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Evaluation of different heterologous prime–boost immunization strategies against *Babesia bovis* using viral vectored and protein-adjuvant vaccines based on a chimeric multi-antigen

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ABSTRACT

Protection against the intraerythrocytic bovine parasite *Babesia bovis* requires both humoral and cellular immune responses. Therefore, tailored combinations of immunogens targeted at both arms of the immune system are strategies of choice to pursue sterilizing immunity. In this study, different heterologous prime–boost vaccination schemes were evaluated in mice to compare the immunogenicity induced by a recombinant adenovirus, a modified vaccinia Ankara vector or a subunit vaccine all expressing a chimeric multi-antigen. This multi-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). Both priming with the adenovirus or recombinant multi-antigen and boosting with the modified vaccinia Ankara vector achieved a high degree of activation of TNFα and IFNγ-secreting $CD4^+$ and $CD8^+$ specific T cells 60 days after the first immunization. High titers of specific IgG antibodies were also detected at the same time point and lasted up to day 120 of the first immunization. Only the adenovirus – MVA combination triggered a marked isotype skew for the IgG2a antibody subclass meanwhile for the other immune traits analyzed here, both vaccination schemes showed similar performances.

The immunological characterization in the murine model of these rationally designed immunogens led us to propose that adenoviruses as well as the bacterially expressed multi-antigen are highly reliable primer candidates to be considered in future experiments in cattle to test protection against bovine babesiosis.

1. Introduction

Bovine babesiosis is a tick – transmitted disease caused by protozoan parasites of the genus *Babesia*. Unlike related apicomplexan parasites of the genera *Plasmodium* and *Theileria*, *Babesia* parasites only infect erythrocytes. Infection of bovines with *Babesia* species occurs primarily in tropical and subtropical areas of the world where the Ixoxid tick vectors, predominantly *Riphicephalus* spp., are found [1]. In Argentina, economic losses due to mortality, costs of treatment and prevention of babesiosis caused by *B. bovis* and *B. bigemina* exceed US$ 38 million per year [2]. All babesial parasites cause anemia, but *Babesia bovis* cause the most virulent disease characterized by strong hemolytic anemia and a high morbidity and mortality in adult bovines [3]. Adaptive immunity against *B. bovis* is characterized by presentation of parasite antigens by antigen presenting cells to $CD4^+$ T lymphocytes as well as the production of neutralizing antibodies against extracellular merozoites and parasite surface antigens located at the erythrocyte membrane [3,4].

In Argentina and many countries, vaccination with attenuated parasite strains either chilled or frozen is one of the control measures currently used. However, major drawbacks of these vaccines are related with their high production costs, distribution logistics and the risk of contamination with pathogenic organisms during their production, especially when splenectomized calves are used. Therefore, there is an urgent need to replace these vaccines with new, safer and equally effective alternatives.

Non replicative viral vectors are actually one of the best vaccine platforms that induce cellular immunity as well as strong humoral responses, both of which are required to achieve protection against

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B. bovis [1,5]. One of these vectors is the non-replicating and highly attenuated modified vaccinia virus Ankara (MVA) which is actually one of the most promising vaccine vectors in human and veterinary vaccines [6,7]. Furthermore, immunogenicity studies have shown that MVA vectored vaccines are most potent when used in heterologous boost vaccination following a strong priming agent expressing the same antigen [8–10]. Other viral vectors that have gained interest among vaccine researchers as potent inducers of T-cell and antibody responses are recombinant adenoviruses (rAd) which have demonstrated immune efficacy and safety in clinical applications, making it an excellent choice of vector for delivery of vaccine antigens [11–14].

Previously, we have conducted pre-clinical studies in mice showing that a priming vaccination with a cocktail of three immunodominant *B. bovis* antigens (MSA-2c, RAP-1 and HSP20) as recombinant proteins followed by boosting with a recombinant MVA (rMVA) virus expressing a fusioned version of these three proteins as a chimeric multi-antigen triggers a strong humoral and cellular immune response. This response was characterized by high titers of specific IgG2a antibodies and high percentages of IFNγ secreting $CD4^+$ and $CD8^+$ – specific T cells upon specific stimulation [15].

Here we further test the hypothesis that the use of adenoviral vectors as antibody-triggering agents will improve the magnitude of humoral immune responses developed by protein in-adjuvant candidates keeping the rMVA as booster. We have also evaluated both schemes in a longer prime–boost time frame in order to optimize a future vaccine regime in bovines.

2. Materials and methods

2.1. Cells and culture media

Baby hamster kidney (BHK-21, ATCC CCL-10™) and Human embryonic kidney 293 [HEK293] (ATCC® CRL1573™) cells were maintained in Dulbecco's modified medium (D – MEM) supplemented with 2% fetal bovine serum (FBS; Internegocios, Buenos Aires, Argentina), 100 mM antibiotic/antimycotic (GIBCO), 100 mM L-glutamine (Sigma – Aldrich, St. Louis, MO, USA). Spleen cells for functional assays were cultured as described [15].

2.2. Generation of recombinant protein – rMABbo – and recombinant adenovirus, rAd

In order to obtain the recombinant protein – rMABbo – as a single chimeric polyprotein, PCR amplification was performed using the previously described MVA transfer vector as a template and the *MABbo F – MABbo R* primer pairs [15].

The PCR product was cloned using the pCR®8/GW/TOPO® TA Gateway cloning kit and the MABbo fragment was further transferred directionally into the pDEST™ 17 vector (both by Invitrogen Corp, Carlsbad, CA, USA). *E. coli* strain BL21 AI™ (Invitrogen) was used as a host for rMABbo expression and protein purification as described in Supplementary Data. The recombinant Surface Antigen 1 from *Neospora caninum*, SAG_1, used in this work as a non-related subunit vaccine control was purified as previously described [16]. Both SAG_1 and rMABbo purified proteins were subsequently treated with Polimixine B – Sepharose (BioRad) to remove endotoxins.

For the generation of the rAd, the *ViraPower Adenoviral Expression System™* (Invitrogen) was used following the manufactured instructions with minor modifications. Briefly, the MABbo fragment was directionally transferred from the pCR®8/GW/TOPO® TA into the pAd/CMV/V5-DEST vector (Invitrogen) using LR Clonase® (Invitrogen). This plasmid was purified using a standard phenol/chloroform extraction and digested with *Pac*I restriction enzyme (New England Biolabs, MA, USA) following manufacture's instruction. Five to 10 μg of purified plasmid was mixed with 18 μl of Lipofectamine 2000™ in 500 μl of Opti – MEM® media (both reagents from Invitrogen) and transfected into a 90% confluence HEK 293A cells cultured 24 h prior to use in 6 – well plate (Nunc). Eight hours after transfection, culture media was removed and replaced with D – MEM supplemented with 2% FBS. Visible regions of cytophatic effect were observed at 11–13 days post-transfection. Adenovirus – containing cells and media were harvested followed by three cycles of freeze/thawing and centrifugation at 2000*g* for 15 min in order to pellet the cell debris. To increase virus titers, the supernatant containing viral particles was used to infect fresh HEK 293A cells. Finally, the rAd was concentrated by ultracentrifugation through a 25% w/v sucrose cushion at 60,494*g* during 3 h at 4 °C and stored at −80 °C in NET buffer (10 mM Tris–HCl pH: 8, 1 mM EDTA, 200 mM NaCl) until use. The number of viral particles was determined by end-point dilution assay in HEK 293A cells. As a control, the pAd/CMV/V5-GW/lacZ vector (Invitrogen) was digested with *Pac*I and transfected into HEK 293A cells to produce lacZ bearing adenovirus.

In order to verify the MABbo expression by rAd-infected cells, a Western Blot (WB) using mouse anti-rMABbo and rabbit anti-HSP20 polyclonal antibodies was performed. Besides, an Indirect Immunofluorescence Assay (IFA) assay was performed in HEK 293 cells using an anti HSP20 polyclonal antiserum (Supplementary data).

2.3. Immunization protocols

Male BALB/c ($H\text{-}^{2d}$) mice of 6–8 weeks old were used ($n = 5$ per group). The time interval between prime and boost was 30 days in group I and 60 days in group II. Doses and routes *per* mouse were: 1×10^7 Plaque Forming Units (PFU) of rMVA or wtMVA intraperitoneally (i.p.); 1×10^9 PFU of rAd or wtAd intramuscularly (i.m.); 20 μg/mouse of rMABbo or $rSAG_1$ (as control) in Freund's complete adjuvant (Sigma–Aldrich) subcutaneously (s.c). Serum samples were collected by tail vein bleeding at day 0, 15, 30, 45, 60 for group I and once at day 120 for group II respectively and stored at −20 °C until use. Mice were sacrificed at day 60 or 120 (groups I and II respectively) and spleen cells were obtained for cellular immune response assays as previously described [15]. 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).

2.4. Antibody measurement, IFNγ cytokine detection and intracellular cytokine staining (ICS)

IgG, IgG1 and IgG2a levels in serum samples were measured by indirect ELISA using rMABbo (10 ng/well) as described previously [15]. 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 of total viable spleen cells were assessed by commercial capture ELISA (OptEIA, Becton Dickinson Labware – BD – Oxnard, CA, USA) following the manufacturer's instruction. Freshly isolated spleen cells were 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) or with rMABbo (10 μg/mL). Concanavalin A (1 μg/mL, Sigma–Aldrich) or RPMI 1640 medium (Invitrogen) were used as positive or negative controls respectively. Cell supernatants were harvested after 72 h of stimulation and stored at −80 °C until analyzed. For functional characterization, splenocytes were stimulated *in vitro* as above, except that Brefeldin A (2 μg/mL; Sigma–Aldrich Inc.) was added for the last 5 h to facilitate

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intracellular IFNγ and TNFα accumulation. Then, the cells were washed twice with staining buffer and were subsequently incubated with anti-mouse $CD4^+$ – FITC (GK1.5 clone; BD) and anti-mouse $CD8a^+$ – PE™ (53–6.7 clone; BD). Then, cells where fixed in 1% formaldehyde, permeabilized in 0.5% saponin/1% FBS and stained intracellularly with anti-mouse IFNγ – APC™ antibody (XMG1.2 clone) and rat anti-mouse TNFα – PerCP – Cy™ 5.5 (MP6-XT22 clone; BD). Flow cytometry analysis was performed in a FACS Calibur cytometer (BD) acquiring up to 350,000 events in a live lymphocyte gate and data was further analyzed using Cell Quest software (BD).

2.5. Statistical analysis

Statistical analysis was carried out using *Graph Pad Prism* (La Jolla, CA) software version 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.

3. Results

3.1. Full length expression of rMABbo in rAd-infected cells and E. coli

The expression of rMABbo was evidenced in the cytoplasm of the rAd – infected HEK 293A cells by IFA using antibodies against HSP20, which is the third antigenic fragment situated in the C – terminus region of the chimeric MABbo (Fig. 1A). The rMABbo expression by the virally infected cells was also confirmed by WB. A reactive band of approximately 72 kDa was only detected in the protein extracts from rAd – infected HEK 293A cells using mouse anti sera against the rMABbo (Fig. 1B). This band corresponds to the size of the full length chimeric multi-antigen.

Purification of the *E. coli* recombinant multi-antigen using denaturing conditions by Ni – agarose chromatography gave a high purification yield as shown in the SDS – PAGE analysis. The purified protein was obtained as a clear band corresponding to the expected molecular weight of 72 kDa (Fig. 1C, lane 3) in a concentration of 1.2 mg/mL.

The presence of the main immunoreactive epitopes in each of the 3 antigens that compose the rMABbo chimeric protein was confirmed by WB using antibodies against MSA-2c, RAP-1 and HSP20 respectively (Fig. 1D). To further verify if the chimeric multi-antigen maintained the B cell epitopes present in the native parasite proteins, we evaluated the ability of the polyprotein to be recognized by antibodies present in serum from naturally (lanes 1–6, Fig. 1E) and experimentally (lanes 7–9, Fig. 1E) *B. bovis* infected cattle. For this, bovine serum samples were tested by WB using purified rMABbo. All 9 sera positively recognized a protein band of 72 kDa corresponding to the expected size of the chimeric multi-antigen.

3.2. Immunogenicity evaluation combining viral – vectored immunogens and rMABbo protein

Firstly, we evaluated both humoral and cellular immune responses elicited in immunization protocols with an interval of 30 days between the prime and the boost. High and similar titers (>1:6400) of specific IgG antibodies for the rMABbo were detected in both regimes: rAd – rMVA and rMABbo – rMVA at 30 days post boost (Fig. 2A). We further characterized the antibody response by analyzing the IgG isotypes. Priming with rAd and boosting with rMVA induced a high ratio (>3) of IgG2a to IgG1 for the multi-antigen, compared with the rMABbo – rMVA scheme (Fig. 2B) suggesting a strong induction of Th1-biased response.

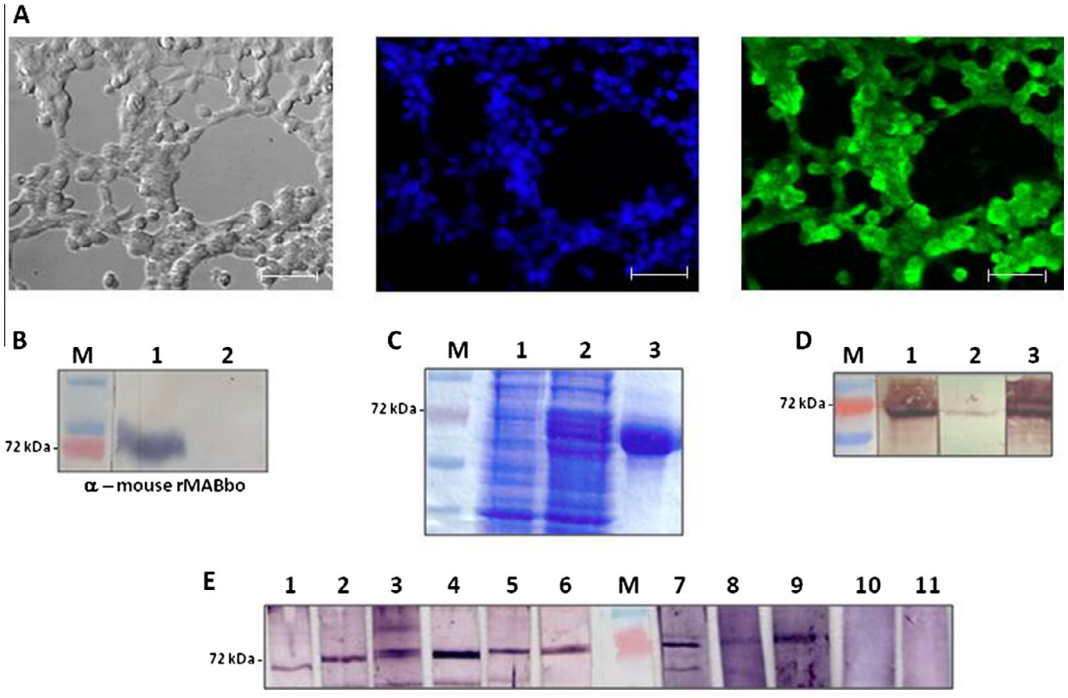

Fig. 1. Characterization of rAd and rMABbo immunogens. In figures throughout this work, 'rAd', 'wtAd' and 'rMABbo' refer to 'recombinant adenovirus' 'wild type adenovirus' and 'recombinant *B. bovis* multi-antigen' respectively. (A) IFA in HEK 293A cells infected with rAd (right). Middle panel: stained nuclei using TO-PRO®3-Iodide. Left panel: contrast resolution. White bars indicate 100 μm. (B) Western blot analysis (WB) of rAd – infected HEK 293A cells using polyclonal anti rMABbo serum. M: molecular weight marker; lane 1: rAd – infected cells; lane 2: wtAd – infected cells. (C) 12% SDS-PAGE analysis showing expression or rMABbo. Lane 1: uninduced control; lane 2: level of expression 4 hs post-induction with 0, 2% arabinose; lane 3: purified rMABbo after elution with 250 mM Imidazole. (D) WB analysis of purified rMABbo using bovine MSA-2c (lane 1), mouse anti RAP-1 (lane 2) or rabbit anti HSP20 (lane 3) polyclonal sera. (E) WB analysis of purified rMABbo with sera from experimentally (lanes 1–6) or naturally (lanes 7–9) *B. bovis* infected cattle. Lanes 10 and 11 were probed with sera from non-infected cattle.

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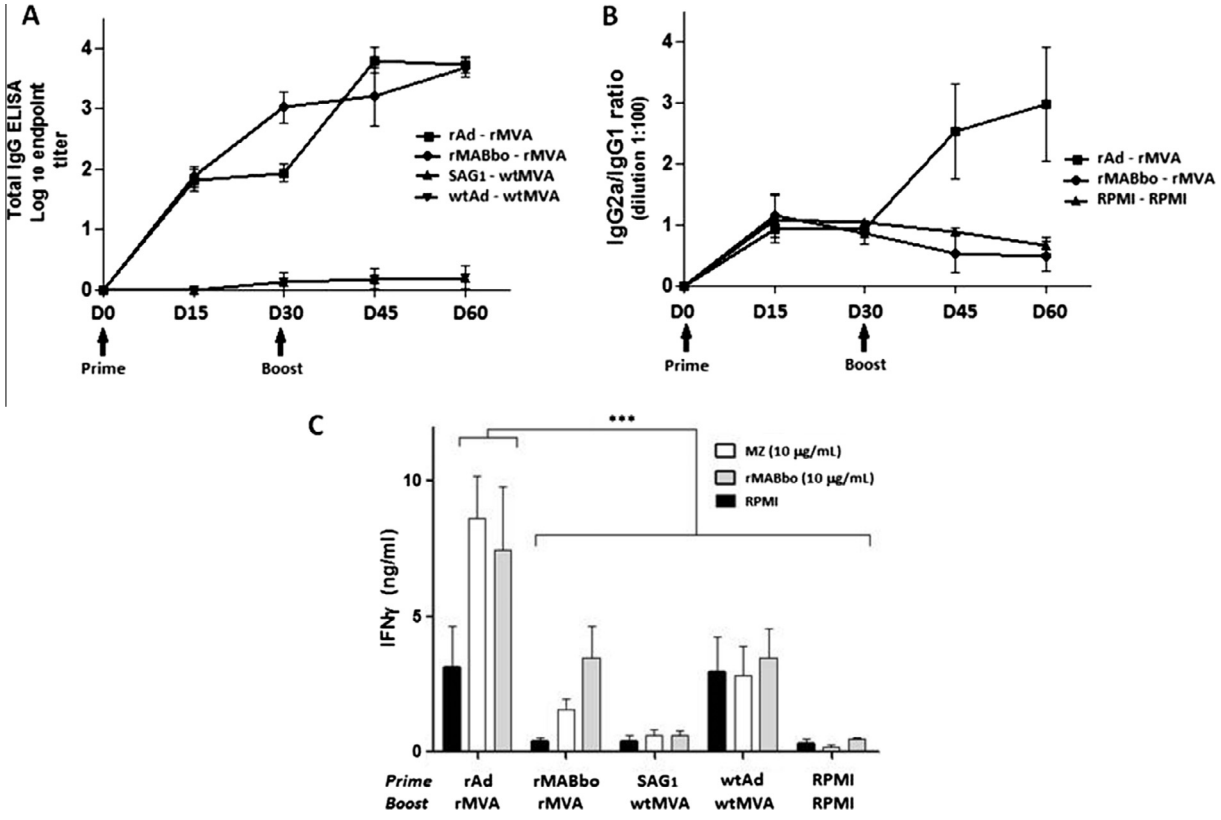

Fig. 2. Immune response evaluation of prime–boost regimes. In all cases BALB/c mice were immunized with 10^7 PFU of rMVA, i.p. route, 10^9 PFU of Ad, i.m. route and 20 μg of rMABbo, s.c. route. All mice received a prime at day 0 with a subsequent boost on day 30. Blood was collected each 15 days gap, and sera sample were obtained for indirect ELISA assays. Pre immune sera were used as negative control. Mice were sacrificed at day 60, spleen cells were obtained for *in vitro* restimulation and levels of IFNγ were determined by capture ELISA. (A) Time course kinetics of total IgG response using rMABbo as antigen. (B) Comparison of IgG2a/IgG1 subclass ratio in prime–boost schemes. (C) Levels of secreted IFNγ in culture supernatants determined by sandwich ELISA. ***$p < 0.001$ compared between the indicated groups.

We also determined the amount of secreted IFNγ by antigen-stimulated splenocytes from immunized mice. A significant three-fold increase of this cytokine ($p < 0.001$) was detected in supernatants from the rAd – rMVA group compared with rMABbo – rMVA and control groups, when specific stimuli were used (Fig. 2C).

We further characterized the profile of the Th1 response by identifying the source of 2 main cytokines of this response: IFNγ and TNFα. As determined by intracellular cytokine staining and flow cytometry, we observed that both heterologous regimes triggered a nearly fourfold increase of the percentages of $CD4^+$ T cells secreting either IFNγ and TNFα in comparison with control groups ($p < 0.001$ one-way ANOVA with Bonferroni post-test; Fig. 3A and B). Minimal unspecific percentages of $CD4^+$ T cells secreting these cytokines were detected in control groups restimulated with RPMI.

The magnitude of the T – cell response can also be visualized by calculating the number of antigen-specific $IFN\gamma^+$ and $TNF\alpha^+$ secreting $CD4^+$ T cells – per million splenocytes. Similar numbers of IFNγ and TNFα antigen-specific $CD4^+$ T cell subpopulations were reached between rAd – rMVA and rMABbo – rMVA regimes (Fig. 3C and D).

In addition, we performed the detection of polyfunctional T cells producing both $IFN\gamma^+$ and $TNF\alpha^+$ by intracellular cytokine staining. As shown in Fig. 4A, both heterologous regimes showed high percentages of double-stained $CD4^+$ T cells in comparison with the control groups ($p < 0.001$ one-way ANOVA with Bonferroni post-test). As expected, the number of double-stained T $CD4^+$ positive cells was lower than the single-stained ones (Fig. 4B). Similarly, frequencies of single cytokine secreting $CD8^+$ T cells ($IFN\gamma^+$ and $TNF\alpha^+$) were similar between both immunization groups ($p < 0.001$ one-way ANOVA with Bonferroni post-test, Figs. S1 and S2).

3.3. Humoral and cellular responses in different times for prime–boost regimes

In order to determine if the interval of prime and boost would alter the induction of humoral and cellular immune responses, another group of mice was immunized with the same vaccine schemes but at a longer interval (60 days between prime and boost instead of 30). Results of both groups are summarized in Table 1.

Antibody titers at the end of the experiment maintained their values independently of the immunization interval in either the rMABbo – MVA scheme or the rAd – rMVA one. Total secreted IFNγ was almost the same in both immunization schemes at day 120 in group II, showing low levels of this cytokine. On the other hand, in group I at day 60 high levels of IFNγ were found in the rAd – rMVA scheme (Table 1). Regarding frequencies of antigen – specific $CD4^+$ – $IFN\gamma^+$ T cells, values at day 120 in both groups were drastically reduced compared with their counterparts at day 60 (Table 1).

4. Discussion

With worldwide distribution of the *Ixoxidae* family tick vectors, babesiosis is the second most common blood-borne disease of free living animals [17]. Climate change may only be partly responsible for the change in distribution of ticks but other local factors i.e. new technology applied to crop production that has displaced cattle industry towards tropical zones in the north of Argentina, have

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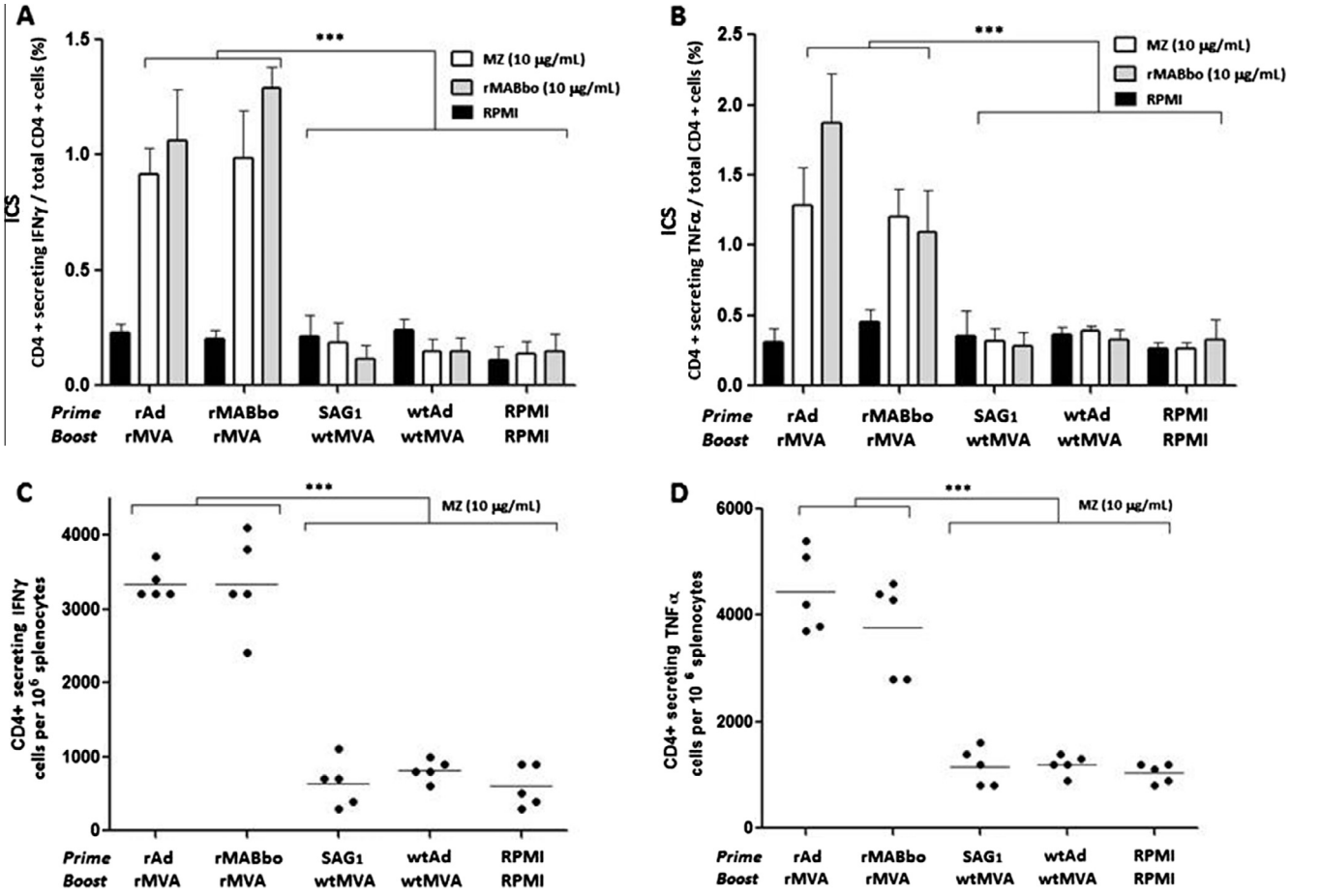

Fig. 3. $CD4^+$ T cells responses by heterologous prime–boost immunization. (A) and (B) Frequency of $IFN\gamma^+$ – $CD4^+$ or $TNF\alpha^+$ – $CD4^+$ T cells. Cells were labeled with anti CD4 – FITC markers and stained intracellularly with an anti IFNγ–APC and TNFα–PerCyP 5.5 antibodies. In all cases, a minimum of 350,000 events was acquired. Results are expressed as mean ± SD. Unless noted, results indicated with asterisks were statistically significant $^{***}p < 0.001$ with one-way ANOVA and Bonferroni post-test. (C) and (D) Numbers of antigen-specific $IFN\gamma^+$ – secreting $CD4^+$ or TNFα-secreting $CD4^+$ 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 $CD4^+$ – IFNγ or $CD4^+$ – $TNF\alpha^+$ cells were calculated from this value and expressed *per* million splenocytes.

also be taken into account. In this complex scenario, there is an urgent need for effective and safer recombinant vaccines against bovine tick-borne pathogens such as *Babesia* spp. but development has proved unsuccessful so far.

Viral vectors have been established as an extremely safe and efficient system for many vaccine developments in human research and also became suitable for industrial scale production. Thus, there is an obvious usefulness of these systems for novel vaccine approaches also in veterinary medicine.

In previous studies we have shown that an heterologous delivery regimen of a prime with a cocktail of three *B. bovis* recombinant antigens (MSA-2c, RAP-1 and HSP20) and a boost with rMVA induced a strong activation of $CD4^+$ – $IFN\gamma^+$ antigen – specific T cells and IgG2a antibodies the murine model [15]. The murine model was used to evaluate immunogenicity of different prime–boost schemes because the ease of its manipulation facilitates the screening of putative vaccine candidates before their evaluation in cattle.

In the present study, using the same model, we primarily sought to assess whether the priming immunization with a novel recombinant adenovirus carrying the MABbo multiantigenic sequence could enhance the humoral immune response in comparison with the previously used subunit – protein cocktail. In this case and for practical reasons, we have cloned and expressed the rMABbo antigen as a single polyprotein instead of the 3 separate antigens used before.

As a first characterization of both new immunogens obtained here, we have confirmed the rMABbo expression in the cytoplasm of rAd – infected HEK 293A cells using antibodies against HSP20 (the third protein fragment in the chimera) indicating the complete expression of the whole polyprotein after viral infection. We have previously demonstrated the expression and conservation of B – cell epitopes of rMABbo in rMVA infected cells [15].

Regarding the rMABbo protein, epitope conservation of the 3 protein fragments that compose this multi-antigen was confirmed when antibodies against individual MSA-2c, RAP-1 or HSP20 proteins recognized a unique band of 72 kDa corresponding to the expected size of the full polyprotein. This result confirms that immunodominant B – cell epitopes of the individual antigens kept a proper folding and remained exposed even after the 3 gene fragments were fused to yield a single polyprotein. To further confirm this result, antibodies present in sera from natural or experimentally *B. bovis* infected bovines recognized the same single band of 72 kDa in Western blot indicating that B – cell epitopes present in *B. bovis* merozoites are maintained in the proper conformation in the chimeric recombinant antigen. In addition to this, the rMABbo could be expressed and purified with high yields, allowing an adequate degree of purity for further uses.

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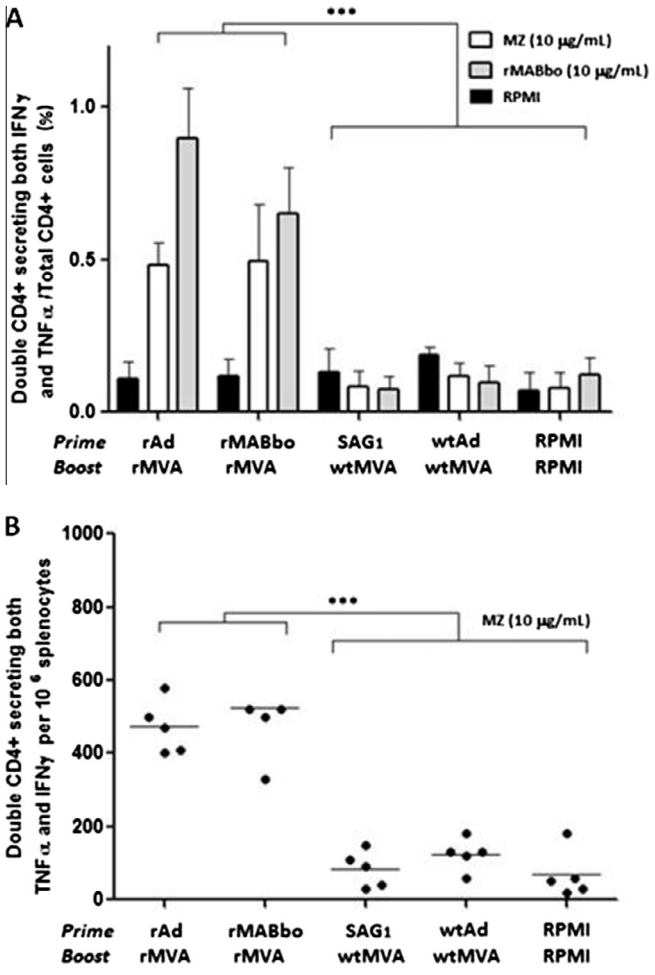

Fig. 4. $CD4^+$ T cells producing both $INF\gamma^+$ and $TNF\alpha^+$. (A) Frequency of $CD4^+$ T cells positive for the two cytokines 30 days after final immunization. (B) Numbers of antigen-specific $IFN\gamma^+$ – TNFα secreting $CD4^+$ T cells per 10^6 splenocytes calculated from data of (A).

In the immunization experiments, our results showed that the differences between both priming agents were only in the proportion of specific IgG2a isotypes and the amount of IFNγ secreted by spleen cells only in group I which was boosted 30 days after priming. The higher amount of secreted IFNγ and the marked bias against the IgG2a antibody subclass in the rAd – rMVA scheme could be related to the fact that IFNγ is involved in the murine IgG2a class switch by B cells during pathogen elimination [18–20]. This significant enhancement of the IgG2a antibody subclass production only in the viral vector scheme is encouraging since previous experiences with subunit vaccines against *B. bovis* in bovines that failed to protect against challenge showed a balanced response between both IgG subclasses [21,22].

There are some previous reports on the neutralizing ability of antibodies against each antigen that compose our chimeric polyprotein. Murine and bovine antibodies directed against a recombinant version of MSA-2c, significantly neutralized *B. bovis* [23,24]. In the case of RAP-1, Norimine et al. have demonstrated that bovine antibodies against the recombinant antigen did not have any effect on merozoite replication [25]. Regarding HSP20, there are no reports about the neutralization capacity of antibodies against this antigen.

With regard to the characterization of T cell populations that secreted Th1 cytokines, our results showed that neither the frequency of $CD4^+$ and $CD8^+$ – T cells secreting only – IFNγ or TNFα nor polyfunctional cells secreting both cytokines showed significant differences between the two heterologous vaccination schemes used here.

Likewise, relative frequencies of mono and/or polyfunctional $CD8^+$ T cells *per* million splenocytes were similar to their $CD4^+$ counterparts. This balanced $CD4^+/CD8^+$ T cell response was also reported for adenoviral – MVA vaccines against malaria in primates and humans [26,27].

In bovine babesiosis, control of infection is mediated by destruction of infected erythrocytes by splenic macrophages and by neutralizing antibodies directed against extracellular merozoites. Both of these immune mechanisms depend on $CD4^+$ T cells [1]. In this sense, relative frequencies of mono and polyfunctional $CD4^+$ T cells secreting Th1 cytokines obtained in both vaccination schemes (between 0.5% and 1.8%) are encouraging with values similar or even higher than what was previously reported for other experimental vaccines using Ad – MVA coding for *Plasmodium* antigens that showed promising efficacy after challenge [26,28]. In murine leishmaniasis and human malaria, the proportions of $CD4^+$ T cells that are polyfunctional for IFNγ, TNFα, and IL-2 have been found to be a marker of the protective efficacy of the $CD4^+$ T cell response [29,30].

Even though *B. bovis* is host-specific for bovines, the magnitude and quality of the $CD4^+$ response obtained by both vaccine schemes in mice is promising for testing our candidates in the bovine model. Correlations in terms of immunoresponsiveness between both models are difficult to pose. For example, in contrast with reports in bovines, in murine babesiosis, cellular immunity but not gamma interferon is essential for resolution of *Babesia microti* infection in BALB/c mice [31]. Other report shows an increased susceptibility to murine babesiosis with age similarly to what is observed in human or bovine babesiosis [32].

Previous attempts of vaccination against *B. bovis* in cattle using protein-in-adjuvant formulations have been disappointing [21,22,33]. Improvements in vaccine protection are clearly necessary as well as the identification and optimization of a protective immunological response.

Vaccination and challenge experiments in bovines followed by detailed immunological characterization of mono and polyfunctional antigen-specific T cells may allow to determine the threshold level needed for significant efficacy.

In conclusion we have provided a detailed description of the immunogenicity of adenovirus, poxvirus and adjuvant – protein vaccination regimes in the murine model. The results obtained in this study led us to propose that adenoviruses as well as the

Table 1
Intervals comparison of both prime–boost immunization schemes. Animals from group I were sacrificed at day 60 and animals from group II were sacrificed at day 120, for endpoint time IgG ELISA and cellular immune response assays. R: IgG2a/IgG1 ratio.

Intervals between prime and boost	rAd – rMVA				rMABbo – rMVA			
	Log 10 IgG titers	R	IFNγ (ng/mL)	% $CD4^+$ – IFNγ	Log 10 IgG titers	R	IFNγ (ng/mL)	% $CD4^+$ – IFNγ
Group I: 30 days	3.5	3	8***	1	3.5	1	2	0.9
Group II: 60 days	4	1.5	2.5	0.002	3.5	1	2.1	0.0017

*** $p < 0.001$ with one-way ANOVA and Bonferroni post-test.

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bacterially expressed multi-antigen are highly reliable primer candidates that induce high percentages of $CD4^+$ and $CD8^+$ T cells with a Th1 cytokine profile. Both priming agents showed similar responses and deserve to be considered for future experiments in cattle including challenge with *B. bovis* parasites, since bovines are the only biological model to test protection against bovine babesiosis.

Conflict of interest statement

José Manuel Jaramillo Ortiz and Silvina Elizabeth Wilkowsky are named inventors on patent application covering the sequence of chimeric multi-antigen including in the viral vectors, subunit-vaccines and immunization regimes. Act No. 20150101988 – R/No. 3263. Instituto Nacional de la Propiedad Industrial, Argentina.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.05.053.

References

[1] Brown WC, Norimine J, Knowles DP, Goff WL. Immune control of Babesia bovis infection. Vet Parasitol 2006;138:75–87.
[2] Späth EJA, Guglielmone AA, Signorini AR, Mangold Atilio. Estimating of direct economic losses caused by the tick Boophilus microplus and associated diseases in Argentina. Therios 1994;23:116–9.
[3] Brown WC, Palmer GH. Designing blood-stage vaccines against Babesia bovis and B. bigemina. Parasitol Today (Personal Ed) 1999;15:275–81.
[4] Florin-christensen M, Suarez CE, Rodriguez AE, Flores DA, Schnittger L. Vaccines against bovine babesiosis : where we are now and possible roads ahead. Parasitol Today 2014:1563–92.
[5] Draper SJ, Cottingham MG, Gilbert S. Utilizing poxviral vectored vaccines for antibody induction-progress and prospects. Vaccine 2013;39:4223–30.
[6] Draper SJ, Heeney JL. Viruses as vaccine vectors for infectious diseases and cancer. Nat Rev Microbiol 2010;8:62–73.
[7] Sánchez-Sampedro L, Perdiguero B, Mejías-Pérez E, García-Arriaza J, Di Pilato M, Esteban M. The evolution of poxvirus vaccines. Viruses 2015;7:1726–803.
[8] Sheehy SH, Duncan CJ, Elias SC, Choudhary P, Biswas S, Halstead FD, et al. ChAd63-MVA-vectored blood-stage malaria vaccines targeting MSP1 and AMA1: assessment of efficacy against mosquito bite challenge in humans. Mol Ther: J Am Soc Gene Ther 2012;20:2355–68.
[9] Sheehy SH, Duncan CJ, Elias SC, Collins K, Ewer KJ, Spencer AJ, et al. Phase Ia clinical evaluation of the Plasmodium falciparum blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors. Mol Ther: J Am Soc Gene Ther 2011;19:2269–76.
[10] Sheehy SH, Duncan CJ, Elias SC, Biswas S, Collins K, O'Hara G, et al. Phase Ia clinical evaluation of the safety and immunogenicity of the Plasmodium falciparum blood-stage antigen AMA1 in ChAd63 and MVA vaccine vectors. PLoS One 2012;7:e31208.
[11] Coughlan L, Mullarkey C, Gilbert S. Adenoviral vectors as novel vaccines for influenza. J Pharm Pharmacol 2015;67:382–99.
[12] Tan WG, Jin H-T, West EE, Penaloza-MacMaster P, Wieland A, Zilliox MJ, et al. Comparative analysis of simian immunodeficiency virus gag-specific effector and memory CD8+ T cells induced by different adenovirus vectors. J Virol 2013;87:1359–72.
[13] Penaloza-MacMaster P, Provine NM, Ra J, Borducchi EN, McNally A, Simmons NL, et al. Alternative serotype adenovirus vaccine vectors elicit memory T cells with enhanced anamnestic capacity compared to Ad5 vectors. J Virol 2013;87:1373–84.
[14] Russell WC. Update on adenovirus and its vectors. J Gen Virol 2000;81:2573–604.
[15] Jaramillo Ortiz JM, Del Médico M, Zanetti F, Molinari M, Gravisaco M, Calamante G, et al. Vaccine strategies against Babesia bovis based on prime-boost immunizations in mice with modified vaccinia Ankara vector and recombinant proteins. Vaccine 2014;32:4625–32.
[16] Wilkowsky SE, Bareiro GG, Mon ML, Moore DP, Caspe G, Campero C, et al. An applied printing immunoassay with recombinant Nc-SAG1 for detection of antibodies to Neospora caninum in cattle. J Vet Diagn Invest 2011;5:971–6.
[17] Hunfeld KP, Hildebrandt A, Gray JS. Babesiosis: recent insights into an ancient disease. Int J Parasitol 2008;38:1219–37.
[18] Bossie A, Vitteta ES. IFN-y enhances secretion of IgG2a from IgG2a-committed LPS-stimulated murine 6 cells : implications for the role of IFN-7 in class switching. Cell Immunol 1991;104:95–104.
[19] Waki S, Uehara S, Kanbe K, Nariuch H, Suzuki M. Interferon-gamma and the induction of protective IgG2a anti bodies in non-lethal Plasmodium berghei infections of mice. Parasite Immunol 1995:503–8.
[20] Huang S, Hendriks W, Althage A, Hemmi S, Bluethmann H, Kamijo R, et al. Immune response in mice that lack the interferon-y receptor. Science 1991;991:21–4.
[21] Hines SA, Palmer GH, Jasmer DP, Goff WL, Mcelwain TF, Hines SA, et al. Immunization of cattle with recombinant babesia bovis merozoite surface antigen-1. Infect Immun 1995;63(1):349–52.
[22] Antonio Alvarez J, Lopez U, Rojas C, Borgonio VM, Sanchez V, Castañeda R, et al. Immunization of Bos taurus steers with Babesia bovis recombinant antigens MSA-1, MSA-2c and 12D3. Transboundary Emerging Dis 2010;57(1–2):87–90.
[23] Dominguez M, Echaide I, Torioni de Echaide S, Mosqueda J, Cetrá B, Suarez CE, et al. In silico predicted conserved B-cell epitopes in the merozoite surface antigen-2 family of B. bovis are neutralization sensitive. Vet Parasitol 2010;167:216–26.
[24] Wilkowsky SE, Farber M, Echaide I, Torioni de Echaide S, Zamorano P, Dominguez M, et al. Babesia bovis merozoite surface protein-2c (MSA-2c) contains highly immunogenic, conserved B-cell epitopes that elicit neutralization-sensitive antibodies in cattle. Mol Biochem Parasitol 2003;127:133–41.
[25] Norimine J, Suarez CE, Mcelwain TF, Florin-christensen M, Brown WC. Immunodominant epitopes in Babesia bovis rhoptry-associated protein 1 that elicit memory CD4 (+) -T-Lymphocyte responses in B. bovis-immune individuals are located in the amino-terminal domain. Infect Immun 2002;70:2039–48.
[26] Capone S, Reyes-Sandoval A, Naddeo M, Siani L, Ammendola V, Rollier CS, et al. Immune responses against a liver-stage malaria antigen induced by simian adenoviral vector AdCh63 and MVA prime-boost immunisation in non-human primates. Vaccine 2010;29:256–65.
[27] De Barra E, Hodgson SH, Ewer KJ, Bliss CM, Hennigan K, Collins A, et al. A phase Ia study to assess the safety and immunogenicity of new malaria vaccine candidates ChAd63 CS administered alone and with MVA CS. PLoS One 2014;9:e115161.
[28] Goodman AL, Epp C, Moss D, Holder AA, Wilson JM, Gao GP, et al. New candidate vaccines against blood-stage Plasmodium falciparum malaria: prime-boost immunization regimens incorporating human and simian adenoviral vectors and poxviral vectors expressing an optimized antigen based on merozoite surface protein 1. Infect Immun 2010;78:4601–12.
[29] Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJ, van Gemert GJ, et al. Protection against a malaria challenge by sporozoite inoculation. New Engl J Med Original 2009:468–77.
[30] Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 2007;7:843–50.
[31] Clawson ML, Paciorkowski N, Rajan TV, La Vake C, Pope C, La Vake M, et al. Cellular immunity, but not gamma interferon, is essential for resolution of Babesia microti infection in BALB/c mice. Infect Immun 2002;70(9):5304–6.
[32] Habicht GS, Benach JL, Leichtling KD, Gocinski BL, Coleman JL. The effect of age on the infection and immunoresponsiveness of mice to *Babesia microti*. Mech Ageing Dev 1983;23(3–4):357–69.
[33] Fish L, Leibovich B, Krigel Y, McElwain T, Shkap V. Vaccination of cattle against *B. bovis* infection with live attenuated parasites and non-viable immunogens. Vaccine 2008;26(Suppl 6):G29–33.