Immunotherapy with combined DNA vaccines is an effective treatment for M. bovis infection in cattle

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\begin{abstract}
Mycobacterium bovis causes tuberculosis in cattle. Because current therapies inadequately prevent disease spread, new therapies for M. bovis infections are urgently needed. We treated M. bovis-infected cattle with combinations of DNA vaccines, resulting in significantly reduced pulmonary bacterial loads compared with the untreated group. Further, the pathological changes of lungs were also slight and the lesions were limited in animals treated with the combined DNA vaccines, whereas untreated animals had extensive lung fibrosis and consolidated lesions. This is the first report of DNA vaccine used to treat M. bovis. Thus, combined DNA vaccines may provide enhanced immunotherapeutic efficacy for treatment of bovine tuberculosis.
\end{abstract}

\section{1. Introduction}
Bovine tuberculosis (TB, caused by Mycobacterium bovis) is an infectious disease that affects not only animal health but also the economic value of cattle and cattle-derived food products. Furthermore, this disease is transmissible to other species, including humans. Bovine TB can progress insidiously through a herd of cattle without any obvious early-stage clinical indications [1].

Experimental M. bovis Bacillus Calmette-Guerin (BCG) vaccination studies of cattle over the past decade have shown that the efficacy of this vaccine varies [2]. For example, there is evidence that pre-sensitization of animals with environmental mycobacteria impairs the efficacy of BCG [3]. Because M. bovis is an intracellular pathogen, the relevant antigens are those that preferentially induce the Th1 response believed to be crucial for immune protection [4] in mice and humans [4–8]. DNA vaccination may be particularly suited to achieving such a shift to Th1 response because of its natural features [9,10]. A DNA vaccine can switch the immune response from one that is relatively inefficient (i.e., results in bacterial stasis) to one that kills bacteria [11,12]. Thus, this DNA vaccine – initially designed to prevent infection by M. tuberculosis – also has pronounced therapeutic action in mice [11,12]. Vaccination of mice with plasmid DNA expressing the mycobacterial protein HSP65 following chemotherapy was effective in preventing the reactivation of intravenously transmitted M. tuberculosis [13]. Immunotherapy using a plasmid DNA encoding the mycobacterial 85A antigen or the IL-12N220L DNA vaccine combined with conventional chemotherapy was also highly effective in preventing TB reactivation in mice [14]. The Ag85A DNA vaccine, when administered in conjunction with chemotherapy, also was effective in preventing reactivation of aerogenically transmitted M. tuberculosis [15]. Further, the Ag85B DNA vaccine was immunotherapeutic for mouse TB, whereas the MPT64 DNA vaccine was not [16]. Importantly, the Ag85A DNA vaccine has played a primary role in the treatment of multi-drug-resistant TB [17]. Recently, a combined DNA vaccine encoding the mycobacterial antigens Ag85B, MPT64, and MPT83 along with chemotherapy showed strong potential for preventing TB reactivation in mice [18]. It may be a promising strategy for controlling M. tuberculosis infection.

In a previous study, we demonstrated that cattle vaccinated with a combination of the Ag85B, MPT64 and MPT83 DNA vaccines had improved immune response [19]. In the present study, we compared the BCG vaccine to two DNA vaccine combinations to evaluate whether combined DNA vaccines provide improved therapeutic efficacy against M. bovis infection.

\section{2. Materials and methods}
\subsection{2.1. Animals and bacterial strains}
The M. bovis strain used in this study was obtained from the Beijing TB Therapeutic Tumor Institute. Escherichia coli DH5α and TOP10 strains were used for cloning and were grown on LB medium. For expression of histidine-tagged antigens, the E. coli
BL21 (DE3)/pLysS strain (Invitrogen, Carlsbad, CA, USA) was transformed with pET22b-based expression vectors (Invitrogen). Male calves (6–9 months old) were obtained from a TB-free herd of cattle in Xinjiang, China. Prior to purchase, animals were tested for reactivity to the purified protein derivative (PPD) of bovine tuberculin, and nonresponsive animals were selected. The cattle were housed in an experimental shed in an isolation unit.

2.2. DNA vaccines and recombinant protein preparations

The pJW4303 vector, kindly provided by Dr. J. Mullins of Stanford University, was used to create all DNA vaccine constructs in this study. The coding regions for antigens Ag85B, MPT64, MPT83 and HSP65 were amplified by PCR from M. tuberculosis H37Rv chromosomal DNA using different primers designed based on the corresponding genome sequence database. Primers for amplification of the genes encoding Ag85B, MPT64, MPT83 and HSP65 have been described [13,19]. All antigen-coding regions were individually fused to the tissue-plasminogen activator signal sequence. To facilitate plasmid construction, the genes were amplified with a 5′ primer containing an Nhel restriction site and a 3′ primer designed with a BamHI site. Each PCR product was initially cloned into the Top10 strains and then into the eukaryotic expression vector pJW4303. All DNA constructs were verified by commercial DNA sequencing (Invitrogen). DNA for immunization was purified using the Mega plasmid DNA kit (Qiagen, Chatsworth, CA) and was diluted in saline solution to a final concentration of 1–2 μg/μl. Histidine-tagged antigens were expressed and purified as described [19].

2.3. Treatment of an established infection via immunotherapy with DNA vaccines

Twenty-four TB-free calves (i.e., non-immunoreactive for Ag85B, MPT64, MPT83 and HSP65) were divided into four groups (six calves per group) for vaccination studies. All calves were subjected to intratracheal pathogen challenge using 1 × 10⁷ CFU of virulent M. bovis. The first group remained untreated for the duration of the study. The second group received 1 × 10⁶ CFU of BCG vaccine at 16 and 22 weeks post-infection (BCG group). The third group was subjected to intramuscular vaccination using 1500 μg of a combined DNA vaccine (500 μg each of plasmids encoding Ag85B, MPT64 and MPT83, dissolved in saline) at 16, 18, 20 and 22 weeks post-infection. The fourth group was subjected to intramuscular vaccination using 1500 μg of a different combined DNA vaccine (500 μg each of plasmids encoding Ag85B, MPT64 and HSP65, dissolved in saline) at 16, 18, 20 and 22 weeks post-infection. For bacterial culture, tissue samples were homogenised in a Tenbroeck grinder (Wheaton, Millville, NJ), decontaminated in 0.75% cetylpyridium chloride for 1 h, centrifuged at 3500 × g at 3500 × g for 20 min, and processed for isolation of mycobacteria. Serial ten-fold dilutions of tissue homogenates prepared from the lung were plated on Lowenstein-Jensen medium. Bacterial counts in the lungs were monitored at 16 weeks post-infection (one calf per group) and 34 weeks post-infection (five calves per group) to analyse the clearance of cultivable organisms as described [19] (Fig. 1).

2.4. PPD test

To verify that animals selected for the study were TB-free, animals were inoculated with 0.1 mg bovine PPD (PPD-bovine, China Institute of Veterinary Drug Control, Beijing, China) in the right side of the neck before infection. Skin thickness at the site of injection was determined before and 72 h after inoculation. As a standard interpretation of the test, positive results were recorded when the increase in skin thickness to bovine PPD was ≥4 mm.

2.5. Interferon-γ assay

Whole-blood samples were taken at 0, 20, 22, 30 and 34 weeks post-infection with virulent M. bovis, and whole blood culture assays were performed in 96-well plates (0.2 ml/well) by mixing 0.1 ml heparinised blood, collected from the jugular vein of each animal, with an equal volume of the purified Ag85B, MPT64, MPT83 and HSP65 antigens (final protein concentration 10 μg/ml, 2.5 μg/ml of each; groups 3 and 4). For samples from group 1 and 2 animals, 20 μg/ml bovine PPD and a mixture of antigens were used. An aliquot of blood incubated without antigen served as the non-stimulated negative control. Supernatants were harvested after 24 h of culture at 37 °C in a humidified 5% CO₂ incubator, and levels of interferon-γ (IFN-γ) were determined using the BOVIgam™ Bovine γ-Interferon Test (CSL, Victoria, Australia). Results are expressed as optical density indices calculated by dividing the OD₄₅₀ obtained from cultures stimulated with antigen by the OD₄₅₀ obtained from cultures without antigen.

2.6. Isolation and FACS analysis of T cell sub-populations

At 30 weeks post-infection (8 weeks after the completion of four immunotherapy treatments), peripheral blood mononuclear cells (PBMC) were obtained by Histopaque-1077 (Sigma, Poole, UK) gradient centrifugation from heparinised blood in each group. After incubating the PBMCs for 24 h at 37 °C under 5% CO₂ without control or with the mixture of antigens (at a final concentration of 5 μg/ml of each antigen for groups 1, 3, and 4, or 20 μg/ml PPD for the BCG group), PBMCs were then washed with ice-cold PBS, and the cells (1 × 10⁶ per labelling) were incubated for 30 min at room temperature with anti-bovine CD4-FITC (CC8) and anti-bovine CD8-PE (CC58) (5 μg/ml each) or with anti-WC1 (CC15) (Serotec Ltd., Oxford, UK). Cells were then washed twice with PBS and resuspended in PBS containing 4% paraformaldehyde at 4 °C. Samples were subjected to FACS analysis using a FACSscan calibrator (BD Corp, San Jose, CA, USA). The typical forward and side scatter lymphocyte gate together with green and red fluorescence gates were set to exclude dead cells and contaminating monocytes-macrophages from the analysis.

2.7. Antigen-specific antibody determination by enzyme-linked immunosorbent assay

Polystyrene 96-well plates (Nunclon, Denmark) were coated with 100 μl (10 μg/ml) of the three antigens separately in 0.1 M carbonate/bicarbonate buffer (pH 9.6) overnight at 4 °C. The plates were then washed five times with a solution of 0.05% Tween 20 in PBS saturated with skim milk protein for 2 h at 37 °C. Serum samples (100 μl per well) were diluted in a two-fold series with
1:100 PBS-Tween 20 buffer containing 0.5 M NaCl (PTN) and were then added to each of duplicate wells. Each plate was incubated at 37 °C for 2 h with continuous shaking and washed as described [19]. Horseradish peroxidase–conjugated sheep anti-bovine IgG or IgG2 or IgG1 (1:5000–1:10000 or 1:1000–1:2000) (Serotec Ltd., Oxford, UK) in PTN were added separately to each well and incubated at 37 °C for 2 h. After adding substrate solution (Chemicon, Harrow, UK), plates were incubated at 37 °C for another 10 min. The reaction was then stopped by addition of 50 μl of 2 M H₂SO₄, and colour development was measured as the OD₄₅₀ using a microplate reader (Bio-Rad, Model 550, Japan). The results are expressed as an optical density index (ODI), i.e., the ratio between OD₄₅₀ of a test sample and OD₄₅₀ of a day 0 animal.

2.8. Necropsy procedure

Calves were subjected to extensive postmortem examination at 16 and 34 weeks post-infection (0 and 12 weeks after the completion of the vaccination regimen). The lungs were initially palpated to detect nodules, and lesions were confirmed as TB-positive by histopathological examination. Samples were collected from any lesions observed in organs. Tissue samples for histopathological analyses were fixed in neutralization buffer containing 10% formalin, embedded in paraffin, sectioned (6 μm per section) and stained with haematoxylin and eosin. All tissue samples were coded and evaluated by a single board-certified pathologist who had no knowledge of animal treatment.

2.9. Statistical analyses

Bacterial count data were analysed by analysis of variance using log₁₀-transformed data. Intradermal tuberculin data and IFN-γ data were analysed by the Student’s t-test. A value of P < 0.05 was considered statistically significant. The proportions of cattle with lung lesions in the different treatment groups were compared using Fisher’s exact test.

3. Results

3.1. Combined DNA vaccines induce stronger antigen-specific IFN-γ production in whole bovine blood compared with BCG-treated calves

T cell responses in infected and uninfected animals were measured as the release of IFN-γ from whole blood stimulated with the Ag85B, MPT64, MPT83, or HSP65 antigens individually (for in the combined DNA vaccine) or with PPD (for BCG-vaccinated animals) or with a mixture of all four antigens (for untreated animals) at 0, 22, 30, 34 weeks after infection. The production of IFN-γ from calves receiving therapy with the combined DNA vaccines encoding Ag85B, MPT64 combined with MPT83 or HSP65, or Ag85B, MPT64, or HSP65 antigens individually (for in the combined DNA vaccine) or with PPD (for BCG-vaccinated animals) or with a mixture of all four antigens (for untreated animals) at 0, 22, 30, 34 weeks after infection. The production of IFN-γ from calves receiving therapy with the combined DNA vaccines encoding Ag85B, MPT64 combined with MPT83 or HSP65 (groups 3 and 4, respectively) was significantly improved at 22 weeks post-infection (upon completion of the vaccination schedule) (P < 0.05). Furthermore, IFN-γ production in groups 3 and 4 was significantly higher than that of the untreated group at 30 and 34 weeks post-infection (8 and 12 weeks after the completion of the vaccination schedule, respectively) (P < 0.05 or P < 0.01 or P < 0.001). IFN-γ production from calves receiving therapy with BCG was lower than that from those receiving the two combined DNA vaccines at the same time points (Fig. 2).

3.2. Combined DNA vaccines produce stronger specific CD4+, CD8+ or WC1γδ T cell responses

PBMCs isolated from blood samples from animals stimulated with various antigens were used to determine the T cell type responses to immunotherapy at 30 weeks post-infection (8 weeks after the completion of the vaccination schedule). The mean percentage of CD4+ and CD8+ T cells from calves that had received therapy with either of the combined DNA vaccines was higher in PBMC cultures re-stimulated with the mixture of specific antigens compared to the untreated group (Fig. 3). In particular, the mean percentage of CD4+ and CD8+ T cells in calves treated with the combined DNA vaccine encoding Ag85B, MPT64 and MPT83 was ~1.5-fold higher than the untreated group. The mean percentage of WC1γδ T cells from calves that had received therapy with either of the combined DNA vaccines was higher in PBMC cultures re-stimulated with the mixture of specific antigens compared to the untreated group. The mean percentage of WC1γδ T cells from calves that had received BCG therapy was higher in PBMCs re-stimulated with PPD compared to the untreated group, but lower than that from calves treated with either of the combined DNA vaccines (Fig. 3).

Fig. 2. IFN-γ concentrations in supernatants from calf whole blood cultures following M. bovis infection. Whole-blood samples from the four treatment groups (see Fig. 1) were obtained at 0, 22, 30 and 34 weeks following M. bovis infection, mixed with the indicated antigen(s), and subjected to IFN-γ analysis. Data represent mean optical density indices (ODI) ± SE. The ODI reflects the OD₄₅₀ ratios of antigen-stimulated cultures to unstimulated cultures. Ag85B, MPT64 combined with MPT83 or HSP65 were used as antigens for group 3 and 4, respectively. PPD and the mixture of all antigens were used as antigens for the BCG and untreated group, respectively. The values of *P < 0.05 or **P < 0.01 were considered to be significant.
3.3. Combined DNA vaccines induce strong antibody responses characterised by the IgG2 isotype after treatment

Antibody titers for antigens Ag85B, MPT64 and MPT83, or Ag85B, MPT64 and HSP65, were determined in sera harvested from treated calves at 30 weeks post-infection by enzyme-linked immunosorbent assay. Higher levels of antigen-specific antibodies were observed for the combined DNA vaccine groups compared with the BCG and untreated groups. The levels of specific antibodies response to Ag85B, MPT64 and MPT83 were detected at 1:3200 dilution in treated calves. Antibody titers for Ag85B and MPT83 were 4- to 8-fold higher for the combined DNA vaccine groups compared with the BCG group. In addition, the calves in the untreated group elicited low humoral responses to PPD. A strong humoral response with a prevalent IgG1 isotype is generally associated with a Th2-type immune response, whereas induction of an IgG2 isotype indicates a Th1-oriented immune response. In our study, the combined DNA vaccines induced a strong humoral response characterised by the IgG2 isotype, indicating a Th1-type immune response. The BCG vaccine, however, induced a strong humoral response that was polarised towards the IgG1 isotype, thus indicating a Th2-type immune response (Table 1).

3.4. Bacterial loads in lungs are reduced in calves treated with combined DNA vaccines

At 16 weeks and 34 weeks post-infection (0 and 12 weeks after completion of the vaccination schedule, respectively), lung lesions from calves were harvested and homogenised. Bacterial loads were counted by plating serial dilutions of the homogenates onto Lowenstein-Jensen agar plates. At 16 weeks post-infection, the mean bacterial counts from lung lesions of all four groups were similar (3.21–3.86 log10 CFU/g of tissue; Fig. 4). At 34 weeks post-infection, however, the mean bacterial counts from lung lesions of calves from the two combined DNA vaccine groups were significantly reduced compared to counts for the untreated group (P < 0.05 or P < 0.01). Furthermore, lesions from the combined DNA vaccine groups displayed mean bacterial counts more than 10-fold lower than those from the BCG group. There was no significant difference in mean bacterial counts in lesions from animals treated with either combined DNA vaccine (Fig. 4).

3.5. Combined DNA vaccines reduce lung pathology

Gross pathological observation and histopathological examination of cattle lungs following sacrifice indicated that animals in the immunotherapy groups had higher resistance to M. bovis compared with the control group (Fig. 5). We observed that some of the lung parenchyma was compromised with fibrosis, presenting a cellular infiltrate containing mainly lymphocytes with a few macrophages, at 16 weeks post-infection in the calves treated with either of the combined DNA vaccines (Fig. 5A and D). However, lungs from animals in these two groups were less compromised by infiltration of lymphocytes and epithelioid macrophages by 34 weeks post-infection (12 weeks after the completion of four times immunotherapy) (Fig. 5B, C, E and F). Importantly, lungs from untreated or BCG-vaccinated calves exhibited similar histopathology, being substantially compromised with numerous lesions (data not shown).

Table 1
A specific antibody production of IgG, IgG1 and IgG2 in calves treated with the combined DNA vaccines or BCG at 30 weeks post-infection (8 weeks after the completion of four times immunotherapy).

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen(s)</th>
<th>IgG2</th>
<th>IgG1</th>
<th>IgG2/IgG1</th>
<th>Antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined DNA vaccine</td>
<td>Ag85B</td>
<td>1:800</td>
<td>1:400</td>
<td>2</td>
<td>1:3200</td>
</tr>
<tr>
<td></td>
<td>MPT64</td>
<td>1:800</td>
<td>1:200</td>
<td>4</td>
<td>1:3200</td>
</tr>
<tr>
<td></td>
<td>MPT83</td>
<td>1:400</td>
<td>1:200</td>
<td>2</td>
<td>1:3200</td>
</tr>
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<td>Combined DNA vaccine</td>
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<tr>
<td></td>
<td>MPT64</td>
<td>1:400</td>
<td>1:100</td>
<td>4</td>
<td>1:3200</td>
</tr>
<tr>
<td></td>
<td>HSP65</td>
<td>1:400</td>
<td>1:200</td>
<td>2</td>
<td>1:1600</td>
</tr>
<tr>
<td>BCG</td>
<td>PPD</td>
<td>1:200</td>
<td>1:400</td>
<td>0.5</td>
<td>1:400</td>
</tr>
<tr>
<td>Untreated group</td>
<td>PPD</td>
<td>1:100</td>
<td>1:400</td>
<td>0.25</td>
<td>1:400</td>
</tr>
</tbody>
</table>

Mixture sera from six calves from each group was analysed 8 weeks after post-infection for antigen-specific antibody production of IgG, IgG1, and IgG2. The three antigens or bovine PPD elicited the same antibody titers in two independent experiments.
4. Discussion

Therapeutic effects of DNA vaccination against *M. tuberculosis* have been reported [12–18], and thus DNA vaccination may provide an alternate immunotherapeutic regimen to treat TB. The Th1 response, which is dominated by antigen-specific T lymphocytes that produce IFN-γ and is cytotoxic towards infected cells, is reportedly required for maximally effective anti-mycobacterial immunity in mice and humans [20]. Because the Th2 response is abundant during infection with *M. tuberculosis* [21,22], a shift in the balance towards the Th1 response may therefore be required for immunity towards mycobacteria. DNA vaccines can achieve such a shift particularly because of the 'adjuvant' effect [23]. Our results indicate that calves treated with combined DNA vaccines elicited a strong humoral response characterised by the IgG2 isotype, thus indicating a Th1-type immune response; notably, the antigen-specific IgG2 to IgG1 ratio increased by 2- to 4-fold, whereas the ratio did not exceed 1.0 after BCG vaccination (Table 1). Significantly enhanced IFN-γ production was also observed for combined DNA-vaccinated calves compared with untreated calves at 30 and 34 weeks post-infection (8 and 12 weeks after completion of the vaccination schedule) (Fig. 2). Thus, it is possible that DNA vaccines encoding *M. tuberculosis* antigens can boost the population of existing antigen-specific T cells during treatment and stimulate production of new antigen-specific T cells. These T cells would then promote an efficient Th1 immune response, including IFN-γ production, and eventually inhibit the growth of *M. bovis*.

Antigen-specific CD4+ and CD8+ T cells contribute to the formation of TB granulomas and to the arrest of mycobacterial growth mainly due to the Th1-type response. It is well documented that IFN-γ production by CD4+ T cells is essential for the protective response and that IFN-γ activates macrophages by enhancing their ability to kill bacteria [24]. However, the CD8+ T cell response is also necessary for immunity; β2-microglobulin-deficient mice, which lack functional CD8+ T cells, are highly susceptible to *M. tuberculosis* [24,25]. Our data demonstrated a higher mean percentage of CD4+ and CD8+ T cells among cattle treated with either of the combined DNA vaccine regimens compared to untreated cattle (Fig. 3). There is strong evidence from animal and human studies that CD4+ and CD8+ T cells are necessary for protective immunity against *Mycobacterium* infections. It is likely that CD8+ T cells also play an important role in cell-mediated immune responses in cattle, particularly because γδ T cells constitute a large proportion of lymphocytes in the peripheral circulation of ruminants [26].

In vivo depletion of WC1γδ T cells in *M. bovis*-challenged cattle suggested a role for WC1γδ T cells in the Th1 bias of developing immunity towards mycobacteria, possibly via innate production of IFN-γ [26,27]. We observed a significantly higher mean percentage of WC1γδ T cells among cattle treated with either of the combined DNA vaccine regimens compared to untreated cattle (Fig. 3). Together, these data provide evidence for the involvement of WC1γδ T cells in the immune response to *M. bovis* infection. Importantly, both of the combined DNA vaccine regimens used in this study effectively suppressed *M. bovis* infection, as indicated by a 10- to 100-fold decrease in bacterial loads from lungs of treated cattle compared to untreated cattle by 34 weeks post-infection.
(12 weeks after completion of the vaccination schedule). In summary, our study provides strong evidence that immunotherapies comprised of combined DNA vaccines increase the efficacy of the immune response against *M. bovis* infections in cattle.

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**References**


