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BOVINE TUBERCULOSIS

Mycobacterium bovis Δmce2 double deletion mutant protects cattle against challenge with virulent *M. bovis*

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SUMMARY

A *Mycobacterium bovis* strain deleted in *mce2A* and *mce2B* genes (*M. bovis* Δmce2) was tested as an experimental vaccine in cattle challenged with a virulent *M. bovis* strain. Three-and-a-half-month old calves ($n = 5$ to 6 per group) were vaccinated and challenged with a virulent strain of *M. bovis* by the intratracheal route 9 weeks after vaccination. A non-vaccinated group and a group vaccinated with BCG were included as controls. Blood samples were collected to measure IFN-γ by an interferon-gamma release assay (IGRA), cytometry and cytokine responses of bovine purified protein derivative (PPD) restimulated peripheral blood mononuclear cells (PBMCs). The IGRA test showed IFN-γ values similar to pre-vaccination except for the animals vaccinated with *M. bovis* Δmce2, where a significant increase was observed at 30 days post-vaccination. The expression of IL-2R on $CD4^+$ cells in response to PPD from the animals vaccinated with Δmce2 increased at 15 days post-vaccination compared to cells from non-vaccinated group. Vaccination of cattle with *M. bovis* Δmce2 induced the highest ($P < 0.05$) expression of IFN-γ and IL-17 mRNA upon PPD stimulation of PBMCs compared to vaccination with BCG or that for the non-vaccinated group. There was a weak positive correlation between the production of these proinflammatory cytokines post-vaccination and reduced pathology scores post-challenge. The animals were euthanized and necropsied 100 days after challenge. The group vaccinated with *M. bovis* Δmce2 displayed a significantly lower histopathological score for lesions in lungs and pulmonary lymph nodes than for the other groups ($P < 0.05$). A marked positive reaction to tuberculin intradermal test was observed post-vaccination in animals vaccinated with *M. bovis* Δmce2 compared to those vaccinated with BCG or the non-vaccinated group. In contrast, after challenge, non-vaccinated animals had greater skin test responses than the vaccinated animals. In summary, *M. bovis* Δmce2 is a promising vaccine candidate to control *M. bovis* pathogenesis in cattle.

1. Introduction

Bovine tuberculosis (BTB) is an infectious disease caused by *Mycobacterium bovis*, which affects cattle as well as a wide range of other mammals, including humans.[1] *M. bovis* is a member of the *Mycobacterium tuberculosis* complex (MTBC), which also includes *M. tuberculosis, Mycobacterium africanum, Mycobacterium canetti, Mycobacterium microti, Mycobacterium caprae* and *Mycobacterium pinnipedii.*

In Latin America, especially in South America, meat and milk are essential products for internal consumption and exportation. Therefore, eliminating diseases that limit the trade of animals and their products is a priority in this region. The population of cattle in

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Latin America has been estimated in 450 million head, 51 in Argentina, 189 in Brazil, 4.1 in Chile, 13.5 in Venezuela and 30 in Mexico.[2] About 262 million cattle live in countries with more than 1% prevalence of BTB.[2]

Conventionally, the control of BTB is based on a test and slaughter strategy, which has reduced the incidence and prevalence of the disease in developed countries, except in those with wildlife reservoirs such as the United Kingdom and New Zealand. However, this method is costly and lengthy to be applicable to most of the developing world. Vaccination of cattle represents an alternative intervention strategy to reduce the impact of BTB on livestock productivity and human health in developing countries. The only currently available vaccine against human tuberculosis is *M. bovis* bacillus Calmette-Guérin (BCG). BCG and other vaccines have been used in cattle in a large number of experiments and trials with variable efficacies as reviewed by Buddle et al.[3] and Waters et al.[4]

The construction of *M. tuberculosis* mutants has allowed the identification of several genes responsible for *M. tuberculosis* complex pathogenicity. These mutants are then tested for multiplication in the lungs of mice or guinea pigs. Several studies using this functional genomic approach have reported the development of gene knock-out mutants with different levels of attenuation, leading to the concept that rationally-attenuated live and replicating mutants of *M. tuberculosis* are potential vaccine candidates against tuberculosis.[5,6] Whereas the vaccine currently in use, BCG, lacks more than 100 genes compared to its parent strain, the use of *M. tuberculosis* complex strains attenuated in virulence genes provides the advantage that only a few selected genes are targeted, making them better candidates as vaccines.[7,8]

In 1993, the description of a DNA fragment from *M. tuberculosis* that encodes proteins mediating entry into mammalian cells led to the discovery of the *mce* operon[9] which is now recognized as a group of four major operons. While the virulence of *mce* mutants in mouse infection models seems to differ depending both on the route and dose, along with the susceptibility of the mouse strain used, in the majority of studies loss of one or more of the *mce* operons usually results in attenuation.[10–14] In one case, however, an attenuated mutant was still able to cause reactivation.[15]

In this study we investigated the capacity of a *M. bovis* strain deleted in two genes in the *mce2* operon, *mce2A* and *mce2B*, to protect cattle against an intratracheal challenge of a virulent *M. bovis* strain, as previously determined in mice and cattle virulence assays.[16,17] The mutant strain protected these animals, as shown by reduced lung pathology, compared to unprotected controls. This candidate is thus a new potential vaccine that should be further tested.

2. Methods

2.1. Bacterial strains and culture media

Δmce2 was derived from *M. bovis* NCTC 10772 as previously described.[18] The strain used for challenge was *M. bovis* 04-303, which is an isolate obtained from a wild boar with tuberculous lesions and has been shown to produce tuberculous lesions in cattle.[17] Both isolates were grown at 37 °C in Middlebrook 7H9 (BD, USA) liquid medium plus 0.5% Tween 80 enriched with 0.4% pyruvic acid, and 1% albumin dextrose complex (ADC). Viable bacteria in the inocula were enumerated with the Live/Dead BacLight™ Bacterial Viability kit (Invitrogen, Molecular Probes, Carlsbad, California).

2.2. Vaccination, challenge and sampling schedule

All the animals used in this study were negative to the tuberculin skin test and showed absence of *in vitro* gamma interferon (IFN-γ) response to both avian tuberculin PPD (PPDA) and bovine tuberculin PPD (PPDB) at the beginning of the experiment. The results shown in this study are part of a trial that included another candidate vaccine whose results will be separately published. Animal experimentations were performed inside the biosafety BSL3 facilities for large animals of the National Institute of Agricultural Technology (INTA), Argentina, in compliance with the regulations of the Ethical Committee of INTA (CICUAE) and the biosafety protocols as authorized by the National Service of Agricultural and Food Health and Quality (SENASA). Three groups of five to six Holstein-Fresian calves (3–4 months of age) were inoculated subcutaneously in the side of the neck with 10^6 colony-forming units (CFU) of either *M. bovis* Δmce2 or BCG Pasteur 1173P2 suspended in phosphate buffered saline (PBS). Sixty days after vaccination, animals were infected with an *M. bovis* 04-303 field strain by intratracheal instillation of 10^6 CFU as described previously.[17] The previous study established that this was an appropriate dose to produce reproducible tuberculous lesions in cattle.[17] The schedule is shown in Figure 1.

2.3. IGRA interferon gamma (IFN-γ) release assay

Heparinized blood samples were dispensed in 200 μl aliquots into individual wells of a 96-well plate. Wells contained whole blood plus 20 μg/ml *M. bovis* PPD (Prionics, Switzerland), 20 μg/ml *Mycobacterium avium* PPD (Prionics), or 4 μg/ml of ESAT6 or CFP10 recombinant antigens. Blood cultures were incubated for 18 h and plasma was harvested and stored at −80 °C. IFN-γ concentrations in stimulated plasma were determined using a commercial ELISA-based kit (Bovigam™; Prionics). Absorbance of standards and test samples were read at 450 nm. The optical density (OD) for the PBS controls, which was usually approximately 0.1 OD units, was used to normalize individual readouts and to calculate optical density indexes (ODIs), where the results obtained by antigen stimulation were divided by the results for the PBS-stimulated cultures. Duplicate samples for individual antigens were analyzed.

2.4. Necropsy

The animals were euthanized with the permission of the Institutional Animal Care and Use Committee (IACUC-CICVyA) of the Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria 100 days post-challenge. Necropsy was conducted paying special attention to typical tuberculosis lesions at lungs, liver, pulmonary lymph nodes (tracheobronchial and mediastinal lymph nodes), prescapular lymph nodes and cranial lymph nodes (submandibular, retropharyngeal, and parotid) were examined and sampled. Lymph nodes were systematically examined and kept for subsequent analysis irrespective of the presence or absence of macroscopic lesions in these organs. Macroscopic lesions were converted to scores according to Wedlock et al.[19] Two pieces were collected from every sample, one for microbiological culture and PCR and the other for histological analysis.

2.5. Cytometry

For flow cytometry determinations, 2×10^6 cells were incubated either with or without PPDB. To evaluate the expression of CD4 (MCA 1653A647, IgG2a), CD8 (MCA837PE, IgG2a), and CD25 (MCA2430F and MCA2430PE) surface markers, cells were stained with fluorescent-conjugated monoclonal antibodies (AdDSerotec, Oxford, UK). Stained cells were analyzed in a FACScalibur cytometer (BD, Franklin Lakes, NJ, USA) using Cell Quest software. Analysis gates were set on lymphocytes according to forward and side scatter. The expression of IL-2R (CD25) as a marker of T cells activation was analyzed in $CD4^+$ and $CD8^+$ populations. Percentages of

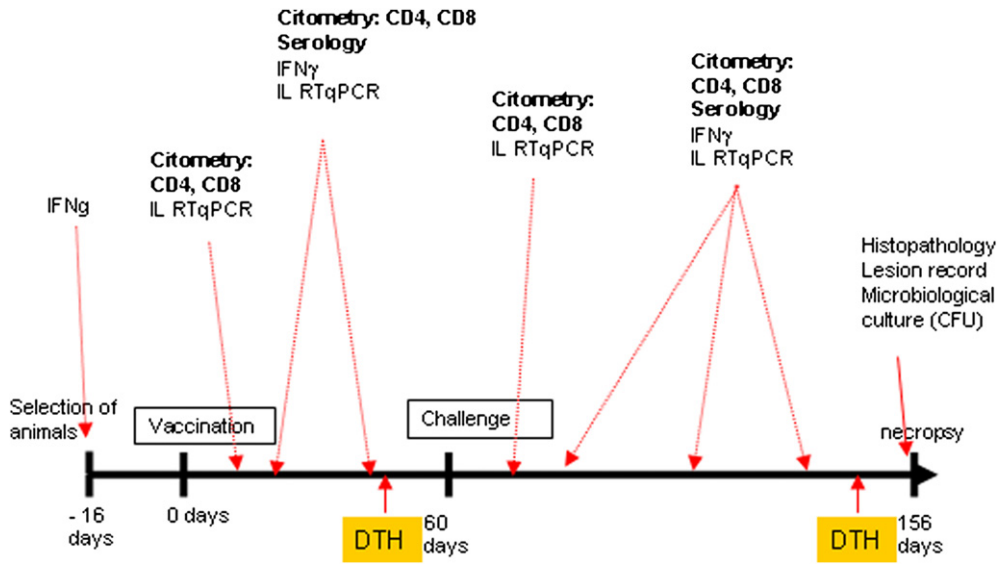

Figure 1. Vaccination, challenge and sampling schedule. The figure shows the sampling schedule after vaccination and after challenge. IFN-γ: Interferon gamma release assay. IL RTqPCR: reverse transcriptase real time PCR for cytokines. Cytometry for CD4, CD8 and CD25 markers. Histopathology with hematoxylin and eosin and Ziehl-Neelsen staining. Lesion register and record. Microbiological culture (CFU) on Stonenbrink media.

IL-2R-expressing cells were calculated as the ratio of $CD4^+$ or $CD8^+$ cells expressing CD25 and total $CD4^+$ or $CD8^+$ cells.

2.6. Expression of cytokines in PBMCs stimulated with PPDB

Cytokine mRNA expression analysis were performed in peripheral mononuclear blood cells. Briefly PBMCs were isolated from heparinized blood by gradient centrifugation over histopaque 1077 (Sigma–Aldrich) following the manufacturer's protocol and incubated at 37 °C with PPDB.[20] Total RNA was extracted from PPDB-stimulated PBMCs using a commercial kit (RNAeasy, QIAGEN). The quality and quantity of the RNA and the synthesis of cDNA were assayed as described previously.[20]

The mRNA of the cytokines (IL-2, IL-4, IL-10, IL-17, and IFN-γ) was quantified by qPCR using specific primers (Table 1). qPCR results were analyzed using the REST software as described previously.[21] For each animal, the pre-immune condition was used as the calibrator and *gadph* was used as a reference gene. Data were analyzed using the Mann–Whitney test.

2.7. Differential PCR

A differential PCR, which distinguishes if the *mce2* deletion is present or not, was applied to identify if the *M. bovis* isolates obtained after necropsy in the *M. bovis* Δmce2-vaccinated group were the vaccine strain or the challenge *M. bovis* 04-303. Two primer pairs (Table 2) were designed to amplify either an internal or external to the deleted region.[16] If the *mce2A* was deleted (Δmce2), the internal pair of primers should not amplify and the external primer pair amplifies 170 bp. In the presence of *mce2A* (Δmce2), the internal and external primer pairs amplify bands of 1800 bp and 167 bp, respectively.

Table 1
Primers for real time PCR.

Primers	Sequences	Amplicon Tm (°C)
GAPDH	**F**: ATCTCTGCACCTTCTGCCGA **R**: GCAGGAGGCATTGCTGACA	79
IL-2	**F:**CGTGCCCAAGGTTAACGCTA **R:**CCATTGAATCCTTGATCTCTCTGG	76
IL-4	**F**: TGCCCCAAAGAACACAACTG **R**: GCACGTGTGGCTCCTGTAGAT	78
IL-10	**F:**GGAAGAGGTGATGCCACAGG **R:** AGGGCAGAAAGCGATGACAG	84
IFN-γ	**F:**AGCTGATTCAAATTCCGGTGG **R:** GATTTTGGCGACAGGTCATTC	78
IL-17	**F:**GGACTCTCCACCGCAATGAG **R**: GGTCCACCTTCCCTTCAGC	81

2.8. Skin test

All animals were tested for skin tuberculin test reactivity both before the *M. bovis* challenge (30 days postvaccination) and 3 months after the challenge. Animals were intradermally injected with 0.1 ml of PPDB and the thickness of the caudal fold tuberculin skin test was measured using calipers immediately before the injection and again 72 h later. PPDB (32,500 IU/ml) was obtained from the National Service of Agricultural and Food Health and Quality (SENASA, Buenos Aires, Argentina).

2.9. Bacteriology

Stomacher macerated organ and lymph nodes samples were cultured on Lowenstein–Jensen and Stonebrink media for mycobacteria after decontamination by Petroff method (4% sodium hydroxide).[22] Cultures were inspected weekly for 90 days when Ziehl-Neelsen staining was performed on any of the colonies. Tissue pools consisted of three types of sources: lungs, pulmonary lymph nodes (mediastinal and tracheobronchials) and cranial lymph nodes (submandibulars and retropharyngeal). Each tissue was assayed separately for each animal.

Table 2
Primers for differential PCR.

Primers	Sequence
Mce2A	F:GAAGACCGAGCTGACTATGG R:ATGTAGCGAGGATTCACGTC
Mut. mce2 del.	F:ACCCGAACTTCAATCTCACG R:ACCTCACCTATCGCATGGTC

2.10. Histopathology

Lungs and lymph nodes (with or without lesions compatible with TB) were collected, fixed in 10% formaldehyde, sectioned and embedded in paraffin. Five μm-thick sections were stained with hematoxylin and eosin and Ziehl Neelsen for histopathological examination. Granulomas were classified as I to IV according to the criteria of Wangoo et al.[23] Histopathological lesions were scored as follows: score 0: without pathological changes; score 1: pneumonia and peribronchial infiltration; score 2: most of granuloma type I; score 3: granuloma type I and II; score 4: most of granuloma type II, score 5: granuloma type II and III; score 6: most of granuloma type III; score 7: granuloma type III and IV; score 8: most of granuloma type IV; score 9: granuloma type IV and cellular infiltration in bronchioles.

2.11. Statistical analyzes

Lesion scores, histopathological scores, tuberculin skin test responses, qPCR, cytometry and IFN-γ responses were analyzed using the Mann–Whitney test. The proportion of animals with lesions in the lungs and pulmonary lymph nodes were analyzed using Fisher's Exact test. A value of $P < 0.05$ denotes statistical significance. Statistical analyzes were performed using GraphPad prism 5.03 software (GraphPad Software, San Diego California USA).

3. Results

3.1. M. bovis Δmce2-vaccinated animals had fewer lesions after challenge than non-vaccinated animals

Animals were euthanized 3 months after challenge and necropsied. The extent and severity of lesions was converted to quantitative values by applying a score table.[19] Upon close inspection of lungs the non-vaccinated group had a significantly higher macroscopic lung tuberculous lesion score compared to those of the two vaccinated groups (Figure 2A). The exception was one non-vaccinated animal that showed no lesions at all. Very few animals in the study presented lesions in the cranial lymph nodes (Figure 2C). When the lymph node scores and total lesion scores per organ (lungs and lymph nodes) and per animal were compared, the *M. bovis* Δmce2-vaccinated group had again the lowest scores but these score values were not significantly lower compared to both the non-vaccinated and BCG-vaccinated group (Figure 2D). The number of animals with visible lesions in lungs and lymph nodes was lowest for the group vaccinated with *M. bovis* Δmce2, but no significant differences were detected between the vaccinated groups (Table 3).

Five of the animals from the non-vaccinated group developed a variable number of macroscopic lung lesions, from 14 small lesions in one individual to large areas of confluent lesions affecting whole lung lobes in another. A high prevalence of type IV granulomas surrounded by type I was observed indicating the active proliferation of *M. bovis* with abundant cellular infiltration in the bronchioles, as well as numerous small medium and large lesions in pulmonary lymph nodes. A single animal presented a large number of small and medium lesions in cranial lymph nodes.

In the BCG-vaccinated group, only 2 animals exhibited lesions: (2 and 17 lesions) in lungs with sizes ranging from 2 to 10 mm in diameter with prevalence of type III granulomas, some type IV granulomas surrounded by abundant type I granulomas and pneumonia. All animals showed small lesions in pulmonary lymph nodes (6-100 lesions/lymph node). A single animal had 7 small lesions in cranial lymph nodes. Finally, in the *M. bovis* Δmce2-vaccinated group a single animal presented 1 macroscopic encapsulated lesion of 10 mm with small type II granulomas in lungs.

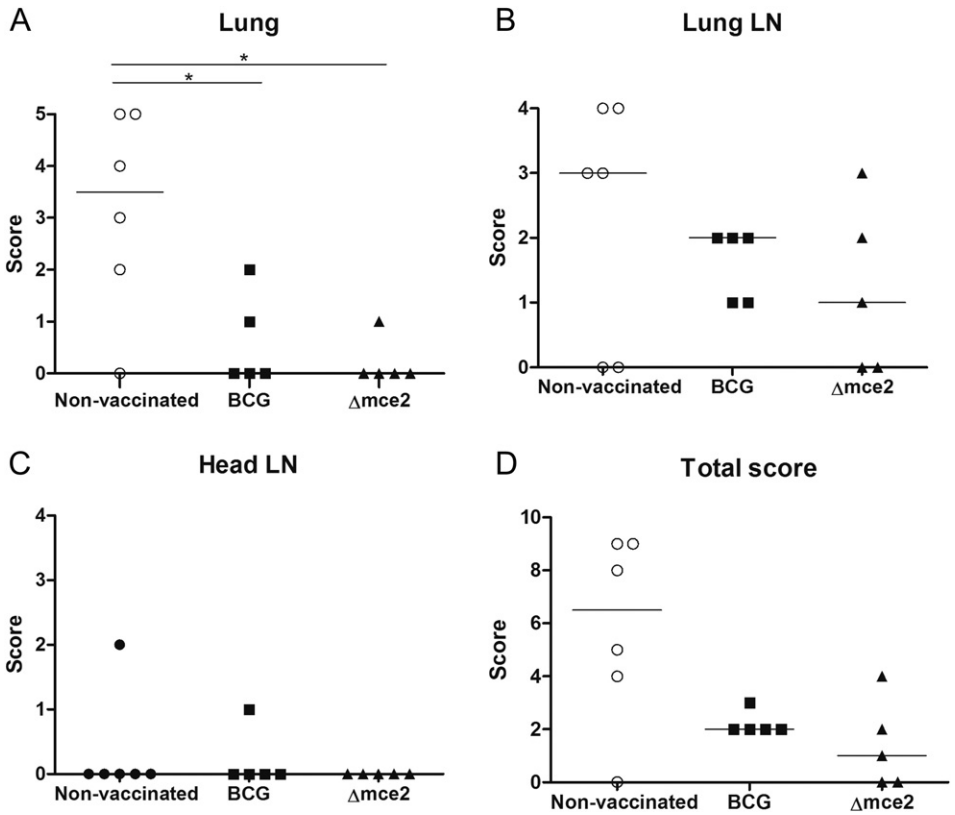

Figure 2. Macroscopic lesions for the vaccinated and non-vaccinated groups. Protective efficacy as measured by gross pathology in vaccinated and non-vaccinated groups. Median of pathology scores of lung parenchyma (A) pulmonary lymph nodes (B) cranial lymph nodes (C) and total score (cranial lymph nodes, pulmonary lymph nodes and lungs) (D). Pathology scores for individual animals are plotted. Horizontal lines indicate median values. Significance was determined by Mann–Whitney test: * statistically significant $P < 0.05$.

Table 3
Proportion of animals with macroscopic lesions in lungs and lymph nodes (LN).

Group	Animals with lesions in the lungs	Animals with lesions in pulmonary LN	Animals with lesions in cranial LN	Animals with lesions in lungs and LN
Non-vaccinated	5/6	5/6	1/6	4/6
BCG	2/5	5/5	1/5	2/5
Δmce2	1/5	3/5	0/5	1/5

(P value between 5/6 v 1/5 (lung) was $P = 0.08$, Fisher's Exact test).

Only 3 animals of this group showed small lesions in pulmonary lymph nodes and no lesion in cranial lymph nodes were observed.

Both vaccinated groups had significantly reduced histopathological scores in lungs compared to the non-vaccinated group. If total scores are compared, the *M. bovis* Δmce2-vaccinated group had significantly ($P < 0.05$) lower histopathological scores compared to the other groups (Figure 3).

3.2. Vaccinated animals had a reduced bacterial load compared to non-vaccinated animals

In the non-vaccinated group *M. bovis* was isolated or identified from pulmonary lymph nodes or lungs of most of the animals: In four out of 6 animals *M. bovis* was detected by culture and/or direct PCR in lungs (Supplementary data Table 4). In addition, in this experimental group *M. bovis* was isolated from at least one pulmonary lymph node in all animals (data not shown). In the BCG-vaccinated group, in 2 out of 5 animals *M. bovis* was isolated and/or detected by PCR in the pulmonary lymph node homogenates. In addition, two animals gave positive results by PCR but not isolated by culture in lung tissue and retropharyngeal lymph node (Supplementary data Table 4). In only two samples from the pulmonary lymph node homogenates of Δmce2-vaccinated group, *M. bovis* was isolated. In this last group, negative results were observed in culture or direct PCR in tissues of lungs and three samples from retropharyngeal lymph nodes gave positive PCR reaction with negative cultures. In all cases the *M. tuberculosis* complex identity was confirmed by IS-6110- based-PCR on colonies (data not shown).

3.3. IFN-γ responses

In the non-vaccinated group all IFN-γ responses were negative (ODI <2.0) before challenge except for one animal with high PPDA reactivity at 30 days post-vaccination (dpv) for unknown reasons. After challenge, IFN-γ responses were increased significantly, (Figure 4) from 40 days post-challenge (dpch), with the PPDB reactivity higher than the PPDA (data not shown). The BCG-vaccinated animals presented lower values of IFN-γ responses for PPDB than the Δmce2-vaccinated animals after vaccination ($p < 0.01$). The BCG-vaccinated group showed increased IFN-γ responses at 40 dpch and these values remained high (ODI around 15) throughout the study (Figure 4). In contrast, a value of 25 ODI of IFN-γ response was observed in the *M. bovis* Δmce2-vaccinated group at 30 dpv. At 60 dpv IFN-γ responses were still elevated. After challenge, IFN-γ responses for PPDB were significantly higher in Δmce2-vaccinated group than in those of the non-vaccinated and BCG-vaccinated groups. Similar trends were observed for ESAT6 and CFP10 restimulation after vaccination. As expected, the BCG-vaccinated group showed a low reactivity to ESAT6 and CFP10, indistinguishable to that

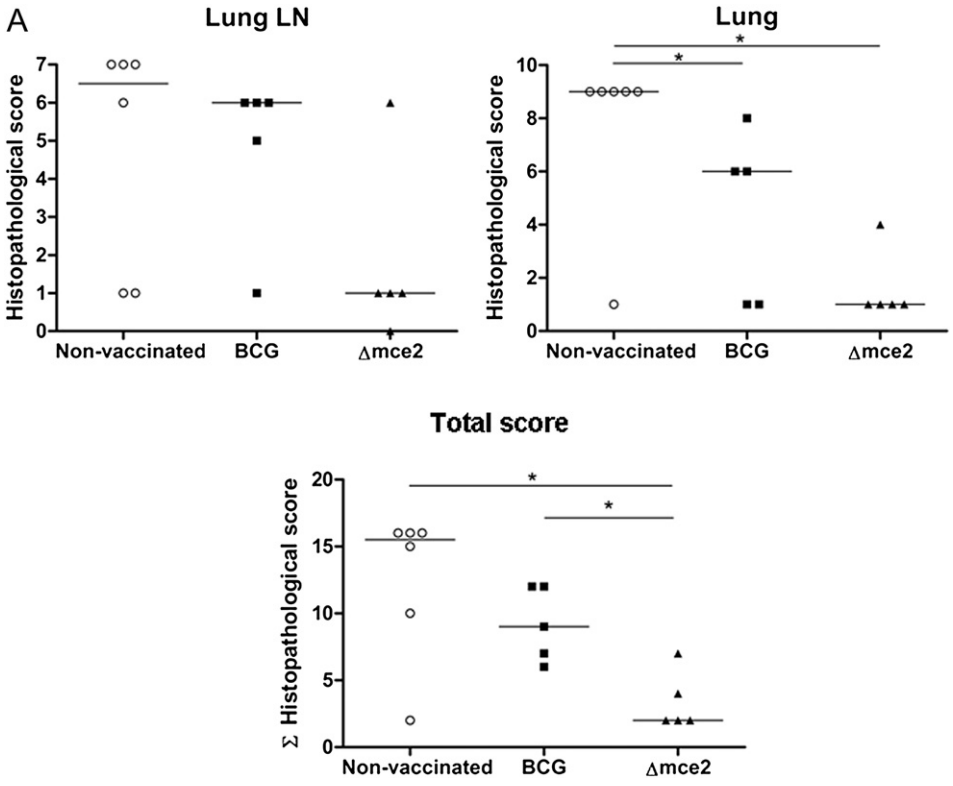

Figure 3. Histological lesions of the vaccinated and non-vaccinated groups. A. scoring of microscopic tuberculous lesions in lungs and lymph nodes. Histopathology scores in lungs of individual animals in vaccinated and non-vaccinated groups are plotted. Histopathology scores were established using the scoring system described in Material and methods. Horizontal lines indicate median values. Significance was determined by Mann–Whitney test, *statistically significant $p < 0.05$. B. (Supplementary material) representative microscopic lesions from cattle. Gross pathology (A) and histopathology (B and C) in lungs. (A) Arrows indicate lesions. Only one animal from the Δ*mce2*-vaccinated group presented a small lesion in lungs. (B and C) Images of hematoxylin and eosin stained lung sample; images 4X. Non-vaccinated and BCG-Pasteur groups showed advanced stage granuloma. The arrow indicates type II granuloma from the Δ*mce2*-vaccinated group. (C) Pneumonia.

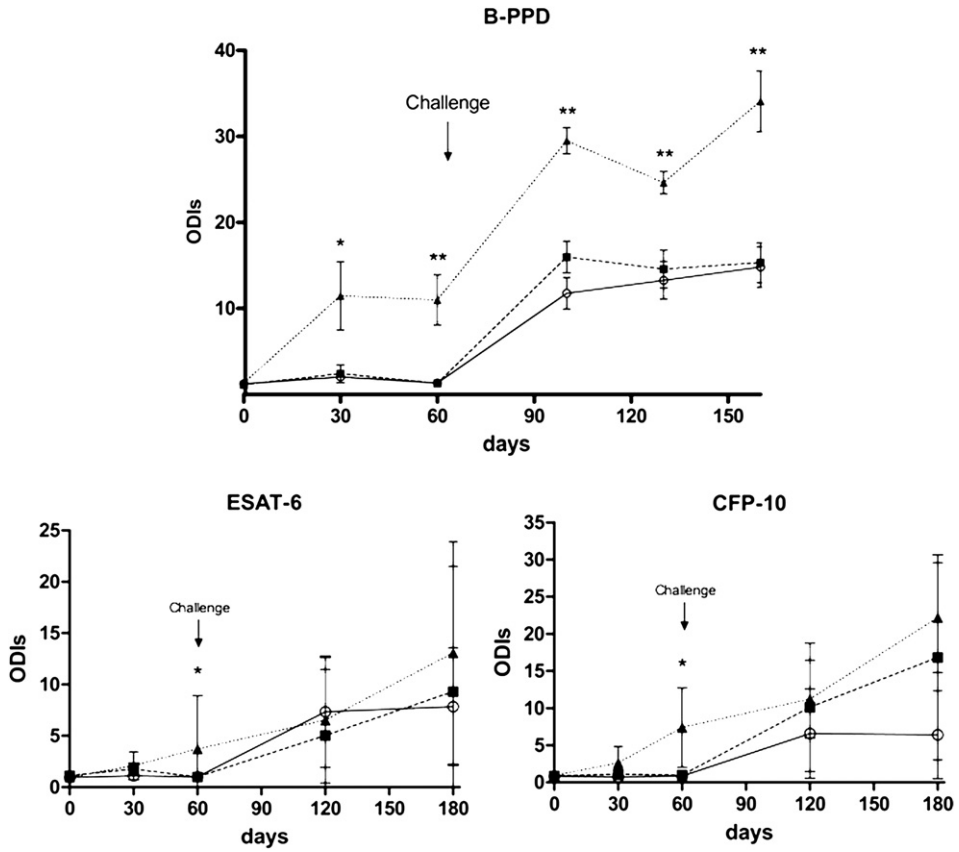

Figure 4. Response of *M. bovis*-infected animals to antigens. IFN-γ release in response to *M. bovis* antigens PPDB, PPDA, ESAT6, CFP10 in blood from animals vaccinated with BCG Pasteur (squares), Δmce2 (triangles) or non-vaccinated (open circles) at different time points (0, 30, 60, 100, 130 and 150 days post-vaccination). Results are expressed as ODIs. The arrow indicates the time of challenge. Significance was determined by Mann Whitney test, *statistically significant $p < 0.05$ and **statistically significant $p < 0.01$.

of the non-vaccinated group. After challenge the response to ESAT6 and CFP10 increased in the three groups (Figure 4).

3.4. Activation of CD4 and CD8 cell subsets

PBMCs samples were analyzed using flow cytometry to determine the expression of CD25 (IL-2 receptor) in $CD4^+$ and $CD8^+$ cell subsets. Samples from individual animals were incubated with PPDB or medium alone for 48 h and then the detection was performed using monoclonal antibodies against CD4, CD8 and CD25 surface markers.

The expression of IL-2R was increased upon stimulation with PPDB at 15 days post-vaccination in $CD4^+$ cells from animals vaccinated with Δmce2 compared to those from non-vaccinated group ($P < 0.05$) and the highest percentages of expression was reached during this period prior to challenge (Figure 5A). Although this activation was higher than the one for the BCG group, the difference was not statistically significant (Figure 5).

After challenge there were no differences among groups. Non-vaccinated and *M. bovis* Δmce2-vaccinated groups recorded significant increased activation compared to post-vaccination period ($p < 0.01$ and $p < 0.05$ respectively). The highest mean percentage of expression of CD25 in $CD4^+$ cells was obtained in the *M. bovis* Δmce2 group at 70 days post-infection.

The expression of IL-2R on CD8 cells was not significantly altered during the entire study. Percentages of $CD8^+CD25^+$ cells increased slightly after challenge with the virulent strain, and like in $CD4^+$ cells, the group of animals vaccinated with Δmce2 reached the highest values of CD25 expression but this difference was not statistically significant (Figure 5B).

3.5. Expression of cytokines in PBMCs after vaccination and challenge

To compare the immune response profile of cattle vaccinated with the candidate vaccine *M. bovis* Δmce2 to that of animals vaccinated with BCG or the non-vaccinated group, cytokine mRNA levels were measured after stimulating the cells with PPDB at different points after vaccination and challenge (Figure 6). Values for sequential samples were normalized to values before inoculation for each animal. The expression of the proinflammatory cytokines IFN-γ and IL-2 and the anti-inflammatory cytokines IL-4 and IL-10 was assessed. The mRNA level of IL-17 expression was also quantified, a previous report has proposed it as a biomarker of vaccine protection against BTB.[24]

Two weeks post-vaccination, only the group vaccinated with *M. bovis* Δmce2 responded to PPDB stimulation with significant production of both IFN-γ and IL-2 mRNA as compared to the values for BCG-vaccinated and non-vaccinated group ($P < 0.005$ and $P < 0.05$, respectively) (Figure 6A and B). This high expression of IL-2 mRNA is consistent with the highest frequency of $CD4^+$ and $CD8^+$ cells expressing the IL-2 receptor (IL-2R) detected at two weeks post-vaccination in stimulated PBMCs of the *M. bovis* Δmce2-vaccinated group (Figure 5). At one month after vaccination, the expression of IL-2 in PBMCs from both vaccinated groups significantly increased upon stimulation. Surprisingly, the PBMCs of the non-vaccinated calves also responded to the PPDB stimulation with IL-2 mRNA expression at this time point (Figure 6B).

Stimulation with PPDB induced significantly greater IL-4 gene expression in animals vaccinated with *M. bovis* Δmce2 than in those vaccinated with BCG at two weeks post-vaccination ($p < 0.001$) (Figure 6C). Significantly lower levels of IL-10 expression were

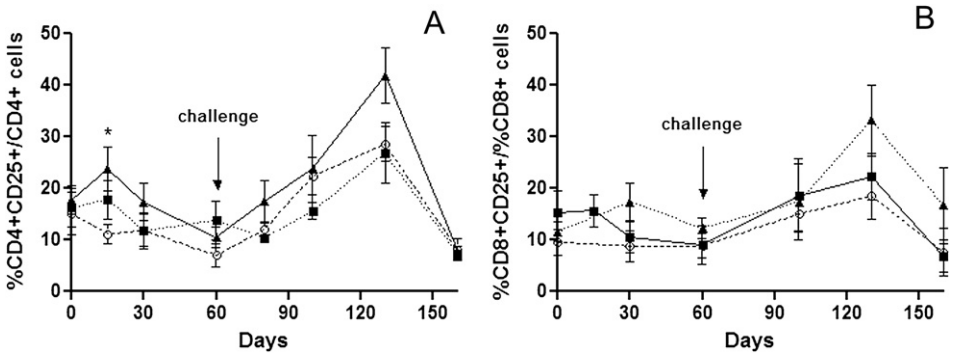

Figure 5. T-cell populations. Percentages of lymphocyte cell subsets $CD4^+$ (A) and $CD8^+$ (B) expressing CD25 of PPDB stimulated-PBMCs from animals vaccinated with BCG Pasteur ($n = 5$,square), Δmce2 ($n = 5$,triangle) or non-vaccinated ($n = 6$, open circle) at different points after vaccination (15, 30 and 60 days) and after challenge (20, 40, 70 and 100 days). The arrow line indicates the time of challenge. Data were analyzed using Mann–Whitney test for comparison between groups (*statistically significant $P < 0.05$ and **statistically significant $p < 0.01$). The means ± SEM are indicated.

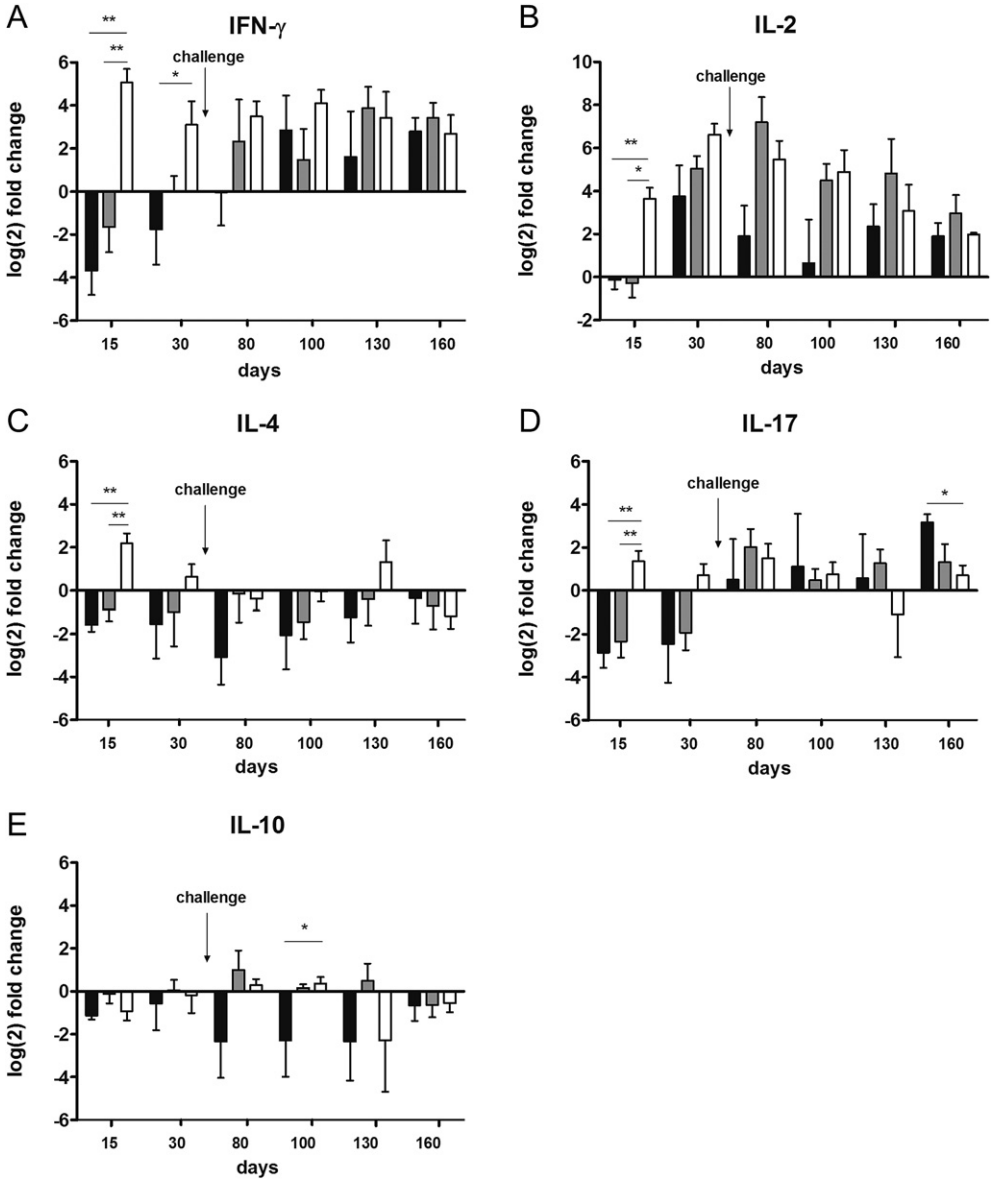

Figure 6. Cytokine mRNA expression. Relative cytokine gene expression. Gene expression of IFN-γ (A), IL-2 (B), IL-4 (C), IL-17 (D) and IL-10 (E) was measured in PPDB-stimulated PBMCs from animals vaccinated with BCG Pasteur (grey), Δmce2 (white) or non-vaccinated (black) at different time points (15 and 30 days post-vaccination and 20, 40, 70 and 100 days post-challenge). The arrow indicates the time of challenge. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with E correction, using *gadph* mRNA expression as a reference gene and the pre-immune condition as the calibrator. Data were analyzed using Mann–Whitney test (*statistically significant $P < 0.05$ and **statistically significant $P < 0.01$). Bars indicate the average fold change ± SEM.

induced in the non-vaccinated group compared to both vaccinated groups after challenge at day 40 (Figure 6E). This downregulation of IL-10 expression is delayed in the Δmce2-vaccinated group and the levels of this anti-inflammatory cytokine were equivalent between vaccinated and non-vaccinated groups after 70 days post-infection.

Importantly, at two weeks post-vaccination, PBMCs obtained from cattle vaccinated with *M. bovis* Δmce2 responded to PPDB stimulation by expressing more IL-17 mRNA than cells from animals vaccinated with BCG ($p < 0.01$) (Figure 6D). Stimulation of cells from the BCG group failed to induce IL-17 production at any point in time after vaccination or prior to challenge, finally there was an upregulated expression of IL-17 in non-vaccinated group at the end of the study.

3.6. Tuberculin test

The median tuberculin skin test responses were significantly greater for the Δmce2-vaccinated group compared to that for both the BCG and non-vaccinated groups (Figure 7, $P < 0.05$). In contrast, after challenge, non-vaccinated animals showed higher skin thickness increases than the vaccinated animals, although these differences were not significant (Figure 7).

3.7. Safety and attenuation

Only *M. bovis* compatible with the challenge strain (*M. bovis* 04–303) and not with vaccine strain were identified by a specific PCR directed to *mce2A* gene and performed on colonies obtained after necropsy (data not shown). In addition, no lesions in the prescapular lymph nodes were observed in the *M. bovis* Δmce2 or BCG-vaccinated group.

3.8. Correlations between immune parameters and protection

Extending our analysis, we compared IL-17 and IFN-γ expression after vaccination in relation to the pathology found at necropsy. A weak statistically-significant negative correlation was identified between both IL-17 ($P = 0.0109$) and IFN-γ ($P = 0.0315$) mRNA expression patterns upon PPDB stimulation at two weeks post-vaccination and disease severity (pathology scores) with a low r^2 of 0.43 and 0.35 for IL-17 and IFNɣ, respectively (Figure 8A and B).

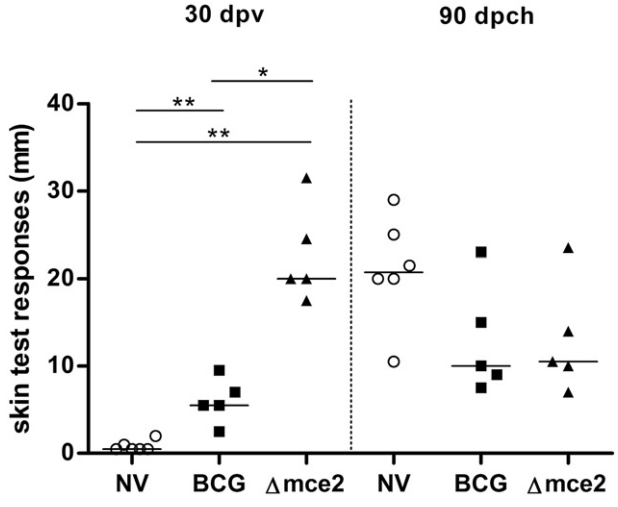

Figure 7. Tuberculin skin test responses to PPDB in animals vaccinated with BCG Pasteur ($n = 5$, squares), Δmce2 ($n = 5$, triangles) or non-vaccinated ($n = 6$, circles). Values indicate skin thickness increase in response to PPDB at one month post-vaccination (1 mpv) and prior to challenge, and three months post-challenge (3 mpch). Horizontal lines indicate mean values. Data were analyzed using Mann–Whitney test,*$P < 0.05$, **$P < 0.01$.

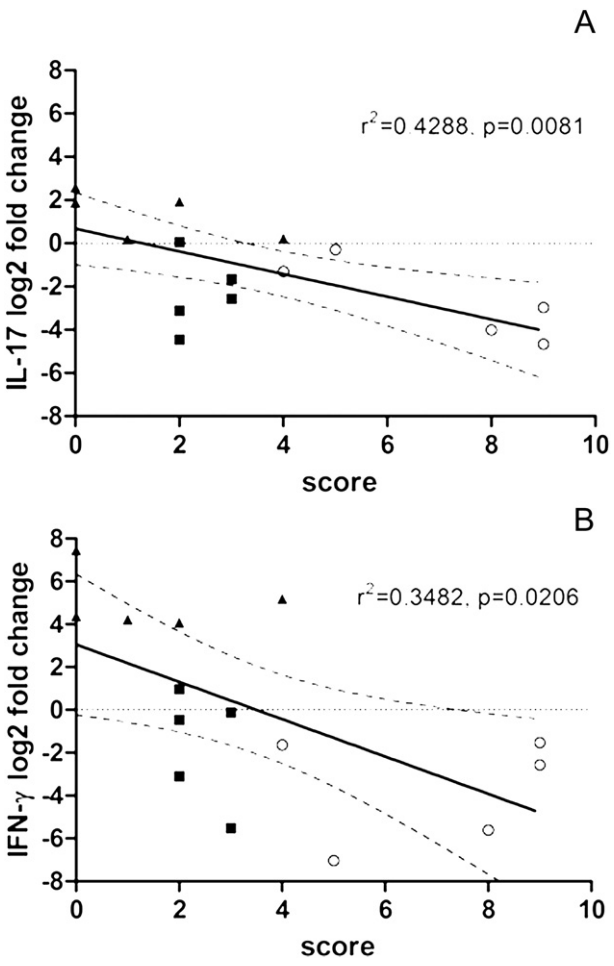

Figure 8. Correlation of IL-17 (A) and IFN-γ (B) mRNA expression at 15 days post-vaccination in PPDB stimulated-PBMCs with disease severity. Results are expressed as mean increases in mRNA expression of individual animals (Animals vaccinated with BCG Pasteur; squares, Δmce2; triangles, or non-vaccinated; circles) in relation to the corresponding total pathology scores observed at necropsy. Animals were infected 56 days after vaccination and sacrificed 100 days post-challenge. Solid lines in panels A and B indicate linear regression; dashed lines indicate 95% confidence intervals. Values for r^2 and p of linear regression analysis are indicated.

4. Discussion

In this study we demonstrated that *M. bovis* Δmce2 conferred better protection than BCG as observed from the significantly lower histopathological lesion scores when compared to the results obtained in the BCG group. In addition, the macroscopic lung score of lesions was significantly lower for the *M. bovis* Δmce2 and BCG-vaccinated groups than the non-vaccinated group. A lower number of animals vaccinated with *M. bovis* Δmce2 presented lesions in lungs and pulmonary lymph nodes compared to those from the BCG-vaccinated group, although this difference was not significant.

Only few papers have described the use of rationally attenuated wild-type *M. bovis* mutants as experimental vaccines to control bovine tuberculosis. For example, Waters et al.[25] tested an RD1 deleted *M. bovis* in young cattle and found reduced TB lesions and histopathological scoring. Nevertheless, the relative protection compared to non-vaccinated was similar to that conferred by BCG. These researchers have used scoring tables similar to that we used here.[23] In the study of Waters et al. it has been found that the most elevated IFN-γ responses, after challenge, occurred in the non-vaccinated group. In contrast, we observed that after challenge, the Δmce2 group had greater responses than that for the BCG-

vaccinated and non-vaccinated groups in response to PPD. Importantly, we observed that the *M. bovis* Δmce2-vaccinated group but not the BCG-vaccinated group showed a peak of IFN-γ response after vaccination. It is also important to note that *M. bovis* Δmce2, as opposed to the RD1-deleted *M. bovis* or to BCG, conserves ESAT6 and CFP10, two powerful T-cell antigens. Consistently with our findings, previous studies reported low or null IFNγresponses in cattle vaccinated with BCG in spite of a protective effect observed.[26]

By comparative analysis between the expression of different cytokines and total pathology scores of individual animals, we found a weak negative correlation between both IL-17 and IFN-γ after vaccination and the disease severity observed at necropsy. Such predictors or surrogates of protection could greatly facilitate vaccine development, as they would allow the estimation of vaccine efficacy without the need to infect animals with *M. bovis*; further studies, however, are required. We found that in PBMCs incubated with PPDB the correlation between IL-17 levels and protection was at the post-vaccination stage but not at the post-challenge stage. These results confirm the findings of Vordermeier et al.[24] who reported that high IL-17 and IFN-γ responses in vaccinated cattle prior to challenge correlates with protection against *M. bovis*. However, Vordermeier et al.[24] showed a positive correlation between the level of IL-17 and protection after challenge but only when cells were stimulated with a purified antigen (Ag85), but not in cells stimulated with PPDB.

At the end of this study, the expression of IL-17 in the non-vaccinated group was significantly higher than in the vaccinated groups. In a previous experimental infection we reported that the levels of this cytokine were increased in animals with macroscopic lesions compared to animals with no disease progression.[27] This finding supports the role proposed for IL-17 in the dissemination of *M. tuberculosis* complex to secondary disease sites[28] and that the induction of mature granuloma formation in a mouse model of TB requires IL-17 production by γδTCR cells.[29]

The downregulation of IL-10 in non-vaccinated animals after challenge could be a factor of disease progression, since the anti-inflammatory action of IL-10 is repressed by the disease; the association of decreased IL-10 levels and exacerbated pathology has been previously addressed by Thacker et al.[30]

A live vaccine has to be avirulent to be evaluated and accepted as a potential candidate. We have previously observed that *M. bovis Δmce2* is attenuated in two mice models[11,14] and in cattle.[18] However, contradictory results exist regarding the impact of the *mce2* gene mutation on *M. tuberculosis* complex virulence.[11,13,14,31] In this study, only *M. bovis* isolates compatible with the challenge strain (*M. bovis* 40–303) and not with the vaccine Δmce2 were detected and differentiated by PCR in tissues from lung and lymph nodes, suggesting that the vaccine strain fails to disseminate. The absence of lesions in the prescapular lymph nodes (the draining lymph nodes from the subcutaneous route of vaccination) in the Δmce2-vaccinated group is one important indication of the reduced virulence of this candidate vaccine.

Given the essential protective role of $CD4^+$ and $CD8^+$ T cells to control tuberculosis infections,[32] it is important that any candidate vaccine is capable of stimulating these cell populations. Therefore, the most effective vaccination strategies should be those that stimulate $CD4^+$ T-and $CD8^+$ cell responses to produce Th1-associated cytokines. In accordance with these requirements, the candidate Δmce2 vaccine elicited stronger T-cell responses in cattle, with activation of $CD4^+$ and $CD8^+$ following stimulation with the *M. bovis* specific antigen PPDB when compared to the non-vaccinated group. The group of animal vaccinated with Δmce2 reached the highest values of activated $CD8^+$ cells throughout the study, although the difference among groups was not statistically significant. This lack of CD8 *in vitro* activation may be explained because we used soluble antigens (PPD) for re-stimulation of PBMC biasing the response to CD4.

Positive reactions of IFN-γ release by PBMCs restimulated with tuberculin and DTH (delayed-type hypersensitivity)-tuberculin skin test responses were observed in animals vaccinated with Δmce2, prior to the challenge. The positive DTH response in the Δmce2-vaccinated group may be a disadvantage for the acceptation of this potential vaccine. However, it is important to take in consideration that the high DTH response together with elevated IFN-γ production in the Δmce2-vaccinated group may be the priming that prepares the animals to resist challenge with *M. bovis*. A strategy to differentiate cattle vaccinated with a strain such as Δmce2 would be to delete a gene expressing an immunogenic protein that could then be incorporated in a differential diagnosis test.[33]

This work demonstrates that *M. bovis* Δmce2 is a promising candidate vaccine. However, other tests including animal challenged for longer periods of time after vaccination or involving TB-free animals vaccinated with this vaccine and subsequently exposed to other animals should be performed to further evaluate this candidate.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2013.02.004.

Role of authors

Federico Carlos Blanco made cytometry, qRT-PCR, generation of the mutant strain, necropsy, processing of tissues, design of experiments and analysis of results María Verónica Bianco made IFNg tests, statistical analysis, histopathology, processing of tissues, necropsy, design of experiments and analysis of results Sergio Garbaccio made selection of animals, inoculations, skin test measurements, and necropsy and analysis of results Virginia Meikle made IFNg tests, inoculations, processing of tissues and necropsy María José Gravisaco made cytometry and analysis of results Valeria Montenegro made selection of animals, inoculations, skin test measurements, blood sampling processing of tissues and necropsy Edgar Alfonseca made necropsy, processing of tissues and analysis of results Mahavir Singh prepared recombinant antigens, and made analysis of results Soledad Barandiaran Necropsy and processing of tissues Ana Canal made histopathology and analysis of results Lucas Vagnoni planned animal tests, sampling, feeding, necropsy and disposal Bryce Buddle advice the team for inoculation and necropsy in situ and made analysis of results Fabiana Bigi obtained the funds, planned the assay, and analized the results Angel Cataldi obtained the funds, planned the assay, analized the results and wrote the manuscript.

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