

**Immunisation of cattle against *Babesia bovis* combining a multi-epitope modified vaccinia Ankara virus and a recombinant protein induce strong Th1 cell responses but fails to trigger neutralising antibodies required for protection.**

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## **Abstract**

Protection against the intraerythrocytic protozoan parasite *Babesia bovis* depends on both strong innate and adaptive immune response that depends on the presentation of parasite antigens to CD4<sup>+</sup> T-lymphocytes by professional antigen-presenting cells. Secretion of Th1 cytokines by CD4<sup>+</sup> T cell is also very important for isotype switching to IgG<sub>2</sub>, the best opsonising antibody isotype in cattle, to target extracellular parasites and parasite antigens displayed at the erythrocyte surface. In the field of vaccinology, heterologous prime-boost schemes combining protein-adjuvant formulations with a modified vaccinia Ankara vector expressing the same antigen have demonstrated the induction of both humoral and cellular immune responses. It has been previously demonstrated that MVA-infected dendritic cells can present antigens in the context of

MHC II and activate CD4<sup>+</sup> T cell. These results support the use of the MVA viral vector for a pathogen like *Babesia bovis*, which only resides within erythrocytes. In this study, 13-15-months-old Holstein-Friesian steers were immunised with a subunit vaccine as a prime and a modified vaccinia Ankara vector as a boost, both expressing a chimeric multi-antigen (rMABbo – rMVA). This antigen includes the immunodominant B and T cell epitopes of three *B. bovis* proteins: merozoite surface antigen - 2c (MSA - 2c), rhoptry associated protein 1 (RAP - 1) and heat shock protein 20 (HSP20). Responses were compared with the *Babesia bovis* live attenuated vaccine used in Argentina (R1A). Eleven weeks after the first immunisation, all bovines were challenged by the inoculation of a virulent *B. bovis* strain. All groups were monitored daily for hyperthermia and reduction of packed cell volume. Both the rMABbo – rMVA and R1A vaccinated animals developed high titers of total IgG antibodies and an antigen-specific Th1 cellular response before and after challenge. However, all rMABbo – rMVA steers showed clinical signs of disease upon challenge. Only the R1A live vaccine group developed an immune response associated with *in vitro* neutralising antibodies at a level that significantly inhibited the parasite invasion. The lack of protection observed with this recombinant formulation indicates the need to perform further basic and clinical studies in the bovine model in order to achieve the desired effectiveness. This is the first report in which a novel vaccine candidate against *Babesia bovis* was constructed based on a recombinant and rationally designed viral vector and evaluated in the biological model of the disease.

**Key words:** Modified vaccinia Ankara vector - multi-antigen - bovine babesiosis - prime-boost-vaccine-bovine

## 1. Introduction

*Babesia bovis* and *B. bigemina* are the main etiological agents of bovine babesiosis, a tick-borne disease caused by these intraerythrocytic protozoan parasites. The disease affects the livestock industry in tropical and subtropical areas where the Ixodidae ticks are present (Bock et al., 2004). Between both *Babesia* species, *B. bovis* causes the most severe form of the disease characterised by hyperthermia, hemolytic anemia and nervous signs

which causes high morbidity and mortality in adult cattle (Brown and Palmer, 1999). The current view of the adaptive immune response against *B. bovis* involves a mixed T and B cell response characterised by the activation of CD4<sup>+</sup> Th1-lymphocytes and the production of neutralising antibodies against extracellular merozoites and parasite surface antigens located at the erythrocyte membrane.

In addition, IFN $\gamma$  is required for activating macrophages to produce babesiacidal molecules and for enhancing an opsonizing IgG<sub>2</sub> antibody response (Brown and Palmer, 1999; Brown et al., 2006).

Since the mid-sixties, vaccination of calves with live attenuated *Babesia* parasites has been widely used for the prevention of the disease. These vaccines offer a high degree of protection and are routinely used as control measure for bovine babesiosis in Australia (Callow, 1977), Argentina, Israel and South Africa (Florin-Christensen et al., 2014).

Even though live vaccines against babesiosis are highly protective after a single dose, they present a number of inherent shortcomings including high costs of production, distribution logistics and the risk of contamination with pathogenic organisms during their production (de Waal et al., 2006). Additionally, even when vaccines based on passages through splenectomised calves can be replaced by culture-derived organisms, the long-term cultivation may result in loss of virulence and immunogenicity (de Vos, 1978). Hence, the development of stable, safer and equally protective vaccines against bovine babesiosis would be of a great benefit for countries where the disease is enzootic.

Several approaches using recombinant *B. bovis* antigens as subunit vaccines were evaluated in cocktails or separately in tick-susceptible *Bos taurus* breeds (Hines et al., 1995; Hope et al., 2005; Fish et al., 2008; Alvarez et al., 2010). Although in some of these trials the animals developed strong immune responses including *in vitro* neutralising antibodies, Th1 cells and a significant reduction of parasitaemia, these traits were not sufficient to prevent clinical signs of the disease after challenge with virulent strains. These results indicate the need to include new combination of antigens or the use of novel strategies to enhance the protective immune response against *B. bovis*.

New expression platforms based on non-replicative viral vectors are gaining more interest in the field of human health due to their ability to induce strong B and T cellular responses. In addition, this technology is becoming increasingly adopted in the field of veterinary vaccinology as an alternative to conventional formulations based on live-attenuated pathogens (Draper and Heeney, 2010).

One example of these vaccine vectors is the non-replicative and highly attenuated modified vaccinia Ankara vector (MVA), a DNA poxvirus capable of transiently expressing recombinant antigens in the host cells in the classical context of MHC I presentation. However, Thiele et al., (2015) have shown *in vitro* evidence that MVA-infected dendritic cells are able to directly transfer viral proteins into MHC II through the endogenous presentation pathway via proteasomes and autophagy and that these cells can efficiently activate CD4<sup>+</sup> T cell.

Recombinant MVA-based vaccine candidates against apicomplexan parasites of the genera *Plasmodium* and *Theileria*, both taxonomically related to *Babesia* spp., have been already described in preclinical and clinical trials with promising results (Gharbi et al., 2011; Sebastian and Gilbert, 2016). Moreover, these trials have demonstrated that MVA-vectored vaccines are more potent when used in heterologous boost vaccination following a strong priming agent expressing the same antigen such as adenoviruses or protein-in-adjuvant formulations (Sheehy et al., 2011, 2012; Ogwang et al., 2013).

Previously, we have performed preclinical trials in mice showing a synergistic effector response elicited by a prime vaccination with a recombinant multi-antigen, which comprises a fusion version of three *B. bovis* antigens (MSA - 2c, RAP - 1 and HSP20, named here as rMABbo), followed by a boost with a recombinant MVA (rMVA) expressing the same full-length construction. In the murine model this response was characterised by high titres of specific IgG antibodies and a high degree of activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells secreting both IFN $\gamma$  and TNF $\alpha$  (Jaramillo Ortiz et al., 2016).

Taking into account the aforementioned results in mice, we have moved forward and evaluated the same heterologous prime-boost scheme in cattle, challenged with the *B.*

*bovis* virulent S2P strain. Previous studies have demonstrated that Holstein-Friesian cattle are highly susceptible to *B. bovis* infection (Mangold et al., 1993); therefore, we have used the same breed to evaluate the performance of the vaccine candidate. A detailed characterisation of both humoral and cellular immune responses were also analysed and compared with the response to the protective *B. bovis* live attenuated vaccine.

## **2. Materials and methods**

### **2.1 Vaccine vectors and recombinant proteins**

In order to obtain the recombinant protein – rMABbo – as a single chimeric polyprotein, gene fragments containing B and T cell epitopes of *msa-2c*, *rap-1* and *hsp20* genes of *B. bovis* (GenBank AY052542.1, AF030062.1, and AF331455.1, respectively) were sequentially cloned and ligated to obtain a single open reading frame encoding the three *B. bovis* antigens (Jaramillo et al., 2014). The rMABbo protein expression and purification was described previously (Jaramillo Ortiz et al., 2016).

The wtMVA used as control was purified with the same protocol. The bacterially expressed recombinant protein (rMABbo) along with the recombinant surface antigen 1 from *Neospora caninum* (rSAG<sub>1</sub>) used in this work as a non-related subunit vaccine control were purified as described previously (Wilkowsky et al., 2011; Jaramillo Ortiz et al., 2016). After purification using affinity chromatography, both rMABbo and rSAG<sub>1</sub> proteins were quantified using the BCA colorimetric kit (Pierce, Rockford, IL), treated with Polimixine B – Sepharose (BioRad) to remove endotoxins and stored in aliquots at - 80°C until use.

### **2.2 Ethic statements**

The Secretary of Agroindustry of the Ministry of Production and Labour (Permit #28/16) approved the evaluation of the rMVA (considered as a genetically modified microorganism) in cattle. The experiment was carried out under guidelines of the Institutional Committee for the Use and Care of Experimentation Animals (CICUAE – INTA

protocol # 43/2015). Animals were housed in plot field facilities at *Instituto Nacional de Tecnología Agropecuaria*, INTA – Castelar (34°35'48.8"S 58°41'01.5"W) and were kept for a week before starting the experiment for adaptation and handling. Trained and experienced animal technical assistants and care workers were in charge of the bovines.

At the end of the trial, all the animals were euthanised by intravenous administration of sodium pentobarbital 40 %/sodium diphenylhydantoin 5 % Euthanyle® (Brouwer SA, Argentina) according to manufacturing instructions in compliance with the approved protocols.

### 2.3 Animal selection, immunisation protocol and challenge procedures

Healthy and even-weighted 13 to 15-month-old Holstein steers were selected from a farm located in the province of Buenos Aires, a tick-free area of Argentina below the parallel 30°S. The selection criteria included animals that resulted negative to the intradermal reaction for tuberculosis (skin thickness < 5 mm following injection of PPD 32,500 UI/mL; batch #86/2017 provided by *Servicio Nacional de Sanidad Agroalimentaria* - SENASA). Selected steers were also negative to *B. bovis* by indirect ELISA (Jaramillo Ortiz et al., 2018) and to *ves1-a* PCR (Bilgiç et al., 2013). The specificity of this PCR was previously using genomic DNA of *B. bovis*, *B. bigemina* and *Bos taurus* as template and proved to be specific only for *B. bovis* (de la Fournière et al., 2014). The 20 steers were randomly assigned into four groups (n=5/group). The subunit vaccine formulations and both the attenuated and the virulent *B. bovis* strains were inoculated subcutaneously. The recombinant (rMVA) and wild type (wtMVA) were inoculated intramuscularly.

One group (rMABbo – rMVA) received a dose of rMABbo (100 µg/animal) in Montanide ISA 61 VG® adjuvant (Seppic, Paris, France) and 42 days after the first immunisation a boost of  $1 \times 10^{10}$  PFU/animal of the rMVA. As positive control, another group (R1A live vaccine) was inoculated at day 42 with  $10^7$  *B. bovis* infected-erythrocytes of the R1A

vaccine strain currently used in Argentina. Two additional groups were included as negative controls: the rSAG<sub>1</sub> – wtMVA group received 100 µg/animal of rSAG<sub>1</sub> in the same Montanide adjuvant and 42 days after the first immunisation a boost of  $1 \times 10^{10}$  PFU/animal of wtMVA. The second group (RPMI – RPMI) received only 5mL of RPMI culture media/animal, at day 0 and day 42. Seventy-seven days after the first immunisation, all animals were challenged with an injection of  $10^7$  erythrocytes infected with the virulent *B. bovis* S2P strain.

Both the R1A and S2P *B. bovis* formulations were prepared for this trial with the routine procedure of the live vaccine formulation thoroughly maintained at 4°C. The immunisation scheme is summarised in Fig. 1.

#### 2.4 Clinical monitoring and sampling

After vaccination at day 42 with the live attenuated vaccine, the R1A live vaccine group was monitored from days 42 - 56 (also called as D0 – D14 of the post-vaccination monitoring period) for hyperthermia above 39.5 °C and reduction of packed cell volume (PCV) since the use of the attenuated vaccine in adult bovines in Argentina is only recommended under strict veterinary supervision (supplementary table S1). Parasitaemia was determined in thick and thin blood smears from the tail tip, stained with Giemsa. All clinical parameters were measured at day 0 and then daily in all groups from day 4 - 14 after challenge infection at day 77. At the day of the challenge, blood samples were taken just before inoculating the virulent S2P *B. bovis* strain.

Blood samples used in this work were aseptically collected by jugular venipuncture in heparinised Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ, USA), for peripheral blood mononuclear cells (PBMC) isolation and without anticoagulant for the separation of sera. During blood extractions, bovines were manually retained (< 3 min/animal).

Parasiticidal treatment of any affected animal was based on the criteria of a drop of more than a 50 % in PCV and/or a rise of rectal temperature (RT)  $\geq 41^\circ\text{C}$  on three consecutive

days. Animals with any of these clinical signs were treated by intramuscular administration of diacetate – 4, 4 diazamine dibenzamidine trihydrate (Ganaseg<sup>®</sup>, Novartis, Argentina) according to manufacturing instructions in compliance with the approved protocols.

#### **2.4 PBMC isolation, *in vitro* culture and restimulation**

Jugular blood samples collected at days 77 and 97 of the experimental protocol were centrifuged at 300 x *g*, 30 min. The buffy coat was diluted three times in 1X PBS 0.05 % acid-citrate - dextrose solution/2 % fetal bovine serum (FBS, Internegocios, Argentina). Then, the PBMC were layered over a Ficoll-Hipaque density gradient (GE Healthcare, Sweden) and centrifuged at 450 x *g*, 30 min. The white layer corresponding to the PBMC was aspirated and the cells were washed by centrifugation at 400 – 500 x *g* three times in 1X PBS, 0.05 % acid-citrate-dextrose solution/2 % FBS. Cells were counted in a Neubauer chamber and cultured in 24-well cell culture plates (2 x 10<sup>6</sup> cells/well, Greiner bio-one, USA) over night (ON) at 39 °C and 5 % CO<sub>2</sub>. The culture medium used was RPMI 1640 (Invitrogen, USA) supplemented with 2 mM L - glutamine (Sigma – Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, 0.1 mg/mL streptomycin (both Gibco, Grand Island), 50 mM 2 - mercaptoethanol (Sigma) and 10 % (v/v) FBS (Gibco). For cytokine measurement, PBMC cells were restimulated with a soluble R1A *B. bovis* merozoite lysate (MZ, 30 µg/mL) obtained as previously described (Jaramillo Ortiz et al., 2014). Concanavalin A (3 µg/mL, Sigma) and RPMI medium were used as positive and negative controls, respectively.

#### **2.5 Antibody measurement**

Serum samples collected at days 0, 56, 77, 97 and 150 of the experimental protocol were analysed in duplicate in two different indirect ELISAs. The MZ - ELISA uses a crude R1A *B. bovis* merozoite lysate (de Echaide et al., 1995) as antigen whereas the rMABbo - ELISA uses the recombinant multi – antigen, rMABbo (Jaramillo Ortiz et al., 2018). For the titration of the total IgG, serum titres of the rMABbo - ELISA were expressed as the Log<sub>10</sub> of the reciprocal of the highest serum dilution that at least duplicates the optical density



(OD) values obtained using negative (pre -immune) sera. This rMABbo - ELISA was also performed for the determination of IgG isotypes using specific anti-IgG<sub>1</sub> or anti IgG<sub>2</sub> antibodies (Serotec - Biorad) as shown in Table 1. For the comparison between the MZ and rMABbo ELISAs, readings were recorded when the *B. bovis* strong positive control serum (C++) reached an absorbance of 1.0 in 10 min. Accordingly, the OD<sub>450</sub> of each serum was expressed as a percentage of positivity related to the C++ according to the formula = (mean serum OD<sub>450</sub> x 100)/mean C++ OD<sub>450</sub>).

## 2.6 Intracellular cytokine staining (ICS)

For functional characterisation of the CD4<sup>+</sup>, CD8<sup>+</sup> and WC1<sup>+</sup> cells, PBMC cells were stimulated *in vitro* as mentioned in section 2.4, except that Brefeldin A (2 µg/mL; Sigma) was added for the last 6 h to facilitate intracellular cytokine accumulation. Following incubation, the PBMC cells were washed twice with staining buffer (1X PBS, 0.01 % Na<sub>3</sub>N, 10 % FBS) and were subsequently incubated with the respective anti-bovine monoclonal antibodies (CD4 – PeCy7, CD8 – PeCy7 or WC1 – FITC, Table 1). Then, cells were fixed in 2 % formaldehyde, pH [7.2] permeabilised in 0.5 % saponin/1 % FBS and stained intracellularly with anti-bovine IFNγ - AF647 and TNFα - AF488 antibodies (Table 1). Flow cytometry was performed acquiring 30,000 events in the live lymphocyte gate using a FACS Aria Fusion™ cytometer (Becton Dickinson) and further analysed using FlowJo 7.6.1 software (Tree Star, USA).

## 2.7 DNA extraction and real time PCR

The quantification of parasites was performed by real time PCR using the StepOnePlus Real -Time System (Applied Biosystems). To this end, a set of *B. bovis* specific primers based on the rhoptry associated protein (*rap - 1*) gene sequence was designed (Guillemi *et al.* 2019, manuscript in preparation). Blood samples from days 0, 6, 9, 11, 14 and 21 after challenge were chosen as sampling dates in order to cover the monitoring period. In particular, blood samples from days 9, 11 and 14 were chosen due to the coincidence with

the moment when the PCV values decreased significantly. Genomic DNA was extracted from 400 µl of blood using the ADN PuriPrep - S kit (INBIO Highway, Argentina) according to the manufacturer instructions, eluted in 100 µl of elution buffer and stored at -20°C until use. The amplification reaction was performed using 5 µl of SYBR Green master mix (Roche Applied Science), 1 µl of each primer (5µM) and 3 µl of genomic DNA. Three technical replicates were carried out for each sample.

Conditions used for the amplification were as follow: 95°C/15 min and 40 cycles of 95 °C/30 s, 62 °C/1 min. A melting curve program was used to evaluate the specificity of amplification. Each assay included the standard curve (6 points) and a non-template control. Standard curve was prepared using serial 10-fold dilutions of a known amount of genomic DNA of *B. bovis* diluted in ultrapure water. The *B. bovis* load was calculated using the calibration curve methodology previously described (Kralik and Ricchi, 2017). The cut off cycle threshold (Ct) value was set to 29 based on reliable limit of detection of the assay. The qPCR efficiency was 90.7 % and the detection limit obtained was 113 infected – erythrocytes/mL of blood.

## **2.8 *In vitro* neutralization assay**

Inhibition of invasion of erythrocytes by *B. bovis* merozoites was essentially carried out as previously described (Wilkowsky et al., 2003). Briefly, 5 - 5.5 ×10<sup>5</sup> live free merozoites from the S2P strain (purified from infected erythrocytes by a Percoll gradient) were incubated in triplicate in 96-well plates with pools of sera from each immunisation group taken at day 0, 77 and 97 and incubated for 30 min at 4°C (time 0). All sera were heat-inactivated previously for 30 min at 56°C and diluted 1:5 in M199 culture medium (Invitrogen). After incubation, M199 medium (60 %), normal bovine sera (40 %) and erythrocytes (5 % final PCV), were added to obtain a total volume of 170 µl per well. Non-treated merozoites were incubated in normal conditions as a control of parasite viability. The plates were finally incubated at 37° C in 5 % CO<sub>2</sub> atmosphere.

The percentages of parasitised erythrocytes were determined at 96 h by microscopic examination of 2500 erythrocytes in Giemsa-stained smears prepared from each well.

Results from D77 and D97 were relativised to D0 in order to express the inhibition of growth.

## 2.8 Statistical analysis

Statistical analysis was carried out using Graph Pad Prism (La Jolla, CA) software version 6.0 for Windows. ANOVA was used for comparing ICS data with Bonferroni post-test. Values of  $p < 0.001$ ,  $p < 0.05$  and  $p < 0.01$  were taken to be statistically significant.

One-tailed Student's *t*-test from three independent experiments was used for comparing *in vitro* neutralization assay, PCV and temperature data among the groups. Values of  $p < 0.05$  were taken to be statistically significant.

## 3. Results

### 3.1 Clinical response to vaccination and challenge

After vaccination of steers with the live R1A vaccine, the animals exhibited an expected PCV mean value drop. However, this decrease was transient and it was the only clinical sign of babesiosis detected (Supplementary Fig. S1 A and B and Supplementary table 1). As thin and thick blood smears resulted negative during this period, the presence of parasites was confirmed by PCR. Three out of the 5 steers vaccinated with the R1A strain showed a positive band of 125 bp between day 4 and day 8 post-vaccination (Supplementary Fig. S1 C) that corresponds to the expected size of specific *B. bovis ves1 - α* gene fragment. Even though it was not possible to detect the PCR product in animal no. 155 and no. 286, these steers showed a transient drop of its PCV levels during this period (Supplementary table 1).

None of the steers vaccinated with R1A required parasitocidal treatment during a monitoring period of 14 days post-vaccination, after which they gradually recovered

normal PCV values. Steers immunised with the recombinant vaccine rMABbo – rMVA as well as both negative control groups did not exhibit any effect associated with the vaccine formulations throughout all post vaccination period and had normal clinical parameters at the time of challenge (data not shown).

The challenge of bovines with the S2P virulent strain led to a rapid onset of clinical signs in rMABbo – rMVA, rSAG<sub>1</sub> – wtMVA and RPMI – RPMI groups. As shown in Fig. 2A, all these groups showed a remarkable decrease in the PCV levels between days 7 to 13 post - challenge (between day 81 and day 87 of the first immunisation, respectively). The average PCV reduction of each group was  $38.36 \pm 9.91$  % in rMABbo – rMVA,  $39.24 \pm 7.32$  % in rSAG<sub>1</sub> – wtMVA and  $35.58 \pm 9.33$  % in RPMI – RPMI group with no statistical significance among these groups (Supplementary table 2S A,  $p > 0.05$ ). In contrast, the R1A live vaccine group showed a low decrease in the PCV levels during these days  $12.66 \pm 4.3$  % in comparison with the other groups (Supplementary table S2 B,  $p < 0.01$ ).

None of the R1A vaccinated animals showed a considerable decrease in their PCV levels during this monitoring period. Hyperthermia was observed between days 7 to 13 after challenge, reaching a maximum of  $40.22 \pm 0.95$  °C ,  $40.57 \pm 0.70$  °C and  $40.18 \pm 0.89$  °C in rMABbo – rMVA, rSAG<sub>1</sub> – wtMVA and RPMI – RPMI groups respectively with no significant differences among them ( $p > 0.05$ ; Figure 2B).

Although most of the animals were clinically affected by the challenge, only one steer (#237 in the rSAG<sub>1</sub> – wtMVA group) reached a drop of 50 % in the PCV and needed treatment at D 10 post-challenge (Table 2S B). The other 14 steers remained with clinical signs of babesiosis during a period of 5 – 6 days, after which they gradually recovered to normal conditions without requiring drug treatment. Once recovered, all animals maintained normal values of RT and PCV until the end of the experiment.

Given that thin and thick blood smears resulted negative during the monitoring period after challenge, parasitaemia was monitored by quantitative PCR. As shown in Table 2, not only the rMABbo – rMVA but also both control groups reached a peak of infected-erythrocytes between days 9 and 11 after challenge. This fact coincided with the sharp

decrease in the PCV values during these days. Regarding the animals from R1A live vaccine group, the qPCR could detect infected-erythrocytes at day 0 of the challenge, which can be attributed to the presence of parasites from the live vaccine. Infected-erythrocytes were also detected at D6 after challenge; however, no parasites were detected in this group between 9 and 11 critical days, suggesting a rapid clearance of circulating parasites.

In consequence, a solid protection against the virulent strain was shown by the group vaccinated with the *B. bovis* R1A strain with no clinical sign of babesiosis during the post-challenge monitoring period (Fig. 2 A and B, and Table 2S A).

### 3.3 Humoral response induced by the prime-boost strategy

In order to characterise the antibody response elicited by the vaccination schemes, we evaluated serum samples from steers of all groups using the two ELISAs. Fourteen days after the second immunisation, high titres of specific IgG were detected only in the rMABbo - rMVA group using the rMABbo as antigen (Fig. 3A). After challenge, a secondary antibody response was observed in this group, with increasing IgG levels until the end of the experiment (day 150). When these sera were tested in immunoblotting assays using a crude *B. bovis* lysate, no specific band which would correspond to any of the three antigens was detected (data not shown).

Regarding the group vaccinated with *B. bovis* R1A strain, a much lower response against the rMABbo was observed before challenge, however, the antibody titres increased after it and remained so until the end of the trial, when they reached the same titre as the rMABbo – rMVA group. Regarding both negative control groups, the steers did not show seroconversion until day 97 (20 days post-challenge).

We further characterised the antibody response using the rMABbo - ELISA by measuring the IgG<sub>1</sub> and IgG<sub>2</sub> bovine isotypes as an indication of bias towards the Th1 response (IgG<sub>2</sub>/IgG<sub>1</sub> values greater than 1 are indicative of a Th1 type response). As shown in Fig. 3B, only the prime with rMABbo and the boost with rMVA produced a ratio of IgG<sub>2</sub>/IgG<sub>1</sub> above 1 at day 77. However, there is no statistical significance at this point amongst the

groups and this ratio decreased in our candidate-vaccinated group throughout the time until reaching a value of 1 at the end of the experiment. The control groups did not show any predominance of IgG<sub>2</sub> over IgG<sub>1</sub> throughout the experiment.

Serological analysis using the MZ - ELISA showed that after challenge, only the steers vaccinated with the R1A strain rapidly developed antibodies against merozoite antigens reaching a mean positivity value of 26.1 % above the cut-off point (>25) at day 56 and then continued increasing throughout the trial (Fig. 4B). Antibodies against the crude fraction were very low in the rMABbo – rMVA group until they reached positivity values of up to 60 % at the end of the trial (day 150, Fig. 4B).

Finally, we wanted to assess the functionality of the antibodies generated by the recombinant scheme by testing the ability of these antibodies to block the parasite invasion to erythrocytes. Using the S2P *B. bovis* virulent strain in the *in vitro* neutralisation assay, only the R1A live vaccine group developed *in vitro* neutralising antibodies at a level that significantly inhibited the erythrocyte invasion (Table 3,  $79.4 \pm 16.8$  % in the R1A live vaccine group vs.  $44.4 \pm 13.7$  % in the rMABbo – rMVA at day 97;  $p < 0.05$ , *t*-student test).

### 3.5 Cellular response in the immunisation schemes

To characterise the CD4<sup>+</sup>, CD8<sup>+</sup> and WC1<sup>+</sup> immune response elicited by vaccination, PBMC isolated from each animal were *in vitro* stimulated with a soluble merozoite lysate of the R1A strain for ICS and subsequent flow cytometry analysis. The IFN $\gamma$  and TNF $\alpha$  cytokines associated with Th1 immune response were measured in all groups before and after challenge (day 77 and 97 post -prime, respectively).

The rMABbo – rMVA heterologous regime triggered in bovines an elevated proportion of antigen-specific CD4<sup>+</sup> T cell secreting IFN $\gamma$  in both time points, compared to control groups (Fig. 5A and B,  $p < 0.001$ , one-way ANOVA with Bonferroni post-test).

The percentages of these cells at day 97 were between 2 and 3 times higher compared to day 77. Regarding the R1A live vaccine group, lower but significant percentages of CD4<sup>+</sup> T cell secreting IFN $\gamma$  were observed after challenge, in comparison with the rMABbo – rMVA

group (Fig. 5B,  $p < 0.01$ , one-way ANOVA with Bonferroni post-test). Minimal percentages of unspecific CD4<sup>+</sup> T cell secreting IFN $\gamma$  were detected in all groups when cells were restimulated with RPMI (data not shown)

We have also characterised the functionality of the non-classical  $\gamma\delta$  WC1<sup>+</sup> T cell, a subset of importance in the bovine immune response. As shown in Fig. 6A and B, the heterologous rMABbo – rMVA showed significant percentages of  $\gamma\delta$ WC1<sup>+</sup> producing IFN $\gamma$  before and after challenge ( $p < 0.05$ , one-way ANOVA with Bonferroni post-test). Meanwhile the values of CD4<sup>+</sup> T cell increased up to 3 times after challenge (Fig.5 A and B), the levels of  $\gamma\delta$  WC1<sup>+</sup>cells decreased up to 50 % at day 97. Minimal percentages of unspecific WC1<sup>+</sup> T cell secreting IFN $\gamma$  were detected in all groups when cells were restimulated with RPMI (data not shown).

For further evaluation of the immune response after challenge, we performed the detection of polyfunctional CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing both IFN $\gamma$  and TNF $\alpha$  using the same methodology. Interestingly, we found that rMABbo – rMVA group produced higher percentages of double-stained CD4<sup>+</sup> T and CD8<sup>+</sup> T cells in comparison with the control groups (Fig. S2 A,  $p < 0.001$  one-way ANOVA with Bonferroni post-test), whereas the R1A live vaccine group only showed high percentages of double-stained CD4<sup>+</sup> T cell when compared to negative controls (Fig. S2 B). Minimal percentages of unspecific CD4<sup>+</sup> T cell secreting IFN $\gamma$  and TNF $\alpha$  were detected in all groups when cells were restimulated with RPMI (data not shown).

Regarding CD8<sup>+</sup> T cell, we observed that both rMABbo – rMVA and R1A live vaccine group elicited high frequencies of CD8<sup>+</sup> - IFN $\gamma$  T cell before challenge (Fig. S3A,  $p < 0.001$ ). However, these frequencies decreased after challenge and only the group receiving rMABbo – rMVA showed lower but significant differences in the frequencies of single and double - stained cells in comparison with the control groups. (Fig. 3SB,  $p < 0.05$  one-way ANOVA with Bonferroni post-test). Minimal percentages of unspecific CD8<sup>+</sup> T cell secreting IFN $\gamma$  and TNF $\alpha$  were detected in all groups when cells were restimulated with RPMI (data not shown).

#### 4. Discussion

The present study aimed to determine whether a heterologous prime – boost vaccination scheme delivering the rMABbo *B. bovis* polyprotein as a subunit vaccine and the rMVA encoding the same multiepitope sequence was able to protect cattle against a highly virulent *B. bovis* S2P strain. The outcome of our rMABbo – rMVA regime was directly compared to the protection induced by the R1A live attenuated vaccine. Even though few previous reports have shown an approach to elucidate the T cell profiles in protected or unprotected animals with the *Babesia* live vaccine (East et al., 1997; Hope et al., 2005), the lack of in depth immunological characterisation of the response against this vaccine in adult bovines, made it relevant to be included as an objective of this study.

It has long been known that purebred *Bos taurus* cattle are notably more susceptible to *B. bovis* infection than *Bos indicus* or cross-bred animals (Bock, 1999). Young calves vaccinated between 4 to 10 months old do not exhibit visible clinical signs to the infection with the attenuated *B. bovis* vaccine strain, whereas animals older than one year require strict clinical surveillance to control potential severe reactions (Fish et al., 2008). In this trial, we have used *B. taurus* Holstein breed steers of approximately 13 months of age in an attempt to use more susceptible hosts for our recombinant vaccine. According to this, in our experiment the R1A vaccinated bovines had a transient decrease in PCV values although all animals recovered normal values and no babesiacidal treatment was required in this group.

The highly pathogenic S2P *B. bovis* strain used in our challenge has been isolated from a natural outbreak in the north of Argentina and has also been used as challenge strain in previous experiments (Mangold et al., 1993). Here, the S2P strain produced a lower than expected drop in PCV values (around 30 % on average) in all animals, excluding the R1A vaccinated steers. The dose and route used here ( $10^7$  infected erythrocytes, subcutaneous) allowed us to monitor the progression of the infection for a longer period, which was required to analyse the immune response over time. In addition, we attempted



to avoid that the strong challenge usually used for these experiments, may conceal any minimal protective immune response generated by our multi-epitope vaccine. Based on our previous experience, challenging of bovines with higher doses of this strain ( $10^8$  infected erythrocytes) caused acute and severe signs in a short period after the infection become patent (3 - 5 days, I. Echaide, personal communication). Similarly, other authors reported that when 10 to 12 month-old Angus heifers were challenged with an intravenous injection containing  $10^8$  parasite-infected erythrocytes of an Australian strain (Hope et al., 2005) or Mexican strain (Alvarez et al., 2010), all animals showed a rapid onset of clinical disease by days 4 - 6 and needed to be treated.

The choice of a sublethal dose of the virulent strain resulted in negative blood smears in all bovines during the monitoring period after challenge. Taking into account the subjectivity in the microscopy observation as well as the technical training to properly identify the parasite, we measured the parasitaemia using an own-developed qPCR targeting the *rap - 1* gene. This qPCR was highly sensitive, more precise and capable of detecting up to 100 copies of *rap - 1* per mL of blood. This sensitivity threshold is between values obtained by previously described qPCR studies targeting the multi-copy cytochrome B (Giglioti et al., 2016) and the single copy *msa - 2c* genes of *B. bovis* (Ramos et al., 2011).

The presence of infected-erythrocytes in the R1A live vaccine group was detected by qPCR at day 0 and 6 days after challenge. Considering that at day 0, blood samples were taken just before inoculation of the S2P strain, this positive signal would indicate that the source of parasites was due to the R1A vaccination *per se*. We cannot determine if the positive results at day 6 in this group were due to the vaccine or the challenge strain since the target if the qPCR is identical in both strains. It is worth mentioning that even though the traditional PCR could not detect parasitic DNA in two animals (no. 155 and no. 286) after vaccination with the R1A strain, the qPCR was able to detect the presence of infected-erythrocytes in all animals of this group 35 days after vaccination (day 77 of the experiment, day 0 of challenge), showing the persistence of parasites. Similar results were

reported by Pipano et al., (2002) where parasites from vaccinated bovines could be transmitted to susceptible animals up to 47 months after vaccination.

Interestingly, although the R1A group had the highest average parasitaemias, bovines achieved a rapid clearance of the parasitic burden after challenge, which resulted in a recovery of normal haematocrit and temperature values. This rapid reaction would involve mechanisms associated with the memory immune response, which was recalled upon challenge.

This protective response did not occur in our recombinant vaccine group, where the peak of parasitaemia coincided with the time of the lowest PCV levels. The control group (RPMI - RPMI), showed a more gradual decrease in the PCV levels and this finding correlates with the low numbers of infected erythrocytes detected by the qPCR.

Regarding immunisation of bovines with the rMABbo - MVA scheme, the vaccine was well tolerated and highly immunogenic at inducing both antibodies and T cell responses. Similar level of responses using the same scheme was previously obtained in immunised mice (*i.e.* specific IgG antibodies between  $\log_{10}$  2.5 - 3.5 titres and between 1 - 1.5 % of specific CD4<sup>+</sup> - IFN $\gamma$ <sup>+</sup> T cells, Jaramillo Ortiz et al., 2016). In this sense, the murine approach reinforces the usefulness of this model in preclinical studies for host-restricted pathogens such as *B. bovis*.

In spite of the fact that the recombinant vaccine was able to induce strong immune responses, the expected protection was not achieved: the rMABbo - rMVA vaccinated steers developed clinical signs of babesiosis after challenge, exhibiting significant decreases in PCVs and elevated hyperthermia similar to the control groups. This lack of protection was observed in each animal even in those which developed high levels of antigen-specific CD4<sup>+</sup> and WC1<sup>+</sup> T cells achieved by the rMABbo - rMVA inoculated steers in comparison with the R1A live strain vaccinated ones. A possible explanation for this finding could be that T cell epitope-specific responses recalled after challenge might differ from those induced by immunisation with the live *B. bovis* vaccine, which has a broader repertoire of epitopes exposed. Moreover, the levels of antigen-specific CD4<sup>+</sup>

polyfunctional T cell, a trait that has been associated with protection in clinical models of malaria and leishmaniasis (Darrah et al., 2007; Roestenberg et al., 2009) were similarly high in both rMABbo – rMVA and in the fully protected *B. bovis* R1A vaccinated group. According to these findings, the role of CD4<sup>+</sup> T cell as a correlate to the protective immunity against *B. bovis* remains to be fully clarified (Norimine et al., 2003; Brown et al., 2006).

Interestingly, apart from the expected CD4<sup>+</sup> T cell response, we have also detected high percentages of CD8<sup>+</sup> antigen-specific T cell not only in the recombinant vaccine group but also in the live vaccinated animals. It is well documented that vaccine trials using recombinant MVAs are able to elicit a strong CD8<sup>+</sup> T cell response in vaccinated individuals (Ramshaw and Ramsay, 2000; Taracha et al., 2003; Bull et al., 2014). However, the current view about immunity against *B. bovis* (a strictly intraerythrocytic parasite) does not involve a CD8<sup>+</sup> T cell response (Brown et al., 2006). In spite of this, Goff et al. (2002) have demonstrated the participation of CD8<sup>+</sup> T cell against the parasite in the spleen of adults and calves during acute babesiosis. Other reports regarding infection only with blood - stage *Plasmodium* have clearly demonstrated the induction of CD8<sup>+</sup> T cells, suggesting a cross-presentation from dendritic cells (Lundie et al., 2008; Imai et al., 2010). Our findings would add evidence in this sense by showing the involvement of CD8<sup>+</sup> blood-derived lymphocytes secreting IFN $\gamma$  as well.

Another key finding of our trial was the induction of antigen-specific  $\gamma\delta$  WC1<sup>+</sup> T cell producing IFN $\gamma$  in the response against the recombinant vaccine. These cells were induced at high levels after vaccination with the rMABbo - MVA scheme and were also detected after challenge. These cells interact with infectious agents in a unique way via the  $\gamma\delta$  TCR and WC1 acting as co-receptors and pathogen recognition receptors and were reported in the vaccine response against important zoonotic bacteria such as *Leptospira*, *Borrelia* and *Mycobacteria* spp (Telfer and Baldwin, 2015). Further studies will be necessary to assess which particular WC1 molecule modulates the interaction with *B. bovis* and the role of the regulatory T cells (T regs) of the MVA-vaccinated bovines since it has been demonstrated

that this viral vector is capable of inducing T regs that limits the number of effector T-lymphocytes. Particularly, Cubillos-Zapata et al., (2011) have reported that bovines uses the  $\gamma\delta$  T cells as the main regulatory subset of the immune response.

We hypothesised that the Th1 cell response could be enhanced by using the novel Montanide™ ISA 61 VG adjuvant in the subunit vaccine immunization. According to the manufacturer's data sheet and previous reports, this mineral-oil formulation skew the IgG response towards the IgG<sub>2</sub> isotype (Aucouturier et al., 2001; Khorasani et al., 2016). The IgG<sub>2</sub> bovine isotype is much more efficient than IgG<sub>1</sub> not only in the pathogen opsonisation but also in the activation of NO-production and killing by neutrophils (McGuire et al., 1981), processes that contribute to the immune control the *B. bovis* infection (Brown et al., 2006). However, in our experiment there was no significant increase in the IgG<sub>2</sub>/IgG<sub>1</sub> ratio. A possible explanation would be that one dose of rMABbo was not sufficient to enhance the desired levels of IgG<sub>2</sub> or this adjuvant is not suitable for this type of immunization scheme.

On the other side, the presence of neutralising antibodies was the only differential feature present in the protected bovines compared to our recombinant vaccine group. The role of neutralising antibodies in protective immunity has been characterised for various hemoparasites such as *Theileria parva* and *Plasmodium falciparum* (Egan et al., 1999; Morzaria and Nene, 2006). In our design, we have included some antigens that have been reported to induce *in vitro* neutralising antibodies. Even though there is no report of *B. bovis* HSP20 showing its seroneutralising competence, the widely conserved antigen MSA - 2c was previously demonstrated to neutralise *in vitro* bovine erythrocyte invasion (Wilkowsky et al., 2003). Norimine et al., (2003) have previously shown that using the full-length RAP - 1 or RAP - 1 NT protein resulted insufficient to induce neutralising antibodies.

The negative results of the indirect MZ-ELISA in the recombinant group could be explained due to the fact that the 3 antigens were not sufficiently represented in the crude lysate used for this test or the antibodies against the recombinant antigen are not able to react to their native counterparts.

However, there are some piece of evidence proving that the rMABbo protein maintains discontinuous B-cell epitopes also present in the parasite but a single immunization of bovines induces low levels of antibodies against these epitopes. Supporting this point, we have previously demonstrated that approximately 78% of naturally *B. bovis*-infected cattle were positive to the rMABbo - ELISA (Jaramillo Ortiz et al., 2018). Here, our results are also in agreement with these previous data since sera from R1A - vaccinated animals reacted against the rMABbo at low titres before challenge with a gradual seroconversion after the second parasite inoculation with the virulent strain.

In summary, we consider that this low antibody levels induced by a single subunit immunization would explain, to certain extent, the negative results in the *in vitro* antibody-based assays (MZ-ELISA and seroneutralisation) and also the lack of protection. Accordingly, it would be interesting to evaluate the reactivity of the antibodies against native antigens after more than one subunit vaccine immunisation and/or using different adjuvants.

In view of these findings, we support the hypothesis that the chimeric rMABbo antigen conserve discontinuous and immunodominant B-cell epitopes that develop a strong antibody response only detectable after repeated exposures to the pathogen *i.e* during tick- biting. The protective role of these antibodies should be evaluated again using the *in vivo* bovine model.

## Conclusion

The development of effective and new generation vaccines against complex pathogens, such as *Plasmodium*, *Theileria* and *Babesia* has been disappointingly difficult. Progress in vaccine development will require a better understanding of the mechanisms of immunity in natural hosts and the antigens or epitopes that induce the protective mechanism. Basic knowledge is necessary to understand the immunological memory response elicited by the live attenuated vaccine. The outcomes presented here could shed light on new vaccine approaches against apicomplexan parasites such as *B. bovis*.

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### Conflicts of interest

José Manuel Jaramillo Ortiz and Silvina Elizabeth Wilkowsky are named inventors on patent application “Chimeric polypeptide, vaccine against babesiosis, immunisation methods, detection methods and kit” WO 2016209859 A1.

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## Figure legends.

**Figure 1. Schematic representation of the experimental design.** Twenty highly susceptible Holstein males (13 to 15-month-old) were divided randomly into 4 groups of 5 steers each and immunised at day 0. Group 1 received 100 µg/animal of the rMABbo in Montanide ISA 61 VG® adjuvant, subcutaneously (sc); group 2: RPMI only (sc); group 3: 100 µg/animal of the heterologous rSAG1 in Montanide ISA 61 VG® adjuvant and group 4 RPMI only (sc). Groups 1 and 3 were inoculated at day 42 with of  $1 \times 10^{10}$  PFU/animal of rMVA intramuscularly (im) or  $1 \times 10^{10}$  PFU/animal of wtMVA (im), respectively. As positive control, group 2 received one dose of  $10^7$  *B. bovis* infected erythrocytes (im). Group 4 received RPMI only (im) as a boost. Seventy – seven days after the first immunisation, all animals were challenged with a S2P *B. bovis* virulent strain. The monitoring periods of 14 days are indicated in curly brackets. Blood and sera sample were collected at days D0, D42, D56, D77, D97 and D150 respectively.

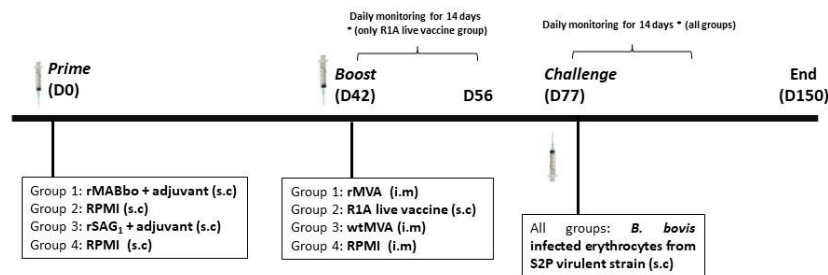


Figure 1

**Figure 2. Clinical response profiles after challenge with the S2P *B. bovis* virulent strain.**

(A) Time-course of the packed cell volume percentage (PCV). The mean percentage values of each animal in all groups were calculated at each time point. (B) Time – course of the mean rectal temperature (°C) of all experimental groups. The dotted line indicates the maximal normal value of the rectal temperature with values above 39.5 °C indicating hyperthermia.

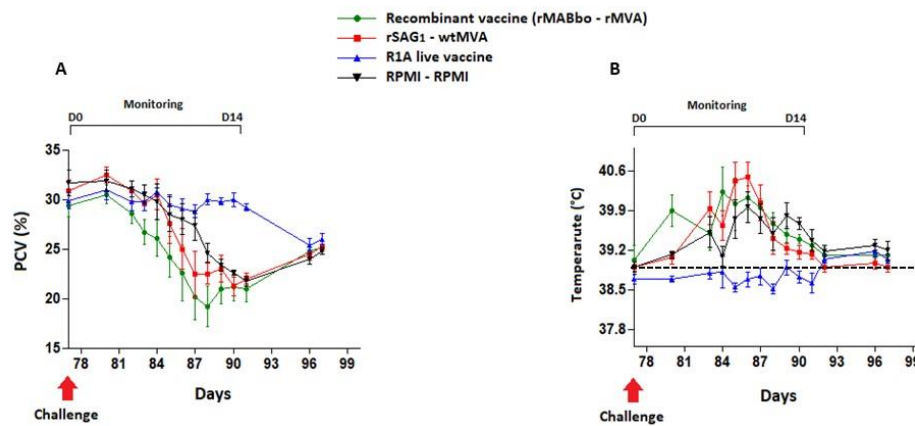


Figure 2

**Figure 3. Humoral immune response to the prime-boost regimes at days 0, 56, 77, 97 and 150.** Vaccination schemes were as described in Fig. 1. (A and B) Time course of total bovine IgG response and IgG<sub>2</sub>/IgG<sub>1</sub> subclass ratio in the prime-boost schemes assessed by indirect ELISA using the rMABbo as antigen. Each time point depicts the group mean value  $\pm$  SD.



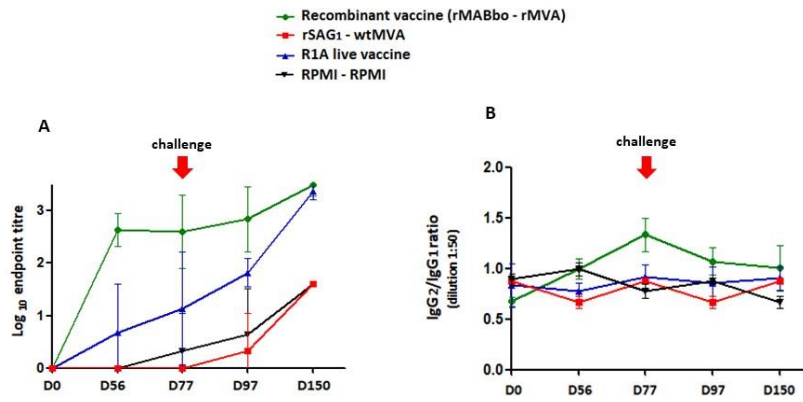


Figure 3

**Figure 4. (A and B) Antibody levels against *B. bovis* using the rMABbo or a R1A crude merozoite lysate as antigen, respectively.** Dotted line indicates the cut off value of each ELISA ( $\geq 35\%$  and  $\geq 25\%$  respectively). The optical density (OD) of each serum was expressed as a percentage of positivity related to the C++ according to the formula = (mean serum OD x 100)/mean C++ OD).

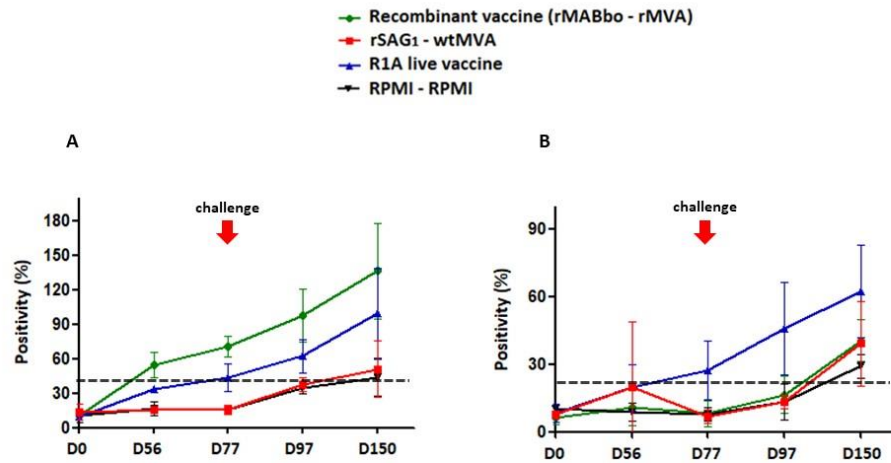


Figure 4

**Figure 5. CD4<sup>+</sup> T cell responses before and after challenge.** (A and B) Frequency of IFN $\gamma$ <sup>+</sup> secreting CD4<sup>+</sup> T cell before or after challenge. Cells were labeled with anti CD4 - PE/Cy7 and stained intracellularly with an anti IFN $\gamma$  - AF647 antibody. In all cases, a minimum of 300,000 events was acquired. The points represent the individual animal level of CD4<sup>+</sup> T cell after PBMC restimulation with soluble R1A *B. bovis* merozoite lysate (MZ, 30  $\mu$ g/mL). Results are expressed as mean values  $\pm$  SD, and those labeled with asterisks were statistically significant \*\*\* $p$  < 0.001 and \* $p$  < 0.01 compared between indicated groups with one-way ANOVA and Bonferroni post-test.

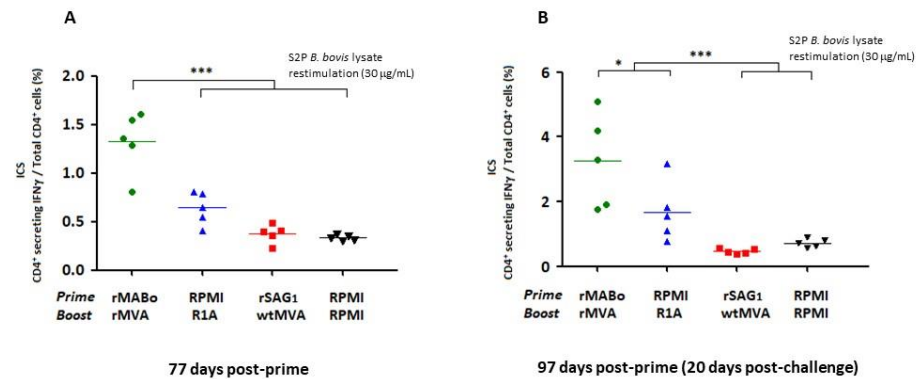
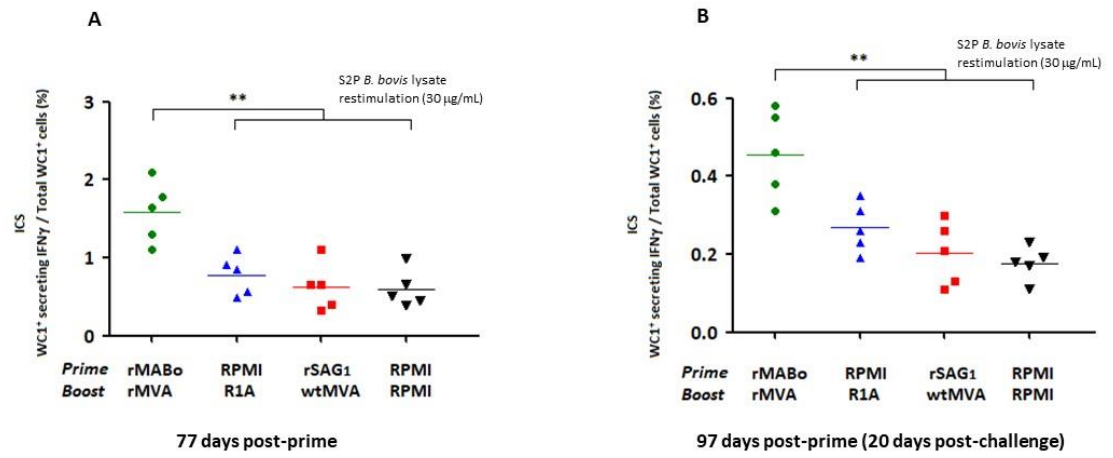


Figure 5

**Figure 6. WC1<sup>+</sup> T cell responses before and after challenge.** (A and B) Frequency of IFN $\gamma$ <sup>+</sup> secreting WC1<sup>+</sup> T cell before or after challenge. Cells were labeled with anti WC1 - FITC and stained intracellularly with an anti IFN $\gamma$  - AF647 antibody. The points represent the individual animal level of WC1<sup>+</sup> T cell after PBMC restimulation with soluble R1A *B. bovis* merozoite lysate (MZ, 30  $\mu$ g/mL). \*\*  $p < 0.05$  compared between indicated groups with one-way ANOVA and Bonferroni post-test.



## Table

**Table 1. Monoclonal antibodies (mAbs) against cell surface markers, intracellular cytokine or immunoglobulin used in this study.** FITC: fluorescein isothiocyanate; PE/Cy7: phycoerythrin - cychrome 7; AF: alexa fluor; HRP: horseradish peroxidase.

**Table 2. Detection of *B. bovis* using the qPCR method after challenge.** D: Days post-challenge. iRBCs: infected erythrocytes per mL of blood. Neg: negative.\* The individual values for each bovine vaccinated with R1A at day 0 (D0) were: #49: 130 iRBCs/mL; #155: 12,525 iRBCs/mL; #286: 245,000 iRBCs/mL; # 394: 212,000 iRBCs/mL; #293: 27,500 iRBCs/mL.

**Table 3. *In vitro* inhibition growth by sera sample from steers immunised with different vaccine formulations.** Pooled sera were added (1:5, final concentration) to wells containing S2P *B. bovis*-infected erythrocytes that were cultured *in vitro*. Results represent means  $\pm$  SD of three independent experiments. \*  $p < 0.05$  compared between the rMABbo - rMVA and R1A live vaccine with one-tailed Student's *t*-test.

Table 1

mAbs	conjugated	Target	Clone	Host	Isotype	Binding site	Dilution	Source
Anti - CD4	-	Bovine	CC8	Mouse	IgG2a	cell surface	1/200	MCA1653GA (AbD Serotec, UK)
Anti - CD8	-	Bovine	CC63	Mouse	IgG2a	cell surface	1/400	MCA837GA (AbD Serotec, UK)
Anti - IgG2a	PeCy7	Mouse	Polyclonal	Goat	IgG	CD4 or CD8	1/200	ab130787 (Abcam, USA)
Anti - WC1	FITC	Bovine	CC15	Mouse	IgG2a	cell surface	1/400	MCA838F (AbD Serotec, UK)
Anti - IFN $\gamma$	AF647	Bovine	CC302	Mouse	IgG1	intracellular	1/400	MCA1783A647 (AbD Serotec, UK)
Anti - TNF $\alpha$	AF488	Bovine	CC327	Mouse	IgG2b	intracellular	1/200	MCA2334A488 (AbD Serotec, UK)
Anti - total IgG	HRP	Bovine	Polyclonal	Mouse	IgG	IgG	1/3000	sc - 2350 (Santa Cruz Biotech, USA)
Anti - IgG1	HRP	Bovine	IL - A60	Mouse	IgG1	IgG1 isotype	1/2500	MCA2440 (AbD Serotec, UK)
Anti - IgG2	HRP	Bovine	IL - A73	Mouse	IgG1	IgG2 isotype	1/2500	MCA2441 (AbD Serotec, UK)

Table 2.

	<b>D0</b>	<b>D6</b>	<b>D9</b>	<b>D11</b>	<b>D14</b>	<b>D21</b>
	iRBCs/ml	iRBCs/ml	iRBCs/ml	iRBCs/ml	iRBCs/ml	iRBCs/ml
<b>rMABbo - rMVA</b>	Neg	Neg	<b>1.2x10<sup>3</sup></b>	<b>4.3x10<sup>2</sup></b>	Neg	Neg
<b>rSAG1 - wtMVA</b>	Neg	Neg	<b>2.1x10<sup>3</sup></b>	<b>8.0x10<sup>2</sup></b>	Neg	Neg
<b>R1A live vaccine</b>	<b>1.0 x10<sup>5</sup>*</b>	<b>1.8 x10<sup>5</sup></b>	Neg	Neg	Neg	Neg
<b>RPMI - RPMI</b>	Neg	Neg	<b>1.0 x10<sup>2</sup></b>	<b>4.3x10<sup>2</sup></b>	Neg	Neg

Table 3.

<b><i>In vitro</i> growth inhibition assay (%)</b>		
Groups / Days	<b>D77</b>	<b>D97</b>
<b>rMABbo - rMVA</b>	(19.1 ± 5.6)	(44.4 ± 13.7)
<b>rSAG1 - wtMVA</b>	(6.1 ± 3.2)	(5.9 ± 3.3)
<b>R1A live vaccine</b>	(26.3 ± 16.5)	(79.4 ± 16.8)*
<b>RPMI - RPMI</b>	(8.3 ± 2.5)	(12.5 ± 3.1)