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Protection of Mice with a Divalent Tuberculosis DNA Vaccine Encoding Antigens Ag85B and MPT64

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Abstract DNA vaccine may be a promising tool for controlling tuberculosis development. However, vaccines encoding single antigens of mycobacterium did not produce protective effect as BCG did. In the present study, we evaluated the immunogenicity and protective efficacy of a divalent DNA vaccine encoding two immunodominant antigens Ag85B and MPT64 of *Mycobacterium tuberculosis*. We found that both humoral and Th1-type (high IFN-γ, low IL-4) cellular responses obtained from the divalent DNA vaccine group were significantly higher than that conferred by BCG. RT-PCR results showed that antigens were expressed differentially in various organs in divalent DNA vaccine group. The survival rate for mice treated with the divalent DNA vaccine after challenging with high doses of virulent *M. tuberculosis* H37Rv was significantly higher than that of the BCG group or any of the single DNA vaccine group. Significant differences were also found between the single and divalent DNA vaccinated mice in terms of body, spleen and lung weight. Bacterial loading decreased about 2000-fold in lungs and about 100-fold in spleens of divalent DNA vaccinated mice when compared with that of the control group. We conclude that our divalent DNA vaccine may be a better choice for controlling tuberculosis disease in animals.

Key words tuberculosis; divalent DNA vaccine; immunogenicity; protection

Tuberculosis (TB) remains to be a major challenge to the public health in the world. It is estimated that, throughout the world, 15 individuals are affected by TB and 6 of them die from it every minute [1]. Drug resistance and coinfection with HIV, which utilizes the host immune system in replication, complicate the situation. BCG (Bacille Calmette-Guerin), the most widely used vaccine against TB, has proven its value in preventing systemic childhood disease development, particularly in control of *Mycobacterium meningitis* [2]. However, its efficacy against the most prevalent form of adult TB is questionable [1]. Currently, a number of new TB vaccines including subunit, auxotrophic, recombinant BCG, and DNA vaccines are being developed [3–7]. For *Mycobacterium tuberculosis*, secreted and surface-exposed cell wall proteins are major antigens recognized by the host protective immune response system, so several of the proteins have been evaluated as candidates for developing DNA vaccines in experimental systems [5,8,9]. The Ag85 complex comprises three closely related components, Ag85A, 85B, and 85C, which together constitute 20%–30% of all proteins present in the supernatant of short-term cultures and thus represent one of the most promising groups of extracellular antigens [10]. Vaccination with naked plasmid DNA encoding Ag85B stimulated strong humoral and cell-mediated immune responses and conferred significant protection to C57BL/6 (B6) mice challenged by live *M.tuberculosis* H37Rv [11]. MPT64 is present in *M. tuberculosis*, in virulent *M. bovis* and also in some strains BCG. It was able to elicit T cell responses and to enhance protective efficacy in a mice model as well [11].

Previous work showed that immunization with plasmid DNA expressing different antigens in combination with adjuvant was more efficient than that of the naked DNA [12–14]. It was demonstrated that DNA vaccines boosted by purified polypeptides were also able to enhance protective immunity in different experimental systems [15,16]. However, none of the vaccines tested so far, including the

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combined DNA vaccine with 10 different plasmids, has been found superior to BCG [12,17,18]. IFN-γ plays a major role in protecting against this intracellular bacteria, but Ig1/Ig2a ratio depends on different antigens [19,20]. Both antigens Ag85B and MPT64 eliminate strong Th1-type cytokine responses.

In the current work, we fused two genes encoding immunodominant antigens Ag85B and MPT64 into one expression vector and evaluated the immunogenicity and protective efficacy in vaccinated mice. Our results showed that the levels of IFN-γ in mice immunized by the divalent DNA vaccine were significantly higher than that of BCG group. Lung histopathology and organ CFU experiments also indicated that our divalent DNA vaccine resulted in better protection of the experimental model animals.

Materials and Methods

Animals and bacterial strains

Female C57BL/6 mice weighing 18–20 g were obtained from Animal Center of the Academy of Military Medical Sciences. They were raised under standard conditions, and fed with commercial mouse chow and water in the Center of Tuberculosis Research, 309th Military Hospital. *M. tuberculosis* H37Rv and *M. bovis* BCG strains were obtained from the Center of Tuberculosis Research. *E. coli* DH5α was grown on LB medium and used for cloning.

Preparation of DNA templates

Bacilli were harvested by scraping the surface of cultures gently and by washing the surface with lysis buffer (50 mM pH 8.0 Tris, 50 mM EDTA, 25% sucrose, 500 μg/ml lysosome). Washed bacilli were resuspended in lysis buffer (2 ml solution for each gram of wet bacilli pellet with the addition of proteinase K and Tween 20 to final concentrations of 5 mg/ml and 0.2% respectively) and the mixture was incubated in a shaker at 37 °C for 14 h. Sodium dodecyl sulfate (SDS) was then added to a concentration of 1% and the suspension was held at 50 °C for 15 min. The sample was diluted to 5 ml with lysis buffer and extracted with phenol twice followed by chloroform. DNA was precipitated with 0.6 volume of isopropanol and 0.1 M pH 5.0 sodium acetate. Genomic DNA isolated and redissolved in ddH_2O was used as template to amplify target sequences.

DNA vaccine construction and purification

Genes encoding mycobacterial antigens were amplified from *M. tuberculosis* H37Rv chromosomal DNA with *EXTaq* (TakaRa, Dalian, China) and primers designed from the *M. tuberculosis* genome sequence database. The construct was generated to encode antigens fused to a TPA (tissue plasminogen activator) signal sequence. To facilitate the plasmid construction, the 5′ primer for gene of Ag85B has a *Nhe*I restriction site and its 3′ primer contains an *Eco*RI restriction site. The 5′ primer for gene of MPT64 has an *Eco*RI restriction site and its 3′ primer contains a *Bam*HI site. Both PCR products were cloned together into the same eukaryotic expression vector pJW4303 (kindly provided to us by Prof. J. Mullins of Stanford University). The constructions of two single DNA vaccines were described elsewhere [18]. The recombinant plasmids encoding single or two antigens were transformed into *E. coli* DH5α cells and the cells were cultured on solid LB medium containing 100 μg/ml Ampicillin. The DNA constructs were sequenced for confirmation of their reading frame before being used for vaccination. Endotoxin-free plasmids DNA were prepared and purified using the Qiagen EndoFree plasmid Maxi kit (Qiagen, Chatsworth, USA). The endotoxin concentrations in the final vaccine preparations were assessed using the QCL-1000 limulus amebocyte lysate kit (BioWhittaker, Walkersville, USA). In general, less than 50 endotoxin units per mg of plasmid DNA were detected in the vaccine preparations.

DNA vaccination

Recombinant plasmids were purified and prepared by adjusting the plasmids to final concentration of 1 mg/ml of DNA in phosphate-buffered saline (PBS). Groups of C57BL/6 mice were injected intramuscularly in the tibialis anterior muscle of each hind leg on day 1, 21, and 42 with a total of 100 μg plasmid DNA. The mice in the negative control group were immunized with same amount of empty vector DNA. The mice in the positive control group were vaccinated subcutaneously with 5×10^6 CFU of BCG on the first day.

Humoral and cytokine response analysis

Serum levels of Ag85B- and/or MPT64-specific antibodies were determined 3 weeks after the DNA injections to mice, using an indirect ELISA. Microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with recombinant Ag85B and/or MPT64 (both in the form of recombinant 6×His fusion protein) at a concentration of 10 μg/ml in carbonate/bicarbonate buffer (pH 9.6). ELISA was carried out as previously described [17,18]. Sera from three individual mice were pooled and

analyzed. The end point titer was defined as the highest dilution of serum that gave an absorbance value that exceeded 0.050 and was two-fold greater than that of the matched dilution of unvaccinated mouse sera. Mice injected with same amount of empty vector DNA were used as the control. Vaccinated mice were sacrificed 3 weeks after the last DNA injection. Spleens removed aseptically from three mice of each group were pooled for analysis of cytokine responses to recombinant protein Ag85B and/or MPT64 (10 μg/ml) in vaccinated mice and to PPD (10 μg/ml) in BCG [17,18]. Antigen-stimulated IFN-γ and IL-4 obtained from spleen cells were measured by sandwich ELISA using paired MAbs (Pharmingen, USA) according to the manufacturer's instructions. Recombinant IFN-γ and recombinant IL-4 (Both from Pharmingen, USA) were used as standards and the detection limit for the assay is about 15 pg/ml.

In vivo expression analysis by RT-PCR

Three weeks after the third immunization, organs were taken out from three mice of divalent DNA group for RNA extraction. Total RNA was isolated from tissues by extraction in Trizol Reagent (Gibco BRL, Grand Island, NY, USA) and alcohol precipitation procedure followed DNase I treatment (Gibco BRL, Grand Island, NY, USA) to avoid genomic and plasmid DNA contamination. Ag85B- and MPT64-specific primers were used to analyze transcript levels in different organs. Total RNA (5 μg) was reversely transcribed using oligo (dT) primers and reverse transcriptase (Gibco BRL) in a final volume of 20 μl according to the manufacturer's instructions. PCR amplification was carried out using 1 μl of cDNA preparation and specific primer pairs of *M. tuberculosis* Ag85B, MPT64 genes. The PCR system contains: 1 μl 10mM 5′ and 3′ primers each, 5 μl of 10×buffer, 0.5 μl *EXTaq* at 1 U/μl, 4 μl 2.5 mM dNTP and 1 μl sample. The final volume was 50 μl and the PCR condition is: 30 cycles of (denaturation at 94 °C for 1 min, annealing of primers to target cDNA at 58 °C for 30 s, and extension at 72 °C for 1 min). β-actin was used as internal control. PCR products were visualized by ultraviolet illumination after electrophoresis in 1.0% agarose gel containing ethidium bromide.

Evaluation of the protective efficacy of DNA vaccines against intravenous challenge

Eight weeks after receiving the final DNA immunization, mice were challenged with 10^6 *M. tuberculosis* H37Rv by intravenous injection via lateral vein. All surviving mice were sacrificed 8 weeks after the challenge for assessment of bacterial growth. Lungs and spleens were removed aseptically, and half of these organs were homogenized separately in 5 ml 0.04% Tween 20-PBS using a Seward Stomacher 80 blender (Tekmar, Cincinnati, USA). The homogenates were diluted serially in the Tween 20-PBS solution, and 50 μl aliquots were plated on Middlebrook 7H11 agar (Difco, Detroit, USA). Samples from BCG-vaccinated mice were plated on Middlebrook 7H11 agar containing 2 μg/ml 2-thiophenecarboxylic acid hydrazide to inhibit the proliferation of BCG while allowing normal growth of *M. tuberculosis*. The number of CFU in infected organs was determined 4 weeks after incubation at 37 °C in sealed plastic bags.

Histopathological analyses

Lung tissues from other halves were perfused and fixed with 10% paraformaldehyde in PBS and then embedded in paraffin for sectioning. The fixed tissue sections were stained with hematoxylin and eosin (H&E) reagent and were evaluated in light microscopy by a pathologist without prior knowledge of the treatments.

Statistical analysis

Student's *t*-test was performed to analyze the animal protection, body and organ weight. A value of $P<0.05$ showed significant difference.

Results

Divalent DNA vaccines induced high level antibody production

Antibody titers against the mycobacterial antigens were determined in sera harvested from immunized mice 3 weeks after the third injection. Recombinant proteins were used for detection of antigen-specific IgG and for cytokine analysis as well. As shown in Table 1, the divalent DNA vaccine produced a high IgG response specific to antigens Ag85B and MPT64 while in the BCG treated group, PPD-specific antibody titer was only 1:800 after the immunization. The DNA-Ag85B treated mice group also stimulated quite strong IgG response while DNA-MPT64 immunized mice produced weak IgG responses throughout the whole experimental period. Similar antibody titers against MPT64 were stimulated in divalent DNA and DNA-MPT64 immunized mice groups showing that there were no competition between Ag85B and MPT64 in divalent DNA group. Mice treated with empty DNA vector produced no antigen specific antibody at all.

Table 1 Antigen-specific humoral response to different vaccines*

Vaccines	Antigens	IgG titers		
		After 1st immunization	After 2nd immunization	After 3rd immunization
Divalent DNA	Ag85B	1:800	1:102,400	1:102,400
	MPT64	1:100	1:800	1:6400
DNA-85B	Ag85B	1:12,800	1:51,200	1:102,400
DNA-64	MPT64	1:200	1:800	1:3200
BCG	PPD	1:800	1:800	1:800
DNA vector	Ag85B+MPT64	0	0	0

*C57BL/6 mice were immunized three times at 3-week interval with 100 μg of plasmid DNA (in 50 μl of saline). Mice were bled 3 weeks after each injection and sera collected from three mice in each group were pooled. Each data point represents the average of 3 measurements and the whole experiment was repeated with similar results (data not shown).

Divalent DNA vaccine resulted in very robust Th1-type cytokine responses

Protective immunity against mycobacterial disease is believed to depend on induction of a Th1-type cellular immune response [19]. In order to assess the effectiveness of our divalent DNA vaccine, we analyzed the cytokine responses in immunized mice using ELISA assay. When stimulated with recombinant antigens Ag85B+MPT64, very robust antigen specific IFN-γ responses were detected in splenocytes of the divalent DNA vaccine treated mice. Animals immunized with BCG or with either of the single DNA vaccine produced about one third or less amount of the IFN-γ as compared with that of the divalent DNA vaccine treated group. Mice vaccinated with the empty DNA vector did not produce significant amount of this cytokine [Fig. 1(A)]. In general, the levels of IL-4 from various groups of mice were two orders of magnitude lower than that of IFN-γ, although the expression patterns were similar [Fig. 1(B)]. BCG also induced a predominant Th1-type response with about 80 times more splenic IFN-γ detected in the splenocytes of BCG vaccinated animals than that of empty DNA vector treated mice. [Fig. 1(A)].

Antigens were expressed differentially in various organs

When antigen-specific primers were used for PCR amplification using total RNA samples prepared from divalent DNA vaccinated mice, Ag85B transcripts were detected in every type of organs used for the experiment. MPT64-specific PCR products were only detected in spleen extracts indicating the possibility that the two

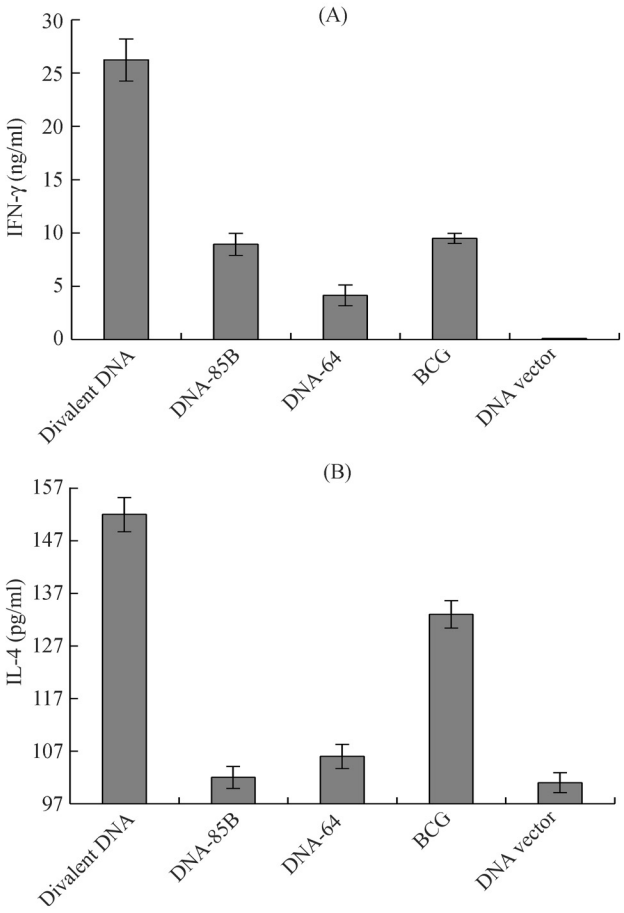

Fig. 1 Antigen-specific IFN-γ (A) and IL-4 (B) responses in spleens of vaccinated mice

Three weeks after the 3rd immunization, spleens were removed aseptically and both IFN-γ and IL-4 concentrations were determined by sandwich ELISA from homogenized spleen tissues collected from 3 mice. In B, y-axis started from the basic value of naive mice (97 pg/ml). Each column represents the mean of triplicate measurements. Error bars indicate the standard errors of the mean.

antigens might accumulated at very different levels in various mouse organs (Fig. 2). In DNA vector treated and non-vaccinated mice, no organs showed detectable expression of the two antigens (data not shown). Mouse β-actin specific primers were included in our PCR reaction as a control measurement for cDNA synthesis from all types of organs indicting that we used equal amount of template RNA samples. No PCR product was detected in the absence of reverse transcriptase (data not shown).

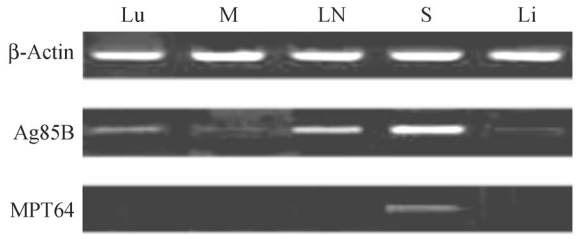

Fig. 2 RT-PCR analysis showing expression of Ag85B and MPT64 RNA in different tissues of divalent DNA immunized mice three weeks after the third immunization

β-actin gene was amplified as RNA quality control. Lu, lung; M, muscle; LN, draining lymph node; S, spleen; Li, liver.

Growth parameters of vaccinated mice challenged with high doses of *M. tuberculosis* H37Rv

The final survival rate was determined 8 weeks after challenging mice with 10^6 CFU *M. tuberculosis* H37Rv. Seven out of nine mice from the divalent DNA treated group survived, while only four out of nine from BCG treated group and two out of nine treated with empty DNA vector survived. Three out of nine mice treated with single DNA vaccine survived (Table 2).

A similar pattern was observed when body, lung and spleen weights from the above five different groups were compared. At the end of our experiments, the average weight of non-treated mice is (27 ± 1.5) g with both lung and spleen weighing less than 2 g. No significant changes were found from divalent DNA vaccine immunized mice compared with non-treated ones suggesting that our vaccination rendered sufficient protection against *M. tuberculosis* in experimental mice (Table 3). A significant loss in total body weight together with higher lung and spleen weight (as a result of organ swelling) was observed from mice treated with the empty DNA vector or single DNA vaccine. In terms of body and organ weight, BCG-treated mice displayed certain degree of protection (Table 3).

Table 2 Survival rate of various vaccinated mice 8 weeks after *M. tuberculosis* challenge

Vaccines	Survived/total
Divalent DNA	7/9
DNA-85B	3/9
DNA-64	3/9
BCG	4/9
DNA vector	2/9

Divalent DNA vaccine resulted in significantly better lung pathology

When lungs from different mice that survived the 8 weeks experimental period were fixed, sectioned and stained with H&E stain for assessment of tissue damage, we found that, in divalent DNA vaccinated mice, the alveolar tissue appeared to be intact with very limited overall lung inflammation. Only mild signs of alveolitis were apparent and small amounts of lymphocytes were observed. No appreciable neutrophil infiltration or necro-

Table 3 Average body, spleen and lung weight of survived mice 8 weeks after challenge with virulent *M. tuberculosis* H37Rv

Vaccines	Average weight of bodies (g)	Average weight of lungs (g)	Average weight of spleens (g)
Divalent DNA	26 ± 1.3*	1.4 ± 0.5**	1.7 ± 0.2*
DNA-85B	20 ± 1.9	2.5 ± 0.3*	2.2 ± 0.3
DNA-64	21 ± 3.3	3.1 ± 0.2	2.3 ± 0.9
BCG	23 ± 1.4*	1.8 ± 0.2*	1.8 ± 0.4*
DNA vector	19 ± 1.7	4.0 ± 0.4	2.6 ± 0.6
No treatment	27 ± 1.5	1.5 ± 0.2	1.7 ± 0.2

**P<0.01, *P<0.05 compared with DNA vector immuinzed mice group.

sis appeared (Fig. 3A&B). Either single DNA group showed more lymphocytes filtrated in whole lung and cellular debris was observed (Fig. 3C&D, Fig. 3E&F). BCG vaccinated mice showed slightly more severe damage in alveolar tissues with relatively large number of lymphocytes and with a small fraction of the lung showing granulomatous lesions (Fig. 3G&H) compared with divalent DNA vaccinated mice lungs. In mice vaccinated with the empty DNA vector, whole lung tissue was consolidated and necrosis was formed. No alvelor tissue was observed indi-

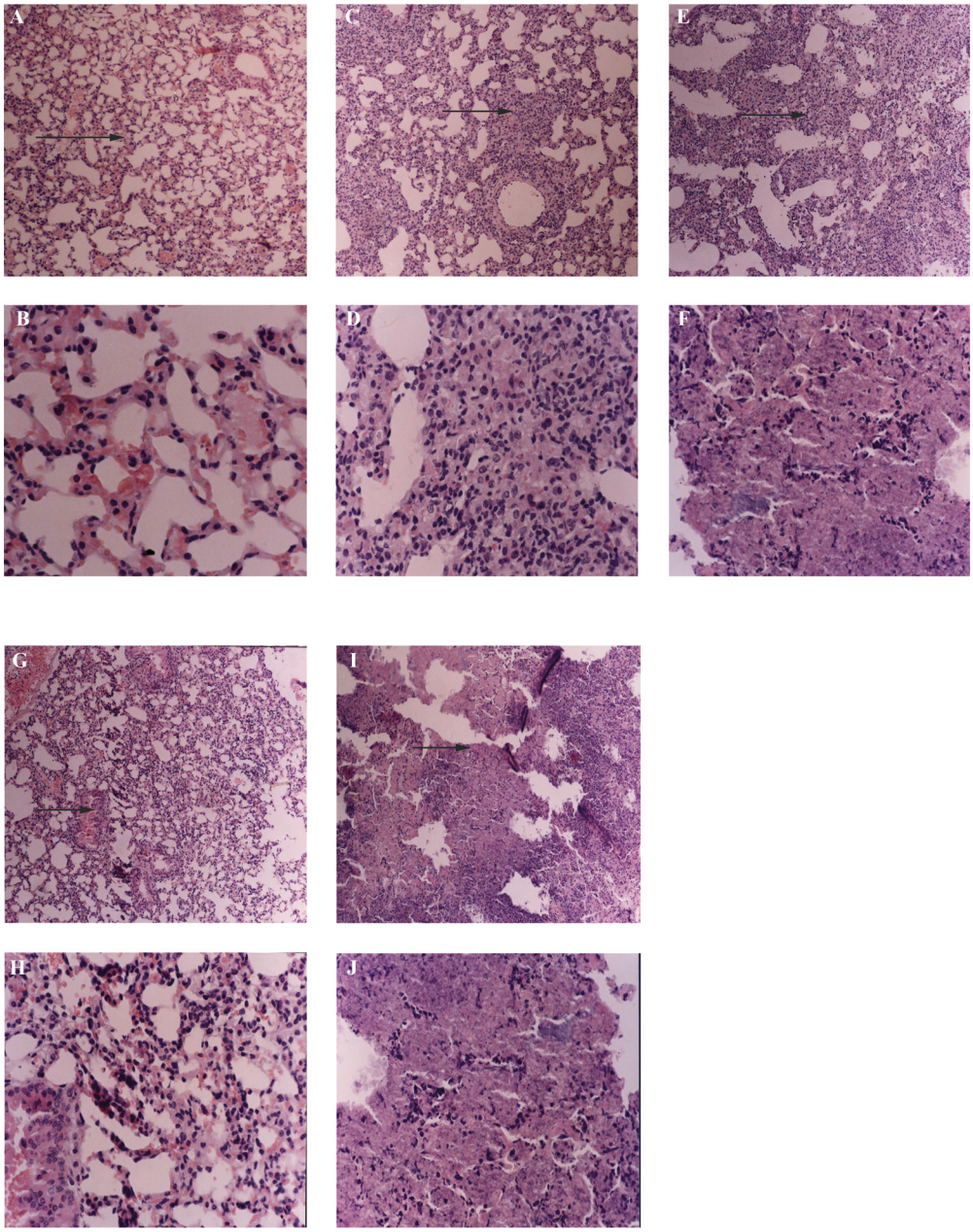

Fig. 3 Representative photomicrographs of lung tissue sections harvested from vaccinated mice 8 weeks after lateral tail vein infection by *M. tuberculosis* H37Rv

The left lobe of the lung was used for fixation before H&E stain. A and B, Lung tissue harvested from divalent DNA vaccinated mice; C and D, Lung tissue from DNA-85B vaccinated mice; E and F, Lung tissue from DNA-64 treated mice; G and H, lung tissue from BCG; I and J, Lung tissue from empty DNA vector. A, C, E, G and I showed large fractions of lungs (magnified ×10). B, D, F, H and J (amplified further from positions of arrows in corresponding sections) showed more detailed cellular structures (magnified ×40).

cating that the functional capability of the lung was severely compromised (Fig. 3I&J).

Bacterial growth is limited in lungs and spleens of experimental mice treated with divalent DNA vaccine

As shown in Fig. 4, the bacterial burden in the lung of mice vaccinated with vector DNA was 1.23×10^8 CFU, similar to that produced using naive mice (data not shown). In contrast, mice vaccinated with the divalent DNA contained two and three orders of magnitudes less bacteria in the spleens and lungs respectively relative to the vector DNA controls. Mice immunized with BCG showed also a marked reduction in lung and spleen bacterial burden when compared with that of the vector DNA controls. Mice treated with DNA-85B and DNA-64 both showed very limited reduction in lung and spleen bacterial burdens.

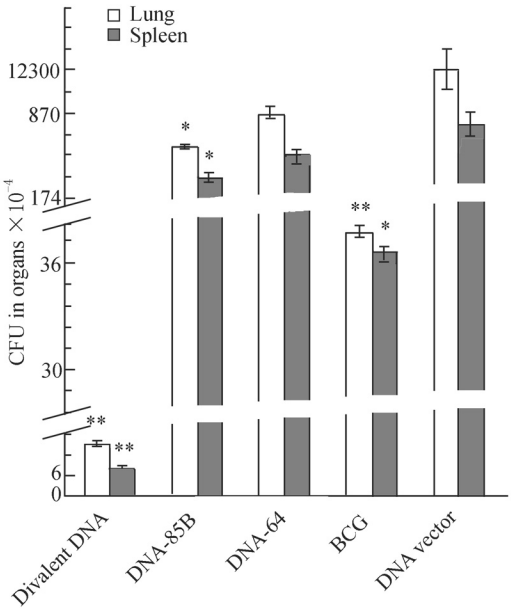

Fig. 4 CFU counts in lungs and spleens from mice vaccinated with different vaccines

**$P<0.01$, *$P<0.05$ compared with DNA vector immuinzed mice group.

Discussion

Antigens Ag85B and MPT64 are major components in culture filtrate of *M. tuberculosis*. The protective efficacy of antigen Ag85B or MPT64 as a monovalent DNA vaccine did not exceed that of live BCG vaccine [11,20]. In fact, a single DNA vaccine developed so far was not superior to that conferred by BCG. Since the report that combinatory DNA vaccines encoding genes of Ag85B and MPT64 were able to induce stronger immune responses than any of the two single DNA vaccines [11], many DNA vaccines were developed using 2, 3, 4 or even 10 different plasmids expressing various antigens [11,12,17,18,20]. Subunit vaccine based on a fusion protein of Ag85B and ESAT-6 was found to induce higher level of protection comparable to that induced by BCG [16]. However, experimental data suggested that only a limited amount of DNA can be taken-up by the immune system so the combinatory effect is likely counteracted by the need for increased DNA dosages [12]. Construction of vaccines that express several antigens in the same plasmid may provide a solution to the problem [9].

In the current work, we find that our divalent DNA vaccine produces high amounts of antigen-specific antibodies together with strong Th1-type cytokine response (Table 1, Fig. 1). The IgG titers of divalent DNA immunized mice increase to very high level only after the second injection and persisted throughout the whole experimental period (Table 1). The amount of IFN-γ was significantly higher from the divalent DNA-treated mice than that of the single DNA vaccine treated mice (Fig. 1). Vaccination with the divalent DNA results also in significantly better lung pathology with much fewer alveolitis, lymphocyte infiltration and granulomatous lesions as compared with that of BCG or empty DNA vector immunized mice (Fig. 3). Accordingly, the CFU counts obtained from spleens and lungs of divalent DNA vaccinated mice are several orders of magnitudes lower than that of the control mice (Fig. 4). In conclusion, vaccination with divalent DNA encoding Ag85B and MPT64 proved to be an efficient method for generating strong and relatively sustained protective immunity to C57BL/6 mice following *M. tuberculosis* H37Rv challenge. Our work may provide new insight in tuberculosis control.

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