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Vaccine strategies against *Babesia bovis* based on prime-boost immunizations in mice with modified vaccinia Ankara vector and recombinant proteins

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ABSTRACT

In this study, a recombinant modified vaccinia virus Ankara vector expressing a chimeric multi-antigen was obtained and evaluated as a candidate vaccine in homologous and heterologous prime-boost immunizations with a recombinant protein cocktail. The chimeric multi-antigen comprises immunodominant B and T cell regions of three *Babesia bovis* proteins. Humoral and cellular immune responses were evaluated in mice to compare the immunogenicity induced by different immunization schemes. The best vaccination scheme was achieved with a prime of protein cocktail and a boost with the recombinant virus. This scheme induced high level of specific IgG antibodies and secreted IFN and a high degree of activation of IFNγ$^+$ CD4$^+$ and CD8$^+$ specific T cells. This is the first report in which a novel vaccine candidate was constructed based on a rationally designed multi-antigen and evaluated in a prime-boost regime, optimizing the immune response necessary for protection against bovine babesiosis.

1. Introduction

Babesiosis is a tick-borne disease caused by intraerythrocytic protozoan parasites of the genus *Babesia*. In particular, *Babesia bovis* infection causes a severe disease characterized by hemolytic anemia and high mortality in adult bovines impairing the livestock industry [1]. Control measures against bovine babesiosis includes chemotherapy and acaricide treatments as well as vaccination with attenuated parasite strains [2]. After vaccination, cattle develop a long-lasting immunity, characterized by rapid activation of memory and effector CD4$^+$ T helper cells that secrete IFNγ, thereby activating phagocytic cells and enhancing antibody production by B cells [1]. However, the inherent disadvantages of these live vaccines include the risk of contamination during their production, the possibility to induce clinical disease when they are administered to older bovines and the need of cold-chain logistics, raising vaccination costs [3,4].

Current trends in vaccinology field focus on developing effective, safe and rationally-designed vaccines capable of inducing a protective immune response without the inconveniences of the use of live strains. In this sense, non-pathogenic viruses engineered to express immunodominant antigens constitute an excellent tool of choice. Particularly, the highly attenuated modified vaccinia Ankara (MVA) virus can be used as a non-replicative vector to express single or multiple antigens of interest *in vivo* [5]. After MVA infection, recombinant proteins are expressed in host cells and presented to the immune system, triggering mainly cellular immune responses. In addition, MVA viruses may also increase immunogenicity acting as nonspecific adjuvants *per se* [6]. Diverse recombinant MVA (rMVA) have been evaluated in preclinical and clinical trials alone or in heterologous prime-boost immunization regimes combined with Adenovirus, DNA or subunit vaccines [7–10].

In the search of vaccine candidates for *B. bovis*, several antigens were evaluated [1]. These antigens include the Rhoptry-Associated Protein 1, (RAP-1), the Merozoite Surface Antigen-2c (MSA-2c) and the small Heat Shock Protein 20 (HSP20) [11–13]. In particular, their B and CD4$^+$ T cells epitopes were shown to be immunodominant and conserved among parasite strains. Some of these antigens were evaluated alone (*i.e.* RAP-1) [14] or in combination

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with other proteins (*i.e.* MSA2-c) [15] as subunit vaccines in bovines but although these approaches induced antibody and $CD4^+$ T-lymphocyte responses that were recalled upon challenge, neither antigen stimulated a protective immune response.

These results pose the need of the development of multivalent vaccines to create broad range protection against several parasite antigens combining different vaccine delivery systems to optimize immunogenicity at both levels of the immune response.

This study describes the development of an rMVA virus expressing a chimeric antigen containing B and T cell epitopes from three antigenic proteins of *B. bovis*: MSA-2c, RAP-1 and HSP20. We also demonstrate that homologous and heterologous prime-boost vaccination schemes combining rMVA and a subunit vaccine cocktail of the three proteins maximized humoral and cellular responses in mice.

2. Materials and methods

2.1. Cells and culture media

Primary chicken embryo fibroblasts (CEFs) were obtained as described before [16]. Baby hamster kidney (BHK-21, ATCC CCL-10™) and Porcine Kidney (PK-15 ATCC CCL-33™) cells were maintained in Dulbecco's modified medium supplemented with 2% fetal calf serum (FCS; Internegocios, Buenos Aires, Argentina). Spleen cells for functional assays were maintained as described [17].

2.2. B. bovis genes and generation of recombinant proteins and rMVA virus

DNA fragments containing B and T cell epitopes of *msa-2c*, *rap-1* and *hsp20* genes (GenBank AY052542.1, AF030062.1, and AF331455.1, respectively) were amplified by a standard PCR using primers listed in Table 1 (Supplementary data) and cloned *in-frame* with a N-terminal His-tag in the pRSET vector (Invitrogen Corp, Carlsbad, CA, USA) for delivery as subunit vaccine. The *rap-1* gene was amplified from nucleotide 20 to 384, while *msa-2c* and *hsp20* were amplified from nucleotides 64 to 708 and nucleotide 117 to 607, respectively. In the case of MSA-2c, PCR amplification and protein purification was performed as previously described [13]. RAP-1and HSP20 were purified under denaturing conditions [18]. For the generation of recombinant MVA clones, DNA fragments of *msa-2c, rap-1* and *hsp20* were sequentially ligated into a pCR-TOPO vector (Invitrogen) in order to obtain a single genetic sequence encoding the three *B. bovis* antigens (multi–antigen: MABbo) and then sub-cloned into a transference vector VT at an *Nhe*I site [16]. This plasmid also contains the expression cassette for the β-glucoronidase enzyme and the flanking sequences of the MVA086R gene (which codifies for the thymidine kinase (TK) enzyme). TV-MABbo was transfected into CEFs previously infected with wtMVA and recombinant viral clones forming blue lysis plaques were subsequently isolated and purified by ultracentrifugation through a 25% w/v sucrose cushion as previously described [16]. The purity of the recombinant viruses and the insertion of the MABbo were confirmed by PCR using *TK1–TK4* [16] and *MABbo* F–*MABbo* R primer pairs, respectively (Table 1, Supplementary data).

MABbo expression was verified by Western blot (WB) using bovine anti-MSA-2c, mouse anti-RAP-1 or rabbit anti-HSP20 polyclonal sera. Bovine sera used in this work proceeded from animals experimentally infected with M2P or S7P *B. bovis* strains and from naturally infected bovines from northeast and northwest Argentina. All cattle sera were kindly provided by INTA–Rafaela, Santa Fe, Arg. and INTA–Mercedes, Corrientes, Arg., respectively, and were serologically positive for *B. bovis* by indirect ELISA. For further confirmation of the MABbo expression, an Indirect Immunofluorescence Assay (IFA) was performed in PK 15 cells (see Supplementary data).

2.3. Immunization protocols

Male BALB/c ($H\text{-}2^d$) mice of 6 to 8 weeks of age were used. The experiments were carried out under guidelines of the Institutional Committee for the Use and Care of Experimentation Animals (CICUAE-INTA protocol no. 22/2011). Mice were divided into 5 groups ($n = 5$ per group). One group (rMVA–P) received a prime of 1×10^7 PFU/mouse of rMVA intraperitonally (i.p) and 14 days after, a subcutaneous (s.c) boost of 50 μg/mouse of a protein cocktail (P). The cocktail comprised the recombinant proteins MSA-2c, RAP-1 and HSP20 in equal amounts in Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). A second group received the reverse regime (P–rMVA) at the same times. A third group was immunized twice with 50 μg/mouse of P in Freund's complete and incomplete adjuvant (P–P), respectively. The fourth group was immunized twice (at day 0 and 14) by i.p injection of 1×10^7 PFU/mouse of rMVA in 1 mL RPMI (rMVA–rMVA). Two control groups received either two doses of 1×10^7 PFU/mouse of wtMVA (wtMVA–wtMVA) or RPMI only (RPMI–RPMI). Serum samples were collected at days 0, 14 and 28 by tail bleeding and stored at −20 °C. Mice were sacrificed 14 days after the last immunization and spleen cells were obtained for IFNγ assays.

2.4. Antibody measurement, cytokine IFNγ production and intracytoplasmatic cytokine staining (ICS)

IgG levels in serum samples were measured by indirect ELISA using MSA-2c, RAP-1 or HSP20 recombinant proteins (2 ng/well). The absorbance was measured at a wavelength of 405 nm. Serum titers are expressed as the $\log_{10}$ of the reciprocal of the highest serum dilution that at least duplicates the optical density (OD) values obtained using negative (pre-immune) sera. Secreted IFNγ levels were assessed by commercial capture ELISA (OptEIA, Becton Dickinson Labware, Oxnard, CA, USA) following the manufacturer's instruction. Freshly isolated splenocytes plated in triplicate at 1×10^6 cells/well in U-bottom 96-well Maxisorp plates (Nunc), stimulated with a soluble *B. bovis* merozoite lysate (10 μg/mL). ConA (1 μg/mL, Sigma–Aldrich Inc.) or medium (RPMI 1640, Invitrogen) were used as positive or negative controls, respectively, and the supernatants were harvested after 72 h and stored at −80 °C until analyzed. For functional characterization, splenocytes were *in vitro* stimulated as above, except that Brefeldin A (2 μg/mL; Sigma-Aldrich Inc.) was added for the last 6 h to facilitate intracellular IFNγ accumulation. Then, the cells were washed twice with staining buffer and were subsequently incubated with anti-mouse $CD4^+$-FITC (GK1.5 clone) and anti-mouse $CD8a^+$-PE (53-6.7 clone) (Becton Dickinson). Then, cells where fixed in 2% formaldehyde, permeabilized in 0.5% saponin/1% FBS and stained intracellularly with anti-mouse IFNγ-APC antibody (XMG1.2clone). Flow cytometry was performed acquiring 50,000 events in a live lymphocyte gate, and further analyzed using Cell Quest software (Becton Dickinson).

2.5. Statistical analysis

Statistical analysis was carried out using GraphPad Prism (La Jolla, CA) version software 6.0 for Windows. One way ANOVA was used for comparing data with Bonferroni post test. Values of $p < 0.05$ were taken to be statistically significant.

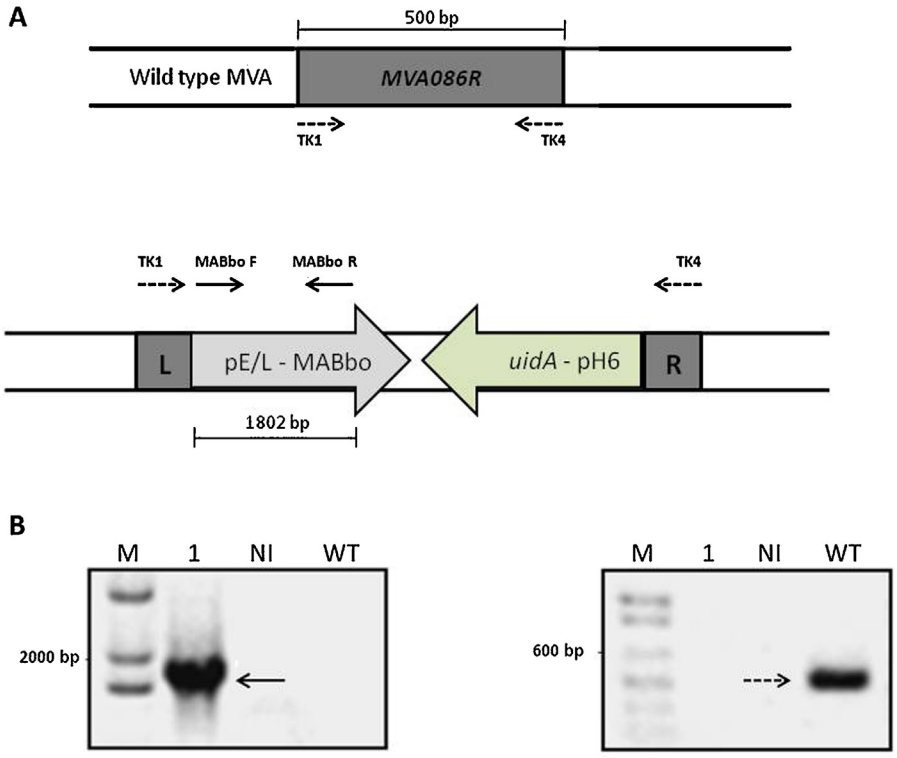

Fig. 1. *Characterization of rMVA.* (A) Schematic representation of the MVA086R gene of the wild type MVA genome (upper panel). In the lower panel, recombinant MVA genome showing the MABbo sequence, the poxviral synthetic early/late promoter (pE/L), the *uid A* gene for β-glucuronidase and the flanking sequences of the MVA086R gene (L and R). In both panels, arrows indicate primers used for PCR and expected sizes of products. (B) PCR using *MABbo* primers forward (F) and reverse (R) (left panel). The *TK*1 and *TK*4 primers were used to analyze the purity of rMVA (right panel). M: DNA size marker; Lane 1: amplification product of DNA from rMVA-infected chicken embryo fibroblasts (CEFs); NI: non-infected CEFs; WT: wtMVA-infected CEFs.

3. Results

3.1. Generation and in vitro characterization of rMVA containing the MABbo

The presence of the MABbo sequence in the rMVA genome and the purity of the recombinant viral stocks were confirmed by PCR (Fig. 1A). A single 1802-bp band corresponding to the expected size of MABbo was observed (Fig. 1B; left box). The stock purity was confirmed by the absence of a PCR product of 550 bp corresponding to the *MVA086R* gene (Fig. 1B; right box).

The ability of the rMVA to successfully express the MABbo was evaluated by confocal microscopy and Western blot (WB). A reactive band of approximately 72 kDa was only detected in the protein extracts from the rMVA-infected CEFs using antisera against the three *B. bovis* proteins (Fig. 2A). This band corresponds to the expected molecular size of the multi-antigen. The expression of MABbo was also evidenced in the cytoplasm of rMVA-infected cells by confocal microscopy using antibodies against HSP20, which is located in the C-terminus of the multi-antigen (Fig. 2B).

To verify if the rMVA-expressed MABbo would be recognized by antibodies present in *B. bovis* infected cattle, bovine sera from experimentally (lanes 1–4, Fig. 2C) or naturally (lanes 5–9, same figure) infected animals were tested by WB using lysates of rMVA-infected cells. All 9 sera positively recognized a protein band of 72 kDa corresponding to the size of MABbo.

3.2. Immunogenicity evaluation of rMVA alone or combined with the recombinant proteins of B. bovis

Firstly, we evaluated the obtained immunogens in a classical scheme of homologous prime-boost vaccination. In the group of mice immunized twice with the protein cocktail (P–P), specific IgGs were clearly detected at day 14 after the first immunization (Fig. 3A). Antibody titers continued increasing after the boost until the end of the experiment (day 28). HSP20 was the most immunogenic protein, with a titer of 12,800, followed by MSA-2c (6,400) and RAP-1 (1,600).

We also determined the amount of secreted IFNγ in splenocyte culture. Low levels of this cytokine were detected in mice immunized with this scheme, with no statistically significant differences in comparison with the control groups (Fig. 3B).

We further vaccinated a group of mice twice with rMVA at the same time points. As a result, a *B. bovis* specific cellular and humoral response was clearly detected 14 days after the boost (Fig. 3B and C). IgG antibodies against the three proteins were detected in this group. However, these IgG titers were similar to those observed for the mice immunized only once with the protein cocktail. A higher production of IFNγ ($p < 0.001$) was observed compared with the group which received two doses of P and control groups (wtMVA or RPMI).

We then evaluated the immunogenicity induced by heterologous prime–boost immunization schemes combining P and rMVA, alternating the order of administration. As shown in Fig. 4A, high levels of specific IgG were observed at day 28 in both vaccinations, comparable with titers acquired in the homologous P–P regime (Fig. 3A). However, priming with P and boosting with rMVA led to the production of higher levels of specific IgG2a for the 3 antigens (Fig. 4B). In general, this effect was observed in both heterologous immunizations suggesting a Th1 profile immune response induced by these schemes. With this in mind, we quantified the levels of IFNγ in splenocyte supernatants. As shown in Fig. 4C, both heterologous regimes induced high levels of this cytokine reaching the

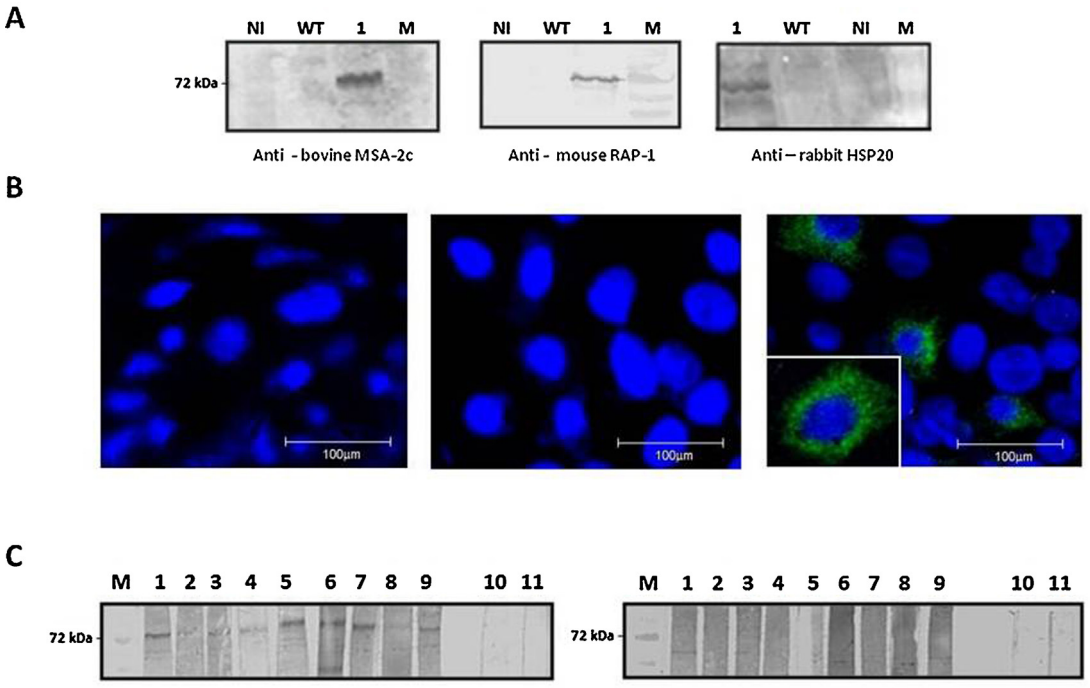

Fig. 2. *Expression analysis of the rMVA.* In figures throughout this study, 'rMVA', 'wtMVA' and 'P' are used in place of 'recombinant MVA', 'wild type MVA' and 'protein cocktail', respectively. (A) Western blot analysis of rMVA-infected CEFs lysate susing different polyclonal antisera (indicated below each figure). All sera reacted with a 72 kDa molecule corresponding to the size of the MABbo. M: Molecular weigth marker; lane 1: rMVA-infected CEFs; WT: wtMVA-infected CEFs; NI: non-infected CEFs. (B) IFA in PK15 cells uninfected (left), infected with wtMVA (middle) or infected with rMVA (right). (C) WB analysis of CEFs lysates infected with rMVA (left) or wtMVA (right) probed with sera from experimentally (lanes 1–4) or naturally (lanes 5–9) *B. bovis* infected cattle. Lanes 10–11 were probed with sera from non-infected cattle.

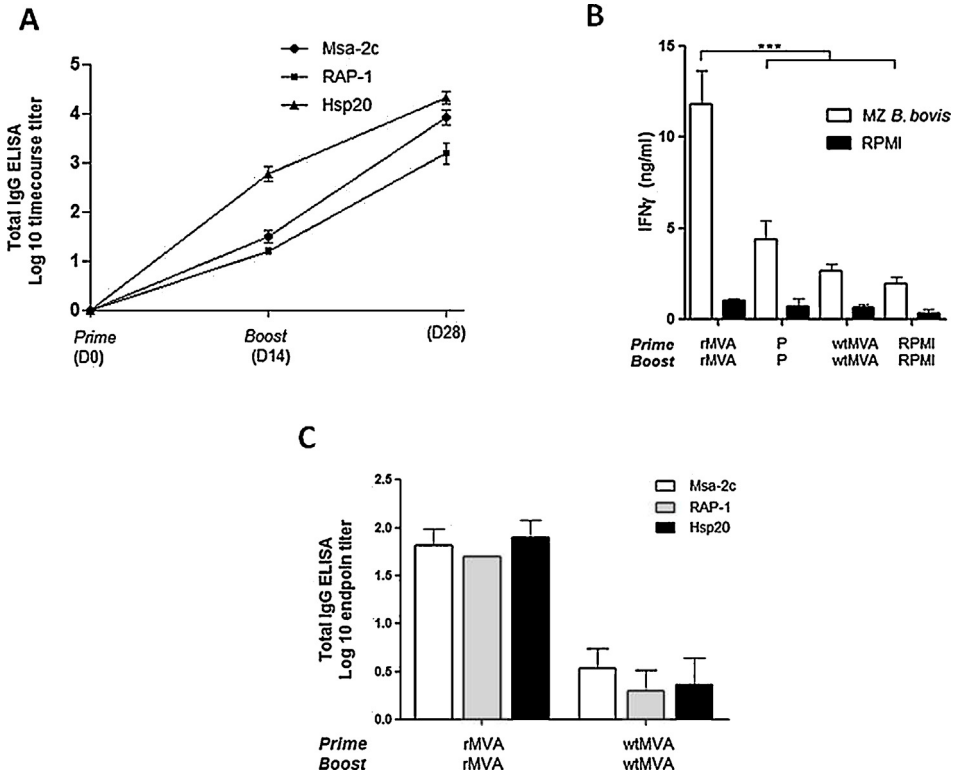

Fig. 3. *Immunogenicity of homologous prime-boost schemes with protein cocktail and rMVA.* In all cases BALB/c mice were immunized with 10^7 plaque forming units (PFU) of MVA, intraperitoneally (i.p.) and 50 μg of P, subcutaneously (s.c.). All mice receiving two vaccinations were primed at day 0 (D0) with a subsequent boost on day 14 (D14). Blood was obtained on D28 and sera were analyzed by indirect ELISA. Pre-immune sera were used as a negative control. (A). Time course kinetics of total IgG response to 'P–P' immunizations. (B) Levels of secreted IFNγ in culture supernatants determined by sandwich ELISA. ** $p < 0.05$ and *** $p < 0.001$ compared between the indicated groups. (C) Comparison of total IgG in mice vaccinated twice with rMVA or wtMVA by indirect ELISA using each of the three proteins contained in the chimeric multi-antigen.

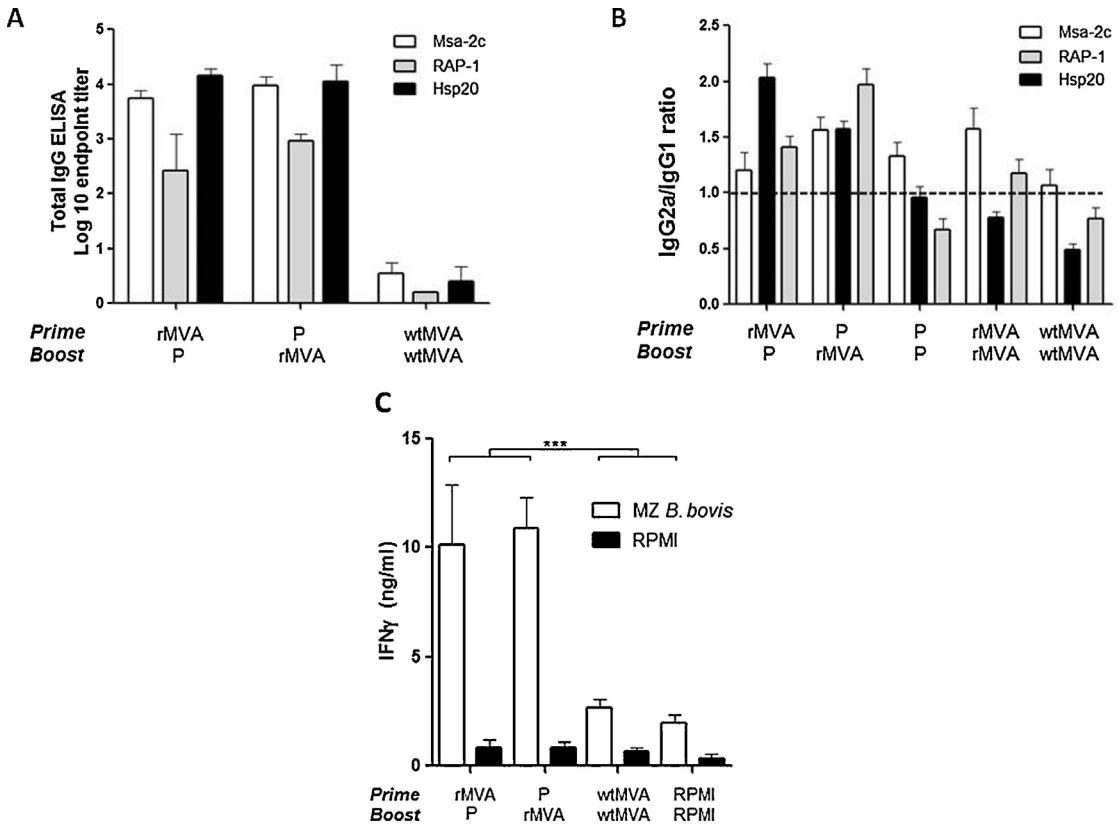

Fig. 4. *Immunogenicity of heterologous prime-boost schemes with protein cocktail and rMVA.* Vaccination regimes were as described in Fig. 3. (A) Total IgG response assessed by indirect ELISA using each of the three proteins contained in the chimera. (B) Comparison of IgG2a/IgG1 subclass ratio in homologous and heterologous prime-boost schemes at day 14 after boost. Dotted line indicates balanced ratio. (C) Levels of secreted IFNγ in culture supernatants determined by sandwich ELISA. Stimuli were as described in Fig. 3B. *** $p < 0.001$ compared between the indicated groups.

values observed when rMVA was used in the homologous regime (Fig. 3C).

3.3. $CD4^+$ and $CD8^+$ T cells response in prime-boost immunogenicity

To identify the source of IFNγ secreted by both homologous and heterologous regimes, we examined IFNγ production by T cells. As determined by flow cytometry, we found that mice immunized with P–rMVA displayed a higher percentage of $IFN\gamma^+$-producing $CD4^+$ and $CD8^+$ T cells than the other immunization groups ($p < 0.01$, ANOVA with Bonferroni post-test) (Fig. 5A and B). Minimal unspecific percentages of both $IFN\gamma^+$-secreting $CD4^+$ and $CD8^+$ T cells were detected in all experimental groups restimulated with RPMI.

The magnitude of the T-cell response can also be visualized by calculating the number of $IFN\gamma^+$-secreting $CD4^+$ and $CD8^+$ lymphocytes per million splenocytes (Fig. 5C and D). Very high frequencies of both antigen-specific T cell subpopulations (>2800 $IFN\gamma^+$ $CD4^+$ and >2100 $IFN\gamma^+$ $CD8^+$) were only reached in the P–rMVA group.

4. Discussion

The aim of this study was to develop new vaccine strategies against *B. bovis* by using rationally designed immunogens and different expression platforms to optimize immune responses at the humoral and cellular levels. In this sense, we have generated, characterized and studied the immunogenicity of a novel recombinant poxvirus that encodes a chimerical polyprotein. This polyprotein included selected portions of 3 antigens of *B. bovis* and was assessed in "prime-boost" schemes in the mice model. We selected epitope—containing fragments of three already well characterized antigens: RAP-1, MSA-2c and HSP20. All 3 proteins were reported as highly immunogenic and conserved among geographically distant strains [11,13,14]. In the case of RAP-1, only the N-terminal region (aa 21 to 236) was included in our chimera, since previous studies demonstrated that the predominant mouse $CD4^+$ T-cells response is directed to epitopes in this region [12]. Although the RAP-1 repetitive C-terminal domain contains B-cell epitopes that could add immunogenicity to the fragment, this domain was deliberately excluded in the construct because the repetitive sequences, although serologically immunodominant, may skew the antibody response away from other domains.

For HSP20, we selected the N-terminal region containing two T helper cell epitopes [19] based on a previous report that demonstrated that this is the most immunostimulatory region for cattle.

Only B-cell epitopes have been reported in MSA-2c [13] and two epitopes have been already characterized [20]. In our chimera, both peptide sequences were included which represent geographically conserved *B. bovis* B-cell epitopes.

One of the advantages of a poxvirus-based vaccine candidate is that it uses the eukaryotic cellular machinery to generate antigens with native conformation. Here, we have demonstrated that the MABbo polyprotein is adequately expressed in rMVA-infected cells and also showed that individual B-cell epitopes are conformationally conserved, since antibodies against MSA-2c, RAP-1 and HSP20 expressed in *Escherichia coli* are able to recognize the cellularly expressed MABbo. This result was further strengthened when antibodies against HSP20 expressed in bacteria (the third protein fragment in the chimera) detected MABbo in the cytoplasm of rMVA-infected PK15 cells.

The immunodominance of the MABbo polyprotein was confirmed when antibodies present in natural or experimentally

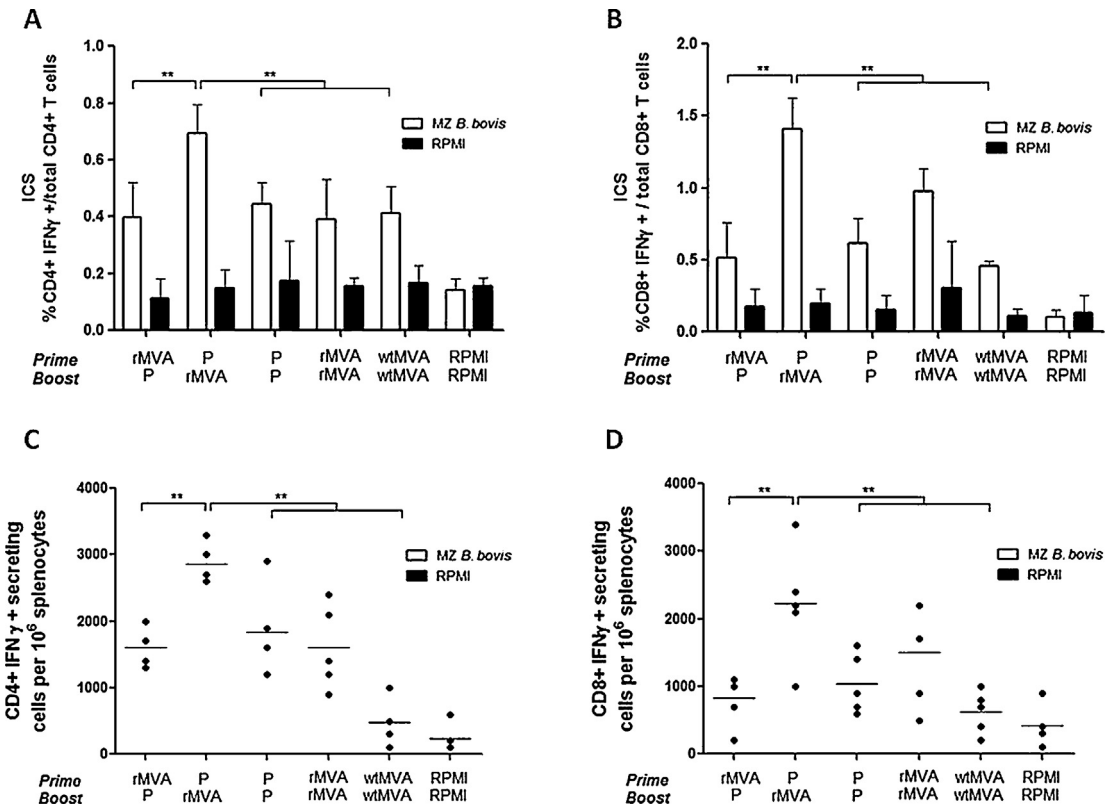

Fig. 5. *CD4⁺ and CD8⁺ T cells responses by homologous and heterologous prime-boost immunization.* ((A) and (B)). Frequency of IFNγ⁺ CD4⁺ or CD8⁺ T cells. Cells were labelled with anti CD4-FITC or anti-CD8-PE markers and stained intracellularly with an anti-IFNγ-APC antibody. In all cases, a minimum of 5×10^4 events was acquired. Results are representative of two independent experiments and are expressed as mean ± SD. The results were statistically significant at ** $p < 0.05$, with ANOVA and Bonferroni post test. ((C) and (D)). Numbers of antigen-specific IFNγ-secreting CD4⁺ and CD8⁺ T cells per 10^6 splenocytes calculated from data of (A) and (B). Total viable cells/spleen were calculated considering the number of viable lymphocytes obtained from FSC and SSC plots. The number of CD4⁺ or CD8⁺-IFNγ⁺ cells was calculated from this value and expressed per million splenocytes.

infected animals recognized a single band of 72 kDa corresponding to the expected size of MABbo. These results indicate that the antigenic portions selected for the chimerical construction kept the reported B-cell epitopes of the individual antigens. We cannot attribute to which of the 3 antigens the antibodies are directed but we hypothesize that they may recognize HSP20 and MSA-2c since previous evidence indicates that these proteins are probably the most immunodominant antigens [11,13].

We carried out a detailed characterization of the immunological responses in mice using homologous and heterologous prime-boost approaches combining a protein cocktail and rMVA. Although the mouse is not a biological model for *B. bovis*, the ease of its manipulation facilitates the screening of the immunogenicity of putative vaccine candidates. The immunization of mice with 2 doses of P elicited strong antibody titers against the 3 proteins. These results are in accordance with previous results [13,14,19,21] that showed that antibodies against the 3 selected antigens as well as T-cell responses against HSP20 and RAP-1 are found in *B. bovis* naturally-infected cattle.

In our experiments, HSP20 and MSA-2c were the most immunogenic proteins and elicited titers that were 10 fold-higher than those with RAP-1. The lower antigenicity of RAP-1 is probably due to the absence of the two B-cell epitopes in the C-terminal region in our construction.

Regarding the homologous immunizations with rMVA, the antibody responses against each individual protein followed the pattern shown by the protein cocktail, with RAP-1 as the less immunogenic antigen. Overall titers were lower than the ones obtained with the protein cocktail but the response against the virally expressed polyprotein was evidenced by the significant differences against wtMVA. This result agrees with recent studies reviewed by Draper et al. [22] showing that recombinant MVA vectors can induce antibodies against poxviral antigens as well as against the encoded transgene.

The importance of IFNγ in the immunity against *B. bovis* is well known since protection against clinical disease relies upon activation of memory and effector CD4⁺ T cells that secrete IFNγ and provide help for the production of protective antibodies [23]. In this study, we quantified the levels of IFNγ (a characteristic cytokine of the Th1 based response) to evaluate the cellular response. The homologous schemes showed remarkably high levels of this cytokine only in the mice that were immunized twice with rMVA. This effect could be attributed to any of the T-cell epitopes present in our chimera.

Considering the high antibody titers elicited by the protein cocktail and the induction of strong levels of IFNγ by rMVA, we sought to explore heterologous prime-boost regimes as to achieve the maximal response at both arms of the adaptive immune response. Either priming with rMVA and boosting with the protein cocktail or *viceversa*, induced similarly high antibody titers, comparable with those achieved by the P–P regime. In both cases the poxvirus functioned equally as a priming agent and also as an effective antibody-boosting platform after the protein priming. Although the mechanism by which the B cell induction by recombinant poxviruses remains elusive, recent evidence suggests that these vectors can be successfully used to prime and boost B cells for effective antibody responses [22].

The ratio of isotype subclasses IgG1 and IgG2a gives an indication of Th2 or Th1 bias of humoral responses, respectively. Characterization of IgG isotypes in homologous and heterologous

immunization schemes showed a clear bias toward a Th1 response with the highest IgG2a levels in both heterologous regimes but with a ratio >1.5 for the three proteins only in the P–rMVA regime. The importance of antibodies in bovine babesiosis was demonstrated in studies with bovines where passively administered immune serum or a mixture of IgG1 and IgG2 was protective against homologous *B. bovis* challenge [24]. The higher IgG2a/IgG1 ratio in both heterologous schemes is probably induced by augmented levels of secreted IFNγ· In our experiments both heterologous vaccinations showed high and similar levels of this cytokine. As expected, the inclusion of the protein cocktail in the heterologous scheme would not be of importance for the induction of cellular immunity since the amount of secreted IFNγ was similar to the rMVA–rMVA regime. Previous data showed similar amounts of secreted IFNγ when mice received a prime with DNA vaccine and a boost with a recombinant MVA expressing a *Leishmania major* antigen in a 28 days vaccination scheme [25].

Although both heterologous regimes showed considerable amounts of secreted IFNγ, characterization of the Th1 cell subpopulations showed that the P prime and rMVA boost was the only scheme that significantly stimulated antigen-specific IFNγ secreting $CD4^+$ and $CD8^+$ T cells. The order in which both immunogens are administered was critical because rMVA priming and protein boosting do not exert the same effect.

There are no reports about the involvement of $CD8^+$ T cells in the immune response against *B. bovis* and current knowledge indicates that these cells do not participate in the protective immune response. In this study, the presence of $CD8^+$-T cells secreting IFNγ is probably triggered by vaccination with rMVA which is well-know that elicit cytotoxicity response.

On the other hand, the role of $CD4^+$ cells in macrophage-mediated inhibition of *B. bovis* replication has been previously reported [23]. In our experiments, a high frequency of *B. bovis*-specific $CD4^+$ T cells (>2500/million splenocytes) secreted IFNγ in the P–rMVA scheme. Although the protective potential of such different quality $CD4^+$-T cells cannot be inferred from this murine immunogenicity model, the level of the response obtained with these combinations deserves in a depth characterization of this scheme in the bovine model.

In summary, we have shown that a three-protein cocktail and the rMVA used in prime-boost regimes are immunogenic for both $CD8^+$ and $CD4^+$ T cells generating promising levels of B and T cell-mediated immunity. The construction of the rMVA demonstrated the ease of building complex poly-epitope vaccines against antigenically complex parasites.

Future studies will determine how well these candidate vaccines will protect against bovine babesiosis in enzootic areas.

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Conflict of interest statement

No competing financial interest exists.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.06.075.

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