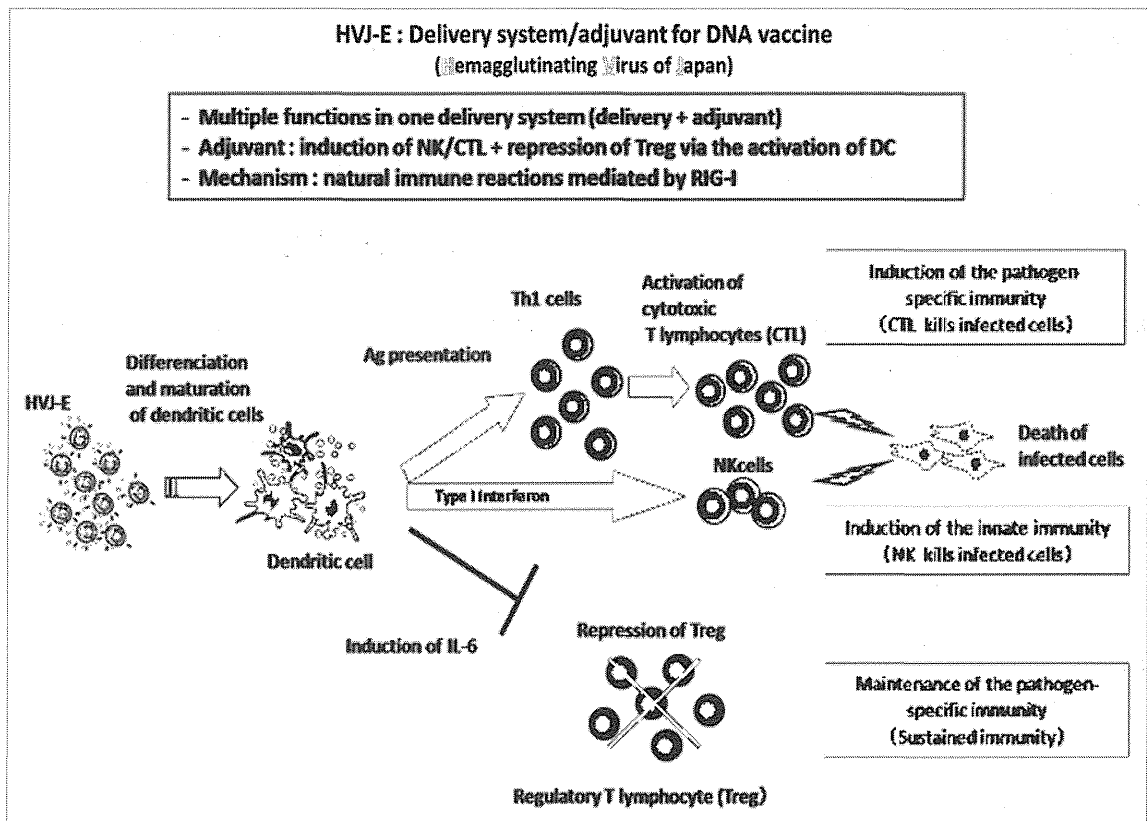

Figure 1. HVJ-Envelope exerts strong adjuvant activity by the induction of CTL/NK and repression of regulatory T cells via the activation of dendritic cells.

was 100 fold more efficient than BCG in the murine model on the basis of the elimination of M. tuberculosis, which is mediated by the induction of CTL.[1,5,6] Furthermore, the HSP65 + IL-12 vaccine delivered by HVJ-envelope was 10,000 fold more efficient than BCG in the murine TB-prophylactic model. This vaccine induced a strong activity of CTL against TB, while BCG vaccine induced little activity of CTL in the same model. It is considered that CTL is most important lymphoid cells for the immunity to TB in chronic human TB diseases. In the present study, we analyzed CTL activity and IFN-γ production after the vaccination with our vaccines. We also evaluated the prophylactic effect in the cynomolgus monkey and mouse models of TB. A nonhuman primate model of TB is an excellent model of human tuberculosis and provides a lot of information for vaccine development. In fact, we previously evaluated the protective effects of HSP65 + IL-12/HVJ vaccine in the cynomolgus monkey model and obtained a data indicating the synergistic effect of the HSP65 + IL-12/HVJ and BCG injected by a prime-boost method.[5-7] The combination of the two vaccines showed a strong prophylactic efficacy in the monkey model infected with M. tuberculosis (100% survival). We have previously obtained a similar data in the monkey model of TB.[5,6,8] In the present study, we examined the production of cytokines (IFN-γ and IL-2) from PBL and revealed the correlation of cytokine levels and efficacy in the monkey model of TB. We also compared the production levels of these cytokines between the combinatorial vaccination (BCG prime and HSP65+IL12/HVJ vaccine boost) group and BCG vaccination group. We also evaluated the potential of other novel vaccines (DNA vaccines encoding granulysin or Ksp37), which were expected to induce the differentiation of CTL against TB. Granulysin is a protein secreted from T cells and NK cells and has an antibacterial effect on TB. Killer-specific secretory protein of 37 kDa (Ksp37) vaccine also showed anti-TB efficacy mediated by the induction of CTL. Synergistic effect on the activation of CTL in vitro was observed by the simultaneous administration of Ksp37- and granulysin-based vaccines. In the present study, we further demonstrated the correlation of the activation of CTL and the efficacy of these novel vaccines (HSP65 + IL-12/HVJ-E DNA vaccine, granulysin vaccine and Ksp37 vaccine) in the mouse and monkey models.

Results

Induction of CTL by HSP 65+IL-12/HVJ DNA vaccine in the mouse model of TB. The advantage of HVJ-Envelope vector is shown in **Figure 1**. (1) HVJ-Envelope is efficient delivery system and functions as an adjuvant for DNA vaccine, (2) It induces CTL and NK cell, (3) It induces a production of IL-6 which suppresses the regulatory T cell (T reg) and (4) It activates the innate immunity by the stimulation of RIG-I signaling pathway.

Mice were immunized three times with the DNA vaccine using HVJ-Envelope every three weeks. Four weeks after last

immunization, H37Rv M.tuberculosis (M.tb) was challenged. Five weeks after the challenge of M.tb, mice were sacrificed and C.F.U. of M.tb in lung, liver and spleen were accessed as reported previously.[2,9-11] The C.F.U. of lungs in BCG vaccine alone group was decreased in 1 log compared with non-vaccinated mice group. The combination of the DNA vaccine (HSP65 DNA + IL-12) and BCG using prime-boost method enhanced the prophylactic efficacy (more than ten thousand fold) in the mouse model of TB (BCG prime-DNA vaccine boost). This regimen (BCG-prime then DNA vaccine boost) strongly increased the number of IFN-γ producing cells as compared with BCG vaccine alone (**Table 1**).

CD8+ CTLs have been considered as critical effectors of protective immunity to M. tuberculosis. This vaccine induced CD8+ CTL against TB, whereas a little or no CTL response was observed in either the naive or BCG-vaccinated mice (**Figs. 2 and 3**). We used HSP65 DNA-transfected syngenic tumor cells as target cells for CTL. This vaccine augmented the induction of CD8+ CD4- CTL against target cells in vivo. On the other hand, a little or no CTL response was observed in BCG-vaccinated mice (**Figs. 2 and 3**).

Furthermore, we revealed that CD8+ T cells as well as CD4+ T cells were necessary for the prophylactic effficacy of this vaccine. The administration of anti-CD8 antibody or anti-CD4 antibody during the whole immunization period decreased the antibacterial immunity against TB and increased the number of C.F.U in lungs (**Table 2**). Simultaneous administration of anti-CD8 and anti-CD4 antibodies resulted in the further increase of the number of C.F.U in lungs (**Table 2**).

T cell activation by HSP 65+IL-12/HVJ DNA vaccine in the monkey model of TB. We used cynomolgus monkeys and prime-boost methods for the evaluation of our vaccines. We immunized monkeys with BCG Tokyo as a prime vaccine, and then immunize them with this vaccine as a boost vaccine. Survival rate of monkeys vaccinated with BCG prime-HSP65+IL-12 DNA boost was 100%. In contrast, survival rate of monkeys vaccinated with BCG alone was 33%. Thus, prime-boost method showed stronger efficacy than the method vaccinated with BCG alone (data not shown).

IFN-γ production from PBL was the highest level in the monkey group immunized with BCG prime-HSP65+IL-12/HVJ DNA vaccine boost (**Fig. 4A**). The strongest production of IL-2 from PBL was also observed in same group (**Fig. 4B**). Thus, in monkey as well as in mouse, we demonstrated that the combination of BCG Tokyo as a prime vaccine and the DNA vaccine as a boost vaccine is suitable method to get the prophylactic efficacies against TB. The prime-boost method efficiently induced the TB-specific CTL and also induced the production of IFN-γ and IL-2.

In Japan, BCG Tokyo vaccine is immunized in all infants. Thus it is expected to function as a prime vaccine. Therefore, we need the administration of novel vaccines (boost vaccines) for adults in Japan. We plan to use similar prime-boost method (BCG prime-DNA vaccine boost) in future clinical trial.

Table 1. ELISPOT assay for IFN-γ antigen-specific responses in the spleens of vaccinated mice following stimulation with HSP65 protein

	Vaccination ($1^{st}/2^{nd}/3^{rd}$)	Number of IFN-γ secreting cells/ (10^6 splenocytes)		
G1	-/-/-	3	±	1
G2	-/-/BCG	13	±	6
G3	DNA*/DNA/DNA	16	±	4
G4	BCG/DNA/DNA	115	±	12

*DNA means HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine. Spleen cell cultures were stimulated with rHSP65 protein for 20 h. The number of IFN-γ-secreting cells specific for rHSP65 protein per million cells was determined individually by ELISPOT assay. Results are expressed as the mean ± S.D. of 6 wells of 3 mice per group. The statistically significant differences of the G1 (naïve) group compared with the G2 (BCG alone group), G3 (DNA / DNA / DNA) or G4 (BCG / DNA / DNA) were observed ($p < 0.01$). The statistically significant difference between G2 group and G4 was observed ($p < 0.01$). The statistically significant difference between G3 group and G4 was observed ($p < 0.01$).

Correlation of IL-2 production and the therapeutic efficacy of HSP65+IL-12/HVJ DNA vaccine in the monkey model of TB. This vaccine showed therapeutic efficacy against MDR-TB and XDR-TB as well as drug-sensitive TB in mice.[9,11,12] Therefore, we confirmed the therapeutic efficacy of the DNA vaccine and T cell responses induced by the DNA vaccine in the monkey model of TB. To establish the model of TB, human TB (5×10^2 CFU) was intratracheally instillated in monkeys. After the TB infection, DNA vaccine was injected intramuscularly 9 times, three times a week. Therapeutic effect was evaluated on the basis of survival, ESR, body weight, immune responses, chest X-ray and PPD skin test.

Injection of the therapeutic vaccine improved the survival of monkeys, as compared with the saline (control) group. No death was observed in monkey group treated with HSP65 +IL-12/HVJ DNA vaccine. In contrast, the survival rate of control saline group was 60% (data not shown). The proliferation of PBL after the stimulation with HSP65 was measured to evaluate the immune response. The proliferation of PBL from the monkeys treated with the DNA vaccine was more augmented than that from the monkeys treated with saline (data not shown). IL-2 production from PBL after the stimulation with killed TB H37Ra antigens was also examined. The level of IL-2 was higher in the DNA vaccine-treated group than that in saline control group (**Table 3A**). In addition, the IL-2 production from PBL after the stimulation with PPD was seemed to correlate with the survival after TB challenge. The level of IL-2 production of died monkeys was significantly lower than that of survived monkeys in saline group at 53days after TB challenge (**Table 3B**). IL-2 production by the stimulation with HSP65 protein was also extremely low level in died monkeys (data not shown). These data suggested that therapeutic efficacy of this vaccine correlate with the level of IL-2 production.

Induction of CTL by 15K granulysin vaccine. Granulysin is protein especially abundant in CTL and NK cells. It is be classified into two major protein products [15kDa (15K) granulysin and 9kDa (9K) granulysin]. Latter granulysin (9K) exhibits

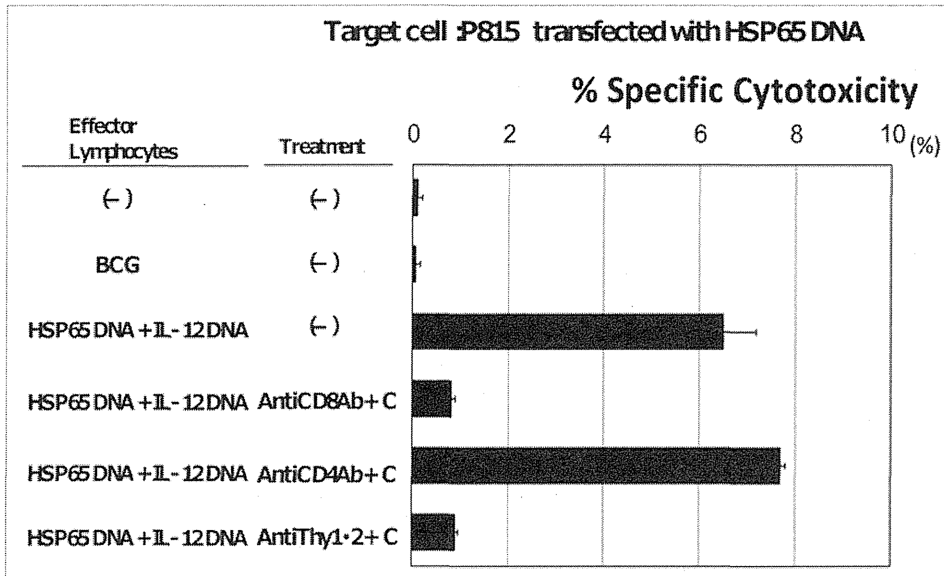

Figure 2. Induction of CD8$^+$ CTL specific for HSP65 protein and M. tuberculosis by vaccination with HVJ-Envelope/HSP65 DNA+IL-12 DNA. Spleen cells from the naïve, BCG-, and HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccinated mice were obtained eight weeks after the final vaccination. Cytotoxicity was assayed as release of radioactivity from ^{51}Cr-labeled P815 target that had been transfected with HSP65 DNA using conventional ^{51}Cr release assay as described in Materials and Methods.

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, NKT cells and γ/δ T cells. However, the precise function of 15K granulysin has not yet been elucidated. We found that 15K granulysin was secreted from CD8 positive CTL, could enter into human macrophages and killed M. tuberculosis in the cytoplasm of macrophages. Expression of 15K granulysin protein and mRNA in CD8 positive T cells in the patients with Tuberculosis was significantly lower than those in the healthy volunteers. Moreover, the induction of 15K granulysin production after the stimulation with PPD antigen was suppressed in the supernatants of PBL from TB patients (data not shown).

Recombinant 15K granulysin protein enhanced the in vitro induction of human CTL in the MLC culture (**Table 4A**). In vivo induction of CTL in the spleen was augmented by the administration of recombinant 15K granulyin into C57BL/6 mice (**Table 4B**). The administration of recombinant 15K granulysin also augmented in vivo induction of CTL in the lymph node and PEC (peritoneal exudate cells) (**Table 4B**).

Synergistic effect was observed by the combination of recombinant 15K granulysin and IL-6-related DNA vaccine (IL-6+ IL-6 receptor + gp130 DNA vaccine). In vivo induction of CTL specific for HSP65 TB antigen was augmented by the combination of two vaccines (data not shown). Two types of granulysin vaccines (recombinant 15K granulysin and 15K granulysin DNA vaccine) showed strong therapeutic efficacy in the mice infected with TB by aerosol challenge, resulted in the decrease of the number of C.F.U. in the lungs, liver and spleen (data not shown).

The granulysin (15K) has function as a CTL differentiation factor (**Fig. 5**). It augmented the differentiation of CTL from precursor CTL into effecter CTL. Thus, this is a first report that reveals the novel function of 15K granulysin (inducer of CTL differentiation). Effector CTLs produce 15K granulysin which induces the differentiation of CTL. Thus, there is a positive feedback loop (or autocrine system) in the regulation of the production of 15K granulysin. Granulysin (15K) might efficiently induce TB-specific CTL and enhance its therapeutic effect against TB infection by means of such a positive feedback loop system (**Fig. 5**).

CTL induction by Ksp37 vaccine. To investigate the immune function of Ksp37 protein, in vitro CTL induction by the addition of recombinant Ksp37 protein was analyzed in murine system. Splenic T cells from C57BL/6 were cultured with alloantigen (BALB/c spleen cells) in the presence or absence of recombinant Ksp37 for five days.

Induction of in vitro CTL differentiation was observed by the addition of recombinant Ksp37 (**Fig. 6**), suggesting that Ksp37 has function as a CTL differentiation factor.

IL-2 production from T cells and spleen cells was augmented by the addition of Ksp37 protein (**Fig. 7A**). Ksp37 also augmented the production of IFN-γ and IL-6 from murine spleen cells in vitro (**Fig. 7B and C**). Thus, Ksp37 is an inducer of multiple types of cytokines (IFN-γ, IL-2 and IL-6).

In order to study the immune function of Ksp37 in vivo, induction of CTL by the administration of Ksp37 DNA vaccine was investigated in murine system. Augmentation of CTL differentiation in vivo in the PEC was observed by the treatment with Ksp37 DNA vaccine (**Fig. 8**). Thus, Ksp37 DNA vaccine functioned as an inducer of CTL in vivo as well as in vitro. Furthermore, synergistic effect on the in vitro CTL induction was observed by the simultaneous treatment of Ksp37 and granulysin vaccines (**Fig. 9**).

Taken together, we established three kinds of novel vaccines and examined their potential in the mouse and monkey models in vitro and in vivo.

We demonstrated that: (1) HSP65+IL-12/HVJ-E DNA vaccine (HSP65 vaccine) had a prophylactic effect against TB. We revealed the induction of CD8$^+$ CTL and CD4$^+$ T cell were required for the protective efficacy of HSP65 vaccine. We also confirmed currently available vaccine (BCG vaccine) induced a little or no TB-specific CTL; (2) We also revealed the 15K granulysin functioned as a cytotoxic T cell differentiation factor (CTL differentiation factor). The production of granulysin is regulated by the positive feedback system. Thus, granulysin-based vaccine might have a strong therapeutic potency against TB, since it is expected to effectively induce TB-specific CTL;

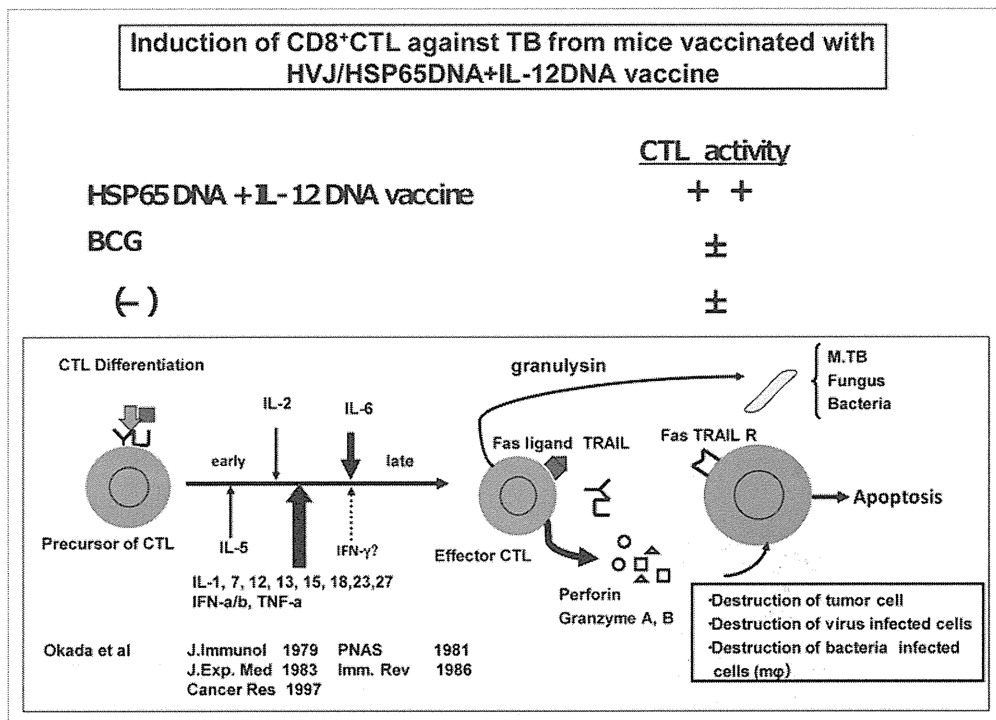

Figure 3. Induction of CD8 positive CTL against TB by HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine, and differentiation of CTL.

(3) We also revealed that Ksp37 acts as a CTL differentiation factor in human and mouse. Ksp37 vaccine augmented the production of IL-2, IFN-γ, and IL-6 from murine T cells and spleen cells; (4) We also demonstrated the synergy of the vaccines, e.g., synergistic effect on the in vitro CTL induction was shown by the combination of Ksp37 and granulysin DNA vaccines.

These data indicate that all of our novel vaccines are able to induce the TB-specific CTL effectively and the combination regimen of those vaccines might provide the new strategy to get very strong protective and therapeutic efficacy against TB.

Discussion

In the present study, we studied the CTL differentiation against TB induced by the administration of novel vaccines (HSP65+IL-12/HVJ-E DNA vaccine, granulysin vaccine and Ksp37 vaccine). We found (1) 15K granulysin functions as a cytotoxic T cell differentiation factor (CTL differentiation factor). The therapeutic efficacy of 15K granulysin vaccine might be mediated by the induction CTL; (2) Ksp37 also acts as a CTL differentiation factor in mice; (3) Ksp37 vaccine induces the production of IL-2, IFN-γ and IL-6 from murine T cells and spleen cells; (4) Synergistic effect on the in vitro CTL induction is expected by the combination of Ksp37 vaccine and granulysin vaccine.

On the other hand, it was demonstrated that HSP65 +IL-12/HVJ-E DNA vaccine (HSP65 vaccine) has a prophylactic efficacy against TB and that the induction of CTL specific for TB is important for the efficacy of the vaccine. By using CD8 antibody and CD4 antibody we found CD8 positive CTL and CD4 positive T cell were required for the protective efficacy of HSP65 vaccine in vitro and in vivo. In contrast, BCG vaccine induced a little or no CTL against TB.

Table 2. The in vivo necessity CD8 positive T cells and CD4 positive T cells for prophylactic efficacy of the HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine

	Vaccination			Antibody	Log_{10} CFU of TB in the lung
	1st	2nd	3rd		
G1	-	-	-	-	6.6 ± 0.3
G2	BCG	DNA	DNA	-	5.3 ± 0.3
G3	BCG	DNA	DNA	αCD8 Ab	5.9 ± 0.1
G4	BCG	DNA	DNA	αCD4 Ab	7.1 ± 0.5
G5	BCG	DNA	DNA	αCD8 Ab+αCD4 Ab	8.4 ± 0.2

*$p < 0.05$ G2vsG3, G4; **$p < 0.01$. G2vsG5, Anti-CD8 antibody and/or Anti-CD4 antibody were injected i.p. every 5 d after the challenge of TB. BCG was used as a prime vaccine and the DNA vaccine was immunized twice (HVJ-Envelope/HSP65 DNA 50 μg + IL-12 DNA50 μg) as boost vaccine. Four weeks after last immunization, 5×10^5 H37Rv were challenged i.v. into mice. (G2-G3: $p < 0.05$); (G2-G4: $p < 0.05$); (G2-G5: $p < 0.01$).

There are increasing evidences which indicate the importance of cytotoxic cells.[3,4] Their role in immunity to TB has been revealed by using knockout mice lacking a gene or genes related to major histocompatibility complex (MHC) class I (e.g., transporter associated with antigen processing-1, CD8, 2m, and MHC class I heavy chain). These genes are involved in the antigen presentation via MHC class I, by cell-transfer experiments and by depletion of $CD8^+$ CTLs with antibodies. $CD8^+$ CTLs play a major role in the control of the latent TB.[1,4,6]

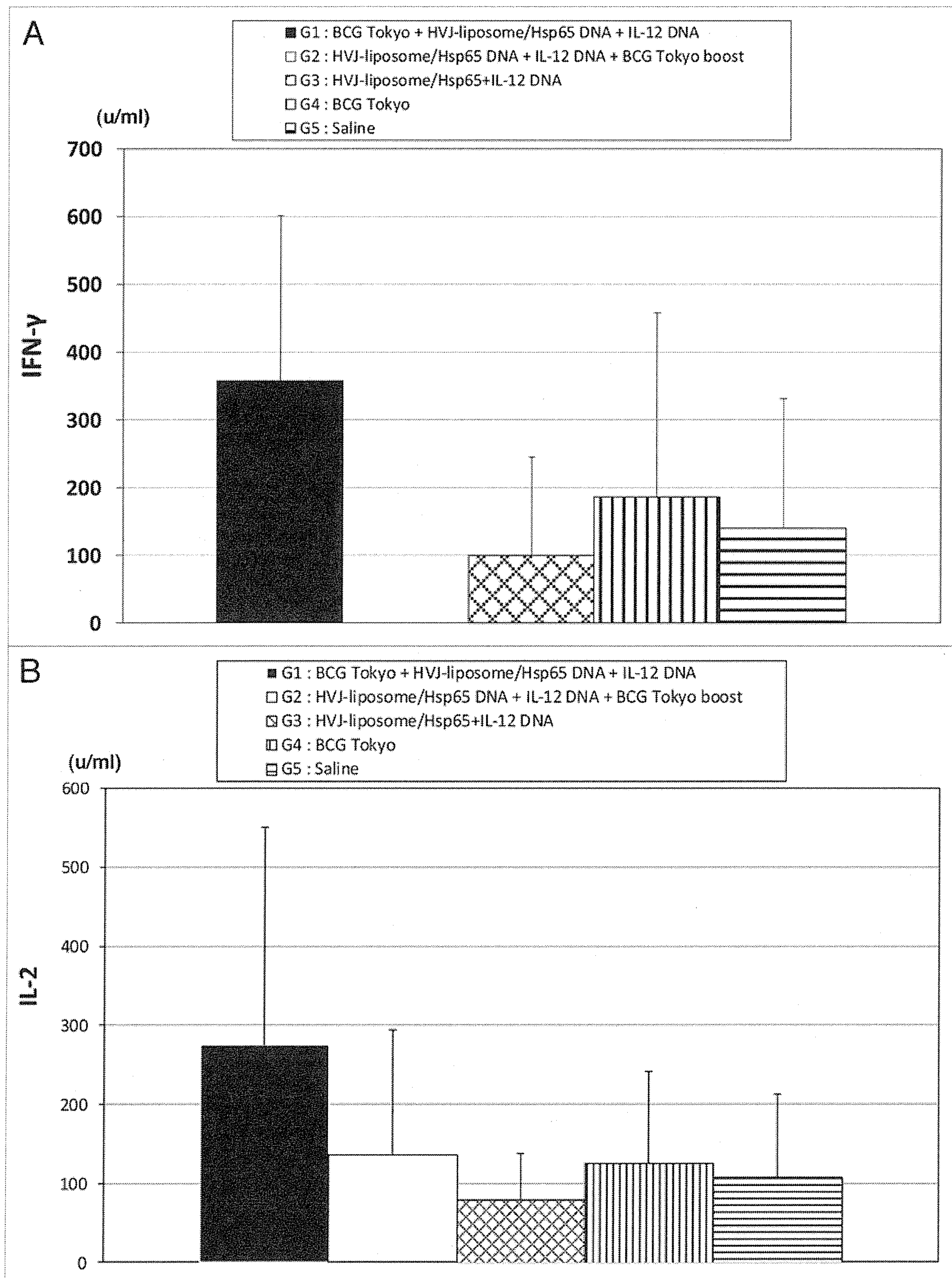

Figure 4. IFN-γ and IL-2 production efficacy of HSP65+IL-12/HVJ and BCG using prime-boost method against TB challenged cynomologus monkeys. Group of animals were vaccinated three times (every three weeks) with (1°) BCG Tokyo, (2°) HSP65 +IL-12/HVJ, (3°) HSP65+IL-12/HVJ = G1(■) BCG prime-HVJ/DNA boost group; (1°) HSP65+IL-12/HVJ, (2°) HSP65 +IL-12/HVJ, (3°) BCG = G2(□) HVJ/DNA prime-BCG boost group; (1°) HSP65+IL-12/HVJ, (2°) HSP65 +IL-12/HVJ, (3°) HSP65+IL-12/HVJ = G3(▩); (1) BCG, (2°) saline, (3°) saline = G4(▥) G4 group animals were vaccinated with BCG once.; (1°) saline, (2°) saline, (3°) saline = G5(▤). (A) PBL from vaccinated monkeys on 56 d or 112 d after 1st immunization, were cultured for three days. IFN-γ activity on 56 d and IL-2 activity (B) on 112 days were assessed by ELISA.

The CTLs other than CD8+ cells might be even more important in humans. It has been reported that there are additional effector modalities, such as granulysin, that are able to kill *M. tuberculosis*.[12] Lymphocyte subsets that recognize antigens presented by molecules other than MHC class 1 were also reported to be involved in the immunity against TB. In addition to MHC class 1 molecule, these lymphocytes are able to utilize HLA-E or group1 CD1 molecules (CD1a, CD1b, etc.) for the recognition of antigens.[6,13,14]

The precise function of 15K granulysin has not yet been elucidated.[15] We found that 15K granulysin was secreted from CD8 positive CTL and entered into human macrophages followed by the killing of *M. tuberculosis* in the cytoplasm.[10,11] The expression levels of 15K granulysin protein and mRNA in CD8 positive T cells of the patients with drug sensitive TB were lower than that of the healthy volunteers. Among the TB patients, the expression level of 15K granulysin protein in CD8 positive T cells of the patients with multi-drug resistant tuberculosis (MDR-TB) was significantly lower than that in the patients with drug-sensitive TB. The expressions of 15K granulysin after the stimulation with PHA-P, ConA, alloantigens or PPD antigens were significantly suppressed in the supernatants of PBL from MDR-TB patients.[10,11] We have currently established 15K granulysin transgenic mice and 9K granulysin transgenic mice.[11] It was demonstrated that 15K granulysin transgenic mice as well as 9K granulysin transgenic mice were resistant to TB infection. The number of TB (C.F.U. in tissues) was decreased in those transgenic mice. As to the induction of anti-TB immunity, differentiation and proliferation of TB-specific CTL was augmented in those transgenic mice. In addition, enhanced production of cytokines was observed.

In the present study, we demonstrated that 15K granulysin has function as a CTL differentiation factor. Granulysin (15K) augmented the differentiation of CTL from precursor CTL into effecter CTL. It has been reported that 15K granulysin is produced from effector CTL. Thus, it is suggested that the production of 15K granulysin is regulated by positive feedback loop system. Large amount of granulysin might enhance the induction of CTL resulting in the increase of the therapeutic efficacy. Recently, it was reported that 15K granulysin activated antigen-presenting cells (APC, dendritic cells or macrophages) and augmented the production of several cytokines.[16,17] Therefore, 15K granulysin is able to activate the immune system effectively, since the target cell of it is both APC and CTL.

Vaccination with BCG prime-HSP65 + IL-12/HVJ boost showed better protective efficacy than BCG alone on the basis of the ESR, chest X-ray findings and immune responses. Importantly, treatment with HSP65 + IL-12/HVJ resulted in an increased survival for over a year.[5]

Significantly higher levels of cytokine production from PBL (IFN-γ and IL-2) were observed in prime (BCG) - boost (HSP65 vaccine) group than those in BCG vaccine group. 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 clinical studies, this vaccine has induced high immune responses in previously BCG-vaccinated individuals.[18]

Table 3. IL-2 production from PBL in the cynomolgus monkeys

(A)	(monkeys) Responder cell	H37Ra Ag	IL-2 (u/ml)
	vaccine-treated	(-)	2 ± 3
	control	(-)	0 ± 0
	vaccine-treated	(+)	13 ± 4
	control	(+)	3 ± 5

(B)	G4 (saline control)	IL-2 production (U/ml) (HSP65 stimulation)		Survival
	ID of monkeys	Baseline	Day53 Post-challenge	
	PR 6847 D	0.0	21.1	Survival
	PR 8018 B	0.0	43.2	Survival
	PR 5368 B	0.0	43.2	Survival
	PR PbB2–4F	0.0	0.0	Death (after 41 d)
	PrZ7–51 AC	0.0	0.0	Death (after 55 d)

(A) Augmentation of IL-2 production from PBL by H37Ra stimulation in the monkeys treated with HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine. (B) IL-2 production from PBL in the survived monkeys and the non-survived monkeys. PBL from monkeys on day 53 after TB challenge were stimulated with HSP65 protein for 3 d. ID (PR PbB2–4F) monkey and ID (PrZ7–51 AC) were died after 41 d and 55 d, respectively.

Table 4. In vitro and in vivo CTL induction by 15K granulysin

		15K Granulysin	% Specific Cytotoxicity		
(A)	In vitro (5day MLC)				
		-	8.0	±	1.0
		+	23.0	±	0.5
(B)	In vivo CTL				
	spleen	-	1.0	±	0.2
		+	11.0	±	0.3
	LN	-	7.2	±	2.5
		+	22.5	±	2.8
	PEC	-	0.5	±	2.0
		+	28.5	±	1.0

(A) In vitro induction of human cytotoxic T cell by the stimulation with recombinant 15K granulysin protein. T cells from human PBL were obtained by nylon-wool column method. An amount of 1×10^6 T cells were cultured with $CESS_{MMC}$ cells (Mitomycic C treated CESS tumor cells) in the presence of 15K granulysin for five days. CTL activity of effector cells was assayed using ^{51}Cr-labbelled CESS cells. Results are expressed as % Specific cytotoxicity ± S.D. (B) In vivo induction of CTL by 15K granulysin. C57BL/6 were injected i.p. with 1×10^7 syngeneic FBL-3 tumor cells and then treated with recombinant 15K granulysin i.p. (5 μg/mouse) six times. Twenty-one days after FBL-3 inoculation, mice were sacrificed, and spleen cells, lymph node (LN) cells and peritoneal exudates cells (PEC) were used as effector cells for CTL. Cytotoxic activity against FBL-3 cells were assessed using ^{51}Cr release method.

Boosting of BCG vaccination with MVA85A reduced the expression of immunoregulatory cytokine TGF-β.[19] Aeras-402 DNA (DNA that expressed 85A, 85B and TB10.4) vaccine is a recombinant adenovirus vector-based vaccine and expected as

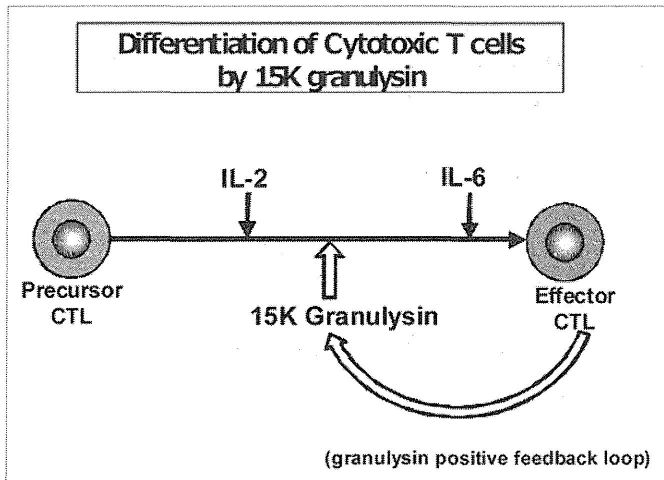

Figure 5. Differentiation of CTL by 15K granulysin and positive feedback loop of 15K granulysin in CTL induction. Precursor CTL are activated by IL-2 in the early stage of CTL induction of five day MLC. On the other hand, IL-6 acts, as a cytotoxic T cell differentiation factor, on the late stage of CTL induction as shown by Okada, et al. (J.I. 1988). 15K granulysin is produced from effector CTL, and induced the differentiation of CTL as a CTL differentiation factor. (Positive feedback loop by 15K granulysin).

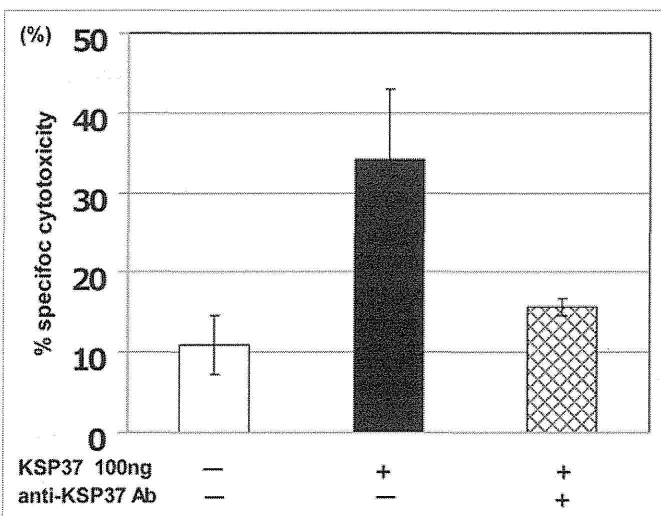

Figure 6. Induction of CTL differentiation by killer-specific secretory protein of 37Kd (Ksp37). Splenic T cell from C57BL/6 mice (H-2^b) were obtained as described in (PNAS Okada et al. 1981), and cultured with uv-treated BALB/c (H-2^d) spleen cells (Mitomycin C treated) in the presence of 100ng/ml rKsp37 and/or an anti-Ksp37 antibody for 5 d. CTL activity against P815 tumor cells (H-2^d) was assessed by using ^{51}Cr assay.

a boosting vaccine for BCG-primed individuals.[6] Several other vaccines use a prime-boost strategy to enhance the immune responses.[20] Recently, Rahman et al. established a plasmid-based vaccine (rBCG/rAd35 vaccine) which enhanced the activation of MHC class I-restricted CD8$^+$ cytotoxic T cell. It is a recombinant BCG (rBCG) expressing a pore-forming toxin and TB antigens (Ag85A, 85B and TB10.4). A non-replicating adenovirus35 encoding the same TB antigens (rAd35) is used as a boost vaccine. The prime-boost method using rBCG and rAd35 vaccines were evaluated in nonhuman primate model.[21] Similar to our BCG prime- HSP65 + IL-12 DNA boost method, the results suggested that activation of CD8$^+$ effector CTL followed by the production of granulysin at the local site were involved in the protective effects of this vaccine (prime-boost). Thus, induction of TB-specific CTL and the augmentation of the cytokine production (IFN-γ, IL-2) might be a critical factor to obtain the prophylactic efficacy.

Furthermore, we established other vaccines (granulysin vaccine and Ksp37 vaccine) which induced the differentiation of CTL against TB. The granulysin secreted from T cells and NK cells has a therapeutic effect against TB.

On the other hand, Ksp37 is expressed selectively in the effector subset of CD8$^+$T cells, CD16$^+$ NK cells and γ/δ T cells.[22] Expression of Ksp37 mRNA was closely correlated with good prognosis of ovarian cancer and gliomas.[23] However, immunological function(s) of Ksp37 is still unclear. Ksp37 showed anti-TB effects by the induction of CTL in the present study. We demonstrated that Ksp37 vaccine also induced murine CTL both in vitro and in vivo. Ksp37 vaccine augmented the production of IL-2, IFN-γ and IL-6 from murine T cells and spleen cells. Synergistic effect on the in vitro CTL induction was observed by the combination of Ksp37 and granulysin vaccines. We also demonstrated the correlation between the activity of CTL induction/cytokine production and the efficacy of these vaccines in the mouse and monkey models.

In conclusion, our data might provide novel strategy to obtain strong protective and therapeutic efficacy. The important factors are the induction of CTL and production of several kinds of cytokines. In addition, the combination of several kinds of vaccines will enhance the efficacy of vaccines, which will be necessary for the vaccine for the severe TBs such as MDR-TB and XDR-TB. Similar to the treatment of cancers or infectious diseases, the regimen of combination therapy will provide useful rationale that is necessary to develop more effective vaccines against TB.

Materials and Methods

Bacteria. *M. tuberculosis* strains H37Rv and *M. bovis* BCG Tokyo, were kindly provided by Dr. I. Sugawara (JATA, Tokyo, Japan). *M. bovis* BCG Tokyo was maintained in synthetic Sauton's medium (Wako Chemicals, Osaka, Japan).[1]

Animals. Inbred and specific pathogen-free female BALB/c mice and DBA/1 mice were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained in isolator cages, manipulated in laminar flow hoods, and used between 8 and 10 weeks of age as described previously.[1]

Plasmid construction. The *HSP65* gene was amplified from *M. tuberculosis* H37Rv genomic DNA, and cloned into pcDNA3.1 (+) (Invitrogen, San Diego, CA) to generate pcDNA-hsp65 (designated as HSP65 DNA) as described previously.[1] The *HSP65* gene was fused with mouse Igκ secretion signal sequence, and pcDNA-Ighsp65 was generated. For construction of the mouse IL-12 (mIL-12) *p40* and *p35* single-chain genes, *mIL12p35* and

mIL12p40 genes were cloned from pcDNA-p40p35,[1] fused and cloned into pcDNA3.1 (+) to generate pcDNA-mIL12p40p35-F (designated as mIL-12 DNA).

HVJ-E vaccination. HVJ-E was prepared as described previously.[1] The HVJ-E complex was aliquoted and stored at -70°C until use. DNA vaccines encoding *M. tuberculosis* HSP65 and IL-12 were encapsulated into HVJ-Envelope or HVJ-liposomes.[24] HVJ-liposomes and HVJ-Envelope were prepared as described previously.[25-29] Groups of BALB/c mice were vaccinated 3 times at 3-week intervals with 100 μL of HVJ-E solution containing 50 μg of pcDNA-IgHSP65 and 50 μg of mIL12 DNA.

Challenge infection of vaccinated animals and bacterial load determination. Mice were challenged by the intravenous route with 5×10^5 CFU of *M. tuberculosis* H37Rv 4 weeks after the third vaccination as described previously.[1]

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. 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 μg i.m.), and then challenged with the *M. tuberculosis* Erdman strain (5×10^2) 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.[5,6,8,9] 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.

Methods for the evaluation of the therapeutic efficacy of the vaccine on the *M. tuberculosis*-infected monkeys. Cynomolgus monkeys were vaccinated nine times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65 + hIL-12/HVJ: 400 μg 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.[5,8,9]

Reagents and antibodies. Fetal calf serum (FCS: lot AGC6341) was obtained from Hyclone (Logan, UT). Anti-L3T4, anti-Lyt2.2 monoclonal antibodies and anti-Thyl.2 antibody were provided.[1,2]

Cell lines. A mouse mastocytoma cell line (P815: DBA/2 origin) was kindly provided by Dr. C. S. Henney (Fred Hatchinson Cancer Res. Center, Seattle).[1] The P815 cells were maintained in RPMI 1640 medium (Flow Laboratories, Inc. Mclean, VA) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 5×10^{-5} M 2-mercaptoethanol.[1,2]

Tuberculosis–specific cytotoxic test using ^{51}Cr release. Eight weeks after the final vaccination, CTL activity of spleen cells and mesenteric lymph node cells from vaccinated mice was assessed by using the ^{51}Cr-release assay. P815 mastocytoma cells, which have the same major histocompatibility complex (MHC) (H-2^d) as BALB/c mice, were transfected with pcDNA-hsp65 and used as HSP65 protein-expressing target cells. A total of 2×10^6 cells/ml effector splenic cells were treated with anti-CD8 antibody, anti-CD4 antibody or anti-Thyl.2 antibody followed

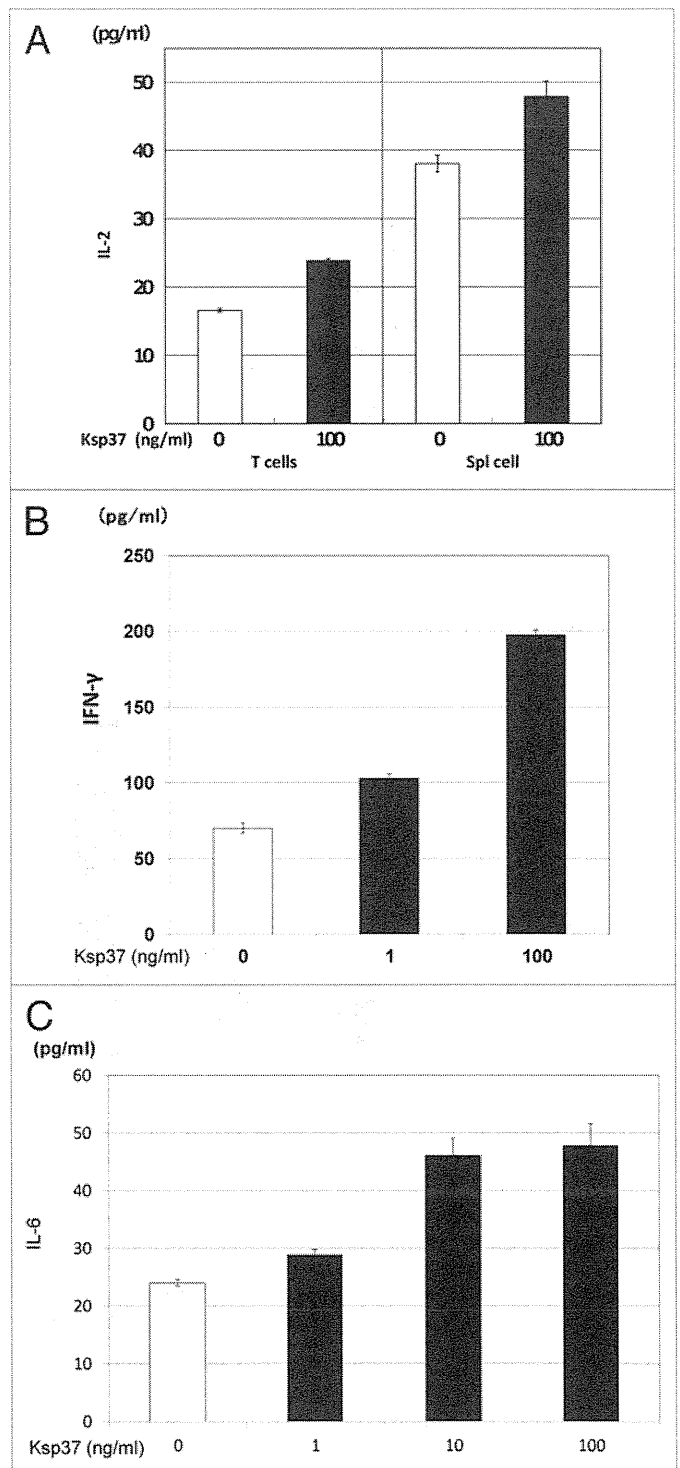

Figure 7. Augmentation of cytokines (IL-2, IFN-γ and IL-6) production by Ksp37 was also observed. (A) Augmentation of IL-2 production from T cells or spleen cells by Ksp37. An amount of 5×10^6 splenic T cells or spleen cells from C57BL/6 mice were cultured with 5×10^5 BALB/c spleen cells (Mitomycin-C treated) in the presence of rKsp37 for two days. IL-2, IFN-γ and IL-6 activities in the supernatants were assessed by ELISA. (B) Augmentation of IFN-γ production from spleen cells by Ksp37. (C) Augmentation of IL-6 production from spleen cells by Ksp37.

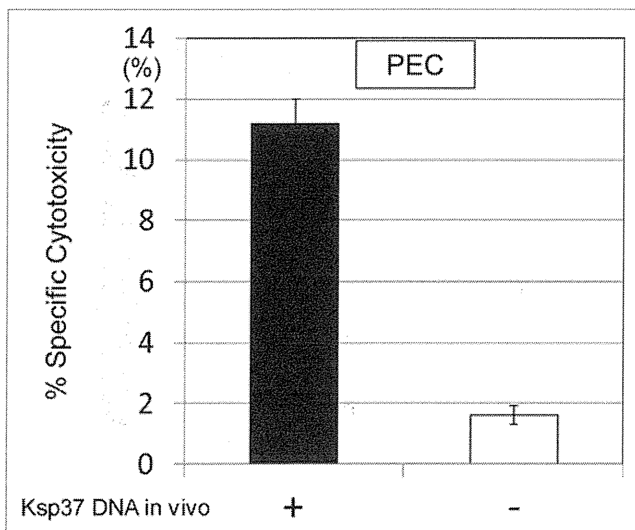

Figure 8. Augmentation of CTL differentiation in vivo by the treatment with Ksp37 DNA was demonstrated. C57BL/six mice were challenged with killed H37Ra antigen (1,000 µg/mouse) in vivo (i.p.) and then treated with Ksp37 DNA (100 µg/mouse, six times) i.m. Twenty-one days after challenge of killed TB antigen, and PEC (peritoneal exudate cells) from these mice were harvested. CTL activity againt TB antigen (HSP65 antigen) was assessed by using ^{51}Cr EL-4 which had been transfected with HSP65 DNA.

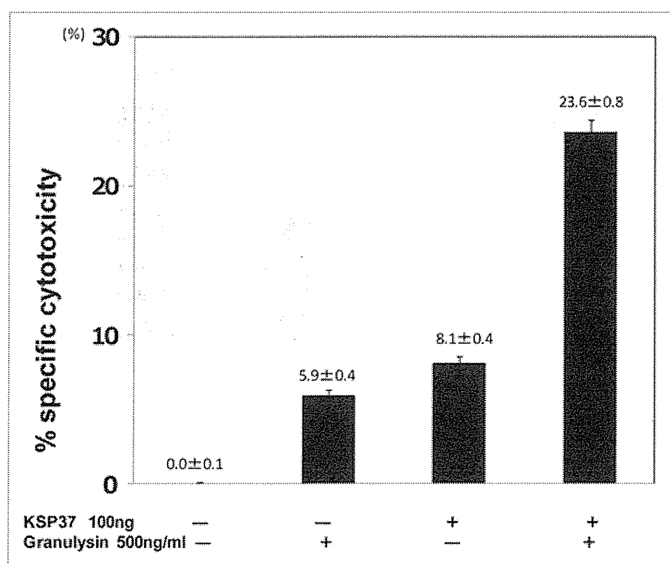

Figure 9. Synergistic efficacy of Ksp37 and granulysin on the in vitro CTL induction. Splenic T cells were cultured with uv-treated BALB/c spleen cells (Mytomycin C treated) in the presence or absence of 100ng/ml Ksp37 protein and 500ng/ml 15K granulysin protein for 5 d. Five days after culture, CTL activity against P815 tumor cells was assessed by using ^{51}Cr release assay.

by complement.[1] ^{51}Cr release was assessed using the ^{51}Cr-release assay[10,30-34] at the Effector:Target (E:T) ratio of 50:1. Spontaneous lysis (with medium alone) and maximum lysis (^{51}Cr release after three cycles of freeze-thaw) were set up for background and targets. Percent specific lysis was determined as: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100.[30-34]

Production of cytokines (IL-2 and IFN-γ). Mouse cytokines were measured in quantitative ELISAs for IL-2 and IFN-γ as described previously.[1,2]

ELISPOT assay. The spleens were removed aseptically from vaccinated mice 3 weeks after the third vaccination. Antigen-specific IFN-γ-producing cells were determined by enzyme-linked immunosorbent spot (ELISPOT) as described previously.[1,2]

Statistical analysis. Tukey-Kramer's HSD tests were used to compare $\log_{10}$ value of CFU between groups following challenge and T cell responses between groups in ELISPOT assay. Student's t-tests were performed to compare T cell responses between groups in T cell proliferation assay and granuloma formation between groups following challenge. A P-value of < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Novel therapeutic vaccines [(HSP65 + IL-12) DNA-, granulysin- and Ksp37-vaccine] against tuberculosis and synergistic effects in the combination with chemotherapy

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Keywords: therapeutic vaccine against, tuberculosis, HSP65 DNA + IL-12 DNA vaccine, granulysin vaccine, Ksp37 vaccine, chemotherapy, Ksp37 transgenic mouse, synergistic therapeutic efficacy, MDR-TB, XDR-TB

Abbreviations: HVJ, hemagglutinating virus of Japan; 15K granulysin, 15 kilodalton granulysin; Ksp37, killer specific secretory protein of 37kDa; MDR-TB, multi-drug registant tuberculosis; XDR-TB, extremely drug resistant tuberculosis; Tg, transgenic

Purpose: Multi-drug resistant tuberculosis (MDR-TB) and extremely drug resistant (XDR) TB are big problems in the world. We have developed novel TB therapeutic vaccines, HVJ-Envelope/HSP65 + IL-12 DNA vaccine (HSP65-vaccine), granulysin vaccine and killer specific secretory protein of 37kDa (Ksp37) vaccine.

Results and Methods: HSP65 vaccine showed strong therapeutic effect against both MDR-TB and XDR-TB in mice. Intradermal immunization of HSP65-vaccine showed stronger therapeutic effect against TB than intramuscular or subcutaneous immunization. Furthermore, the synergistic therapeutic effect was observed when the vaccine was administrated in combination with Isoniazid (INH), which is a first line drug for chemotherapy. The combination of types of vaccines (HSP65- and granulysin- vaccines) also showed synergistic therapeutic effect. In the monkey model, granulysin-vaccine prolonged the survival period after the infection of TB and long-term survival was observed in vaccine-treated group. We examined the potential of two kinds of novel DNA vaccines (Ksp37-vaccine and granulysin-vaccine). Both vaccines augmented in vivo differentiation of CTL against TB. We measured the amount of Ksp37 protein in human serum and revealed that the level of Ksp37 protein of patients with tuberculosis was lower than that of healthy volunteers. Therefore, we established Ksp37 transgenic mice as well as granulysin transgenic mice to elucidate the function of those proteins. Both transgenic mice were resistant to TB infection.

Conclusion: These data indicate the potential of combinational therapy; the combination of two DNA vaccines or combination of DNA vaccine with antibiotic drug. Thus, it will provide a novel strategy for the treatment of MDR-TB.

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. About 500,000 of people around the world are affected by MDR-TB every year. However, there are a small number of effective drugs against MDR-TB. 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 to evaluate the potential of newly developed vaccines as reported by Walsh and Tan.[1] Onset and progress after TB infection in the cynomolgus monkey is very similar to human TB disease.[1-3] SCID-PBL/hu is an IL-2 receptor γ-chain disrupted mouse (NOD-SCID) transplanted with human PBL. It is an in vivo humanized immune model and provides an useful tool for investigating human immune responses to the administration

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Table 1. Therapeutic efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine on TB infection in murine models

1. HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine showed therapeutic efficacy against MDR-TB as well as drug-sensitive TB in mice.

2. Significant prolongation of survival was observed in the XDR-TB infected mice by the treatment with this vaccine.

3. Therapeutic efficacy of this vaccine on chronic TB disease models using a mouse infected with TB in the aerosol chamber was demonstrated.

Therapeutic efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine was evaluated by using mouse models. Therapeutic efficacies of this vaccine on the MDR-TB infection and XDR-TB infection were observed.

of vaccines.[4,5] Transgenic mice that express the components of vaccine also provide a lot of information about novel TB vaccines. Therefore, we used three animal models (cynomolgus monkey model of TB, SCID-PBL/hu mice and transgenic mice) to develop three kinds of novel vaccines against TB.

We have previously evaluated a novel therapeutic TB vaccine which consists of plasmid DNA encoding HSP65 + IL-12 and HVJ-Envelope (HSP65-vaccine) and revealed the efficacy of the vaccine in these animal models.[6-9] However, it is very important to treat the patients with TB completely within a short period of time. Therefore, we improved the regimen of the vaccination. We first examined a combination therapy of vaccination and chemotherapy. In the present study, we used a HSP65-vaccine for the vaccination and first line TB drugs (INH or RFP) for the chemotherapy. We next identified a suitable administration route to increase the efficacy of the DNA vaccine. The result suggested that intradermal vaccination is more suitable for the DNA vaccine than intramuscular or subcutaneous vaccination.

Finally, we developed plasmid-based DNA vaccines encoding the granulysin or Ksp37. Granulysin is a member of the saposin-like protein family and colocalizes with perforin and granzymes in the cytolytic granules of human CTL and NK cells. In the presence of perforin, it has a cytolytic activity against intracellular pathogens in the cytoplasm of infected cells. It also has a cytotoxic effect on tumor cells.[10-12] Granulysin is present in cytoplasm of human CD8 positive cytotoxic T cells and NK cells. It has been suggested that granulysin has a cytolytic activity against M. tuberculosis outside macrophages and reduces the number of M. tuberculosis in the macrophage dependent on the presence of perforin in vitro.[11] However, the precise role of granulysin in vivo has not been elucidated yet. Therefore, we have established granulysin transgenic mice to elucidate in vivo role of granulysin and obtain the information to develop novel vaccines against M. tuberculosis. Transgenic mice of 15K and 9K granulysins were resistant to TB infection in vivo. This is the first report indicating an in vivo role of granulysin in the defense against the infection of TB. In addition, we demonstrated the efficacy of granulysin-vaccine in the monkey model of TB.

Ksp37 is also produced from CTL and NK cells.[13] However, immunological function of Ksp37 has not been elucidated yet. In the first step, we first examined the amount of Ksp37 in the serum of patients with TB. The result indicated that Ksp37 in the serum of patients with TB was lower than that of healthy volunteer. In the next step, we have established Ksp37 transgenic mice to elucidate the in vivo role on Ksp37 in the immunity to the infection of M. tuberculosis. Ksp37 transgenic mouse was resistant to the infection of TB, suggesting the anti-TB effect of Ksp37 in vivo.

To increase the efficacy of the vaccine, we examined the synergistic effect of the combination therapy, in which HSP65-vaccine and first line chemotherapy drug (INH) were administrated. We also examined the synergistic effect of the combination of two kinds of DNA vaccines. HSP65- and granulysin-vaccine were simultaneously administrated in the murine models of TB and efficacy of the vaccines was evaluated in vivo.

In parallel, we investigated the immunological effects of granulysin and Ksp37 and revealed the synergistic effect of two molecules on the induction of TB-specific CTL (manuscript submitted).

These findings demonstrated that granulysin-vaccine and Ksp37-vaccine might provide very useful weapon as TB vaccines, and those efficacy will be enhanced in combination with other DNA vaccine (including HSP65 vaccine) and INH.

Results

Novel therapeutic vaccine of HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine. *Therapeutic effect of the vaccine in murine models of TB.* Therapeutic efficacy of HSP65 DNA + IL-12 DNA vaccine (HSP65-vaccine) was evaluated in murine models. At 30 d after intravenous challenge of MDR-TB, the CFUs of TB in the lungs, spleen, and liver were counted and therapeutic efficacy of HVJ-Envelope DNA vaccine was evaluated.

Table 1 shows the feature of novel TB vaccines; the vaccine consists of a plasmid DNA vaccine expressing mycobacterial heat shock protein 65 (HSP65)+interleukin-12 (IL-12) and a vector (hemagglutinating virus of Japan (HVJ)-liposome or HVJ-envelope). We used the mouse model in our previous study.[6,7] This vaccine showed therapeutic effect against TB in the mouse: (1) This vaccine significantly decrease the number of drug sensitive H37Rv TB in the spleen and the lung of mice (data not shown). This vaccine also decreased the number of MDR-TB in the lungs and spleen of mice, indicating the efficacy of the vaccine in the mouse model of TB. (2) Significant prolongation of survival was observed in the XDR-TB infected mice. The survival period of the mouse treated with this vaccine was prolonged compared with non-vaccinated mice. Those results demonstrated that this vaccine have strong therapeutic effect against XDR-TB. (3) We have established the mouse model of chronic TB disease using an inhalation system (aerosol chamber) for the infection of TB. By using this model, therapeutic efficacy of this vaccine was examined. The vaccine was administered 5 weeks after aerosol infection of TB. Treatment with this vaccine decreased the number of TB in the lung of the mice (data not shown).

Thus, we demonstrated that this vaccine had a therapeutic effect against XDR-TB and MDR-TB as well as drug-sensitive TB.

Synergistic effect of HVJ-Envelop/HSP65 DNA + IL-12 DNA vaccine in combination with INH against TB infection. To enhance the

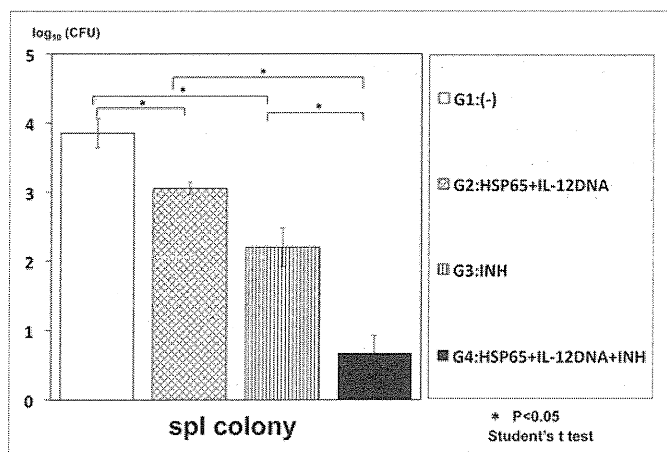

Figure 1. Synergistic therapeutic efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine and INH on TB infection of mice. BALB/c mice were infected with H37Rv TB by using intratracheal aerosol challenge using aerosol chamber. One week after challenge of TB, the vaccine and INH (0.03mg/mouse) were administered 6 times for 3 weeks. Five weeks after TB challenge, mice were sacrified, and CFU of TB in the spleen were evaluated. G1 vs. G2; $p < 0.05$; G1 vs. G3; $p < 0.05$; G3 vs. G5; $p < 0.05$; G2 vs. G5 $p < 0.05$; Student's test.

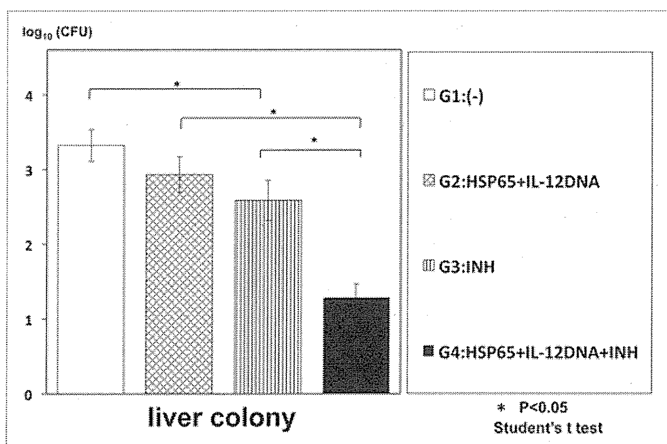

Figure 2. Synergistic therapeutic efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine and INH on TB infection of mice. BALB/c mice were infected with H37Rv TB by using intratracheal aerosol challenge using aerosol chamber. One week after challenge of TB, the vaccine and INH (0.03mg/mouse) were administered 6 times for 3 weeks. Five weeks after TB challenge, mice were sacrified, and CFU of TB in the liver were evaluated. G2 vs. G4; $p < 0.05$; G3 vs. G4; $p < 0.05$; Student's test.

efficacy, we examined synergistic effect of combinational therapy of this vaccine and INH (isoniazid) on the infection of TB. The numbers of TB in the mouse spleen and liver were significantly decreased compared with the monotherapy, indicating the synergistic effect of the combinational therapy (**Figs. 1 and 2**).

We also compared the administration route of the vaccine to improve the regimen of the therapy. We selected three routes (intradermal, intramuscular and subcutaneous injections) for our experiment. The efficacy of intradermal administration (i.d.) was highest among three administration routes (**Fig. 3**).

Therefore, in the monkey model we plan to study the efficacy of intradermal injection of this vaccine, in comparison to i.m administration.

IL-2 receptor γ-chain gene disrupted SCID-PBL/hu. We have very important humanized immune model (SCID-PBL/hu) to study the human T cell immune response in vivo as reported first in Cancer Research 1997.[4] We evaluated the efficacy of novel vaccines in vivo using this humanized immune model.

We used IL-2 receptor γ-chain gene knockout mouse-based model (NOD SCID-PBL/hu) to analyze the human T cell responses to the vaccine (**Table 2**).

The efficacy of HSP65+ IL-12 DNA vaccine was examined in this IL-2 receptor γ-chain gene disrupted SCID-PBL/hu-model and significant decrease of the number of TB in the liver was observed as shown in **Table 2A**. This model shows stronger human CTL induction and proliferation than conventional SCID mouse-based model (CB17-SCID-PBL-hu) (**Table 2B**).

Therapeutic efficacy of this vaccine on monkey model. We are developing a GMP level of DNA vaccine that contains two expression units in one plasmid vector for future clinical trial. In this study, we used a GMP level of DNA vaccine for the evaluation of the potency in the monkey model of TB. Monkeys were intratracheally instillated with 5×10^2 CFU of human TB (Erdman strain). After TB infection, 9 times intramuscular injection of this vaccine (total 400 μg/monkey) was conducted. Therapeutic efficacy was evaluated on the basis of survival, ESR, body weight, immune responses, chest X-ray and PPD skin test. The monkey group treated with HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine showed 100% survival (data not shown). In contrast, the monkey group of control saline showed 60% survival. Thus, the therapeutic DNA vaccine improved the survival rate of TB-infected monkeys, compared with the saline (control).

These data indicated the therapeutic efficacy of a GMP-level of DNA vaccine in TB-infected monkeys.

Efficacy of granulysin-vaccines. *Efficacy of granulysin in transgenic mice.* We noticed the in vivo function of granulysin, since it shows a cytolytic activity against Mycobacterium tuberculosis. The features of granulysin are as follows:

(1) Granulysin is cytolytic molecules expressed by human CTL and NK cells and show the cytolytic activity against a variety of tumors and microbes, including Mycobacterium tuberculosis. Granulysin belongs to the saposin-like protein family that includes amoebapores and NK lysine. Recent studies show that granulysin also has chemoattractant and proinflammatory activities. However, in vivo anti-microbe activity and anti-tuberculosis activity of granulysin has not been elucidated yet.

(2) It has been reported 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.

(3) However, the precise role of granulysin in the in vivo against the tuberculosis infection has not been elucidated yet. Therefore, we have established granulysin transgenic mice to elucidate mechanism of granulysin in vivo. We have established two kinds of transgenic mice by using usual microinjection method: 15K granulysin transgenic mice and 9K granulysin transgenic

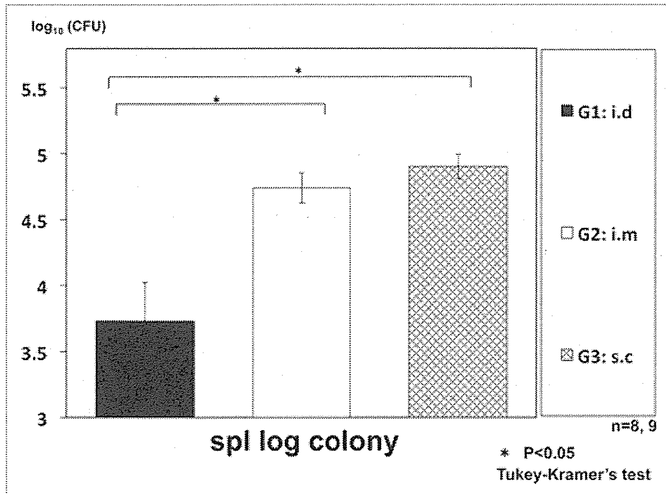

Figure 3. Therapeutic efficacy of intradermal (i.d.) vaccination of HVJ-Envelope/HSP65 DNA + IL-12 DNA, compared with intramuscular (i.m.) or subcutaneous (s.c.) vaccination using intratracheally aerosol infected DBA/1 mice. DBA/1 mice were infected with H37Rv TB by using intratracheal aerosol challenge using aerosol chamber. One week after challenge of TB, 100μg of HVJ-Envelope/HSP65 DNA + IL-12 DNA were administered 6 times for 3 weeks by i.d, i.m, or s.c administration. Four weeks after TB challenge, mice were sacrified, and CFUs of TB in the spleen were evaluated. G1 vs. G2; $p < 0.05$; G1 vs. G3; $p < 0.05$; Student's test.

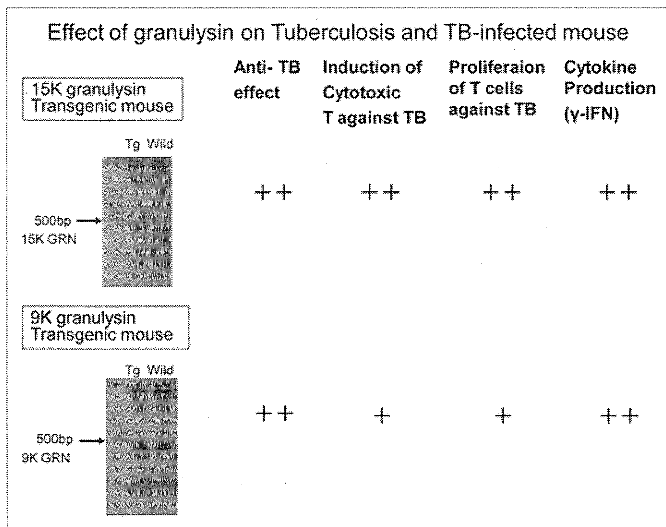

Figure 4. The establishments of 15K granulysin transgenic mice and 9K granulysin transgenic mice. The efficacies of 15K granulysin transgenic mice and 9K granulysin transgenic mice on TB infection were summarized in this Figure. An anti-TB effect, the induction of CTL against TB, the proliferation of T cells against TB and γ-IFN production were augmented in these transgenic mice, compared with wild type C57BL/6 mice.

mice. We measured CFU number of *M. tuberculosis* in the lung four weeks after intravenous injection of TB (5×10^5 /mouse). As shown in **Table 3**, reduction of CFU number was observed in 15K granulysin transgenic mice compared with the normal C57BL/6 mice, indicating the in vivo anti-TB effect of 15K granulysin. As summarized in **Figure 4**, augmentation of immune responses were also observed in 15K granulysin transgenic mice: in vivo induction of cytotoxic T cells against TB, enhanced proliferation of T cells stimulated with TB antigen and augmentation of cytokine production. Furthermore, we examined synergistic effects of the combination of two vaccines. As shown in **Figure 5**, the combination of HSP65-vaccine and granulysin-vaccine showed synergistic effects and 10 times reduction of the CFU number in the liver of TB-infected mice was observed. The number of TB in the liver was significantly reduced by the combination of two vaccines.

Efficacy of granulysin-vaccine in monkey models. We examined the efficacy of granulysin-vaccine in the therapeutic model of TB. The survival rate of granulysin-vaccine (HVJ-Envelope/15K granulysin DNA vaccine)-treated group was 25% (1/4) at 1 y after TB infection (**Fig. 6**). In contrast, all monkeys in saline group were died within 200 d after TB challenge. Thus, survival rate at one year after TB infection was 0% (0/4).

The proliferation of PBL from the monkeys treated with granulysin-vaccine was augmented compared with that of control (saline treated) monkeys (**Fig. 7**). These results indicated the efficacy of granulysin-vaccine in therapeutic models using monkey. Thus, granulysin-vaccine is effective in the monkey as well as the mouse model of TB.

Efficacy of Ksp37-vaccine in therapeutic models. Ksp37 protein is produced from CTL, TypeI helper T cell, γ/δ T cell and NK cell. Ksp37 is composed of 223 amino acids. We analyzed the concentration of Ksp37 in the serum of patients with TB by ELISA. The level of Ksp37 protein in the serum of patients with TB (n = 31) was significantly lower than that of healthy volunteers (n = 60) ($p < 0.05$) (**Fig. 8**). This is first report suggesting the relation between the serum level of Ksp37 and TB disease (**Fig. 8**).

Therefore, we tried to elucidate the in vivo function of Ksp37 protein, especially function as an anti-TB agent in vivo.

In the first step, we have established a Ksp transgenic mouse for the analysis of function in vivo. We measured the CFU number of M. tuberculosis in the lung 3 weeks after TB aerosol infection. In Ksp transgenic mice, the CFU number of M. tuberculosis was decreased compared with that of wild type control mice (**Fig. 9**). This result indicated the anti-TB effect of Ksp37 in vivo.

These finding suggested that Ksp37 produced from CTL and NK cell functions as an important anti- TB factor in humans and mice.

Discussion

In the present study, we evaluated the potential of three kinds of novel therapeutic vaccines (HSP65-, granulysin- and Ksp37-vaccines) in mouse and monkey models of TB. All of the vaccines showed anti-TB effects in therapeutic models. It is noteworthy

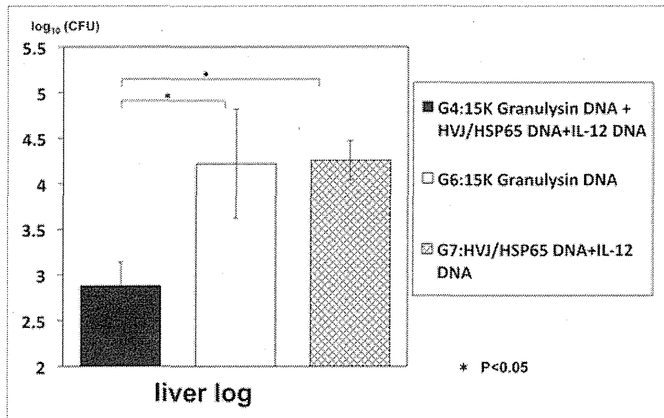

Figure 5. Therapeutic effect of granulysin DNA vaccine on TB-infected (DBA/1) mice DBA/1 mice were infected with H37Rv TB using intratracheal aerosol challenge. One week after challenge of TB, 100μg of HVJ-Envelope/HSP65 DNA + IL-12 DNA and/or 100 μg of HVJ-Envelope/granulysin DNA were injected i.m. into mice 6 times for 3 weeks. Four weeks after TB challenge, mice were sacrified, and CFUs of TB in the liver were evaluated. G1 vs. G2; $p < 0.05$; G1 vs. G3; $p < 0.05$; Student's test.

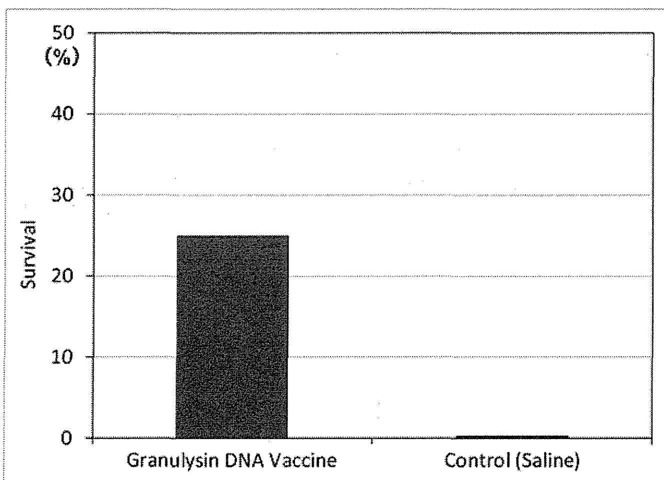

Figure 6. Therapeutic efficacy (survival) of HVJ-Envelope/15K granulysin DNA vaccine, 365 d after TB infection using cynomolgus monkey models. Five × 10^2 M. tuberculosis (Erdman strain) were intratracheally into cynomolgus monkeys as described in Materials and Methods. Four weeks after challenge of TB, 400μg of HVJ-Envelope/15K granulysin were injected i.m. Six times every two weeks. Survival of monkeys treated with this vaccine were evaluated for 1 y (365 d).

that efficacy of novel therapeutic vaccines were demonstrated in monkey models as well as murine models. Thus, this is the leading report of new vaccine against TB. According to our knowledge, only a few therapeutic vaccines against TB have been reported.[14,15] HSP65-vaccine as well as 15K granulysin-vaccine delivered by HVJ-Envelope vector prolonged the survival and augmented the immune responses in the cynomolgus monkey model which closely mimics human TB disease. Thus, we are taking advantage of the availability of multiple animal models and are accumulating essential data on the DNA vaccine/HVJ-envelope in anticipation of initiating a phase I clinical trial.

It is very important to evaluate the long-term survival in a monkey model, as human TB is a chronic infection disease. Thus, it is necessary for the development of effective vaccine to evaluate the long-term survival of monkey.[2,3,7-9] In this study, increase in the survival rate was also observed in HVJ-Envelope/15K granulysin vaccine-treated group, compared with saline-treated group (control group). In addition, it is noteworthy that histopathological improvement was observed in the lung of vaccine-treated monkey (365 d after TB infection). A lot of granulomatous lesions were observed in lung of survived monkey, while a little or no such lesions were observed in lung of saline-treated monkey, which had died of TB within 200 d after TB challenge. Histology of granulomatous lesions observed in this experiment was very similar to human lung TB granuloma by histopathological examinations.

Efficacy of 15K granulysin-vaccine was studied in murine models of TB. We used therapeutic models in this experiment. Furthermore, we examined the synergistic effect of two vaccines (the combination of HSP65- and granulysin-vaccines) in the same therapeutic model. The results indicated the synergistic effect of the combinational vaccination. Therefore, the combination of these therapeutic vaccines might be useful for the development of vaccines against human TB infectious disease. In summary, it was demonstrated that granulysin-vaccine had a therapeutic effect against TB in the mouse and monkey models of TB.

We also elucidated the in vivo function of Ksp37. Ksp37 is expressed in cytotoxic lymphocytes, selectively in the effector subset of $CD8^+$ T cells, $CD16^+$ NK cells and γ/δT cells.[13] Expression of Ksp37 mRNA was closely correlated with good prognosis in ovarian cancer cells and gliomas.[16,17] However,

Table 2. Therapeutic efficacy and Immune responses using IL-2 receptor γ-chain gene disrupted SCID-PBL/hu models

(A)	**Therapeutic efficacy**	
	Treated	CFU of TB (log)
	(-)	6.03 ± 0.06
	HSP65 DNA + IL-12 DNA vaccine	5.40 ± 0.97
(B)	**Immune reponses**	**human CTL and T cell proliferation**
	IL-2 R γ-chain(-/-) SCID PBL-hu	human CTL (+++) human T cell proliferation (+++)
	CB17-SCID PBL-hu	human CTL (+) human Tcell proliferation (+)

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 (50ug i.m.). Ten days after the third vaccination, mice were sacrificed and CFU of TB in the liver of mice were accessed as described in Materials and Methods. One × 10^7 PBL from a healthy human volunteer were injected i.p. into IL-2 receptorγ-chain disrupted NOD-SCID mice. Twenty one days after injection of PBL, mice were challenged with 5 × 10^5 H37Rv i.v. and then treated with vaccine. *Student's t-test was used to compare the CFU of TB of each group ($p < 0.05$). Human immune responses [human CTL activity and human T cell proliferation against alloantigen (CESS cells)] of IL-2 receptor γ-chain (-/-) NOD SCID PBL-hu mice were compared with those of CB17-NOD-SCID PBL-hu mice. (+), weak; (+++), very strong.

Table 3. In vivo anti-TB effect of 15kDa Granulysin Transgenic mouse

mouse	lung Number of M. tuberculosis 4 weeks after TB injection (log)
G1 15K Granulysin Tg	5.3 ± 0.35
G2 Wild Type C57BL/6	6.0 ± 0.5

Mean ± SD, n = 7; *p < 0.05 G1 vs. G2; Tukey-Kramer's HSD; In vivo inhibition of the growth of *M. Tuberculosis* in the 15K granulysin transgenic mice; In vivo anti-TB effect of 15K granulysin transgenic mouse; Five 15 K granulysin transgenic mice and five wild type C57BL/6 mice were injected with 5×10^5 H37Rv M. tuberculosis i.v. Four 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. Student's t-test was used (p < 0.05).

detailed immunological function has not been elucidated yet. We first revealed that the level of Ksp37 protein in the serum of patients with TB was lower than that of healthy volunteer. The result suggested the relation between the serum level of Ksp37 and TB disease. Next, we have established Ksp37 transgenic mice to elucidate the in vivo role of Ksp37 in the defense against the infection of M. tuberculosis. Ksp37 transgenic mice showed in vivo anti TB effect. Thus it was demonstrated that Ksp37 played an important role in anti-TB function in human as well as mice bodies. Finally, we examined the efficacy of Ksp37-vaccine in the mouse model of TB. Similar to granulysin-vaccine, Ksp37-vaccine augmented in vivo differentiation of CTL against TB (data not shown). In addition, simultaneous administration of Ksp37- and granulysin-vaccines induced CTL generation synergistically (data not shown). Therefore, these findings indicate that granulysin- and Ksp37-vaccine might provide very useful weapon as a novel TB vaccine, in the monotherapy or combination therapy.

The HSP65 vaccine showed a significant therapeutic effect against TB, as described previously: (1) Prolongation of survival of mice infected with XDR-TB; (2) Decrease in the CFU of TB in lung, liver and spleen of mice infected with MDR-TB as well as drug-sensitive TB (H37Rv); (3) Decrease in the CFU of TB in organs of mice challenged with TB in the in vivo humanized immune model of SCID-PBL/hu.

Here, we revealed the synergistic effects of the combination therapy of HSP65-vaccine and a first line chemotherapy drug Isoniazid (INH). It is very important to make a suitable regimen, which enables the treatment of the patient with TB to complete within a shorter period. In such circumstances, our data demonstrating the synergistic effect of the combinational therapy using a DNA vaccine and a chemotherapy drug will provide a new strategy for the treatment of TB.

We also revealed the importance of administration route of DNA vaccine. Generally, vaccines are administrated either intradermally (i.d.), intramuscullary (i.m.) or subcutaneously (s.c.). Our data suggested that the intradermal injection is suitable for the administration of our DNA vaccines. Therefore, in the monkey model we plan to conduct the efficacy study of intradermal injection of this vaccine. We will compare the efficacy of intradermal administration to conventional i.m administration.

In the recent study using cynomologus monkeys, it is suggested that i.d. vaccination of HSP65-vaccine showed stronger therapeutic effects against TB than i.m. vaccination on the basis of the prolongation of survival and ESR (Erythrocyte Sedimentation Rate).

DNA vaccine is a relatively new approach of immunization for infectious diseases.[3,4,18-21] We have developed a hemagglutinating virus of Japan envelope (HVJ-Envelope) using inactivated Sendai virus, as a nonviral vector for drug delivery.[22-24] It can efficiently deliver DNAs, siRNAs, proteins and anti-cancer drugs into cells both in vitro and in vivo.[25-27] Therefore, HVJ-Envelope was suitable as an efficient and safe vector for DNA vaccines.

The priority of development of vaccine(s) to prevent reactivation of TB will be increased, since large proportion of the world is latently infected with TB. The combination of HSP65-vaccine with conventional vaccine (BCG) showed synergistic effects in the mouse and monkey models of TB and prolonged the survival of animals. Therefore, it will be important to evaluate the current vaccines as post-exposure vaccines. Combination of several vaccines or combination of vaccines with drugs for chemotherapy might provide a new insight for the prevention of the reactivation of TB.

In conclusion, our data indicated the synergistic therapeutic effect of combination of HSP65-, granulysin- and Ksp37-vaccines or combination of these DNA vaccines and first line chemotherapy drug(s). Combinational therapy using vaccines and antibiotics might provide novel rationale against MDR-TB therapy. Furthermore, the efficacies of HSP65 vaccine and granulysin vaccine were confirmed in the murine therapeutic model for XDR-TB and cynomolgus monkey therapeutic model. These data will provide a rationale for moving this vaccine into clinical trial. HSP65-, granulysin- and Ksp37-vaccines might be useful

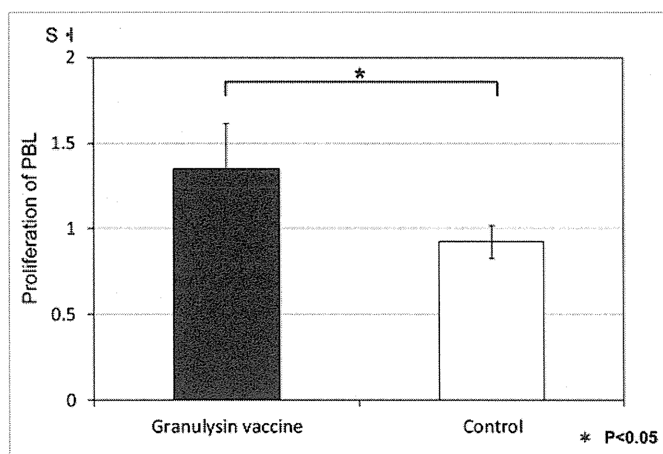

Figure 7. Proliferation of PBL from monkeys vaccinated with HVJ-Envelope/15K granulysin DNA by the stimulation with HSP65 antigen. Five $\times 10^2$ M. tuberculosis (Erdman strain) were intratracheally into cynomolgus monkeys as described in Materials and Methods. Four weeks after challenge of TB, 400μg of HVJ-Envelope/15K granulysin were injected i.m. Six times every two weeks. The proliferation of PBL from monkeys vaccinated with HVJ-Envelope/15K granulysin on 13 weeks after TB challenge were assessed by the ^{3}H-TdR uptake of lymphocyte for 3 d culture.

vaccines against TB including XDR-TB and MDR-TB after the clinical trials.

Materials and Methods

Methods for the evaluation of the therapeutic 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: 400 ug 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.[2,6,7]

The animals were vaccinated with HVJ-Envelope/15K granulysin DNA vaccine 6 times. Four weeks after challenge of TB, 400 μg of HVJ-Envelope/15K granulysin were injected i.m. six times every two weeks. Survival of monkeys treated with this vaccine were evaluated. 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.

Methods for the evaluation of the efficacy of vaccines on the *M. tuberculosis*-infected mice. DNA vaccines encoding *M. tuberculosis* HSP65 and IL-12 were encapsulated into HVJ-Envelope.[3,6,8,28] HVJ-Envelope were prepared as described previouslyl.[4,29] The HVJ-Envelope complex was aliquoted and stored at -70°C until use. Groups of mice were vaccinated three times with 100 μl of HVJ-Envelope solution containing 50 μg of pcDNA-IgHSP65 and 50 μg of pcDNA-mIL12p40p35-F in the tibia both anterior muscles after TB challenge.[28,29] At 30 d after intravenous challenge of *M. tuberculosis* H37Rv and MDR-TB, the number of CFU in the lungs, spleen, and liver were counted and therapeutic efficacy of HVJ-Envelope DNA vaccines was evaluated.[28,29] DBA/1 mice were treated with HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine three times i.m. at 1, 8 and 15 d after the challenge of 5×10^5 CFU MDR-TB i.v. 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). Mice were maintained in isolator cages, manipulated in laminar flow hoods and used between 8–10 weeks of age. All vaccinations and experiments on isolate tissue of animal were done under anesthetic state with sevoflurane. Infected animals were housed in individual micro-isolator cages in a Biosafety Level (BL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee.

Methods for the establishment of SCID-PBL/hu model. IL-2 receptor γ-chain disrupted NOD-SCID-PBL/hu was constructed as described in our previous study.[4,5] CTL activity was assessed using the method as described previously.[30-32]

Methods for the establishment of granulysin transgenic mouse. Either 15K granulysin gene, 9K granulysin gene or secreted 9K granulysin DNA (15K Gra secretory signal DNA was fused into N-terminal of 9K 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. Two types of 15K granulysin Tg mice, 3 types of 9K granulysin Tg mice and 6 types of secreted 9K granulysin Tg mice were made. Granulysin activity was assessed by monoclonal antibody targeting 15K granulysin and 9K granulysin. *Mycobacterium tuberculosis* H37Rv 5×10^5 CFU was intravenously injected to 15K granulysin Tg mice, 9K granulysin Tg mice, wild type (control) mice and normal C57BL/6 mice (8~12weeks).[3,7] From 2 to 12 weeks after injection, these mice were sacrificed. The lungs, the liver and the spleen of these mice were removed, homogenized and cultivated for 14 d on 7H11 agar medium. Then, the number of colony of *Mycobacterium tuberculosis* was measured.[28,29]

Method for the establishment of Ksp37 transgenic mouse. Ksp37 gene were transferred to expressing plasmid DNA

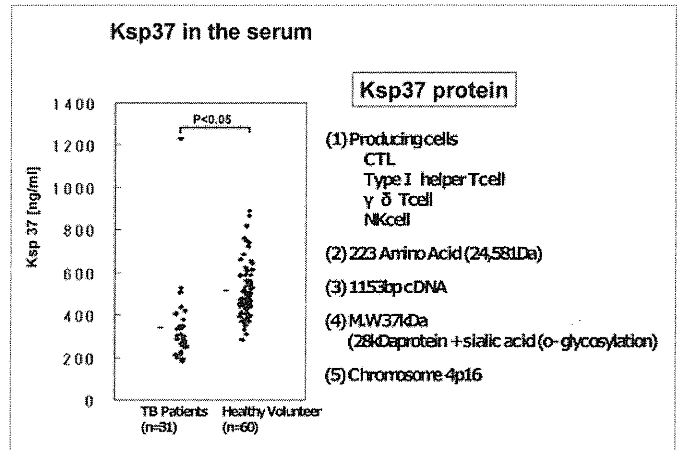

Figure 8. Killer specific secretory protein of 37kDa (Ksp37 protein) in the serum of patients with tuberculosis. Ksp37 protein in the serum of 31 patients with TB and 60 healthy volunteers were assessed by ELISA.

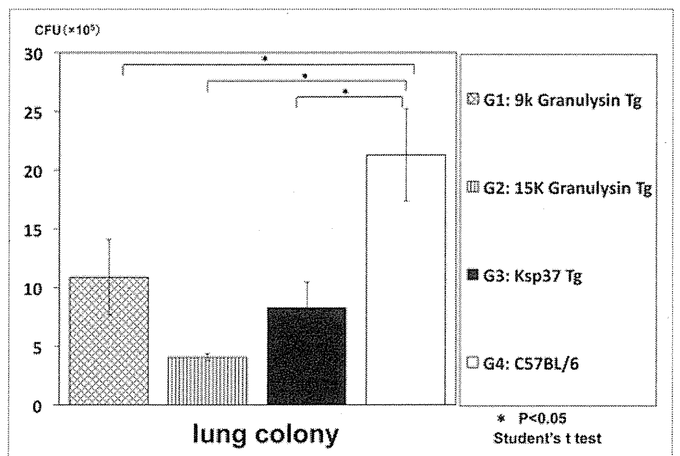

Figure 9. In vivo anti-TB effect of Ksp37 transgenic mice. Ksp37 Tg mice were established as described in Materials and Methods. Ksp37 Tg mice, 15K granulysin Tg mice, 9K graunlysin Tg mice and wild type C57BL/6 mice were infected with H37Rv TB by using intratracheal aerosol challenge using aerosol chamber.

(pCAGGS) having CAG promoter. DNA fragment was injected to pronuclei embryo and grafted to 200 foster parents. Two types of Ksp Tg mice (#13, #14) were made. Ksp activity was assessed by monoclonal antibody targeting Ksp 37.

Reagents. Isoniazid (INH) was obtained from Sigma Co. Ltd (lot No. 117K0712). Rifampicin (RFP) was obtained from Sigma Co. Ltd (lot No. 087K18753). An amount of 0.03 mg/mouse of INH and 0.1 mg/mouse of RFP were administered to mice per os.

Statistical analysis. Student's t tests and Tukey-Krumer's test were used to compare log 10 value of CFU between groups following challenge of TB. Student's t tests were also performed to compare immune responses between groups in T cell proliferation assay. A P-value of < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Clonal expansion of *Mycobacterium tuberculosis* isolates and coexisting drug resistance in patients newly diagnosed with pulmonary tuberculosis in Hanoi, Vietnam

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Abstract

Background: Newly diagnosed patients without anti-tuberculosis (TB) treatment histories have not often undergone drug susceptibility testing (DST), but have received the standard treatment regimen without information about their DST profiles in many countries with inadequate resources.

Methods: We collected 346 clinical isolates from previously untreated patients with smear-positive active TB in Hanoi, the capital of Vietnam. Of these, 339 were tested for susceptibility to four first-line anti-TB drugs, including isoniazid (INH), rifampicin (RMP), streptomycin (SM), and ethambutol (EMB), using the proportion method. A pyrazinamidase (PZase) test was used to assess pyrazinamide (PZA) resistance. Results of the culture-based drug susceptibility tests were confirmed by those from reverse hybridization-based line probe assays (LiPAs) that detected mutations associated with RMP, INH, PZA, and fluoroquinolone (FQ) resistance. To investigate a diversity of these strains, IS*6110*-probed restriction fragment length polymorphisms (RFLPs) were analyzed. Nucleotide sequences for *furA-katG* and *fabG1-inh*A operons, transcription units responsible for INH resistance, were also determined.

Results: Of the isolates tested, 127 (37.5%) were resistant to at least one of the four drugs, which included 93 (27.4%) isolates that were resistant to INH. RFLP analysis identified four clusters defined by similarity of the band patterns, which accounted for 46.1% of the tested isolates. Among the clustered isolates, 37.7% were resistant to INH, most of which (85.4%) carried a g944c mutation, which causes an S315T amino acid substitution, in the *katG* gene.

Conclusions: Our results suggest that drug-resistant strains, particularly those with INH resistance characterized by a single mutation, S315T, are spreading in Hanoi, Vietnam. When RMP resistance is combined with this setting, patients are not easily cured by conventional short-term treatment. We will need to carefully monitor these trends and search for the origins and transmission routes of these strains.

Keywords: Primary drug resistance, Isoniazid, Gene mutation, Restriction fragment length polymorphism, Vietnam

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Background

The drug susceptibility profiles of clinically isolated *Mycobacterium tuberculosis* (MTB) strains, particularly those from previously untreated patients, have not been included in clinical practice in many countries with inadequate resources. A single standard anti-tuberculosis (TB) treatment without information regarding drug susceptibility is prone to failure or relapse, as initial drug resistance increases the chance of acquiring additional drug resistance [1].

Molecular fingerprinting of MTB strains has been used extensively and is crucial for elucidating the transmission routes of drug-resistant TB [2,3]. A rapidly developing large city is often accompanied by overcrowding and a floating population, and it is often not easy to identify the epidemiological link between TB cases. Nevertheless, the molecular epidemiological techniques are useful for providing insights into the spread patterns of MTB on site and can thus aid in enhancing TB control activities in the entire city.

Vietnam is a Southeast Asian country stretching over 1,800 km from north to south. It is one of 22 high-burden countries worldwide, and its TB prevalence remains high (323 per 100,000 in 2011) [4]. Vietnam reported an incidence of 2.7% multi-drug resistant-TB among new cases in a 2006 survey (95% confidence interval: 2.0–3.6) [5].

The northern and southern regions of Vietnam have also been under different health policies for more than 20 years. It remains unclear whether entire profiles of MTB isolates obtained in one area are equally useful throughout the country. An earlier report [6] suggested differences in genotypes and drug susceptibility patterns between isolates obtained in distant regions of Vietnam.

Although the status of primary drug resistance has been reported in some areas of Vietnam [7-9], molecular biological approaches to this issue have not yet been completely exploited. Thus, we analyzed the profiles of drug susceptibility testing (DST), drug resistance genes, and fingerprint patterns of MTB isolates obtained from 339 previously untreated patients with smear-positive active TB in Hanoi, the capital of Vietnam.

Methods

Ethics statement

A written informed consent was obtained from each participant. The study was approved by ethical committees of the Ministry of Health, Vietnam and National Center for Global Health and Medicine, Japan.

Clinical isolates from acid-fast bacilli (AFB)-positive sputum

Clinical isolates were consecutively collected from previously untreated patients with AFB-positive active TB in Hanoi city between August 2007 and August 2008. At least two sputum specimens were collected from each patient; one was for a smear test and the other was used for culture in the Department of Microbiology of the Hanoi Lung Hospital. Specimens were decontaminated and homogenized with 0.5% NALC–2% NaOH and subsequently inoculated on Löwenstein–Jensen media. MTB isolates were transferred to the Molecular Biology Laboratory of the National Lung Hospital and subjected to MTB identification using niacin and nitrate tests, DST, and other molecular epidemiological tests.

Drug susceptibility testing (DST)

DST was performed using the proportion method based on World Health Organization (WHO) guidelines [10]. The test medium contained rifampicin (RMP; 40 μg/mL), isoniazid (INH; 0.2 μg/mL and 1.0 μg/mL), ethambutol (EMB; 2.0 μg/mL), and streptomycin (SM; 4.0 μg/mL). Drug resistance was defined as ≥1% colony growth compared with a drug-free control of Löwenstein–Jensen medium.

Pyrazinamidase (PZase) assay

PZase activity was determined by Wayne's method with minor modifications [11,12]. As a positive control, we used the MTB H37Rv strain that is susceptible to pyrazinamide (PZA) and is positive for PZase. As a negative control, we used the *M. bovis* BCG strain that is resistant to PZA and is negative for PZase.

Isolation of genomic DNA

Genomic DNA from MTB was extracted using the original method described [13], with slight modifications [14]. Approximately 400 μl of a bacterial suspension in TE buffer was heated at 80°C for 20 min to kill bacteria. First, 50 μl of lysozyme (10 mg/ml) was added followed by incubation at 37°C for 1 h. Subsequently, 75 μl of SDS/proteinase K was gently mixed followed by incubation at 65°C for 10 min. In addition, 100 μl of 5-M NaCl and 100 μl of CTAB/NaCl solution were thoroughly mixed and incubated for 10 min at 65°C. An equal volume (approximately 750 μl) of chloroform/isoamylalcohol was added, and the mixture was centrifuged for 5 min at 12,000× g. The aqueous supernatant was carefully transferred to another tube. Total DNA was precipitated in isopropanol and was dissolved in 0.1× TE buffer.

Line probe assays (LiPAs)

Reverse hybridization-based LiPAs were used to confirm the results of DST and to detect mutations associated with resistance to RMP [15], INH [16], PZA [12], and fluoroquinolone (FQ) [17]. To detect mutations associated with RMP resistance, 5 oligonucleotide probes were used to hybridize to wild-type sequences and 4 probes to mutation sequences of the *rpoB* gene. For INH resistance,