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DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation

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Abstract

We investigated the immunogenicity and protective efficacy of DNA vaccine combinations expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) using gene gun bombardment and the hemagglutinating virus of Japan (HVJ)-liposome method. A mouse IL-12 expression vector (mIL-12 DNA) encoding single-chain IL-12 proteins comprised of p40 and p35 subunits were constructed. In a mouse model, a single gene gun vaccination with the combination of Hsp65 DNA and mIL-12 DNA provided a remarkably high degree of protection against challenge with virulent *Mycobacterium tuberculosis*; bacterial numbers were 100-fold lower in the lungs compared to BCG-vaccinated mice. To explore the clinical use of the DNA vaccines, we evaluated HVJ-liposome encapsulated Hsp65 DNA and mIL-12DNA (Hsp65 + mIL-12/HVJ). The HVJ-liposome method improved the protective efficacy of the Hsp65 DNA vaccine compared to gene gun vaccination. Hsp65 + mIL-12/HVJ induced $CD8^+$ cytotoxic T lymphocyte activity against Hsp65 antigen. 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 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*. These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to BCG vaccine.

Keywords: Tuberculosis; DNA vaccine; HVJ-liposome

1. Introduction

Tuberculosis is a major global threat to human health, with about 2 million people dying each year from *Mycobacterium tuberculosis* infections. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG. BCG continues to be widely administered to children in developing countries, yet its efficacy remains controversial, particularly against the pulmonary form of the disease in adults. In recent years, the increasing frequency of drug-resistant *M. tuberculosis* isolates has further complicated the clinical management of this disease. Clearly, a more effective vaccine for the control of tuberculosis is urgently needed.

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It is well established that protective immunity to *M. tuberculosis* depends on both $CD4^+$ and $CD8^+$ T cells [1–6]. Because DNA vaccination results in the generation of cellular immune responses, including those of a Th-1-type response, and protection in animal models of infectious diseases [7,8]. In fact, several human clinical trials have recently been initiated to test the efficacy of DNA vaccines against emerging and re-emerging infectious diseases including hepatitis B [9], malaria [10,11] and HIV infections [12]. DNA vaccination has also shown potential for the development of tuberculosis vaccines in the mouse model [13–16]. However, in a guinea pig model, which is arguably one of the most biologically relevant systems available for studying human pulmonary tuberculosis, DNA vaccines has not proven more efficacious than BCG [17]. The efficacy of any experimental tuberculosis vaccine remains to be evaluated in human clinical trials and, thus, a vaccine against tuberculosis is still anxiously awaited.

Mycobacterial heat shock protein 65 (Hsp65) is a potential target for protective immunity and has been extensively studied [18]. Several groups have reported that *hsp65* DNA vaccines can induce strong protective immune responses in mice against virulent *M. tuberculosis* infections [19–21]. Protection is attributed to the establishment of a cellular immune response dominated by Hsp65-specific T cells that both produce IFN-γ and are cytotoxic towards infected cells. Furthermore, Lowrie et al. have reported that this vaccine reduces bacterial loads in mice infected with *M. tuberculosis* when given therapeutically after infection [22]. Interleukin-12 (IL-12) is a cytokine with a major role in the induction of IFN-γ-dominated immune responses to microbial pathogens. Orme and colleagues have demonstrated the importance of IL-12 in generation of the protective response to tuberculosis [23]. Co-administration of the IL-12 gene, which participates in the induction of IFN-γ dominated immune responses to microbial pathogens, with various tuberculosis DNA vaccines including the *hsp65* DNA [20,24] and 35 K MW DNA [25] may boost the efficacy of these DNA vaccines to levels achieved with BCG in the mouse model, although inhibitory effect rather than synergistic effect on immunotherapy was observed in mice co-administrated with *hsp65* DNA vaccine plus the *IL-12* gene.

In order to explore the preclinical use of tuberculosis DNA vaccine combinations of the *IL-12* DNA with the *hsp65* DNA, we chose 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. In a recent study, highly efficient transfection of muscle cells was observed for several weeks when pcDNA3 plasmid containing the human tumor antigen genes, *MAGE-1* and *MAGE-3*, were encapsulated in HVJ-liposomes and injected intramuscularly into mice [26]. Effective induction of $CD4^+$ T cell responses by a hepatitis B core particle-based HIV vaccine was achieved by subcutaneous administration of HVJ-liposomes in mice [27]. HVJ-liposomes were also very effective as a mucosal vaccine against HIV infection [28]. Thus, it is likely that HVJ proteins may be responsible for inducing a robust immune response. No side effects from repetitive injections of HVJ-liposomes into mice, rats or monkeys were observed.

We designed this study to clarify the clinical feasibility of HVJ-liposome-mediated DNA vaccines for tuberculosis. First, we clarify that co-administration of IL-12 DNA with Hsp65 DNA via gene gun delivery enhanced protection in mice compared with Hsp65 DNA alone. Second, we show that vaccination with HVJ-liposome encapsulated Hsp65 DNA resulted in better protection than did gene gun vaccination. Third, we demonstrate that HVJ-liposome encapsulated Hsp65 DNA and IL-12 DNA induce enhanced protective immunity in the mouse model compared to that seen with BCG. This protective efficacy was associated with the emergence of IFN-γ-secreting T cells upon stimulation with Hsp65 and purified protein derivative. These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to BCG vaccine. We also discuss in this paper the prospects of this HVJ-liposome-based DNA vaccine for testing in primate models [29] and, ultimately, in a clinical setting.

2. Materials and methods

2.1. Bacteria

M. tuberculosis strain 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 medium (Wako Chemicals, Osaka, Japan). For the mouse infection studies, a single colony of *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 (DIFCO Laboratories, Detroit, MI: lot 137971 XA MD) medium supplemented with albumin–dextrose complex and grown at 37 °C until approximately mid-log phase. Aliquots were stored at −80 °C and thawed at 10 days before use, grown to mid-log phase in 7H9 medium.

2.2. Reagents and antibodies

Purified protein derivative (PPD: lot T-3-4) was obtained from JAPAN BCG Co., Ltd. (Tokyo, Japan). Killed *M. tuberculosis* H37Ra (lot 13971XA) was obtained from DIFCO Laboratories. Fetal calf serum (FCS: lot AGC6341) was obtained from Hyclone (Logan, UT). Anti-L3T4, anti-Lyt2.2 monoclonal antibodies and anti-Thy1.2 antibody were kindly provided by Dr. K. Kuribayashi (Mie University, Tsu, Japan) and Dr. E. Nakayama (Okayama University, Okayama, Japan) [30].

2.3. Animals

Inbred and specific pathogen-free female BALB/c mice were purchased from Clea Japan Inc. (Tokyo, Japan). Mice were maintained in isolator cages, manipulated in laminar flow hoods and used between 8 and 10 weeks of age. 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.

2.4. Cell lines

COS-7 cells were kindly provided by Dr. H. Endoh (Jichi Medical School, Tochigi, Japan). COS-7 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. A mouse mastocytoma cell line (P815: DBA/2 origin) was kindly provided by Dr. C.S. Henney (Fred Hutchinson Cancer Research Center, Seattle) [31]. A mouse macrophage cell line (J774.1: BALB/c origin) was kindly provided by Dr. P. Ralph (Sloan Kettering Cancer Inst., New York, NY) [32]. The P815 and J774.1 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 [33,34].

2.5. Plasmid construction

The *hsp65* gene was amplified from *M. tuberculosis* H37Rv genomic DNA by PCR using a set of primers, phsp65-F1 and phsp65-R1, and cloned into the *Bam*HI/*Not*I sites of pcDNA3.1 (+) (Invitrogen, San Diego, CA) to generate pcDNA-hsp65. pcDNA-hsp65 was designated as Hsp65 DNA in this text. For the construction of the *hsp65* gene fused with the mouse Igκ secretion signal sequence, the PCR product was cloned into the *Bam*HI/*Not*I sites of pcDNA-CS87 [35] to generate pcDNA-Ighsp65. pcDNA-Ighsp65 was designated as IgHsp65 DNA in this text. For the construction of the mouse IL-12 (mIL-12) *p40* and *p35* single-chain gene, the *mIL12p35*and *mIL12p40* genes were cloned from pcDNA-p40p35 [35] by PCR using sets of primers, pmIL12p35-F1 and pmIL12p35-R1, and pmIL12p40-F1 and pmIL12p40-R1, respectively, and cloned into pcDNA3.1 (+) to generate pcDNA-mIL12p40p35-F. pcDNA-mIL12p40p35-F was designated as mIL-12 DNA in this text. As a control, pcDNA-EGFP vector expressing the *EGFP* gene was used. Sequences of oligonucleotide primers used are available as request.

2.6. Protein production and antibody preparation

Recombinant Hsp65 (rHsp65) protein was expressed in *E. coli* BL21 (λDE3) and purified by affinity chromatography on Ni-NTA columns (Qiagen).

2.7. Transfection

DNA transfection of COS-7 cells was performed with the PolyFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. After 24 h, supernatant and cells were harvested separately. Immunoprecipitation of cell lysates and supernatants with antibodies were performed as described previously [36]. Rat anti-mouse IL-12p70 (BD Biosciences Pharmingen, San Diego, CA) and mouse anti-rHsp65 polyclonal antibody were used for immunoprecipitation. For IL-12 bioassay, COS-7 cells (1×10^6 cells/plate) were plated into 60-mm cell culture plates and transfected with 2.5 μg of pcDNA 3.1, pcDNA-mIL12p40 + p35, or pcDNA-mIL12p40p35-F using the PolyFect Transfection Reagent. At 48 h after transfection, culture supernatants were collected and stored at −70 °C until use. Various volumes of the supernatants were added to the mouse spleen cells (2×10^6 cells/ml). Murine culture supernatants after 60 h incubation were collected and the level of mouse IFN-γ measured using sandwich ELISA kits (BD Opt EIA™ Set. BD Biosciences Pharmingen), according to manufacturer's instructions.

2.8. Vaccination

2.8.1. Gene gun vaccination

Gold particles coated with plasmid DNAs and their cartridges were prepared as described previously [35]. The abdomen was shaved and gold particles coated with plasmid DNA (1 μg plasmid DNA per shot) was delivered once into the abdomen using a Helios Gene gun (Nippon Bio-Rad Laboratory, Tokyo, Japan) at a helium discharge pressure of 300 psi. A separate group was vaccinated once subcutaneously with 1×10^6 colony-forming units (CFU) of *M. bovis* BCG Tokyo strain.

2.8.2. HVJ-liposome vaccination

HVJ-liposomes were prepared as described previously [37]. The HVJ–liposome complex was aliquoted with 10% DMSO and stored at −70 °C until use. HVJ-liposomes without plasmid DNA was used and designated as Empty/HVJ in this text. Groups of BALB/c mice were vaccinated three times at 3-week intervals with 100 μl of HVJ-liposome solution containing 50 μg of pcDNA-IgHsp65 and/or 50 μg of pcDNA-mIL12p40p35-F in the tibia both anterior muscles. A separate group was vaccinated once with 1×10^6 CFU *M. bovis* BCG Tokyo by subcutaneous injection at four different sites (left upper, right upper, left lower, right lower back) at the same time. HVJ-liposome DNA vaccines encapsulating pcDNA-IgHsp65, pcDNA-mIL12p40p35-F, or combination of pcDNA-IgHsp65 and pcDNA-mIL12p40p35-F was designated as IgHsp65/HVJ, mIL-12/HVJ, and IgHsp65 + mIL-12/HVJ, respectively, in this text.

2.9. 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 3 weeks after the third vaccination as described previously [38]. At 5 and 10 weeks after *M. tuberculosis* H37Rv challenge, the lungs, spleens, and livers were aseptically homogenized by using homogenizer in saline, and serial dilutions of the organ homogenates were plated on Ogawa agar (Kyokuto, Tokyo, Japan) or 7H11 Middlebrook agar (Kyokuto). Plates were sealed up and incubated at 37 °C and the number of CFU was counted 2 or 4 weeks later. Results are converted to $\log_{10}$ values and $\log_{10}$ [mean ± standard deviation (S.D.)] for CFU/organ/animal were calculated for each experimental group.

2.10. Histological analysis

The lungs were obtained from the mice, fixed with 10% buffered formalin, and embedded in paraffin. Each block was cut into 4 μm-thick sections and stained using hematoxylin and eosin. Semi-quantitative morphometric analysis of pathological slides was performed by our modified method of Dascher et al. [39] using a micrometer-attached microscope (Microphot-FXA, Nikon, Japan) [39,40]. The longer axis and minor axis of each granuloma in the field (×4 magnification) were measured. Longer axis to minor axis of each granuloma were multiplied and added up. Three random fields from each tissue section of mice and six random fields of guinea pigs were evaluated, and the average score of the fields was designated as the granuloma index ($\times 10^{-2}$ mm^2). This method for the evaluation of granuloma area is significantly correlated with the granuloma area by other scanning method of hematoxylin and eosin section.

2.11. 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. J774.1 macrophage cells were pulsed with *M. tuberculosis* (killed H37Ra) for 24 h and used as target cells. A total of 2×10^6 cells/ml effector splenic cells were treated with anti-CD8 antibody, anti-CD4 antibody or anti-Thy1.2 antibody followed by complement as described above. ^{51}Cr release was assessed using the ^{51}Cr-release assay [31,33] 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:

$$\left[\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}\right] \times 100.$$

2.12. Proliferative responses of lymphocytes

Vaccinated mice were sacrificed immediately prior to challenge, and 1×10^5 single spleen cells were cultured in a 96-well flat bottom plate (Linbro) with rHsp65 protein (10 μg/ml) or PPD (20 μg/ml) for 60 h at 37 °C, and then pulsed with 1 μCi of [^{3}H]thymidine per well for the final 12 h of incubation [30]. Cells were harvested onto glass wool fiber filters, and [^{3}H]thymidine incorporation was measured using a Liquid Scintillation Counter LSC-6100 (ALOKA Co. Ltd., Tokyo, Japan).

2.13. Production of cytokines (IL-2 and IFN-γ)

Mouse cytokines were measured in quantitative ELISAs for IL-2 and IFN-γ as described previously [38]. Briefly, spleen cells from vaccinated mice were cultured at a concentration of 5×10^6 cells/ml in 200 μl of medium at various antigen concentrations. Culture supernatants were collected 48 h later and the levels of IFN-γ and IL-2 measured using sandwich ELISA kits (BD Opt EIA™), according to manufacturer's instructions.

2.14. ELISPOT assay

The spleens were removed aseptically from vaccinated mice three weeks after the third vaccination. Antigen-specific IFN-γ-producing cells were determined by ELISPOT as described previously [41]. Briefly, ELISPOT plates (MultiScreen IP Filtration plate MAIPS45; Millipore, Bedford, MA) were coated with anti-mouse IFN-γ MAb R4-6A2 (BD Biosciences Pharmingen). Spleen cells from vaccinated mice were suspended to 1×10^7 cells/ml (1×10^6 cells/well). In some experiments, the spleen cells from mice vaccinated with IgHsp65 + mIL-12/HVJ were pre-incubated with anti-CD8 antibody or anti-CD4 antibody (1:50 dilution) for 15 min at 4 °C and then incubated with rabbit complement (1:10 dilution) (Cedarlane, Hornby, Ont., Canada) for 45 min at 37 °C as described previously [30,33]. The cells were placed in five wells into antibody-coated wells, and rHsp65 protein (10 μg/ml) or PPD (10 μg/ml) was added to each well. After 20 h of incubation at 37 °C, cells were removed by washing the plates, and the site of cytokine secretions was detected using biotynylated anti-mouse IFN-γ MAb XMG1.2 (BD Biosciences Pharmingen) and streptavidin-alkaline phosphatase conjugate (BD Biosciences Pharmingen). The enzyme reaction was developed with BCIP-NBT substrate (Vector Laboratories, Inc., Burlingame, CA). Spot-forming cells (SFCs) were enumerated using KS ELISPOT system (Carl Zeiss, Hallbergmoos, Germany).

2.15. 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.

3. Results

3.1. In vitro expression of Hsp65 and IL-12

The DNA vaccines encoding mature and secreted forms of Hsp65 were constructed as Hsp65 DNA and IgHsp65 DNA, respectively. Hsp65 DNA contains the full-length *M. tuberculosis hsp65* gene. IgHsp65 DNA contains the full-length *M. tuberculosis hsp65* gene fused to the mouse Igκ signal sequence. Each construct is driven by CMV promoter and terminated at a bovine growth hormone polyadenylation sequence. Hsp65 DNA or IgHsp65 DNA was transfected into COS-7 cells and cell lysates and supernatants were analyzed for the *hsp65* gene expression. As shown in Fig. 1A, the mature form was detected as a single band in cell lysates (lane 2), whereas the secreted form was detected as a doublet band in cell lysates (lane 3). The doublet of slightly higher molecular weight than the mature form is most likely due to incomplete cleavage of the Igκ signal peptide in COS-7 cells because only a single band corresponding to the mature form was seen when HeLa cells or HepG2 cells were transfected with pcDNA-IgHsp65 (data not shown).

Based on the results of study reporting with high levels of IL-12 expression [42], we constructed a mouse IL-12 expression vector, mIL-12 DNA. The vector encodes mouse single-chain IL-12 protein comprised of p40 and p35 subunits linked by Gly_6Ser polypeptide linkers. As shown Fig. 1A, COS-7 cells transfected with mIL-12 DNA transiently expressed the mouse single-chain IL-12 protein with molecular weight of 80 kDa (lane 8). Quantitative analysis using ELISA showed that the COS-7 cells transfected with the mIL-12 DNA secreted four-fold higher levels of mIL-12p70 (125 ng/ml) than those transfected with from pcDNAmIL-12p40 + p35 (30 ng/ml), which previously constructed as a murine expression vector with IL-12 p40 and p35 expression cassettes in tandem array [35] (data not shown). Consistent with the mIL-12p70 expression level, the supernatant form the mIL-12 DNA transfectant cells induced 3.2-fold higher levels of IFN-γ from murine T lymphocytes than that from pcDNAmIL-12p40 + p35 transfectant cells (Fig. 1B). Thus, the mIL-12 DNA construct expresses biologically active IL-12, indicating that the single-chain IL-12 DNA is an effective DNA vaccine adjuvant capable of inducing primary Th-1 responses.

3.2. Evaluation of the best combination of Hsp65-based DNA vaccines with mIL-12 DNA for vaccine efficacy via gene gun

We compared the protective abilities of two versions of Hsp65-based DNA vaccine (Hsp65 DNA versus IgHsp65 DNA), and combinations with mIL-12 DNA (Hsp65 DNA versus Hsp65 DNA plus mIL-12 DNA, or IgHsp65 DNA versus IgHsp65 DNA plus mIL-12 DNA). Mice vaccinated with Hsp65 DNA, IgHsp65 DNA, and the combination with

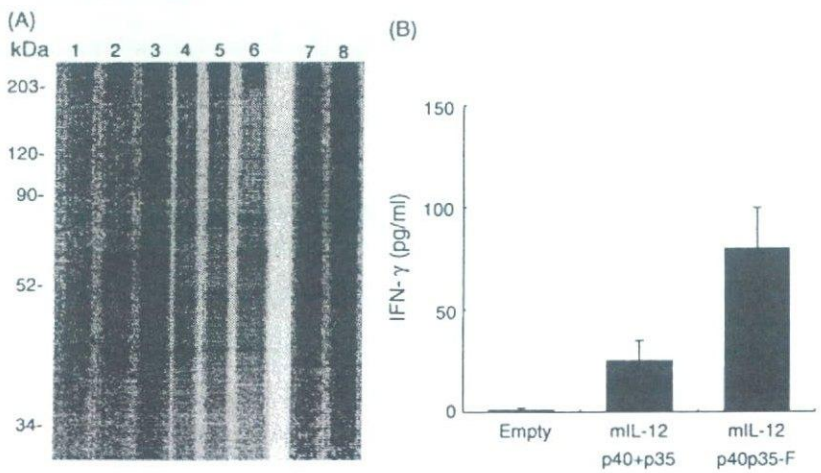

Fig. 1. Expression and biological analysis of Hsp65 and mIL-12. (A) In vitro expression analysis of Hsp65 and mIL-12 from cells transiently transfected with DNA vaccines. COS-7 cells were transfected with EGFP DNA (lanes 1, 4 and 7), Hsp65 DNA (lanes 2 and 5), IgHsp65 DNA (lanes 3 and 6), and mIL-p40p35-F DNA (lane 8). Following metabolically labeling with [^{35}S]methionine, cell lysates (lanes 1, 2, 3, 7 and 8) and supernatants (lanes 4–6) were immunoprecipitated with mouse anti-Hsp65 polyclonal antibody (lanes 1–6) or rabbit anti-murine IL-12p70 antibody (lanes 7 and 8). (B) The biological activities of IL-12 expressed in transfected cell supernatants in vitro. Culture supernatants from COS-7 cells transfected with pcDNA3.1 (no insert empty vector), pcDNA-mIL12p40 + p35 or pcDNA-mIL12p40p35-F were added to the mouse spleen cells (2×10^6 cells/ml) at the final concentration of 4% (v/v) and incubated for 60 h. The levels of mouse IFN-γ were measured using sandwich ELISA kits as described in Section 2.

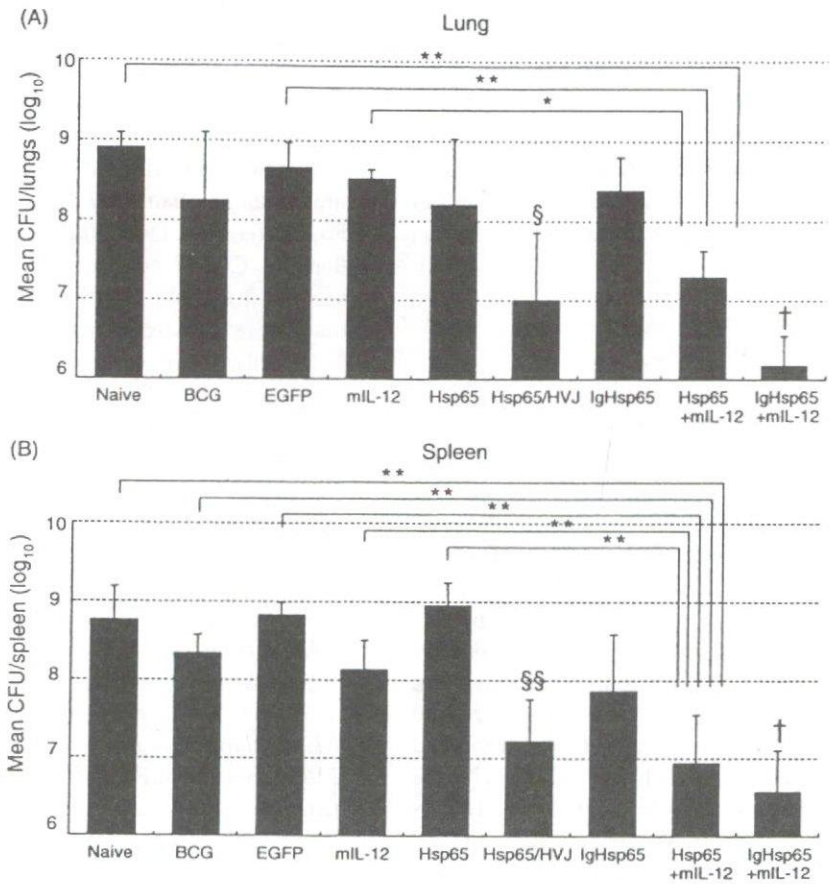

Fig. 2. The effect of a combination of mIL-12 expression vector and Hsp65-based DNA vaccines and comparison of different vaccines on the protective efficacy against challenge with *M. tuberculosis*. Groups of mice were vaccinated once with Hsp65 DNA, IgHsp65 DNA and a combination of mIL12 DNA via gene gun or three times with Hsp65/HVJ via intramuscular route and challenged intravenously with *M. tuberculosis* H37Rv as described in Section 2. Ten weeks after challenge, protection was measured by enumerating bacterial loads (CFU) in the lungs and spleen from vaccinated mice. Reduction of bacterial load was expressed as the mean log_{10} difference in CFU in the organs of the naive and vaccinated mice. The statistical significance of differences between individual groups in the number of CFU was determined by Tukey–Kramer's HSD test ($n = 4$–5). * and **, the statistical significance of differences ($P < 0.05$ and $P < 0.01$) compared to Hsp65 DNA + mIL-12 DNA group, respectively; †, the statistical significance of differences ($P < 0.01$) of IgHsp65 DNA + mIL-12 DNA group compared to the naive, BCG, EGFP DNA, mIL12 DNA, Hsp65 DNA and IgHsp65 DNA groups; §, the statistical significance of differences of Hsp65/HVJ group compared to BCG group ($P < 0.05$) in the lungs; §§, the statistical significance of differences of Hsp65/HVJ group compared to Hsp65 DNA ($P < 0.01$) and BCG ($P < 0.05$) groups in the spleen.

mIL-12 DNA via gene gun were challenged intravenously with *M. tuberculosis* H37Rv. The bacterial loads of the naive and vaccinated mice were compared 10 weeks after challenge (Fig. 2). Consistent with the previous report by Lima et al. [43], gene gun vaccination with Hsp65 DNA alone did not result in significant protective immunity as assessed by the bacterial load in the lungs or spleen. Vaccination with IgHsp65 DNA, which encodes the additional mouse Igκ signal sequence upstream of the *hsp65* gene, did not significantly improve the protective efficacy in the bacterial load in the lungs, although there was a modest decrease in the bacterial load in the spleen. In contrast, the combination with mIL-12 DNA markedly improved the protective efficacy both in the lungs and spleen ($P < 0.01$). In particular, vaccination of IgHsp65 DNA plus mIL-12 DNA conferred the greatest reduction of the bacterial load both in the lungs and spleen. Similar to IgHsp65 DNA plus mIL-12 DNA, the increased protection in the lungs and spleen was also observed in mice vaccinated with Hsp65 DNA plus mIL-12 DNA compared to IgHsp65 DNA alone and mIL-12 DNA alone. Thus, a strong synergistic effect on protection was achieved when Hsp65 DNA was co-administrated with IL-12 DNA. It is notable that the prophylactic effect of IgHsp65 DNA plus mIL-12 DNA in the lungs was more than 100-fold greater than that of BCG. These vaccinations of IgHsp65 DNA plus mIL-12 DNA and Hsp65 DNA plus mIL-12 DNA also exerted the significant reduction in the liver compared to the naive ($P < 0.05$) and control EGFP DNA groups ($P < 0.01$), whereas there was no significant difference of the naive group compared with Hsp65 or mIL-12 group (data not shown). In mice vaccinated with IgHsp65 DNA plus mIL-12 DNA, increased protection in the lungs were also observed at 5 weeks after challenge, which was equivalent to that obtained by vaccination with BCG (data not shown).

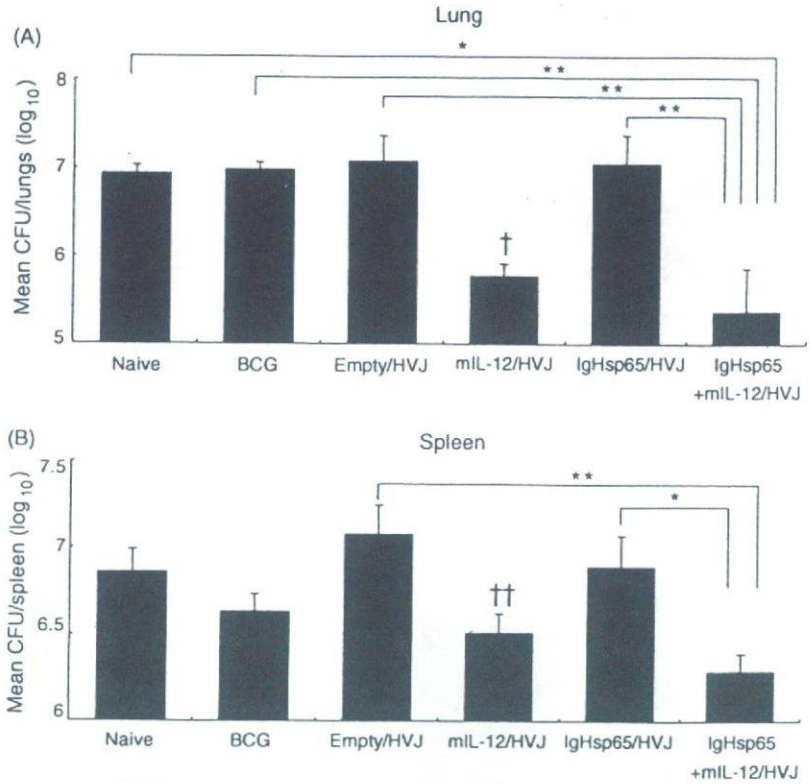

Fig. 3. Mouse protection studies using HVJ-liposome vaccines. Groups of mice vaccinated with HVJ-liposome DNA or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the lungs (A) and spleen (B) from vaccinated mice. Results are expressed as the mean $\log_{10} \pm$ S.D. of CFU. The statistical significance of differences between individual groups in the number of CFU was determined by Tukey–Kramer's HSD test ($n = 4$–5). $^{*}P < 0.05$; $^{**}P < 0.01$; †, the statistical significance of differences ($P < 0.05$) of mIL-12/HVJ group compared to BCG, Empty/HVJ, and Hsp65/HVJ groups in the lungs; ††, the statistical significance of differences ($P < 0.05$) of mIL-12 DNA group compared to Empty/HVJ group in the spleens.

3.3. Comparison of the protective efficacy of gene gun versus HVJ-liposome delivery of Hsp65 DNA vaccines

We next compared methods of DNA vaccine delivery on vaccine efficacy at 10 weeks after challenge. Hsp65/HVJ vaccination and challenge experiments were conducted simultaneously with gene gun experiments. As shown in Fig. 2, Hsp65/HVJ vaccination significantly reduced the bacterial loads as compared to Hsp65 gene gun immunization in the spleen ($P < 0.01$). IgHsp65 gene gun immunization significantly reduced the bacterial loads as compared to Hsp65 gene gun immunization in the spleen ($P < 0.05$, data not shown). Therefore, we used IgHsp65/HVJ for further experiments.

3.4. Protective efficacy of HVJ-liposome DNA vaccines

At 5 and 10 weeks after intravenous challenge of *M. tuberculosis* H37Rv, the number of CFU in the lungs, spleen, and liver were determined. Fig. 3 shows the results of bacterial loads 5 weeks after challenge. Vaccination with mIL-12/HVJ group resulted in significant protective immunity in the bacterial as compared to BCG, Empty/HVJ and Hsp65/HVJ groups in the lung ($P < 0.05$) and as compared to Empty/HVJ group in the spleen ($P < 0.05$). Vaccination with IgHsp65 + mIL-12/HVJ induced better protective immunity in the bacterial load both in the lungs and spleens than IgHsp65/HVJ alone and mIL-12/HVJ alone. Thus, the synergistic effect of IgHsp65 DNA and mIL-12 DNA resulted in improving the protective efficacy. At 10 weeks after challenge, the same reduction was also observed in these organs from mice vaccinated with IgHsp65 + mIL-12/HVJ (data not shown). Body weights of vaccinated mice were similar in all vaccinated groups. Tissue weight of lungs, liver, and spleen in the IgHsp65 + mIL-12/HVJ group were slightly lower than that from the naive mice (data not shown). In this experiment, BCG vaccination did not provide significant reduction of the bacterial load compared to the naive group. This may be due to the single-dose of vaccination used usually, the use of BCG Tokyo strain requires a three-dose vaccination to achieve 10 to 30-fold reduction of the bacterial loads compared to a non-vaccinated group. Although, 5 weeks after challenge, no reduction of bacterial loads was observed in IgHsp65/HVJ group compared with the naive control group, we confirmed the increased protection 10 weeks after challenge compared with the naive control group

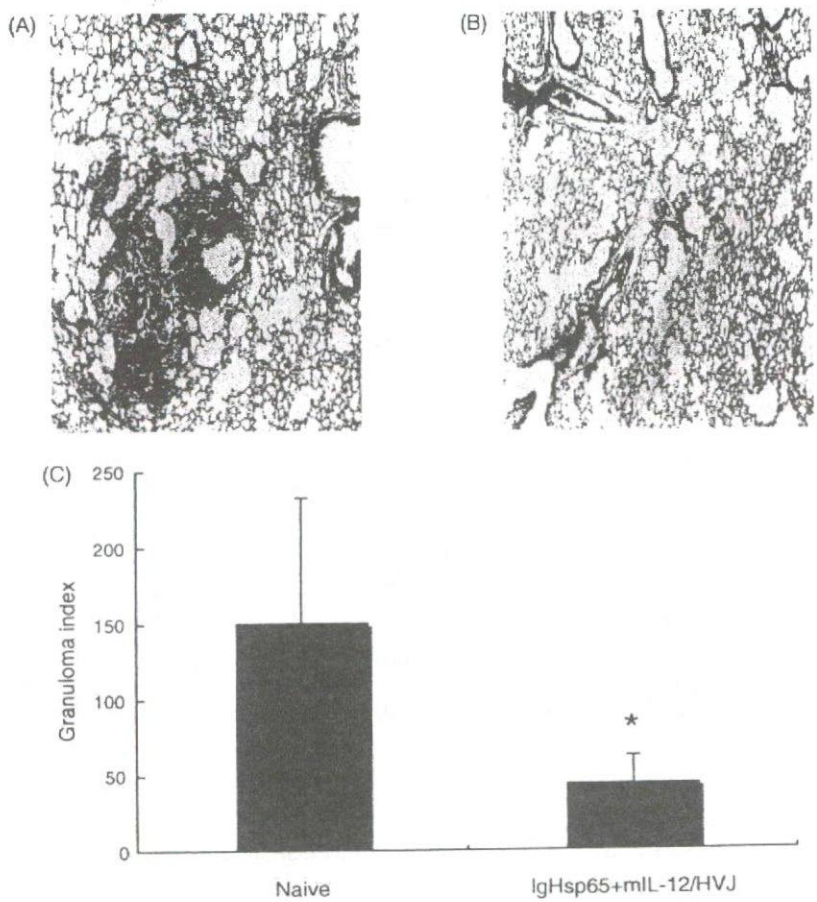

Fig. 4. Histopathological analysis of vaccinated mice 10 weeks after *M. tuberculosis* challenge. Representative photomicrographs of lung tissue sections harvested from the naive control group (A) and from the IgHsp65 + mIL-12/HVJ group (B) are shown (10 weeks after *M. tuberculosis* challenge, hematoxylin and eosin staining, ×10 objective). There were much infiltration of mononuclear cells and extensive parenchymal destruction by large, poorly demarcated granuloma in the lung from the naive control group. In the IgHsp65 + mIL-12/HVJ group, the lungs were less inflamed and only a few granuloma was observed. (C) Granuloma index of the naive control group and the IgHsp65+mIL-12/HVJ group in the lungs. Results are expressed as the mean ± S.D. of triplicates of five mice per group. The statistical significance of differences between the groups was determined by Student's *t*-test. $^{*}P < 0.05$ as compared with the naive control group.

at the same experiments (data not shown). These results indicate that co-vaccination with IL-12 DNA was effective for inducing protective immunity at as early as 5 weeks after challenge.

3.5. IgHsp65 + mIL-12/HVJ vaccination markedly reduced granuloma formation in the lung

In addition to the reduction of bacterial loads, the effects of vaccination on the mice were assessed by histological analysis. The granulomatous lesions in the lungs from IgHsp65 + mIL-12/HVJ mice were significantly less in number and size than from the naive control group (Fig. 4A and B). Quantitative evaluation of the granulomatous lesions clearly shows that IgHsp65 + mIL-12/HVJ vaccinated mice group exhibited significant reduction in granuloma index in the lungs, compared to the naive group ($P < 0.05$) (Fig. 4C). Thus IgHsp65 + mIL-12/HVJ vaccine provided significant protection against the pulmonary pathology caused by *M. tuberculosis* infection.

3.6. HVJ-liposome DNA vaccines generated T-helper response and cytokine production

To investigate lymphocyte proliferative and cytokine responses induced by HVJ-liposome DNA vaccines, spleen cells from vaccinated mice were re-stimulated with antigen in vitro. As shown in Fig. 5, substantial lymphocyte proliferation was observed in response to rHsp65 protein in spleen cells from mice vaccinated with IgHsp65/HVJ or IgHsp65 + mIL-12/HVJ but not with the naïve control. IgHsp65 + mIL-12/HVJ vaccination induced significantly better proliferative response to rHsp65 protein than did IgHsp65/HVJ vaccination ($P < 0.01$). In addition to lymphocyte proliferative responses, vaccination with IgHsp65 + mIL-12/HVJ induced elevated levels of IFN-γ and

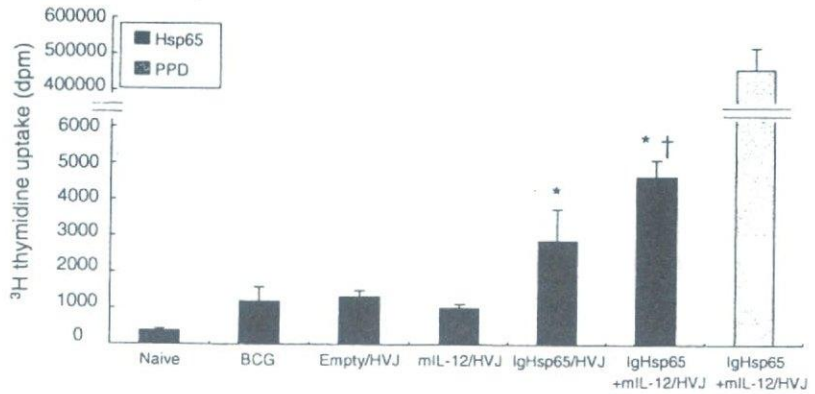

Fig. 5. The effect of vaccination with HVJ-liposome DNA on T cell proliferation. Proliferative responses of splenic lymphocytes from mice vaccinated with IgHsp65/HVJ, mIL-12/HVJ, IgHsp65 + mIL-12/HVJ, BCG, or Empty/HVJ. Incorporation of [^{3}H]thymidine in response to rHsp65 protein (black bars) or PPD (gray bar) was measured as described in Section 2. Results are expressed as the mean ± S.D. of triplicates of three mice per group. The statistical significance of differences between individual groups in T cell proliferation was determined by Tukey–Kramer's HSD test. The statistical significance of differences ($P < 0.01$) compared to the naive and BCG groups are indicated as (*) and (†), respectively.

IL-2 in response to rHsp65 protein, but not with the naive control or BCG group (Fig. 6). In response to PPD, vaccination with IgHsp65 + mIL-12/HVJ markedly increased both IFN-γ and IL-2 production as compared to the BCG group. Moderate but significant levels of IFN-γ and IL-2 were also induced in Hsp65/HVJ vaccination in response to Hsp65 protein and PPD. Thus, the synergistic effect of IgHsp65 DNA and mIL-12 DNA resulted in the strongest response not only to T cell proliferation but also to cytokine production.

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

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

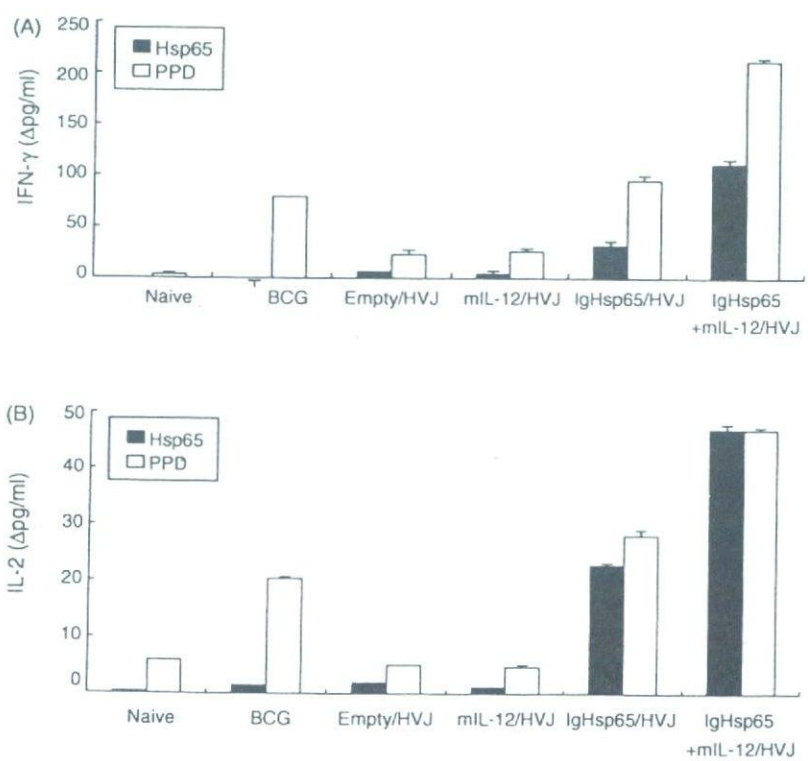

Fig. 6. IFN-γ (A) and IL-2 (B) production in spleen cell culture supernatants from vaccinated mice following stimulation with rHsp65 protein and PPD. Spleen cell cultures were stimulated with rHsp65 protein (black bars) or PPD (white bars) for 48 h, and the levels of IFN-γ and IL-2 production were determined by ELISA. Results are expressed as the mean ± S.D. of duplicates of three mice per group with antigens minus the mean ± S.D. of triplicates of three mice per group with medium alone.

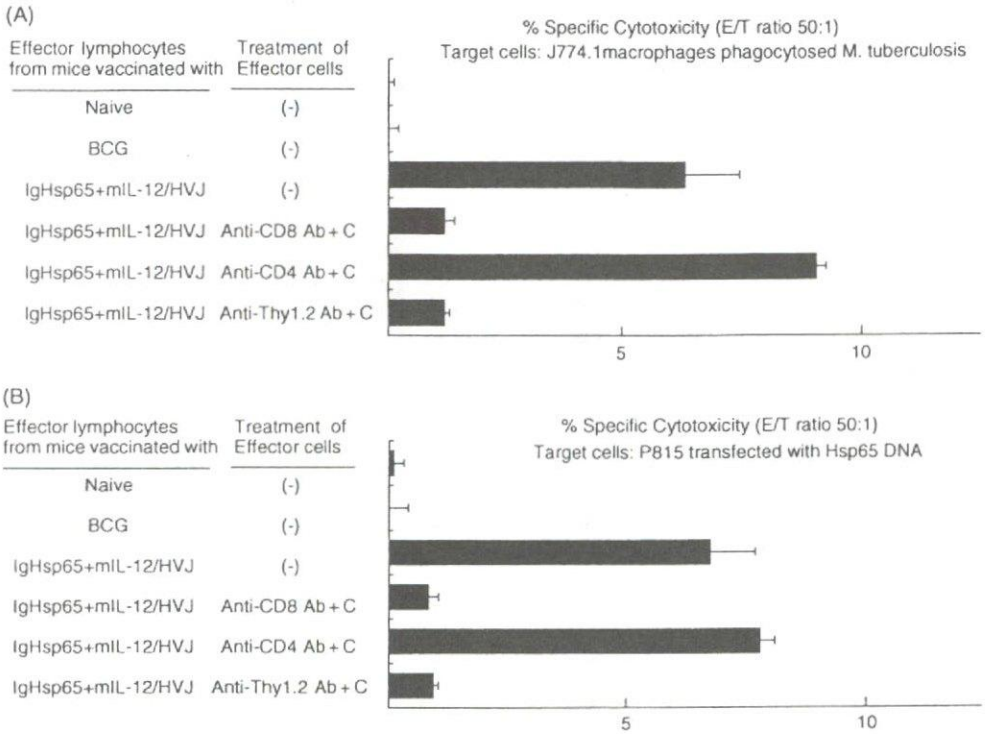

Fig. 7. Induction of $CD8^+$ CTL specific for Hsp65 protein and *M. tuberculosis* by vaccination with IgHsp65 + mIL-12/HVJ. Spleen cells from the naive, BCG-, and IgHsp65 + mIL-12/HVJ-vaccinated mice were obtained 8 weeks after the final vaccination. Cytotoxicity was assayed as release of radioactivity from ^{51}Cr-labeled J774.1 macrophages that had phagocytosed *M. tuberculosis* (killed H37Ra) (A) or from ^{51}Cr-labeled P815 target that had been transfected with Hsp65 DNA (B) using a conventional ^{51}Cr release assay at E:T ratio of 50:1. The effector cells were pre-incubated with anti-CD8, anti-CD4 or anti-Thy1.2 antibody, followed by treatment with complement. Percent specific lysis was determined as: [(experimental release–medium control release)/(maximum release–medium control release)] × 100. Ab: antibody; C: complement; (−), non-treatment.

absence of re-stimulation. As shown in Fig. 7, high levels of Hsp65- and *M. tuberculosis*-CTL specific lysis against J774.1 macrophages phagocytosed *M. tuberculosis* and P815 mastocytomas transfected with Hsp65 DNA were detected in mice vaccinated with IgHsp65 + mIL-12/HVJ, whereas little CTL response was detectable in either the naive or BCG-vaccinated mice. In vitro depletion of $CD8^+$ T cells eliminated the specific lysis. 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-

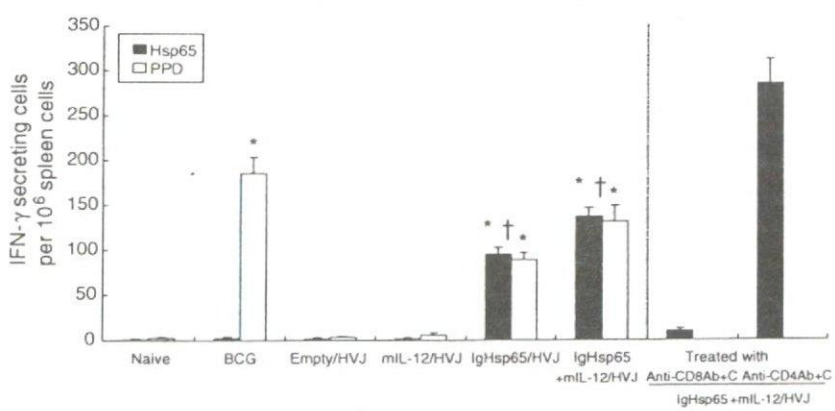

Fig. 8. ELISPOT assay for IFN-γ antigen-specific responses in the spleens of vaccinated mice following stimulation with rHsp65 protein and PPD. Spleen cell cultures were stimulated with rHsp65 protein or PPD for 20 h or pre-incubated with anti-CD8 antibody or anti-CD4 antibody followed by treatment with complement and then stimulated with rHsp65 protein for 20 h. The number of IFN-γ-secreting cells specific for rHsp65 protein (black bars) or PPD (white bars) per million cells were determined individually by ELISPOT assay. Results are expressed as the mean ± S.D. of five-wells of three mice per group. The statistical significance of differences between individual groups in the number of IFN-γ-secreting cells was determined by Tukey–Kramer's HSD test. The statistical significance of differences ($P < 0.01$) compared to the naive and BCG groups are indicated as (*) and (†), respectively.

12/HVJ vaccine induced long-term immune response with strong $CD8^+$ CTL activity.

3.8. ELISPOT assay

In order to determine whether enhanced protection was associated with increased IFN-γ production, the frequency of IFN-γ-secreting cells was enumerated by ELISPOT. Vaccination with IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ resulted in a marked increase of IFN-γ secreting cells following stimulation with rHsp65 protein (Fig. 8). Moreover, the increase of IFN-γ secreting cells was also seen in IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ groups following stimulation with PPD. These results indicate that vaccination with IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ activated antigen-specific T cells producing IFN-γ. Depletion of $CD8^+$ cells from responder cells by treatment with anti-CD8 antibody and complement almost abrogated the IFN-γ producing cells. In contrast, an increase in the number of IFN-γ producing cells was observed in the responder cells when treated with anti-CD4 antibody and complement. BCG vaccination resulted in significant increase of IFN-γ secreting cells following stimulation with PPD but not rHsp65 protein. These data indicate that the protective efficacy of IgHsp65 + mIL-12/HVJ is strongly associated with the emergence of IFN-γ-secreting cells upon stimulation with Hsp65. Taken together, vaccination with IgHsp65 + mIL-12/HVJ capable of augmenting T cell activation and frequency of IFN-γ-secreting cells proves to reduce bacterial burden and pathology in the lungs—all to an extent greater than those achieved by vaccination with BCG.

4. Discussion

In the first stage of this study, we evaluated the protective efficacy of Hsp65 DNA vaccines via gene gun vaccination. One of the significant findings of the present study is that a single gene gun vaccination with the combination of IgHsp65 DNA and mIL-12 DNA led to a remarkably high degree of protection against intravenous challenge infection with virulent *M. tuberculosis*; bacterial numbers declined exponentially in internal organs and were 100-fold lower in the lungs than in BCG-vaccinated mice. Consistent with previous studies [43], gene gun vaccination with Hsp65 DNA alone did not promote reduction in bacterial burden compared to the naive mice. However, co-vaccination of Hsp65 DNA or IgHsp65 DNA plus mIL-12 DNA significantly improved the protective efficacy compared to either Hsp65 DNA alone or IgHsp65 DNA alone. Since the importance of IL-12 in the control of mycobacterial infections has been well documented, these results are consistent with other studies describing an adjuvant effect of IL-12 gene when administrated in combination with various tuberculosis DNA vaccines [20,24,25]. The mIL-12 DNA, which express both p40 and p35 chains as a single molecule, is able to induce four-fold higher levels of IFN-γ from mouse T lymphocytes than mIL12p40 + p35, which has previously been constructed as a murine expression vector with IL-12 p40 and p35 expression cassettes in tandem array [35]. Culture supernatants from the mIL-12 DNA-transfected COS-7 cells were effectively induced IFN-γ from mouse spleen cells. Thus, the improved expression levels of IL-12 DNA and the biologically active IL-12 explain the enhanced protection observed.

The second stage of this study demonstrated the protective efficacy of HVJ-liposome DNA vaccines in mouse and guinea pig models. We originally developed HVJ-liposomes, a viral/nonviral hybrid vector, as a gene transfer vector for cancer gene therapy. HVJ-liposome gene transfer method can deliver DNA directly and efficiently into host cells in vivo by means of the HVJ virus cell fusion machinery. We found that HVJ-liposome-mediated gene transfer was 30–100 times more efficient in gene expression in skeletal muscle than naked DNA transfer (unpublished data) and over three times more efficient in delivering intact oligodeoxyribonucleotide within the nuclei of transfected cells than Lipofectin®, a different cation liposome [44]. In addition to its high transfection efficiency, there are numerous safety advantages of HVJ-liposomes including: (i) no apparent toxicity or inflammation and (ii) repeated gene transfection without reduction of transfection efficiency. In fact, no significant adverse effects were induced in monkeys by intravenous injection of HVJ-liposomes [45]. Using this novel vector, we observed the enhancement of protection conferred by Hsp65 DNA compared to gene gun vaccination. This result is encouraging for the development of a novel tuberculosis DNA vaccine that is applicable both for prophylactic and therapeutic uses with no side-effects after repeated injections.

The most significant finding of this study is that vaccination with IgHsp65 + IL-12/HVJ provided greater protective efficacy than vaccination with BCG. In the mouse model, IgHsp65 + mIL-12/HVJ preferentially triggered a Th1 type T helper response, characterized by elevated levels of IFN-γ and IL-2, and augmentation of lymphocyte proliferation. After challenge, vaccination with IgHsp65 + mIL-12/HVJ 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 directed against Hsp65 and PPD. $CD8^+$ CTL activity against macrophage target cells, which had previously phagocytosed *M. tuberculosis* or expressed Hsp65 protein, was still observed in the spleen cells from mice vaccinated with IgHsp65 + mIL-12/HVJ at 8 weeks after the final vaccination, IgHsp65 + mIL-12/HVJ vaccine capable of augmenting long-term immune response with anti-tuberculosis CTL activity proves IgHsp65 + mIL12/HVJ to be a promising tuberculosis vaccine candidate.

Although the *hsp65* DNA vaccines have been shown to have significant promise as a new prophylactic vaccine against tuberculosis [19,21,46], negative outcomes have also been reported [47,48]. In the case of vaccination with *hsp65* DNA alone, our results are consistent with the previous report

that vaccination with *hsp65* DNA alone did not provide significant protective effect in the bacterial load in the lung either in the mouse model or in the guinea pig model [43,47]. However, as described above, the combination with mIL-12 DNA expressing biologically active IL-12 and the use of HVJ-liposome as a DNA vaccine delivery system remarkably improved the protective efficacy. In addition, our preliminary results of a guinea pig model in the collaborative study with Dr. D. McMurray (Texas A&M University) show that vaccination with IgHsp65 + guinea pig IL-12 (gpIL-12)/HVJ provided better protection against the pulmonary pathology caused by aerosol challenge with *M. tuberculosis* than did BCG vaccination (data not shown). For immunotherapeutic use, *hsp60*/lep DNA vaccine (*hsp65* DNA derived from *Mycobacterium leprae*) has been shown to be effective in a Cornel-type model [22], although others have argued that this vaccine induced progressively severe pulmonary necrosis in the model [48]. In support of the effectiveness, when administered to mice or SCID-PBL/hu mice [49] already infected with *M. tuberculosis*, neither IgHsp65 + mIL-12/HVJ vaccine nor IgHsp65 + human IL-12 (hIL-12)/HVJ vaccine, respectively, resulted in exacerbation of the granulomatous response in the lungs (unpublished data). Moreover, therapeutic administration of IgHsp65 + mIL-12/HVJ resulted in significant reduction of bacterial loads (paper in submission). The pathological parameter of protection included reductions in the mean lung granulomatous lesion score in our study. In parallel with the protective efficacy of HVJ-liposome vaccines on bacterial loads, histopathological analysis shows that mice vaccinated with IgHsp65 + mIL-12/HVJ had fewer and smaller lesions in the lung and significantly less lung granuloma than the naive mice. These results suggest that severe toxicities (Koch phenomenon) could not be induced by this vaccine. One possible explanation for these diverging results may be different *hsp65* DNA construct (secreted form versus cytoplasmic form; derived from *M. tuberculosis* versus *M. leprae*), different mIL-12 DNA construct (p40p35 fusion form versus p40-p35 tandem form), and different vaccine delivery (HVJ-liposome versus gene gun or naked DNA).

In conclusion, we demonstrate the development of a novel HVJ-liposome DNA vaccine encapsulating Hsp65 DNA plus IL-12 DNA. These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to the currently available BCG vaccine. The goal of our study is to develop a new tuberculosis vaccine superior to BCG. To this aim, we believe that the protective efficacy and protective immune responses for vaccine candidates should be addressed in larger animals, such as non-human primates, before proceeding to human clinical trials. Although other DNA vaccine candidates that appear to protect against virulent *M. tuberculosis* in mice better than BCG have failed to provide better protection than BCG in guinea pigs against aerosol challenge of a low dose of virulent *M. tuberculosis* [47,50,51], some of them are being prepared to enter early human clinical trials [52]. More recently, we evaluated the IgHsp65 + hIL-12/HVJ vaccine in the cynomolgus monkey model [29], which is currently the best non-human primate animal model of human tuberculosis. Monkeys were subsequently challenged with virulent *M. tuberculosis* by the intra-tracheal route after the third vaccination. This challenge dose normally causes death from acute respiratory infection within 4–6 months. In this particular experiment, monkeys vaccinated with IgHsp65 + hIL-12/HVJ induced Hsp65-specific T cell proliferation and improvement of chest X-P findings, resulting in an increased survival for over a year, superior to BCG group [29]. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data of the HVJ-liposome DNA vaccine, including the vaccine efficacy and safety, for up-coming Phase I clinical trials.

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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

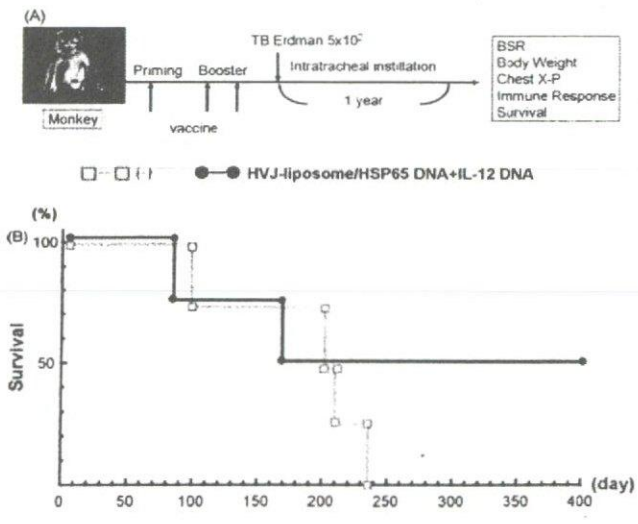

Fig. 1. (A) Evaluation of vaccine efficacy using cynomologus monkey. Groups of animals were vaccinated three times with either the HVJ–liposome combination with HSP65 DNA plus human IL-12 DNA (HSP65 + hIL-12/HVJ: 400 μg i.m.), BCG Tokyo (1×10^6 CFU i.d), or saline (A). One month after the third vaccination, 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.

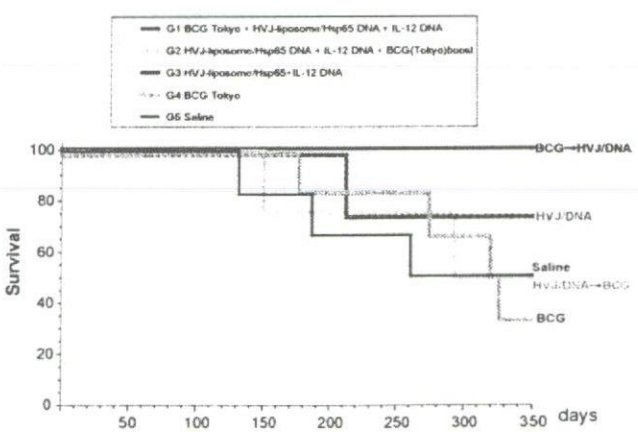

Fig. 2. Protective efficacy of HSP65 + IL-12/HVJ and BCG using priming-booster method against TB challenged cynomologus monkeys. Group of animals were vaccinated three times (every 3 weeks) with (1) BCG Tokyo, (2) Hsp65 + IL-12/HVJ, (3) Hsp65 + IL-12/HVJ = G1 (—) BCG prime-HVJ/DNA booster group; (1) 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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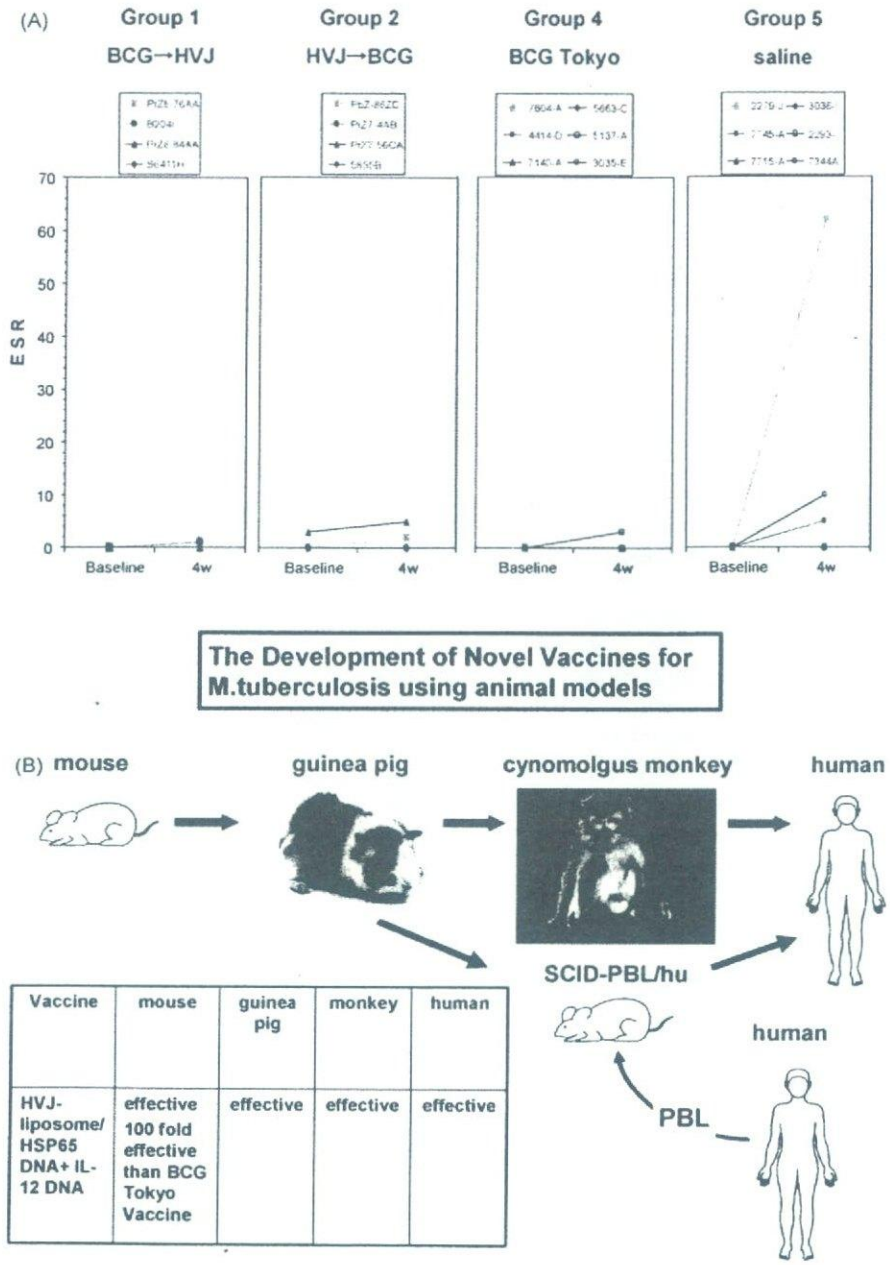

Fig. 3. (A) Improvement of erythrocyte sedimentation rate (ESR) in the cynomolgus monkeys immunized with HVJ–liposome/HSP65 DNA + IL-12 DNA vaccine. Cynomolgus monkeys were immunized and challenged as described in Fig. 2. Elevation of ESR of all monkeys was evaluated every month and maximum values of 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.

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