



## Research Article

# Therapeutic Potential of a Candidate *relA* Deletion Mutant Vaccine of *Mycobacterium Avium* Subsp. *Paratuberculosis*

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### ABSTRACT

Analysis of the immune response to a mutant of *Mycobacterium avium* subsp. *paratuberculosis* with a deletion of *relA* (*MapΔrelA*) revealed that deletion of *relA* interrupts the pathways used by *Map* to subvert the immune response in goats and cattle to establish a persistent infection. The immune response elicited by *MapΔrelA* cleared infection and limited the capacity of wild type *Map* to establish an infection. The findings indicate the mutant should be useful as a vaccine under field conditions. The present study was conducted to explore the therapeutic potential of *MapΔrelA* using a cow with late stage paratuberculosis. The cow was tested negative using *Map* skin test and peripheral blood mononuclear cells were unresponsive to antigen stimulation *ex vivo*. A strong CD4 and CD8 proliferative response was detected one week post vaccination with *MapΔrelA*. Analysis of the cytokine profile for four weeks post vaccination revealed a mixed response with up-regulation of pro-inflammatory and inhibitory cytokine gene expression. The findings indicate further testing of the mutant as a candidate vaccine should include examining analysis of the cellular events associated with restoring a protective immune response as well as its therapeutic potential.

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### INTRODUCTION

Various approaches have been taken to develop a vaccine to *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) that induces sterile immunity against Johne's disease (JD) or prevents disease progression. These approaches have included development of killed whole vaccines, live attenuated, DNA and subunit vaccines and tests in different animal species (Bull et al. 2007; Park et al. 2008; Juste et al. 2009; Kathaperumal et al. 2009; Scandurra et al. 2010; Bastida and Juste 2011; Stabel et al. 2012; Faisal et al. 2013). Although none of the vaccines induced sterile immunity they did slow disease progression and demonstrated that development of a vaccine that induces sterile immunity is feasible (reviewed in (Rosseels and Huygen 2008)). Of interest, the studies also revealed the current vaccines can be used therapeutically to reverse immunopathology (Bull et al. 2007; Juste et al. 2009; Singh et al. 2010), an observation first observed by Koch (reviewed in (Burke 1993)). If animals are not too far advanced in clinical disease, the effect can be dramatic with a reversal of pathology (Singh et al. 2010). Unfortunately, the effect is transient as discovered by Koch (Burke 1993) and noted by Singh et al. (personal communication). The heightened immune response wanes allowing clinical disease to recur. Further studies are needed to determine what aspect of the immune response to *Map* accounts for the therapeutic efficacy and identify a method

that increases the longevity of the therapeutic effect. One approach is to use an attenuated vaccine that minimizes some of the disadvantages associated with the use of killed vaccines. We have developed a deletion mutant that provides an opportunity to explore the therapeutic potential of an attenuated candidate live vaccine. Studies in calves and goats showed deletion of *relA*, a global regulator, interrupts the pathways used by *Map* to establish a persistent infection without reducing its immunogenicity. The mutant, *MapΔrelA*, elicits an immune response that clears infection and limits colonization of bacteria in challenge experiments (Park et al. 2011). The immune response is clearly different than the response elicited by wild type *Map*. The basis for this difference remains to be elucidated. The findings in the present study show the mutant offers an opportunity to characterize the components of the immune response associated with the therapeutic effect of vaccinating animals at the clinical stage of infection.

### MATERIALS AND METHODS

#### *Animals*

A cow infected with *Map* that was scheduled for disposal was obtained from a local dairy. The cow had lost weight and had continuous bouts of diarrhea and shedding bacteria. Clinical evaluation revealed some edema in the jaw and

brisket (Figure 1). Analysis of the immune status showed that the cow was ELISA positive, skin test negative and unresponsive to antigen stimulation *ex vivo* as detected by flow cytometric (FC) analysis. The cow collapsed at 6 weeks after starting the experiment and was euthanized. Necropsy revealed the intestine had deteriorated.



Figure 1: Clinical JD cow used in this study

### Experimental Design

The cow was vaccinated with  $10^9$  of *Map* $\Delta$ *relA* (in 0.5 ml PBS) on the left side of neck behind ear by intra dermal injection. Blood was collected through the jugular vein at day 0 and 1, 2, 3, and 4 wk post vaccination (PV). Feces were collected on the same schedule as blood collection. The blood and feces were used as described below.

All animal treatments and experimental procedures were approved by the Washington State University Institutional Animal Care and Use Committee, USA.

### Detection of *Map* DNA from Blood Samples by Real Time PCR

Ten milliliter of blood was mixed with 40 ml of red blood cell (RBC) lysis buffer (0.87 % Ammonium Chloride, 0.012 % Tris–Base, pH 7.3) in a 50 ml tube, and incubated at 37° C for 15 min. After RBC lysis, the leukocytes were pelleted by centrifugation at 600 xg for 10 min. The pellet was washed with 50 ml of phosphate buffered saline (PBS, pH 7.3), re-suspended in 1 ml of PBS, and transferred in to a 2 ml screw cap tube. Following centrifugation DNA was extracted with DNeasy Blood & Tissue kit with mechanical disruption as previously described (Park et al. 2011). The DNA extraction was done in duplicate at each bleeding time. A real time PCR (RT–PCR) method targeting IS900 sequence described by Irengue et al. (Irengue et al. 2009) was used to detect *Map* DNA from blood samples. Each DNA sample was run in duplicate.

### Quantification of Bacterial Shedding in Feces by RT–PCR

The feces collected at each time point were processed for DNA extraction as previously described (Park et al. 2011). RT–PCR targeting *Map*–specific single copy gene, F57, was used for a correct quantification (Schonenbrucher et al. 2008). DNA extraction was done in duplicate, and the RT–PCR for each DNA sample was conducted in duplicate. A standard curve was generated using *Map* gDNA with 10 fold

serial dilutions, and used for calculating the number of *Map* in the samples.

### Peripheral Blood Mononuclear Cell Isolation and Stimulation with *Map*

Peripheral blood mononuclear cells (PBMC) from blood were isolated and stimulated with live *Map* in 6 well plates for 6 days as previously described (Allen et al. 2009; Park et al. 2011). Total RNA from PBMC was collected on day 3 for analysis of cytokine expression. Live PBMC were collected on day 6 for FC analysis.

### Flow Cytometric Analysis

The same gating strategy, as used in previous studies, was used to analyze the CD4 and CD8 T cell proliferative response to live *Map* stimulation (Allen et al. 2009; Park et al. 2011). In brief, fresh PBMC (time 0) and stimulated PBMC with/without live *Map* (day 6) were labeled with three color combinations of monoclonal antibodies, CD4 (ILA11A, IgG2a), CD45R0 (ILA116A, IgG3), and CD25 (CACT116A, IgG1), or CD8 (7C2B, IgG2a), CD45R0, CD25 (Washington State University Monoclonal Antibody Center, Pullman, WA, USA). As shown in Figure 2, three electronic gates were used to analyze the data. The first two gates were used in side (SSC) and forward (FSC) light scatter to identify and color code resting (G1, non-proliferating, orange) cells and activated (G2, proliferating, blue) cells (Figures. 2A, 2D, 2G). Cells in G2, at T0, included monocytes and large lymphocytes. At day 6, G2 contained activated proliferating T cells. The third gate was placed on CD4 or CD8 positive cells to electronically isolate the cells for analysis. The results are displayed in FSC vs CD45R0 to show the relative frequency of naïve cells (resting cells, orange), and activated (blue) memory CD4 (Figure 2B, 2E, 2H) and CD8 (Figure 2C, 2F, 2I) cells. A FACSort flow cytometer and Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) were used to collect data. FCS Express software ver. 3 (De Novo software, Los Angeles, CA) was used to analyze the data.

### Analysis of Cytokine Expression Profile

Quantitative reverse–transcription RT–PCR (qRT–PCR) was used to compare the relative cytokine transcription of PBMC stimulated with live *Map* at different time points. Total RNA was extracted from PBMC stimulated with live *Map* using TRIzol (Life Technologies, CA). The RNA was treated with Turbo DNA–free kit (Applied Biosystems, TX) to remove genomic DNA contamination. A total of 1 µg of RNA was reverse transcribed to cDNA using High–Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) following the manufacturer's instructions. After dilution (1/20), 5 µl of cDNA was used for each qRT–PCR in total of 20 µl reaction volume. qRT–PCR was performed using StepOnePlus Real–Time PCR System machine (Applied Biosystems, CA). The qRT–PCR conditions and primer information were described previously (Park et al. 2011). The data from each sample were normalized to the control sample (collected before vaccination) using two housekeeping genes ( $\beta$ –actin and GAPDH) to calculate the relative quantification (RQ) of

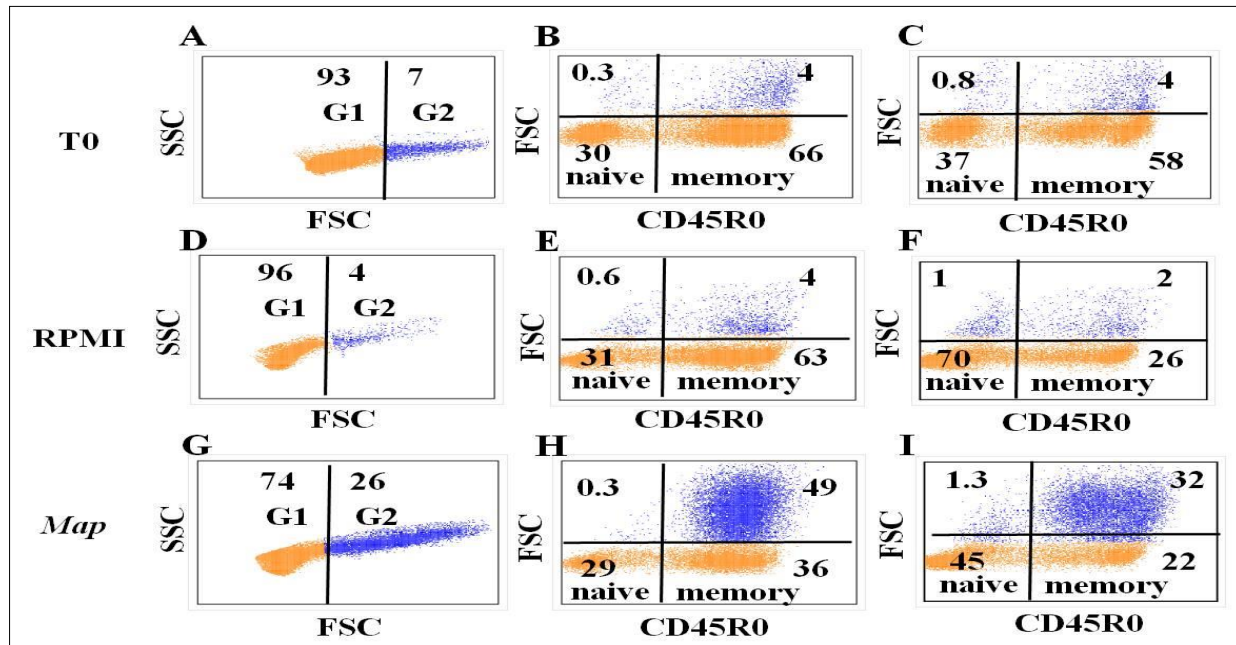


Figure 2: Flow cytometric analysis of PBMC in response to live *Map* stimulation; Data represent the result of FC at 1 wk PV; PBMC were collected at time 0 (A, B, C) or after 6 day culture in RPMI alone (D, E, F) or with live *Map* stimulation (G, H, I); Electronic gates 1 and 2 were placed on small unactivated and large activated cells based on SSC vs FSC and color coded to identify T cells proliferating in response to antigen stimulation (A, D, G); An additional electronic gate was used to isolate CD4 or CD8 T cells to determine the frequency of activated memory CD4 and CD8 memory T cells proliferating in response to live *Map* stimulation; The results are displayed in FSC vs CD45R0 to show the relative frequency of naïve cells (resting cells, orange), and activated (blue) memory CD4 (B, E, H) and CD8 (C, F, I) cells; No naïve CD4 or CD8 cells expressed CD25, while all activated memory CD4 and CD8 cells expressed CD25 (data not shown)

each cytokine gene transcription at different time points (Park et al. 2011).

## RESULTS

### Effect of Vaccination on Blood Circulation and Fecal Shedding of *Map*

Blood was screened before and after vaccination for the presence of *Map* by a RT-PCR targeting IS900 sequence due to its high sensitivity compared to a single copy gene (Li et al. 2005; Irengue et al. 2009). *Map* DNA was detected in blood collected before and at 2 wk PV. *Map* DNA was below detectable level at 1, 3 and 4 wk PV. For a correct quantification of bacterial shedding, we used a RT-PCR targeting a specific single copy gene of *Map*, F57, as described by Schonenbrucher et al. (Schonenbrucher et al. 2008). The F57 RT-PCR was less affected by PCR inhibitors present in the DNA samples compared to IS900 RT-PCR (Park et al. 2014). A large number of bacteria were detected in feces before vaccination ( $1.2 \times 10^8$  *Map* / RT-PCR reaction). Bacterial shedding was reduced after vaccination until 2 wk PV (more than 1 log scale reduction). *Map* shedding was increased slightly at 3 wk PV, but decreased again at 4 wk PV (Figure 3).

### Flow Cytometric Analysis of PBMC Stimulated with Live *Map*

PBMC stimulation with live *Map* failed to stimulate PBMC proliferation before vaccination. We tentatively concluded the immune response was anergic and vaccinated the cow to

determine if anergy could be reversed. A strong CD4 and CD8 response was detected at one week PV (Figure 2). Proliferation was only observed in the cultures with live *Map* (Figure 2D and 2G). The specific memory response of CD4 and CD8 T cells to live *Map* was clearly observed as shown in the panels of CD4 (Figure 2E and 2H) and CD8 (Figure 2F and 2I) gated cells. The proliferating cells expressed CD45R0 and CD25 (data not shown). The proliferative response was similar at 2 and 3 wk PV. Flow cytometry was not conducted at 4 wk PV.

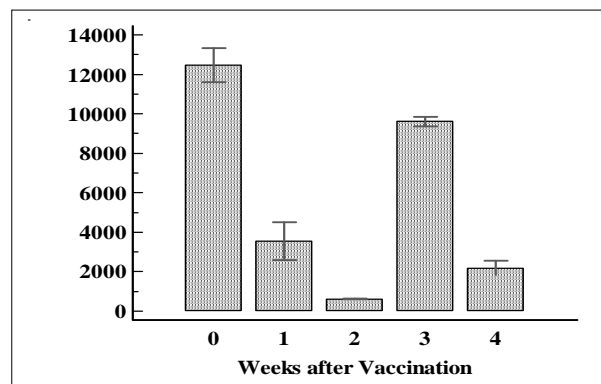


Figure 3: Quantification of *Map* shedding in feces after vaccination. DNA was extracted from feces and *Map* gDNA was quantified by F57 RT-PCR. Data are presented as the average gDNA copy number per RT-PCR reaction

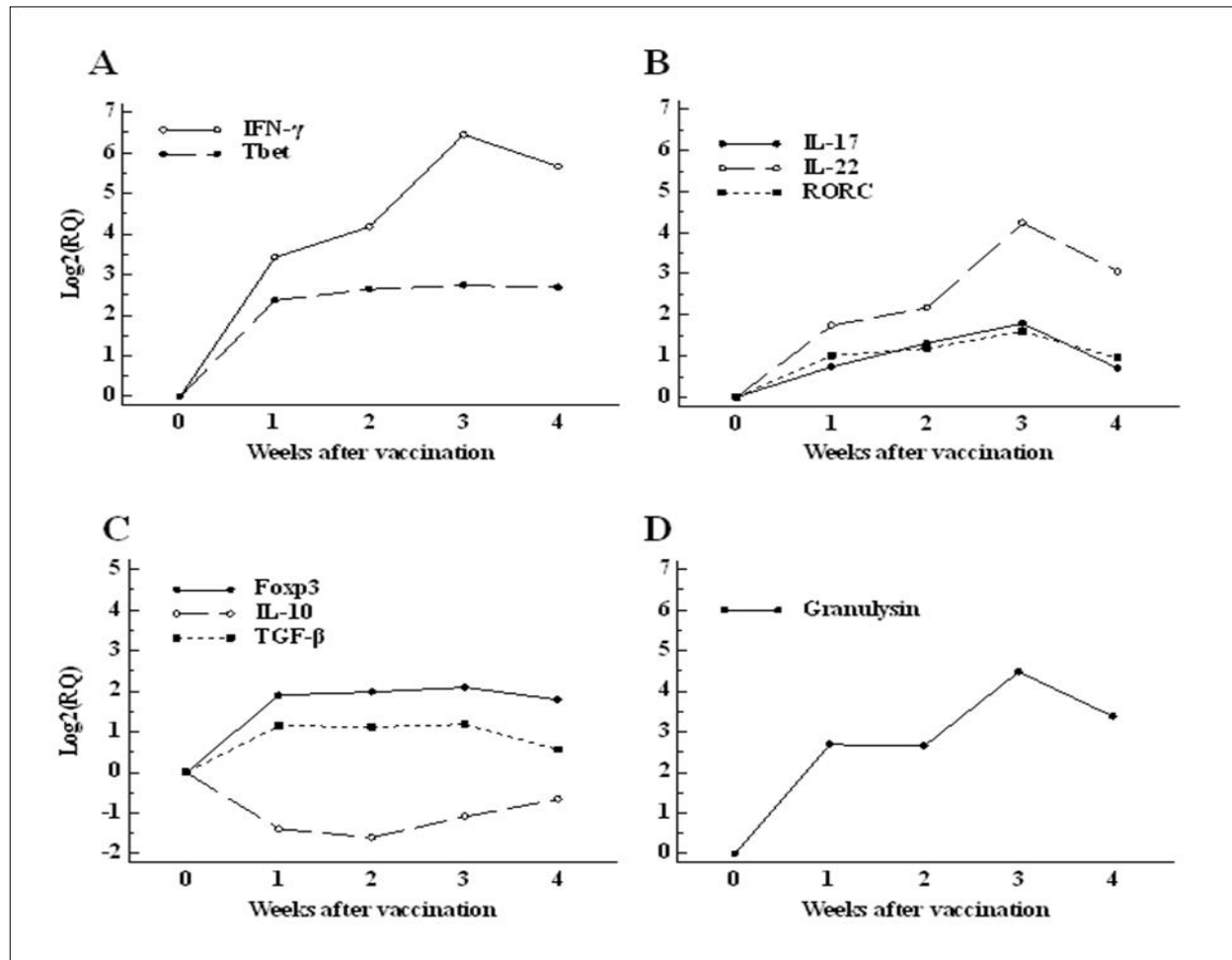


Figure 4: Relative changes of cytokine transcription in PBMC in response to live *Map* stimulation after vaccination; PBMC were collected before and after vaccination and at 1 wk intervals and stimulated with live *Map* for 3 days; qRT-PCR was used to measure the relative expression of cytokine genes; The relative changes of cytokine expression were calculated using the value at before vaccination as the calibrator with two housekeeping genes ( $\beta$ -actin and GAPDH); Data are presented with grouping based on the type of immune response; Th1 (IFN- $\gamma$  and Tbet, A), Th17 (IL-17, IL-22, and RORC, B), Treg (IL-10, TGF- $\beta$ , and Foxp3, C), and granulysin (D); RQ, relative quantification

#### Cytokine Transcription in PBMC Stimulated with Live *Map*

The relative changes in cytokine expression of PBMC in response to live *Map* stimulation were compared before and after vaccination. The important signature cytokines for host immune response to *Map*, such as Th1, Th17, and regulatory T cell (Treg) type cytokines, and a mycobactericidal peptide (granulysin) (Dieli et al. 2001; Gansert et al. 2003), were compared to evaluate the effect of therapeutic vaccination.

The most striking increase in cytokine gene expression after vaccination was IFN- $\gamma$ . The transcription of IFN- $\gamma$  gradually increased until 3 wk PV (fold change: 86.4), and then slightly decreased at 4 wk PV (fold change: 50.5). The transcription of Tbet, a transcription factor regulating development of Th1 responses (Zhu et al. 2012), was simultaneously elevated at 1 wk PV (fold change: 5.2) and the level was maintained until 4 wk PV (fold change: 6.4) (Figure 4A). The changes of transcription of Th17 type

cytokines (IL-17, IL-22) were lower than Th1 type cytokines. The transcription of IL-17, secreted by Th17 cells, and RORC, a transcription factor for Th17 cells (Ivanov et al. 2006), were just slightly elevated after vaccination (1.6 to 3.4 times for IL-17, and 1.9 to 3.0 times for RORC, respectively). However, the effect of vaccination on inducing another Th17 cytokine, IL-22, was clearly evident. The pattern of transcriptional change of IL-22 was similar to that of IFN- $\gamma$ . It increased gradually until 3 wk PV (fold change: 18.9), and then slightly dropped at 4 wk PV (Figure 4B). The change of regulatory cytokine gene expression was not significant at any time point. The transcription of TGF- $\beta$  was slightly up-regulated, while the transcription of IL-10 was down-regulated (Figure 4C). The up-regulation of granulysin gene was also detected after vaccination. The peak increase was observed at 3 wk PV (fold change: 22.3) following the initial increase at 1 wk PV (fold change: 6.3) (Figure 4D).

## DISCUSSION

Efforts to characterize the immune response to *Map* have primarily focused on analysis of the immune response during the early and the latent stages of infection. The cumulative data show that exposure to *Map* invariably leads to infection and development of an immune response that controls but does not eliminate the pathogen, similar to infection with *Mycobacterium tuberculosis (Mtb)* (O'Garra et al. 2013). Although studied extensively, it remains unclear which components of the immune response are critical for controlling infection during this latent stage of infection. Cell mediated immunity (CMI) is involved in maintenance of protection and an increase in humoral immunity is associated with disease progression (Stabel 2000). An increase in antibody titer to *Map* antigens is being used by the dairy industry to predict when animals will develop clinical disease and should be removed from the herd. Few studies have been conducted at the late stage of infection to identify the cellular events associated with a breakdown of protective immunity. The finding that vaccination can transiently reverse immunopathology offers an opportunity to gain insight into how the immune response is being modulated and the components of cellular immunity that are critical for controlling infection (Juste et al. 2009; Singh et al. 2010; Alonso-Hearn et al. 2012). The two most informative studies have shown vaccination can be used at any stage of infection and obtain a therapeutic effect. A field trial showed whole herd vaccination with a commercial vaccine (Silirum, Zoetis), in dairies where *Map* infection was endemic, reduced bacterial shedding at the herd level and improved milk production (Juste et al. 2009; Alonso-Hearn et al. 2012). A more comprehensive study in goats comparing the therapeutic efficacy of a locally developed killed vaccine (Bison type) and a commercial killed vaccine (Gudair, Spain) showed vaccination significantly reduced shedding of *Map*, reduced morbidity and mortality, and reversed clinical signs as well as improved physical body condition (Singh et al., 2010). These observations support the use of therapeutic vaccination to increase the duration of the immune response associated with controlling disease progression and show further studies are warranted to find ways to increase the duration of the therapeutic effect. The finding that therapeutic vaccination reactivates the components of the immune response associated with protection also shows that it might be possible to use vaccination at the late stage of infection to identify the components of the immune response critical for maintenance of protection. The present preliminary study was conducted with this in mind and to determine if vaccination with a *relA* deletion mutant could modulate the immune response similar to killed vaccines. The cow that we obtained to conduct the study was at a very late stage of clinical disease. It had been removed from the herd based on a high titer of antibody to *Map*, severe diarrhea and weight loss, and reduced productivity. The cow was skin test negative and initial testing for an immune response *ex vivo*, using our FC assay, showed there was no proliferative response to antigenic stimulation with live *Map*. qRT-PCR showed expression of cytokine genes associated with inflammatory and suppressive immune responses were not elevated. A proliferative response was observed in CD4 and

CD8 cells one week PV. The response was still detectable at three weeks but cell proliferation was not as robust. Although there were fluctuations, the overall *Map* numbers in blood and feces were reduced following vaccination. Cytokine gene expression was mixed. There was an increase in expression of pro-inflammatory IFN- $\gamma$ , IL-17, IL-22 genes and associated transcription factors, and granulysin, a bactericidal peptide. There was also an increase in expression of the transcription factor Foxp3 and TGF- $\beta$ . There was a reduced expression of IL-10.

The promising findings of the current study are that the live attenuated vaccine reversed the immunosuppressive response of the late staged JD cow and induced a CMI response, as measured by CD4 and CD8 proliferation and increased cytokine gene expression. A recent therapeutic vaccination study in clinical JD cattle using a subunit vaccine (heat shock protein 70) showed increased survival and reduced bacterial shedding. However, the effect of the subunit vaccine was limited. It only induced humoral immunity, but not CMI (Santema et al. 2013). The increase in CMI, in addition to the reduced bacterial shedding, after *Map* $\Delta*relA* vaccination in the current study suggests the mutant has a broader therapeutic potential. The findings also indicate that more extensive studies are warranted to determine if the response elicited by vaccination with *Map* $\Delta*relA* increases the length of the therapeutic effect and also identify the components of the immune response associated with the therapeutic effect. Our initial studies have shown expression of multiple activation molecules are up-regulated on PBMC from experimentally and naturally infected animals following stimulation with PPD, soluble antigens, and live *Map* (Koo et al. 2004; Allen et al. 2009; Allen et al. 2011). Efforts to disrupt the immune response in experimentally infected animals to accelerate disease progression have shown the response is resilient and not readily altered by exposure to high doses of *Map*, immunosuppressive agents, or by depletion of CD4 cells (Stabel et al. 2009; Allen et al. 2012). Analysis has not revealed the basis for this resilience thus far but has shown the response is complex and involves the interplay of subsets of effector CD4 and CD8 cells secreting combinations of IFN- $\gamma$ , IL-17, IL-22, and granulysin (Park et al. 2011), subsets that appear to be involved in transient improvement of the immune response at the clinical stage of infection. Comparison of the response mediated by killed or subunit vaccines and *Map* $\Delta*relA* should show whether the live vaccine is more effective in enhancing the therapeutic effect and elucidate which component of the response restores protective immunity.$$$

Taken together, the findings indicate further testing of the mutant as a candidate vaccine should include examining analysis of the cellular events associated with restoring a protective immune response as well as its therapeutic potential.

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