < 0.01$).

(including junior high school students, high school students and old persons) may be required for the significant improvement of clinical protective efficacy against TB.

In the present study, using very long-term period (4 month interval between prime and boost), protective efficacy of the combination of vaccines was evaluated. In human, long-term interval (5~15 years) between prime vaccine and boost vaccine might be used in the clinical application of a novel TB prophylactic vaccine.

Thus, our results with the HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine in the murine prophylactic model and cynomolgus monkey prophylactic model should provide a significant rationale for moving this vaccine into clinical trial.

On the other hand, we established transgenic mice and a vaccine expressing granulysin. The granulysin expression in the $CD3^+CD8^+$ PBL-T cells of the patients with drug sensitive TB was significantly lower than that of normal volunteer (data not shown). The granulysin expression in $CD3^+CD8^+$ T cells from MDR-TB patients was lower than that in $CD8^+$ T cells from drug sensitive TB patients.

The production of 15 K granulysin was also suppressed in the culture supernatants of PBL from 10 patients with MDR-TB, compared to that from healthy volunteer (data not shown). Thus, these data suggest that granulysin vaccine treatment provides a useful tool to regulate the human TB infection disease.

Two major protein products, 15 K granulysin and 9 K 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.

We found that 15 K granulysin was secreted from CD8 positive CTL, and it could enter human macrophages and kill *M. tuberculosis* in the cytoplasm (data not shown). Therefore, we

Table 1. Efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine against tuberculosis infection using IL-2 Receptor (-/-) NOD-SCID mice (SCID-PBL/hu)

Group	Treated	CFU of TB (log)
G1	(-)	6.03 ± 0.06
G2	HSP65DNA + IL-12 DNA vaccine (10 μg)	5.96 ± 0.15
G3	HSP65DNA + IL-12 DNA Vaccine (50 μg)	5.40 ± 0.97

Therapeutic efficacy of HVJ-envelope/HSP65DNA + IL-12DNA, 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/HSP65DNA + IL-12DNA (50 ug i.m. or 10 ug i.m.). 10 days after the third vaccination, mice were sacrificed and CFU of TB in the liver of mice were assessed as described in Materials and Methods. 1 x 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 challenged with 5 x 10^5 H37Rv i.v. and then treated with vaccine. G1, (-) control; G2, treated with HVJ-envelope/HSP-65DNA + IL-12DNA 10 μg; G3, treated with HVJ-envelope/HSP65DNA + IL-12DNA 50 μg. Student's t-test was used to compare the CFU of TB of each group (G1–G3: $p < 0.05$).

established 15 K granulysin transgenic mice and 9 K granulysin transgenic mice.

It was demonstrated that 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB effect and decrease in the number of TB. Thus, granulysin DNA vaccine therapy and recombinant granulysin therapy might provide a weapon against MDR-TB and XDR-TB.

In conclusion, we have the advantage of the availability of multiple animal models to accumulate essential data on the HVJ-Envelope DNA vaccine and granulysin vaccine in anticipation of a phase I clinical trial.

Materials and Methods

Methods for the evaluation of the prophylactic efficacy of the vaccine on the TB infection of the monkeys. Cynomolgus monkeys were housed in a BSL 3 animal facility of the Leonard Wood Memorial Research Center. All animal experiments were approved by the Leonard Wood Memorial Animal Care and Use Committee and the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. The animals were vaccinated three times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65 + hIL-12/HVJ: 400 ug i.m.), and then challenged with the *M. tuberculosis* Erdman strain (5 x 10^2) by intratracheal instillation. Survival, immune responses (proliferation of PBL and cytokines production), body weight, ESR, PPD skin test and chest X-P findings were examined as described in our previous studies.[3,9,10]

Methods for the establishment of granulysin transgenic mouse. 15 K granulysin gene, 9 K granulysin gene or secreted 9 K granulysin DNA (15 K granulysin secretory signal DNA was fused into N terminal of 9 K granulysin DNA) were transferred to expressing plasmid DNA (pCAGGS) having CAG promoter. DNA fragment was injected to pronuclei embryo and grafted to 200 foster parents. 2 types of 15 K granulysin Tg mice (#3, #16), 3 types of 9 K granulysin Tg mice (#15,