from the immunized mice were treated with Tris-buffered ammonium chloride solution to remove red blood cells as described in our previous report [7].

2.8. Bacterial infection

A seed of *L. monocytogenes* EGD strain was cultured overnight in trypticase soy broth (Beckton Dickinson and Company, Cockeysville, MD) at 37 °C in a bacterial shaker and suitably diluted with PBS. The exact infection dose was assessed retrospectively by plating. Mice were immunized four times with DNA vaccine plasmids as described above, or immunized by a single intraperitoneal injection with a sublethal dose (1×10^4 CFU) of *L. monocytogenes*. One month later, the mice were challenged intraperitoneally with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers of the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates (Beckton Dickinson and Company).

2.9. Statistics

Data from multiple experiments were expressed as the mean ± S.D. Statistical analyses were performed by using StatView-J 5.0 statistics program (SAS Institute, Inc., Cary, NC). Data were analysed with one factor-analysis of variance followed by the Fisher's protected least significant difference (PLSD) test.

3. Results

3.1. Construction of plasmids for DNA immunization

In order to evaluate vaccination with plasmid DNA encoding murine GM-CSF inserted with a dominant Th epitope, we constructed pGM215m plasmid (Fig. 1A). As a control, we prepared another plasmid, p215m, a minigene plasmid for expression of LLO 215–226 peptide alone (Fig. 1A). In order to confirm expression of the GM-CSF-LLO 215–226 protein (GM215) by transfection of pGM215m, we transiently transfected 293T cells with pGM215m or pGM-CSF control plasmid and prepared the culture supernatants. As shown in Fig. 1B, we were able to detect expression of GM215 protein in the culture supernatant of pGM215m-transfected 293T cells by using GM-CSF-specific ELISA.

3.2. Proliferative responses of spleen cells of mice immunized with pGM215m plasmid and the epitope-specific IFN-γ production by the spleen cells

In order to examine the effect of immunization with pGM215m, we performed lymphocyte proliferation assay after immunization of C3H/He mice with the plasmid by using gene gun bombardment. We chose the immunization method as it is an appropriate vaccination route to evaluate the effect of GM-CSF expression plasmid on Langerhans cells

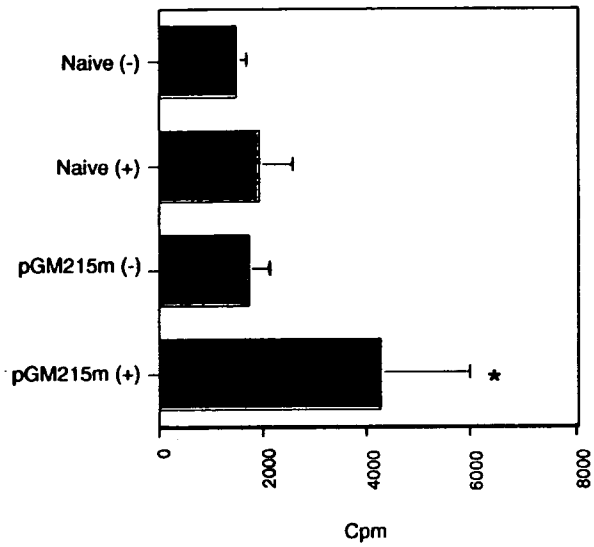

Fig. 2. Specific proliferative responses of spleen cells from pGM215m-immunized mice. C3H/He mice were immunized with pGM215m plasmid by using gene gun four times at 1-week intervals. Spleen cells from the immunized mice were harvested three weeks after the last immunization and cultured in vitro (5×10^5 cells/well) in the presence or absence of 1 μM of LLO 215–226 peptide for 2 days and pulsed with 0.5 μCi of [methyl-^{3}H] thymidine for last 12 h. Results of naïve C3H/He mice are also shown as a control. The values indicate cpm per well. The mean ± S.D. of cpm of four mice per group are shown. Asterisks indicate statistical significance ($p \leq 0.001$) compared with the value of spleen cells of naïve mice without LLO 215–226 stimulation.

and also it is a reliable and reproducible method from our previous experience [8]. As shown in Fig. 2, immunization with pGM215m plasmid induced LLO 215–226-specific proliferative responses of spleen cells from the immunized mice. Immunization with p215m plasmid showed less proliferative responses to LLO 215–226 peptide stimulation, which were not significantly different from those observed in naïve mice (data not shown).

Furthermore, we analyzed IFN-γ amounts in the supernatants of spleen cell culture after 5-day in vitro stimulation with LLO 215–226 peptide. Again, immunization with pGM215m induced higher amounts of IFN-γ than those of mice immunized with p215m after the in vitro stimulation (Table 1). We did not detect IL-4 pro-

Table 1
IFN-γ production by splenocytes from C3H/He mice immunized with pGM215m plasmid

Immunization	Stimulation[a]	IFN-γ (pg/ml)[b]
Naïve	–	85.1
	LLO 215	74.1
pGM215m	–	95.9
	LLO 215	1318.7

[a] Spleen cells of immunized mice (2×10^6 cells/well) were cultured in the absence (–) or presence of 1 μM of LLO 215–226 peptide (LLO 215).

[b] After 4 days, cytokine concentrations in culture supernatants were quantified with sandwich ELISA. The mean of duplicate wells of representative data was shown.

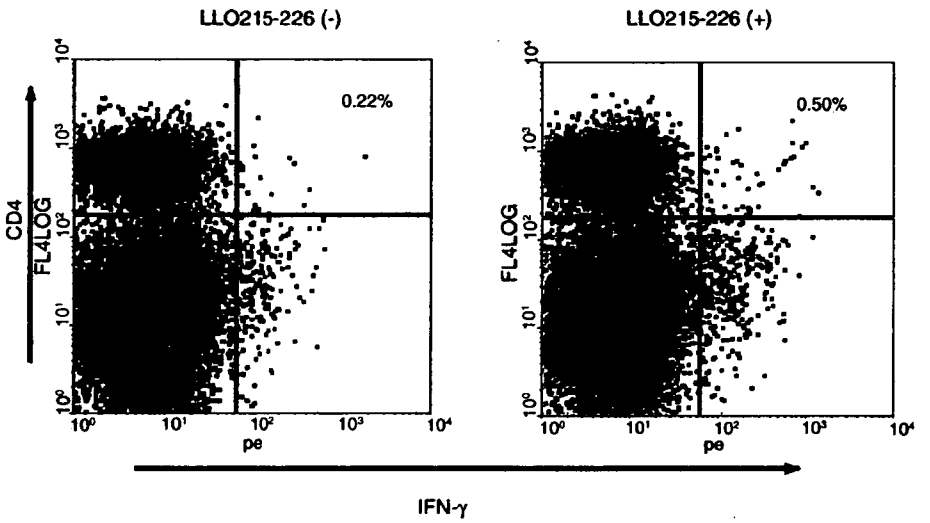

Fig. 3. Induction of LLO 215-specific $CD4^+$ T cells after pGM215m immunization. Intracellular IFN-γ staining on $CD4^+$ T-cell subset was performed using spleen cells after pGM215m immunization in the presence or absence of LLO 215–226 peptide. The percentages of IFN-γ-positive cells in $CD4^+$ T cells are shown.

duction from spleen cells of the immunized mice with ELISA (the detection limit of IL-4 in our ELISA system was approximately 100 pg/ml; data not shown). In addition, we performed intracellular IFN-γ staining with spleen cells of mice immunized with pGM215m. After in vitro stimulation with LLO 215–226 peptide, $CD4^+$ IFN-γ-producing cells was induced in the immune spleen cells (Fig. 3).

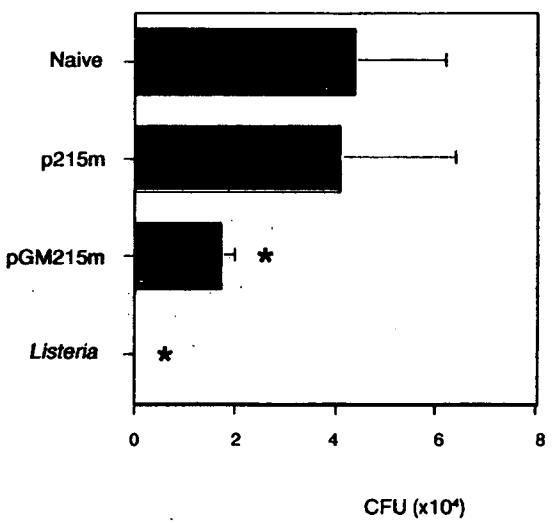

Fig. 4. Protective immunity induced by immunization with pGM215m. Mice were immunized with pGM215m four times at one-week intervals. One month after the last immunization, the immunized mice were challenged intraperitoneally with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers in the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates. Results of naïve mice and mice immunized with a sublethal dose of *L. monocytogenes* are also shown as controls. Results are expressed as the means ± S.D. for three mice for each group. Asterisks indicate statistical significance ($p < 0.05$) compared with the value of naïve mice.

3.3. Induction of protective immunity against listerial infection after immunization with pGM215m plasmid

In order to examine whether the immunity evoked by immunization with pGM215m plasmid is associated with an increased resistance to infection of virulent *L. monocytogenes*, the in vivo protection experiment was carried out. Seventy-two hours after listerial challenge, mice immunized with pGM215m plasmid were sacrificed and CFU from the spleens were counted. As shown in Fig. 4, immunization with p215m did not show significant protective effects. On the contrary, mice immunized with a sublethal dose of *L. monocytogenes* were able to eliminate challenged *L. monocytogenes* from the spleens. Immunization with pGM215m conferred moderate, but significant protective immunity against lethal listerial challenge.

4. Discussion

DNA vaccination may work through direct transfection of antigen presenting cells (APCs), or by secretion of the encoded protein by muscle or skin cells for the uptake by APCs. Therefore, two different strategies have been considered to induce a particular epitope-specific Th by DNA vaccination. One is an intracellular targeting of antigens [9–11]. Another strategy is taking an advantage of secreted proteins. In general, secreted proteins are phagocytosed by APCs and presented on MHC class II molecules. In this study, we used a plasmid expressing murine GM-CSF, a cytokine which is indispensable for development of APCs such as dendritic

cells and macrophages. GM-CSF is one of most studied cytokines for vaccine adjuvants [12,13]. GM-CSF expression plasmid injected into mouse muscle has been reported to lead to a local infiltration of potential APCs [14]. We therefore reasoned that immunization of a gene for GM-CSF-Th epitope fusion molecule may work well for induction of the epitope-specific Th subset.

Several reports showed that immunization with a DNA vaccine co-expressing both antigen and GM-CSF [15] or a bicistronic plasmid DNA for antigen and GM-CSF [16,17] is superior to co-immunization of DNA vaccines for antigen alone and for GM-CSF in terms of strength of the specific immunity induced by the vaccines. Linking antigen and GM-CSF expression closely in vivo may provide a microenvironment suitable for the uptake and presentation of antigen by dendritic cells or macrophages. Immunization of pGM215m plasmid fits this condition.

It has been reported that $CD4^+$ T cells, especially LLO 215–226-specific T cells, are involved in protective immunity against listerial challenge. Verma et al. [18] demonstrated that induction of $CD4^+$ T cell population responsive to LLO 215–226 in vivo elicits partial protective immunity by using *Salmonella* carrier system. They showed one-log order reduction in numbers of the bacterium in spleens and livers of the immunized mice. In another approach, we showed that significant induction of protective immunity to *L. monocytogenes* by immunization with a plasmid DNA expressing LLO 215–226 Th epitope that replaces the class II-associated invariant chain (Ii) peptide (CLIP) of the Ii [10] or immunization with a plasmid encoding LLO 215–216 Th epitope fused with the endosomal/lysosomal targeting signal of lysosome-associated membrane protein (LAMP)-1 [11]. pGM215m immunization shown here was more effective than LLO215–LAMP fusion DNA immunization [11] in terms of induction of the protective immunity (data not shown).

The immunization strategy shown here, i.e., immunization with GM-CSF gene inserted with a double-stranded oligonucleotide encoding a Th epitope, would be applicable to DNA vaccination for induction of CTL or antibodies as a molecular adjuvant for supplying Th. Investigators add a universal Th epitope such as 13 aminio-acid Pan HLA-DR Epitope (PADRE) [19] in multi-CTL epitope plasmid DNA construction for efficient CTL induction [20]. GM-CSF-Th epitope DNA vaccination would be the alternative strategy for induction of Th.

Acknowledgements

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Brief Communication

IFN-γ overcomes low responsiveness of myeloid dendritic cells to CpG DNA

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Summary Dendritic cells (DC) are professional APC that have an extraordinary capacity to prime naive T cells. It has been reported that human DC subsets express distinct toll-like receptor (TLR), which influences their function. In mice, we observed that plasmocytoid DC (pDC) express a higher level of TLR9 compared with myeloid DC (mDC) cultured with GM-CSF. However, we demonstrated that stimulation with IFN-γ is capable of upregulating TLR9 expression in mDC to a level comparable with expression in pDC. Consistent with this observation, IL-12 p40 and IL-6 mRNA expression and IL-12 p70 secretion in response to CpG-oligodeoxynucleotides are enhanced in mDC pretreated with IFN-γ compared with untreated cells. Therefore, TLR-mediated responses of DC subsets may be influenced not only by signals delivered by pathogens but also by regulatory signals from cytokines such as IFN-γ.

Key words: CpG-oligodeoxynucleotide, IFN-γ, myeloid dendritic cell, plasmacytoid dendritic cell, toll-like receptor 9.

Introduction

Dendritic cells (DC) have a pivotal role in the interplay between the innate and adaptive immune responses against pathogens and tumours. They are a unique group of bone marrow-derived leucocytes that are specialized for uptake, transport, processing and presentation of antigen to T cells.[1] Although triggering of T cells into cell cycle progression is a central function of DC, it has also been suggested that DC subsets can influence the subsequent development of these dividing T cells. In humans, CD40 ligand-activated monocyte-derived DC, but not plasmacytoid DC (pDC) produced a large amount of IL-12 and induced Th1 rather than Th2 responses.[2] In mice, pDC ($CD11c^+$ $CD11b^-$ $B220^+$) induced Th1 cells and myeloid DC (mDC) ($CD11c^+$ $CD11b^+$ $B220^-$) induced Th2 cells when activated with CpG-DNA; however, both DC subsets have been shown to induce flexibly Th1 and Th2 cell development depending on antigen dose and differential toll-like receptor (TLR) ligation.[3] It has been reported that a given DC subset can induce either a Th1 or a Th2 response depending on the type of stimulation and pathogen.[4] Therefore, it seems likely that these DC subsets themselves may not have intrinsic capacity to direct either Th1 or Th2 cell development.

Recognition of the pathogen-associated molecular patterns involves members of the TLR family.[5] In humans, 11 TLR have been identified, and distinct DC subsets express different TLR.[6,7] For example, mDC express TLR2 and TLR4 whereas pDC express TLR7 and TLR9, indicating that distinct DC subsets respond to distinct microbial products.[8] In mice, pDC express low levels of TLR2, TLR3 and TLR4, and high levels of TLR7 and TLR9.[9] In contrast, mDC express high levels of TLR4 and low levels of TLR9.[3] Bacterial CpG DNA and CpG-oligodeoxynucleotides (ODN) have been shown to stimulate mammalian immune cells through TLR9.[10] Therefore, pDC produce type I IFN, IL-6 and IL-12 p70 when activated with TLR9 ligand CpG.[3] In contrast, LPS stimulates mDC to produce IL-12 p70.[3]

In this study, we showed that IFN-γ significantly increases TLR9 expression in mDC and thereby overcomes low responsiveness to CpG DNA.

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Materials and methods

Mice

Female BALB/c mice (Japan SLC, Hamamatsu, Japan) were kept under specific pathogen-free conditions and were fed autoclaved food and water ad libitum at the Institute for Experimental Animals of the Hamamatsu University School of Medicine. Two-month-old female mice were used in all experiments. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

Generation of bone marrow-derived mDC and pDC

Bone marrow-derived $CD11c^+$ $CD11b^+$ $B220^-$ mDC were generated as described previously.[11] In brief, bone marrow cells were isolated by flushing femurs with culture medium and red blood cells were lysed using 0.83% ammonium chloride. The cells (1×10^6) were placed in 12-well plates in 2 mL medium with 10 ng/mL GM-CSF and 10 ng/mL IL-4 (Pepro Tech EC, London, UK). The cultures were fed every 2 days by gently swirling the plates, aspirating the medium and adding fresh medium back. At day six, non-adherent cells were collected and resuspended in fresh medium and cultured for an additional 1 day in 12-well plates. $CD11c^+$ $CD11b^-$ $B220^+$ pDC were generated by culturing bone marrow cells in culture medium containing 100 ng/mL Flt3 ligand (R & D Systems, Minneapolis, MN, USA)

for 10 days at 2 × 10^6 cells in 12-well plates in a volume of 2 mL. At day five, 1 mL medium was replaced by 1 mL fresh medium containing Flt3 ligand.[12] The purity after culture was always >85%.

CpG-ODN

Lipopolysaccharide-free phosphorothioate oligodeoxynucleotides (BEX, Tokyo, Japan) were used in all experiments. The nucleotide sequence of CpG-ODN used in this study is 5′-TGACTGTGAACGTTCGAGATGA-3′ (underlining indicates the immunostimulatory DNA sequence).

Preparation of total RNA and semiquantitative RT–PCR

Reverse transcriptase (RT)–PCR was performed as described previously.[13] The sequences of the primers used in this study are as follows: TLR9 forward, 5′-TGTTGCATAAGGCACAGAGC-3′; TLR9 reverse, 5′-CCTGAGCTATTCTGCTGTAGG-3′; IL-12 p40 forward, 5′-GGGACATCATCAAACCAGACC-3′; IL-12 p40 reverse, 5′-CCCAACCAAGCAGAATGCAGC-3′; IL-6 forward, 5′-TATGAAGTTCCTGTGTGCAA-3′; IL-6 reverse, 5′-CTTTGTATCTCTGGAAGTTT-3′; G3PDH forward, 5′-ACCACAGTCCATGCCATCAC-3′; and G3PDH reverse, 5′-TCCACCACCCTGTTGCTGTA-3′.

Real-time quantitative RT–PCR

One μL of total RNA from each sample was used for cDNA synthesis. Nineteen μL of LightCycler mastermix (Fast Start DNA master SYBR Green I; Roche Diagnostics, Basel, Switzerland) was filled in the glass capillaries and 1 μL cDNA was added as a template. Real-time quantitative PCR was performed using a LightCycler rapid thermal cycler (Roche Diagnostics). The manufacturer's LightCycler experimental run protocol was used.

Western blotting

Bone marrow-derived mDC and pDC were stimulated with 5 ng/mL IFN-γ for 12 h. After stimulation, cells were harvested and resuspended in lysis buffer. Protein concentrations were measured by Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were loaded in each lane and separated by SDS–PAGE. Gels were transferred onto Hybond-P membranes (Amersham, Piscataway, NJ, USA) and blotted with antimouse TLR9 antibody (IMGENEX, San Diego, CA, USA).

ELISA

ELISA was performed as described previously.[14] To measure IL-12 concentration, antimurine IL-12 p70 mAb (PharMingen, San Jose, CA, USA) and antimurine IL-12 p40/p70 (PharMingen) were used as capture and detection mAb, respectively.

Results and discussion

First, we investigated the level of TLR9 expression in murine bone marrow-derived mDC in comparison with that in pDC. As shown in Figure 1a,b, both semiquantitative RT–PCR and real-time quantitative RT–PCR analyses revealed that TLR9 mRNA was faintly expressed in mDC. In marked contrast to mDC, TLR9 mRNA was highly expressed in pDC. This observation agrees well with a published report of murine DC subsets with respect to TLR expression.[3] To investigate the effect of IFN-γ on expression of TLR9 mRNA in subsets

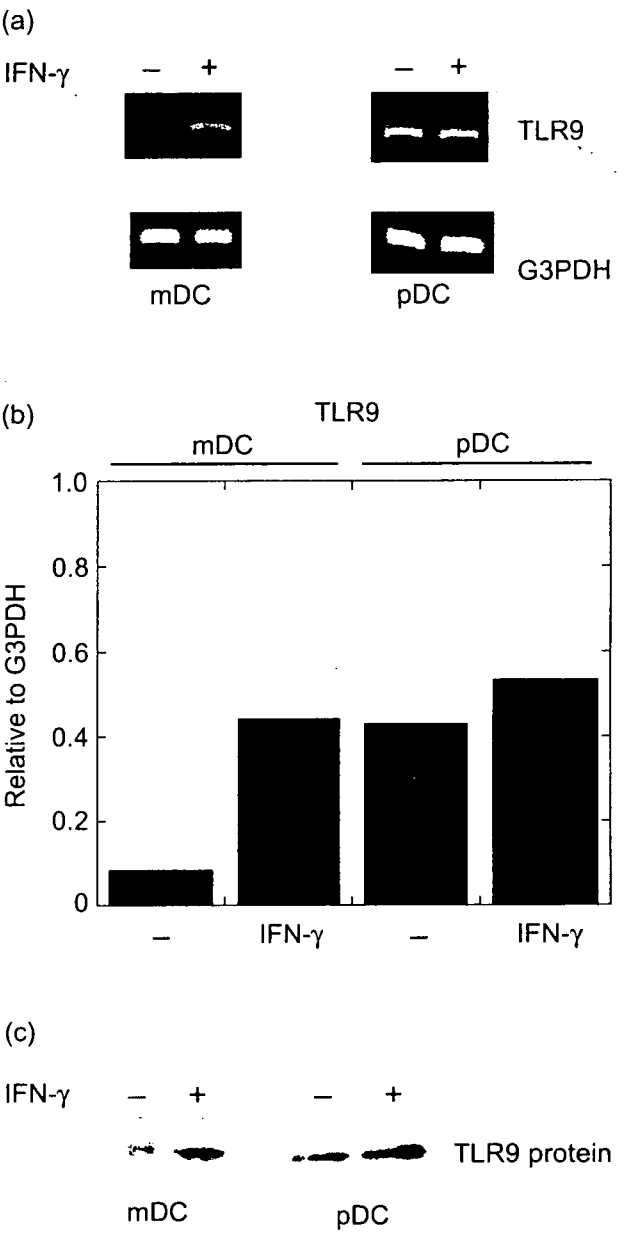

Figure 1 Upregulation of toll-like receptor (TLR)9 expression in myeloid and plasmacytoid dendritic cells (mDC, pDC) by IFN-γ. (a) DC were stimulated with IFN-γ (5 ng/mL) for 6 h and total RNA was isolated for first strand cDNA synthesis. Then, semiquantitative reverse transcriptase (RT)–PCR analysis were performed. (b) IFN-γ mRNA expression was further analysed by quantitative real-time PCR using the same cDNA used in (a). Expression is relative to G3PDH. (c) mDC and pDC were treated with IFN-γ for 12 h. Equal amounts of cell lysates were subjected to SDS–PAGE and immunoblotting for TLR9. Similar results were observed in three independent experiments.

of DC, murine bone marrow-derived mDC and pDC were stimulated with 5 ng/mL IFN-γ for 6 h. After stimulation with IFN-γ, mDC appeared to upregulate TLR9 mRNA expression to a level that was comparable with pDC (Fig. 1a,b). Expression peaked at 12 h and remained constant for 24 h after IFN-γ

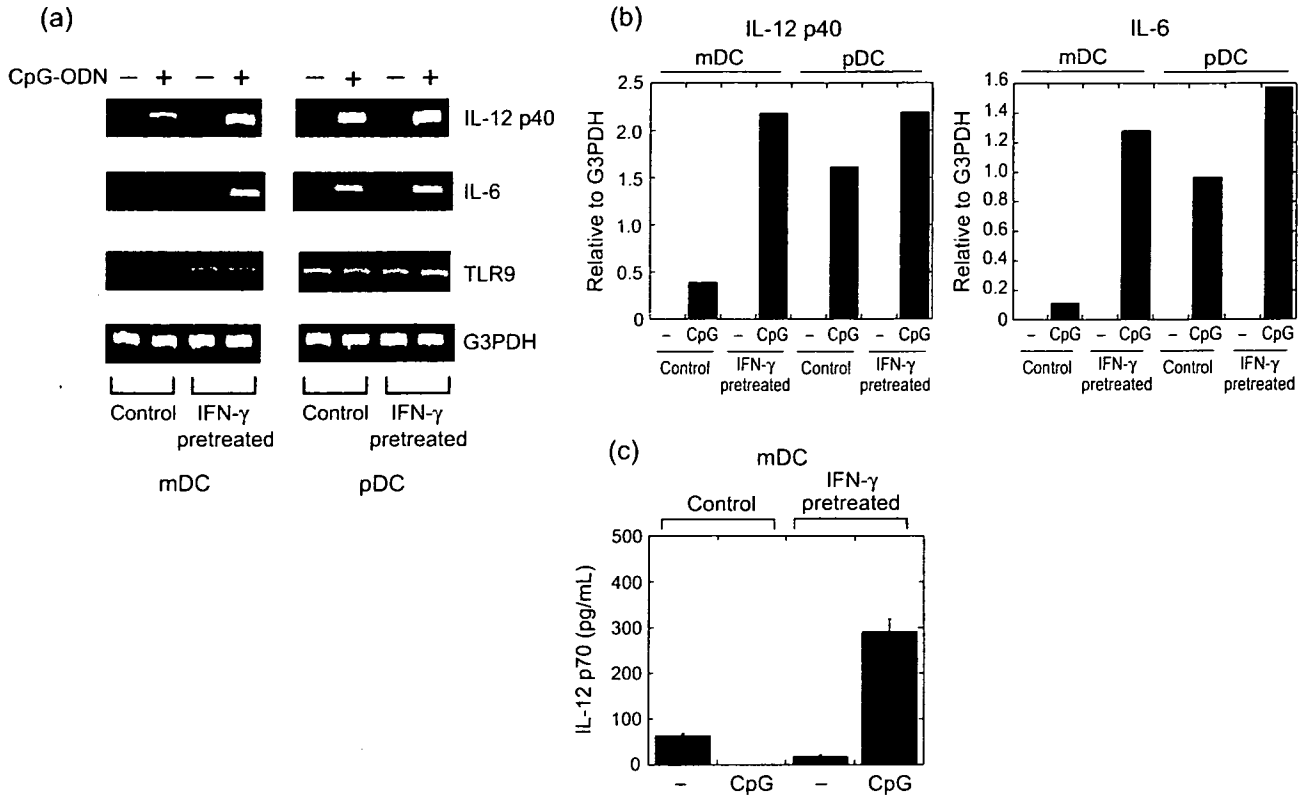

Figure 2 Effect of IFN-γ pretreatment on CpG-oligodeoxynucletide (ODN)-inducible gene expression. (a) Myeloid and plasmacytoid dendritic cells (mDC, pDC) were treated with IFN-γ (5 ng/mL) for 12 h and washed. The cells were incubated for 2 h before stimulation with CpG-ODN for 6 h. Total RNA was isolated and semiquantitative reverse transcriptase (RT)–PCR was performed. (b) IL-12 p40 and IL-6 expressions were further analysed by quantitative real-time PCR using the same cDNA used in (a). Expression is relative to G3PDH. (c) IL-12 p70 protein concentrations were measured by ELISA. Control or IFN-γ pretreated mDC were stimulated with CpG for 4 days and the supernatants were subjected to analysis.

stimulation (data not shown). The effect of IFN-γ was confirmed by the observation that no IFN-γ-induced increase of mRNA levels for TLR9 was observed in mDC and pDC from IFN-γ receptor-deficient mice (data not shown).

Given that treatment of mDC with IFN-γ significantly augmented TLR9 mRNA, we next investigated TLR9 protein expression in response to IFN-γ (Fig. 1c). Control mDC showed only a detectable level of TLR9 protein. Consistent with mRNA expression, TLR9 protein expression in mDC was upregulated after 12 h treatment with IFN-γ.

Because expression levels of TLR were expected to be reflected by their ability to produce cytokines in response to binding of their ligands, we next investigated cytokine mRNA induction of IFN-γ pretreated mDC by CpG-ODN stimulation. As shown in Figure 2a, we observed that IL-12 p40 and IL-6 mRNA expression in response to CpG-ODN was enhanced in IFN-γ pretreated mDC compared with expression in untreated cells. To obtain highly accurate quantitative data, we applied real-time RT–PCR analysis. Real-time quantitative RT–PCR confirmed the results obtained by semiquantitative RT–PCR (Fig. 2b). We also confirmed enhanced IL-12 p70 secretion from IFN-γ pretreated mDC by CpG-ODN stimulation (Fig. 2c). It seems most likely that the enhanced cytokine mRNA and protein expression depends on enhanced TLR9 expression but not on pretreated IFN-γ, because CpG DNA induced IL-12 p40 gene expression has been reported to be independent of STAT1 or IFN consensus sequence binding protein.[15] In addition, DC pretreated with IFN-γ did not induce expression of IL-12 p40 and IL-6 mRNA (Fig. 2a,b).

In humans, TLR9 expression is restricted to plasmacytoid DC.[16] In mice, however, TLR9 is highly expressed on bone marrow-derived and spleen plasmacytoid pDC, and to a lesser extent on GM-CSF cultured mDC.[3] Consistent with this report, we observed high levels of TLR9 mRNA and protein expression in pDC and significantly lower levels of expression in mDC.

Our data indicate that a high level of TLR9 expression is induced in mDC after stimulation with IFN-γ, resulting in acquisition of enhanced responsiveness to CpG. In fact, many studies support a high degree of plasticity in the capacity of DC to prime T cells and drive their functional differentiation.[17] The mechanism of IFN-γ inducible TLR9 in mDC remains unknown and awaits elucidation of the promoter sequence of the TLR9 gene.

Thus, TLR-mediated responses of DC may be influenced not only by signals delivered by pathogens such as LPS and CpG DNA, but also by regulatory signals from cytokines such as IFN-γ.

Acknowledgements

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Evaluation of a novel vaccine (HVJ–liposome/HSP65 DNA + IL-12 DNA) against tuberculosis using the cynomolgus monkey model of TB

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Abstract

We have developed a novel tuberculosis (TB) vaccine; a combination of the DNA vaccines expressing mycobacterial heat shock protein 65 (HSP65) and interleukin 12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ). This vaccine provided remarkable protective efficacy in mouse and guinea pig models compared to the BCG vaccine, on the basis of an induction of the CTL activity and improvement of the histopathological tuberculosis lesions, respectively. Furthermore, we extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis. This novel vaccine provided a higher level of the protective efficacy than BCG based upon the assessment of mortality, the ESR, body weight, chest X-ray findings and immune responses. Furthermore, the combination of HSP65 + IL-12/HVJ and BCG by the priming-booster method showed a synergistic effect in the TB-infected cynomolgus monkey (100% survival). These data indicate that our novel DNA vaccine might be useful against *Mycobacterium tuberculosis* for human clinical trials.

Keywords: HSP65 DNA + IL-12 DNA vaccine; Tuberculosis; Monkey

1. Introduction

Tuberculosis is a major global threat to human health, with about 2 million people dying every year from *Mycobacterium tuberculosis* (TB) infections. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG (BCG), although its efficacy against adult TB disease remains controversial. Therefore, we have recently developed a novel TB vaccine, a DNA vaccine expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ). The vaccine was 100 fold more efficient than BCG in the mouse model on the basis of the elimination of *M. tuberculosis* mediated by the induction of CTL [1]. A nonhuman primate model of TB will provide critical information for vaccine development. In fact, in the previous study we evaluated the protective

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efficacy of HSP65 + IL-12/HVJ in the cynomolgus monkey model, which is an excellent model of human tuberculosis [2,3]. In the present study, we evaluated the synergistic effect of the HSP65 + IL-12/HVJ and BCG using a priming-booster method in the TB-infected cynomolgus monkey. The combination of the two vaccines showed a very strong prophylactic effect in monkeys challenged with *M. tuberculosis* (100% survival), as we have seen previously in the mouse model of TB.

2. Materials and methods

DNA vaccines encoding *M. tuberculosis* HSP65 and human IL-12 were encapsulated into HVJ–liposomes [4]. CTL activity was assessed by 51Cr-release [3,5]. A total of 44 cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial Research Center. 12 monkeys population in first experiment and 32 monkeys population in second experiment are different. The animals were vaccinated three times with either the HVJ–liposome with expression plasmids of HSP65 and human IL-12(HSP65 + hIL-12/HVJ: 400 μg i.m.), BCG Tokyo (1×10^6 CFU i.d), or saline (Fig. 1A). One month after the third vaccination, the monkeys were challenged with the *M. tuberculosis* Erdman strain (5×10^2) by intratracheal instillation. Erythrocyte sedimentation rate (ESR), body weight, chest X-rays, immune responses, DTH reaction against PPD, and survival periods were examined for 12–16 months [2,3].

3. Results

The purpose of this study was to evaluate a TB vaccine we have developed in a nonhuman primate TB model infected with *M. tuberculosis*. To this end, a total of 12 monkeys were in the first experiment vaccinated either with HSP65 + hIL-12/HVJ, BCG, or saline, followed by the TB challenge by intratracheal instillation. Fig. 1B shows the survival rate of the vaccinated monkeys after infection. All monkeys in the control group (saline, $n = 4$) died within 8 months, while 2 monkeys in the HSP65 + hIL-12/HVJ group ($n = 4$) as well as BCG group ($n = 4$) survived more than 14 months post-infection (the termination period of the experiment). Furthermore, in the second experiment using 32 monkeys the protective efficacy of the HSP65 + IL-12/HVJ and BCG using the priming-booster method in the TB infected cynomolgus monkeys was very strong. All four monkeys from the group of BCG-priming and the DNA vaccine (HVJ–liposome/HSP65 + IL-12 DNA vaccine) booster were alive more than 12 months post-infection (Fig. 2). In contrast, only 2 monkeys out of 6 from the BCG Tokyo group

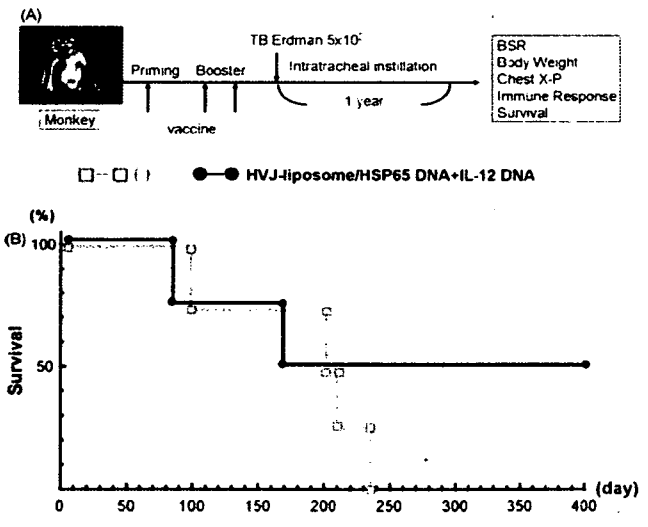

Fig. 1. (A) Evaluation of vaccine efficacy using cynomologus monkey. Groups of animals were vaccinated three times with either the HVJ–liposome combination with HSP65 DNA plus human IL-12 DNA (HSP65 + hIL-12/HVJ: 400 μg i.m.), BCG Tokyo (1×10^6 CFU i.d), or saline (A). One month after the third vaccination, monkeys were challenged with the *M. tuberculosis* Erdman strain (5×10^2) by intratracheally instillation, erythrocyte sedimentation rate (ESR), body weight, chest X-ray, immune responses, DTH reaction against PPD, and survival periods were examined during 12–16 months. (B) Survival of cynomolgus cynomolgus monkeys immunized with HVJ–liposome/HSP65 + IL-12DNA vaccine. Cynomolgus monkey (4 monkeys/group) were immunized three times (every 3 weeks) with (1) HVJ–liposome/HSP65 DNA + IL-12 DNA vaccine (●–●), (2) saline(□–□) as control group as described in Section 2. One month after last immunization, *M. tuberculosis* (Erdman strain 5×10^2) was challenged by intratracheally instillation. Survival was studied more than 14 months.

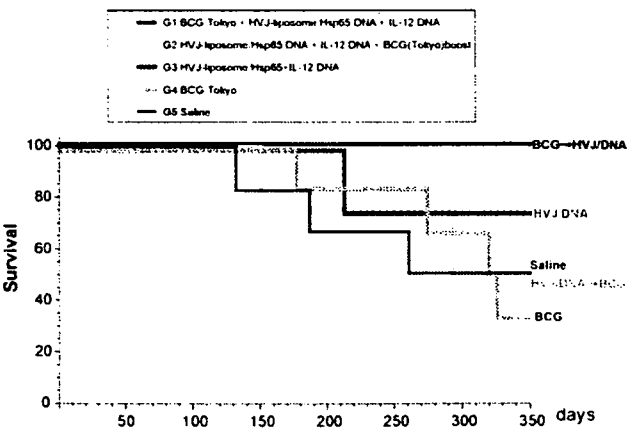

Fig. 2. Protective efficacy of HSP65 + IL-12/HVJ and BCG using priming-booster method against TB challenged cynomologus monkeys. Group of animals were vaccinated three times (every 3 weeks) with (1) BCG Tokyo, (2) Hsp65 + IL-12/HVJ, (3) Hsp65 + IL-12/HVJ = G1 (—) BCG prime-HVJ/DNA booster group; (1) Hsp65 + IL-12/HVJ, (2) Hsp65 + IL-12/HVJ, (3)BCG = G2 ( ) HVJ/DNA prime-BCG booster 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. (—) One month after the third vaccination, monkey were challenged with the *M. tuberculosis*. Kaplan-Meier's method (Logrank test) was used to compare the survival of each groups. (G1–G4, p 0.05; G1–G2, p 0.13; G1–G3, p 0.32; G1–G5, p 0.12; G2–G4, p 0.82; G3–G4, p 0.30; G3–G5, p 0.44; G4–G5, p 0.88).

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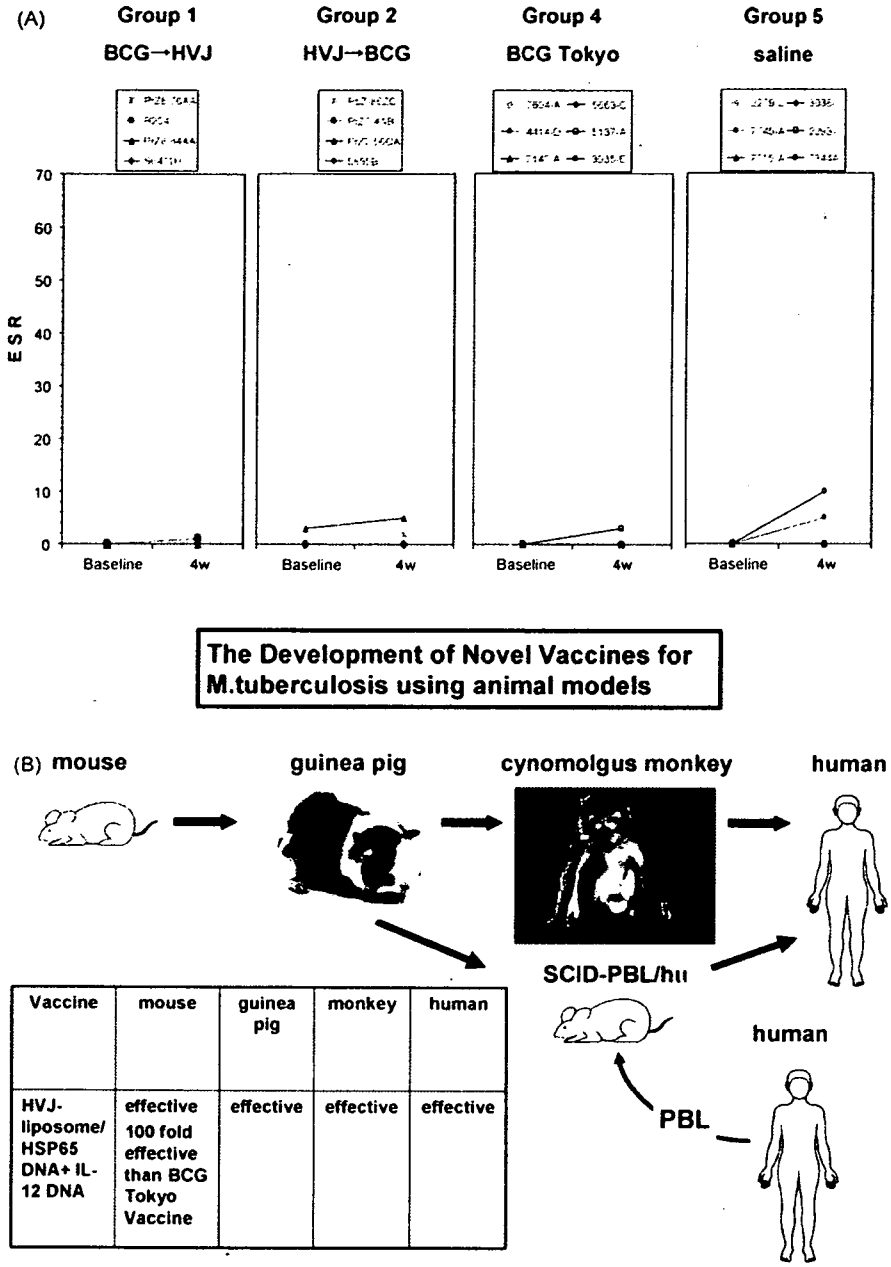

Fig. 3. (A) Improvement of erythrocyte sedimentation rate (ESR) in the cynomolgus monkeys immunized with HVJ–liposome/HSP65 DNA + IL-12 DNA vaccine. Cynomolgus monkeys were immunized and challenged as described in Fig. 2. Elevation of ESR of all monkeys was evaluated every month and maximum values of BSR in each monkeys were shown. (B) The development of novel vaccines for *M. tuberculosis* using animal models.

were alive (33% survival). 50% of the monkeys from the saline control group and DNA vaccine-priming and the BCG Tokyo vaccine booster group, respectively, were alive more than 12 months in the study. In addition, both HSP65 + hIL-12/HVJ improved ESR and chest X-ray findings (Fig. 3A). IL-2 and IFN-γ production were augmented in the group vaccinated with HSP65 + hIL-12/HVJ (data not shown). Furthermore, proliferation of PBL was strongly enhanced. Taken together, these results clearly demonstrate that BCG priming and the HSP65 + hIL-12/HVJ booster could provide extremely strong protective efficacy against *M. tuberculosis* in the cynomolgus monkey model.

4. Discussion

The HSP65 + hIL-12/HVJ vaccine exerted a significant prophylactic effect against TB, as indicated by: (1) extension of survival for over a year; (2) improvement of ESR and chest X-ray findings; (3) increase in the body weight;

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(4) augmentation of immune responses, in a cynomolgus monkey model which closely mimics human TB disease. It is very important to evaluate the long survival period in a monkey model, as human TB is a chronic infection disease. Furthermore, the decrease in the body weight of TB patients is usually accompanied by a progression of the disease [6].

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. 3B). In the present study, it was demonstrated that BCG vaccine priming and HSP65 + h IL-12/HVJ booster could provide extremely strong (100% survival) efficacy against *M. tuberculosis* compared to BCG alone (33% survival) in the cynomologus monkey model. In Japan and other countries, the BCG vaccine is inoculated into human infants (0–6 months after birth). Therefore, BCG priming in infants and HSP65 + h IL-12/HVJ boosters for adults (including junior high school students, high school students and old persons) may be required for the significant improvement of clinical protective efficacy against TB. Our results with the HSP65 + hIL-12/HVJ vaccine in the cynomolgus monkey model should provide a significant rational for moving this vaccine into clinical trials. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data on the HVJ–liposome DNA vaccine in anticipation of a Phase I clinical trial.

Acknowledgements

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NOVEL VACCINATION (HVJ-LIPOSOME / HSP65 DNA+ IL-12 DNA) AGAINST TUBERCULOSIS USING CYNOMOLGUS MONKEY.

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[Abstract]

A novel TB vaccine; a DNA vaccine combination expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12) by using the hemagglutinating virus of Japan (HVJ)-liposome (HSP65+IL-12/HVJ) has been developed. This vaccine provide remarkable protective efficacy in mouse and guinea pig models, as compared to the current by available BCG vaccine. HVJ-liposome / HSP65 DNA + IL-12 DNA vaccination were 100 fold more efficient than parental BCG Tokyo vaccination, on the elimination of M. TB in lungs, liver, and spleen of BALB/c mice. Hsp65+mIL-12/HVJ induced $CD8^+$ cytotoxic T lymphocyte activity against Hsp65 antigen. 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 IFN-γ-secreting T cells and activation of proliferative T cells and cytokines (IFN-γ and IL-2) production upon stimulation with Hsp65 and antigens from *M. tuberculosis*. Our studies were extended to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis, to evaluate the HSP65+IL-12/HVJ vaccine. Vaccination with HSP65+IL-12/HVJ vaccine provided better protective efficacy as assessed by the Erythrocyte Sedimentation Rate, chest X-ray findings, body weight and immune responses (IFN-γ, IL-2, IL-6 production, and lymphocyte proliferation of cynomolgus monkey), 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.

[Introduction]

In order to explore the preclinical use of tuberculosis DNA vaccine combinations of IL-12 DNA with Hsp65 DNA, we choose the viral-based hybrid antigen delivery system hemagglutinating virus of Japan (HVJ)-liposome because this delivery system results in a high transfection efficacy, repeated

gene transfection without reduction of gene transfer efficiency *in vivo*, and no apparent toxicity. These characteristics of HVJ-liposomes support the feasibility of its clinical application not only for cancer gene therapy but also for DNA vaccinations.Researchers have recognized that a nonhuman primate model of TB will be able to provide critical information for vaccine development.

In the present study, we evaluated the protective efficacy of HSP65+IL-12/HVJ in the cynomolgus monkey model, which is an excellent model of human tuberculosis [1]. These vaccines provided a strong prophylactic effect in monkeys challenged with M. tuberculosis as we have seen previously in mice.

[Materials and methods]

DNA vaccines encoding M. tuberculosis HSP65, mouse IL-12 and guinea pig IL-12 were encapsulated with HVJ- liposomes [2]. Groups of animals (mice and guinea pigs) were vaccinated intramuscularly with HVJ-liposome DNA vaccines. Female BALB/c mice and C57BL/6 mice aged 6～8 weeks were infected by intravenous injection with M.tuberculosis H37Rv. DNA vaccination using gene gun (HSP65 DNA + IL-12 DNA) was initiated 14 days before the i.v. injection of M.Tb. IL-12 gene or heat shock protein (HSP65) gene derived from M. tuberculosis was constructed as DNA vaccine into plasmid using CMV promoter [3, 4]. HSP65 gene was also constructed with HVJ-liposome by Professor Kaneda of Osaka University.Eight weeks after the final vaccination, CTL activity of spleen cells from vaccinated mice was assessed by using the ^{51}Cr-release assay [5, 6]. P815 mastocytoma cells were transfected with pcDNA-hsp65 and used as Hsp65 protein-expressing target cells.A total of cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial. Groups of animals were vaccinated three times with either the HVJ-liposome combination with HSP65 DNA plus human IL-12 DNA (400 μg i.m.),, BCG Tokyo (1×10^6 CFU i.d.) or saline. One month after the third vaccination, monkeys were challenged with the M. tuberculosis Erdman strain (5×10^2) by intratracheally instillation, Erythrocyte Sedimentation Rate (ESR), body weight, chest X-ray, immune responses, DTH reaction against PPD and survival periods were examined during 14 months [7].

[Results]

HVJ-liposome DNA vaccines generated cytotoxic CD8$^+$ T cells.

Mice vaccinated with HSP65 + mIL-12/HVJ had significantly reduced numbers of CFU [5] in the lungs, liver and spleen as compared with mice vaccinated with BCG [6]. CTL activity correlated with the protective efficacy of vaccination (Fig 1). Because CD8$^+$ CTLs have been considered critical effectors of protective immunity to *M. tuberculosis*, it was of interest to determine whether a tuberculosis specific response could be induced in the vaccinated mice. We characterized CD8$^+$ T cells specific for Hsp65, PPD, or killed M. tuberculosis by using a conventional ^{51}Cr release assay in the absence of re-stimulation. As shown in Fig. 1, high levels of HSP65-and M. tuberculosis-CTL specific

lyses against J774.1 macrophages phagecytosed M. tuberculosis and P815 mastocytomas transfected with Hsp65 DNA were detected in mice vaccinated with IgHsp65+mIL-12/HVJ, whereas little CTL activity response was detectable in either the naive or BCG-vaccinated mice. In vitro depletion of $CD8^+$ T cells eliminated the specific lyses. Depletion of $CD4^+$ T cells had no effect. Stronger (more than twenty percent) cytotoxicity against Hsp65 was detected in the spleen cells from mice 2 weeks after the last vaccination with IgHsp65+mIL-12/HVJ (data not shown). These results indicate that IgHsp65+mIL-12/HVJ vaccine induced long-term immune response with strong $CD8^+$ CTL activity.

In the guinea pig model, HSP65 + gpIL-12/HVJ provided better protection against the pulmonary pathology caused by pulmonary challenge with TB than BCG vaccination (data not shown).

Protective efficacy of HVJ-liposome DNA vaccine using cynomolgus monkey

The purpose of this study was to evaluate TB vaccine we have developed in a nonhuman primate model of M. tuberculosis infection. To this end, a total of 16 monkeys were vaccinated either with HSP65 + hIL-12/HVJ, BCG or saline, followed by TB challenge by intratracheally instillation. All four monkeys in the control (saline) group died of TB infection within 8 months. In contrast, two monkeys from HSP65 + hIL-12/HVJ groups, respectively, were alive more than 14 months post-infection (the termination period of the experiment). Survival period of the remaining monkeys in this group were much longer than those of saline control group. In addition, HSP65 + hIL-12/HVJ significantly improved ESR and chest X-ray findings. Body weights of the HSP65 + hIL-12/HVJ group also increased significantly, as compared to saline control group. IL-2 and IFN-γ production were augmented in the group vaccinated with HSP65 + hIL-12/HVJ (data not shown). Furthermore, proliferation of PBL was strongly enhanced in the group vaccinated with HSP65 + hIL-12/HVJ in response to HSP65 protein 4 weeks after TB challenge. Taken together, these results clearly demonstrate that both HSP65 + hIL-12/HVJ could provide protective efficacy against M. tuberculosis in the cynomolgus monkey model.

Taken together, these results indicate that the experimental models using cynomolgus monkeys and HSP65+IL-12DNA vaccine might provide new strategies capable of developing new vaccines against tuberculosis.

[Discussion]

Hsp65+mIL-12/HVJ induced $CD8^+$ cytotoxic T lymphocyte activity against Hsp65 antigen. 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 CTL, IFN-γ-secreting T cells and activation of proliferative T cells and cytokines (IFN-γ and IL-2) production.

HSP65 + hIL-12/HVJ vaccine exerted the significant prophylactic effect against TB of monkey, as indicated by: (1) prolongation of survival for over a year, (2) improvement of ESR and chest X-ray findings, (3) increase in the body weight and (4) augmentation of immune responses, in a cynomolgus

monkey model which closely mimics human TB disease. It is very important to evaluate the long survival period in a monkey model, as human TB is a chronic infection disease. Furthermore, the decrease in the body weight of TB patients with TB is usually accompanied by progress of TB disease. Suppression of IFN-γ production, CTL activity and T-cell proliferation has also been observed in patients with TB [9].

Our results with the HSP65 + hIL-12/HVJ vaccine in the cynomolgus monkey model should provide a significant rational for moving this vaccine into clinical trials. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data on the HVJ- liposome DNA vaccine in anticipation of a Phase I clinical trial.

[Acknowledgements]

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Fig.1

(A)

Effector lymphocytes from mice vaccinated with	Treatment of Effector cells
Naive	(-)
BCG	(-)
IgHsp65+mIL -12/HVJ	(-)
IgHsp65+mIL -12/HVJ	Anti -CD8Ab+C
IgHsp65+mIL -12/HVJ	Anti -CD4Ab+C
IgHsp65+mIL -12/HVJ	Anti -Thy1.2Ab+C

(B)

Effector lymphocytes from mice vaccinated with	Treatment of Effector cells
Naive	(-)
BCG	(-)
IgHsp65+mIL -12/HVJ	(-)
IgHsp65+mIL -12/HVJ	Anti -CD8Ab+C
IgHsp65+mIL -12/HVJ	Anti -CD4Ab+C
IgHsp65+mIL -12/HVJ	Anti -Thy1.2Ab+C

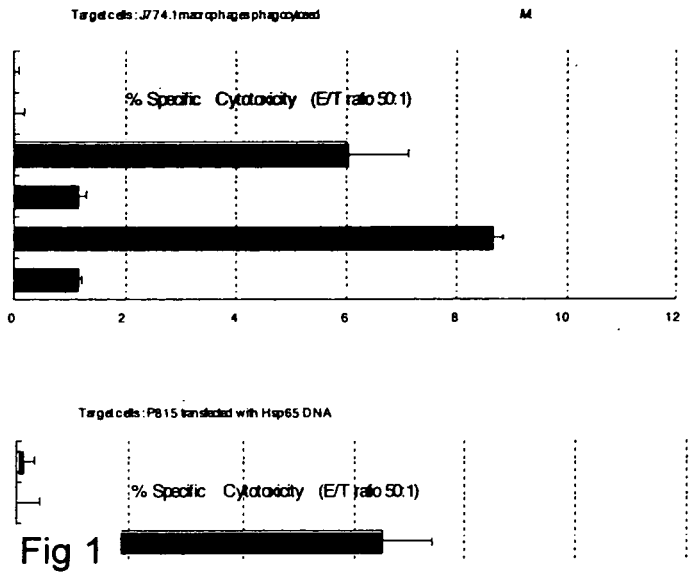

Fig 1

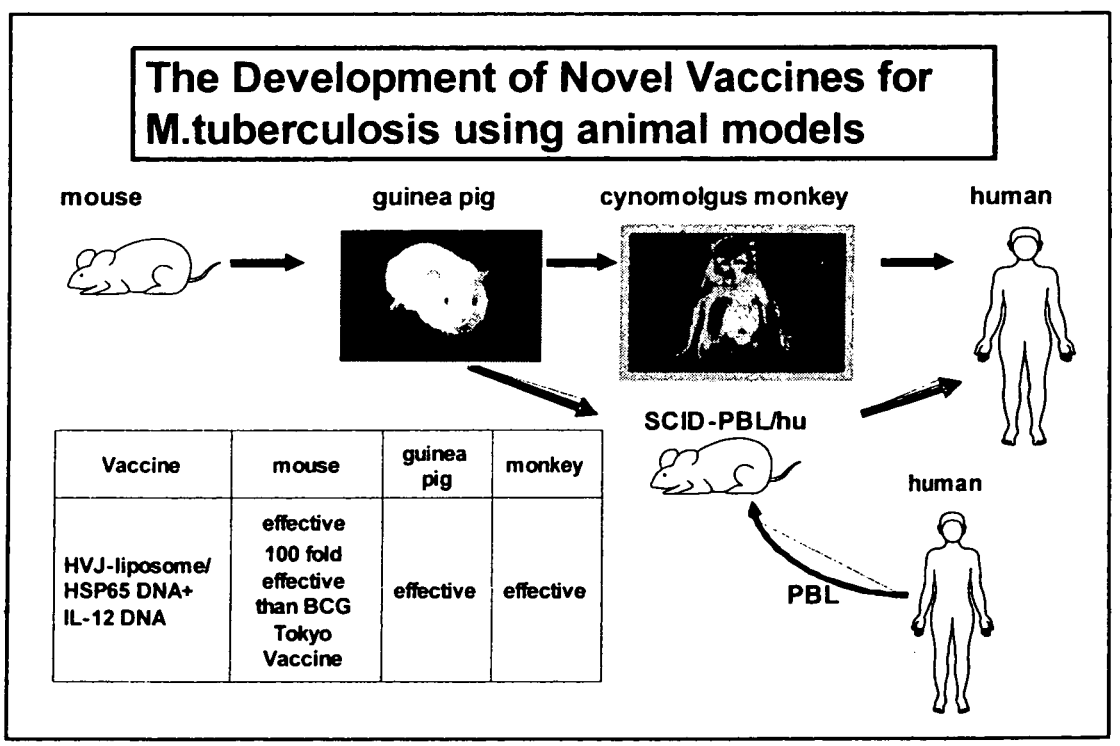

Fig 2

第81回総会教育講演

新しい結核ワクチン

岡田 全司

要旨：1998年，米国CDCおよびACETは新世代の結核ワクチン開発の必要性を発表した。しかしながら，BCGワクチンに代わる結核ワクチンは欧米でも臨床応用には至っていない。われわれはBCGをはるかに凌駕する100倍以上強力な結核予防ワクチン効果を示す新しいDNAワクチン（HVJ-リポソーム/HSP 65＋IL-12 DNA）やリコンビナント72f BCGワクチンを開発した。このワクチンは結核菌抗原特異的なキラーT細胞の分化を増強した。IFN-γ産生T細胞の分化と増殖増強効果も示した。さらに，治療結核ワクチン効果も示した。欧米では治療ワクチンは未開発である。さらに，ヒト結核感染モデルに最も近いカニクイザルを用い，サルでも有効なHSP65 DNA＋IL-12 DNAワクチンを世界に先駆けて開発した。リンパ球増殖反応・サイトカイン産生の増強および胸部X線所見・血沈，体重の改善効果が認められた。また生存率改善・延命効果も認められた。コントロール群の生存率は0～50%であった。一方，このDNAワクチン投与群はpriming-booster法で100%の生存率を示した。このワクチンの臨床応用を計画中である。

キーワーズ：結核ワクチン，HSP65 DNA＋IL-12 DNAワクチン，リコンビナントBCGワクチン，臨床応用，キラーT細胞，カニクイザル

1. はじめに

いまだに世界の3分の1の20億人が結核菌に感染しており，その中から毎年880万人の結核患者が発症し，200万人が毎年結核で死亡している，最大の感染症の1つである（WHOレポート2002年）[1]。結核症に対する宿主の抵抗性は細胞性免疫といって過言ではない。特に獲得免疫（キラーT細胞とTh1ヘルパーT細胞）が重要である。1998年，米国CDCおよびACETは結核に対し，政府・学術機関・企業が一体となってBCGに代わる新世代の結核ワクチン開発の必要性を強く主張する発表をした。しかしながら，BCGに代わる結核ワクチンは欧米でも臨床応用には至っていない。われわれはBCGよりもはるかに強力なDNAワクチンやリコンビナントBCGワクチンの開発に成功した（Table 1, Fig. 1）[1)~4)]。新しい抗結核ワクチン開発と臨床応用の可能性についても述べる[3)~6)]。

2. 新しい結核ワクチン開発

（1）現行のBCGワクチンの有用性

BCGワクチンの評価がWHOによりなされた。すなわち大人（成人）結核に対しては，BCGワクチンは予防効果がないという結論がWHOによって報告された。10万人を超す南インド農民を対象として実施された大規模なcontrolled trial（Chingleput study）では，全く有効性が否定される結果となった（上記WHOの報告）[6]。（ただし，小児における結核性髄膜炎や粟粒結核など播種性のものには十分な予防効果がある。）したがって，成人の結核に対し有効な新しい結核ワクチンの開発が必須である[1)6)]。

（2）BCGワクチンより1万倍強力な結核予防ワクチン

われわれ国立病院機構近畿中央胸部疾患センター臨床研究センターが，画期的な結核の新しいワクチンを開発した。マウスの実験で現行のBCGワクチンを超えるきわめて強力な有効性（1万倍の効果）を確認した。われ

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われはHSP 65 DNA＋IL-12 DNA（HVJ-エンベロープベクター）のワクチンはBCGワクチンよりも1万倍強力な結核予防ワクチンであることを世界に先駆けて明らかにした。これらの研究が国内外よりきわめて高く評価され，当臨床研究センターはWHO（世界保健機関）よりWHO STOP TB Partnershipに選ばれた。また，大阪大学大学院（医学系研究科）・連携大学院にも選ばれた（Table 1）。

（3）新しい結核ワクチン

結核ワクチンは，①サブユニットワクチン，②DNAワクチン，③リコンビナントBCGワクチン（弱毒化結核菌を含む），その他に大別される。

マウスではBCGワクチンをはるかに凌駕する新しい結核ワクチンはきわめて少ない。われわれはHSP65 DNA＋IL-12 DNA予防ワクチンにてBCGワクチンの100倍強力なワクチンの開発に成功した（Table 1，Fig. 1）[1)~4)]。

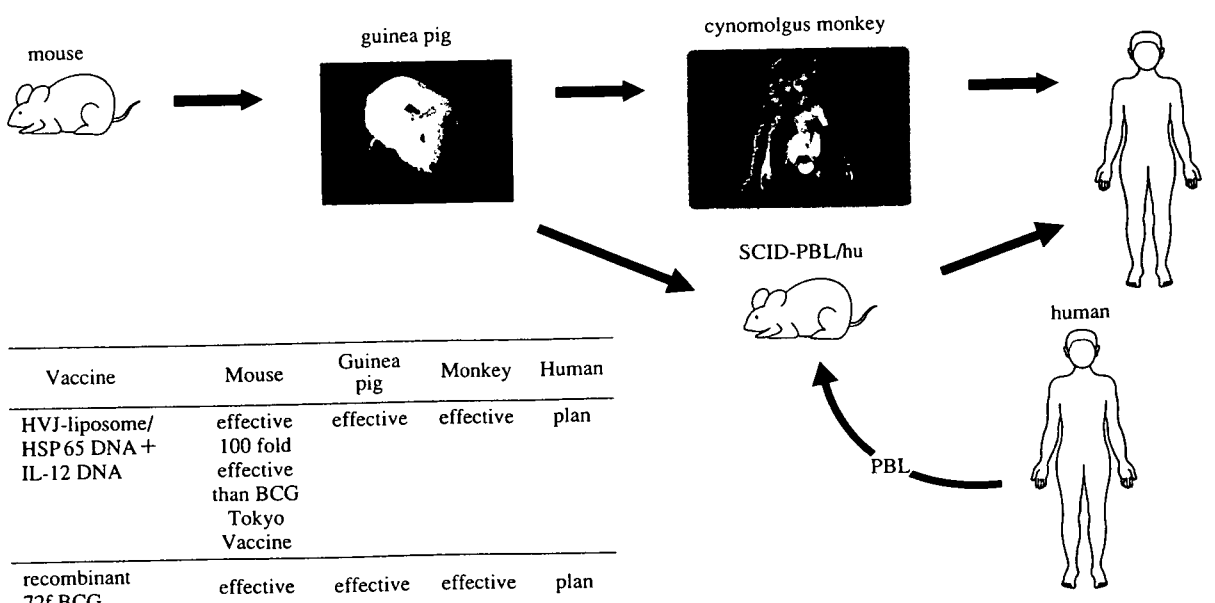
（a）DNAワクチン

われわれはHSP65 DNA＋IL-12 DNAのワクチンが相乗効果を示し，gene gunを用いた遺伝子投与でBCGよりもきわめて強力な（約100倍）結核予防ワクチンであることを明らかにした（自治医科大学吉田博士との共同研究）。IL-12のp35およびp40をCMVプロモーター下流域に挿入した発現プラスミドを作製した。さらに，ヒト型結核菌H37RV由来HSP65 DNAワクチンの作製に成功した（Table 1）[4)]。

HVJリポソームをベクターに用いた場合，HSP65

Table 1 The development of novel vaccines for *M. tuberculosis*

1. DNA vaccine HVJ-liposome/HSP 65 DNA＋IL-12 DNA	more effective than BCG (mouse, guinea pig, cynomolgus monkey)
2. DNA vaccine HVJ-Envelope/HSP 65 DNA＋IL-12 DNA	extremely stronger effect than BCG
3. Recombinant BCG vaccine (1) recombinant 72f BCG (2) recombinant (Ag85A＋85B＋MPB51) BCG	more effective than BCG (mouse, guinea pig, cynomolgus monkey) more effective than BCG (mouse)
4. Therapeutic vaccine IL-6 related DNA	(mouse)
5. Priming-Booster Method BCG (priming)＋Novel vaccine (booster)	(cynomolgus monkey)
6. Novel vaccine (per os) using gene-knock out attenuated Listeria	
7. Novel vectors AAV vector (1000 fold effective expression vector ↑), Adenovirus vector	
→ Selected as WHO STOP TB Partnership and WHO STOP TB Vaccines Working Group	



Vaccine	Mouse	Guinea pig	Monkey	Human
HVJ-liposome/ HSP65 DNA＋ IL-12 DNA	effective 100 fold effective than BCG Tokyo Vaccine	effective	effective	plan
recombinant 72f BCG	effective	effective	effective	plan

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

DNA単独（HVJリポソーム/HSP65）でBCGよりも有効であることをマウスの系で明らかにした（大阪大学医学部金田博士との共同研究）。

さらに，HSP65 DNAとIL-12 DNA両者のDNAワクチンを投与した，HSP65 DNA＋IL-12 DNAワクチンマウスでは，BCG東京ワクチンマウスの肺，肝，脾の結核菌数の100倍以上の減少が認められた。すなわち，HSP65 DNA＋IL-12 DNAワクチンはBCGに比較して100倍以上強力な結核予防ワクチン効果を示した（Fig. 2）。さらに，このHSP65 DNA＋IL-12 DNAワクチンはHSP65タンパク抗原に対する脾リンパ球増殖反応を著明に増幅した（BCGワクチンよりはるかに強い増殖反応）。また，KS-Elispot Assay自動計測器（ELISA Assayの200倍以上の感度）を用いて，HSP65 DNA＋IL-12 DNAワクチンは脾臓のIFN-γ産生細胞数の増強とIFN-γ産生細胞の著しい分化増強を誘導することを明らかにした[4]。

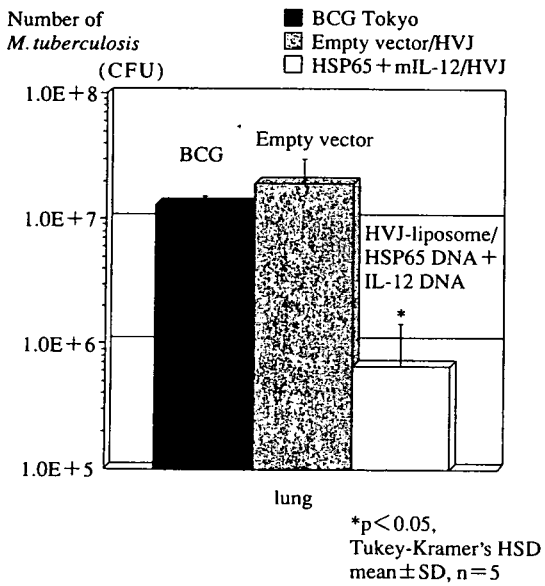


Fig. 2 Prophylactic efficacy of HVJ-liposome/HSP65 DNA＋IL-12 DNA vaccine on TB-infected mice (5 weeks after TB infection)

さらに，結核菌に対するCD8陽性キラーT細胞の分化誘導を増強した。また，結核菌抗原の主要な抗原タンパクであるHSP65タンパク抗原に対するCD8陽性キラーT細胞の分化誘導を著明に増強した。一方，BCGワクチンは結核菌に対するキラーT細胞やHSP65タンパクに対するキラーT細胞誘導活性はほとんど認められなかった（Fig. 3）[4]。

このように，HVJ-リポソーム/HSP65 DNA＋IL-12 DNAワクチンは結核菌に対するキラーT細胞分化誘導，IFN-γ産生細胞分化誘導，T細胞増殖反応増強を介して，BCGワクチンより100倍以上強力な結核予防ワクチン効果を発揮することが示された[1)2)4)]。

アデノウイルスベクターに導入したIL-6関連遺伝子（IL-6遺伝子＋IL-6レセプター遺伝子＋gp130遺伝子）ワクチンは，BCGよりも強力な治療ワクチン効果を示した。

以上のワクチン効果は，キラーT細胞やTh1細胞の分化誘導を増強することによって発揮されることが示された。

新しい結核ワクチンの開発研究が高く評価され，WHO STOP TB Vacciene Working Group Meetingに選出された（Table 1）。

一方，Huygenらは，Ag85AのDNAワクチンを用い，マウスで抗原特異的キラーT細胞（CTL）が誘導されることや，BCG免疫と同等の防御効果が得られることを明らかにした。

（b）リコンビナントBCGワクチン

結核菌は300種以上のタンパク質を分泌するが，α抗原Ag85Bとそのファミリー（85A，Ag85C）DNAをリコンビナントBCGに使用した[1)3)5)6)]。

これらの遺伝子をPNN2シャトルベクター（大腸菌↔好酸菌）に組み込みBCG東京菌に，遺伝子を導入した。われわれはBA51（Ag85A＋Ag85B＋MPB51）リコ

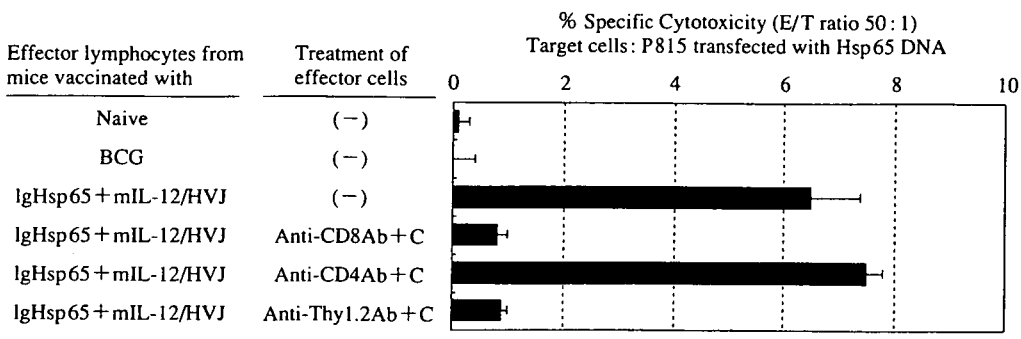


Fig. 3 Induction of $CD8^+$CTL against *M. tuberculosis* in the spleen cells from HVJ-liposome/HSP65 DNA＋IL-12 DNA vaccine