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Evaluation of the immune response to *Anaplasma marginale* MSP5 protein using a HSV-1 amplicon vector system or recombinant protein

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ABSTRACT

Anaplasma marginale is an intraerythrocytic vector-borne infectious agent of cattle. Immunization with the current vaccine, based on parasitized erythrocytes with live *Anaplasma centrale*, shows some constraints and confers partial protection, suggesting the feasibility for the development of new generation of vaccines. The aim of the present study was to assess the effect of sequential immunization of BALB/c mice, with herpesvirus amplicon vector-based vaccines combined with protein-based vaccines, on the quality of the immune response against the major surface protein 5 of *A. marginale*. The highest antibody titers against MSP5 were elicited in mice that received two doses of adjuvanted recombinant protein ($p < 0.0001$). Mice treated with a heterologous prime-boost strategy generated sustained antibody titers at least up to 200 days, and a higher specific cellular response. The results presented here showed that sequential immunization with HSV-based vectors and purified antigen enhances the quality of the immune response against *A. marginale*.

1. Introduction

Anaplasmosis is a relevant vector-borne hemoparasitic rickettsial disease of ruminant livestock, caused by the intraerythrocytic rickettsia *Anaplasma marginale* (Dumler et al., 2001), in tropical and subtropical regions of the world, including South America (Brayton et al., 2009; Guglielmone, 1995; Suarez and Noh, 2011). It is transmitted either biologically by ixodid ticks or mechanically through needles and biting flies. The replication of the rickettsia in erythrocytes of infected cattle, results in anemia, weight loss, abortions, and eventually death. Infection is characterized by sequential cycles of rickettsemia, which occurs as a logarithmic increase in the parasite population, followed by a dramatic decline. Each cycle reflects the emergence of a new generation of bacteria expressing variants of the major surface protein 2 (MSP2) (French et al., 1999).

In cattle that survive clinical disease, the microorganisms persist indefinitely after infection, although at low levels, acting as reservoirs for further transmission (Aubry and Geale, 2011; Kuttler et al., 1984; Vidotto et al., 1994), and remaining protected against subsequent homologous challenge (Aubry and Geale, 2011).

The vaccine against bovine anaplasmosis, currently used in several countries, is based on live *A. centrale*, multiplied in splenectomized calves. Although one dose of this vaccine confers immunity for several years, it has the drawbacks of requiring exhaustive quality control to prevent the presence of other blood-borne organisms in the donor calves and the induction of only partial protection from challenge with virulent *A. marginale*.

New generation vaccines, such as those based on recombinant DNA methodologies, are not available yet for anaplasmosis control (OIE, 2012). Nevertheless, it has been reported that cattle immunized with an outer membrane fraction of *A. marginale* were protected from clinical disease following homologous challenge (Noh et al., 2008; Tebele et al., 1991), and that antibody titers against some members of outer membrane proteins, correlated with protection against anemia (Vidotto et al., 1994).

Several immunization strategies have been experimentally evaluated to protect cattle against anaplasmosis (Brown et al., 1998; McGuire et al., 1994; Tebele et al., 1991); however neither one prevents cattle from becoming persistently infected with *A. marginale*, as reviewed elsewhere (Aubry and Geale, 2011; Kocan et al., 2003).

Despite the advances in vaccine development, further studies are needed in order to evaluate the combination of novel antigen-delivery systems in the improvement of protection of cattle from *A. marginale* infection, and in blocking its biological transmission. Viral vectors represent an interesting delivery tool for DNA vaccines, which have not been extensively studied for anaplasmosis.

In this regard, HSV-1 amplicon vectors have a large transgene capacity (up to 150 kb), which allows the encapsidation of multiple genes or multiple copies of a transgene. Additional advantages of HSV-1 derived vectors include (i) low toxicity and low immunogenicity, in particular when helper virus-free amplicon vectors are used, (ii) high transduction efficiencies both in quiescent and

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proliferating cells from most mammalian species, including antigen-presenting cells in vivo, (iii) genetic stability, and (iv) strong adjuvant effects, very long-lived immune responses, and the capacity of generating both humoral and cellular immune response and mucosal immunity (Cuchet et al., 2007; De Silva and Bowers, 2009; Epstein, 2009; Hocknell et al., 2002; Kaur et al., 2007; Laimbacher and Fraefel, 2014).

In this study, genetic vaccines based on HSV-1 amplicon vectors are explored as vehicles to deliver DNA encoding *A. marginale* Major Surface Protein 5 (MSP5) to assess the immune response generated in mice, in homologous viral vector vaccination, or in combination with recombinant protein.

2. Materials and methods

2.1. Cells and viruses

Vero, MA104, Vero-7b (Krisky et al., 1998; Marconi et al., 1996) and human glioma Gli36 (Kashima et al., 1995) cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 μg/ml). Vero-7b cell line provides the essential ICP4 and ICP27 immediate-early (IE) herpesvirus gene functions *in trans*, to support the in vitro propagation of replication defective HSV-1-LaLΔJ virus (Zaupa et al., 2003), used as helper in the production of the amplicon vectors. For culturing Vero-7b cell line, Geneticin® (500 μg/ml, Invitrogen, CA, Carlsbad, USA) was included in the medium.

2.2. Construction of amplicon plasmids

Amplicon DNA plasmids were constructed using the *pHSVs* plasmid system, as previously described (D'Antuono et al., 2010). The main genetic elements of *pHSVs* are: (i) a transcription unit including the HSV-1 IE 4/5 promoter to support transgene expression and an IRES element followed by EGFP as reporter gene for titration; (ii) a prokaryotic origin of DNA replication (*colE1*) and the ampicillin resistance gene (*AmpR*), to allow the propagation of the plasmids in *E. coli*; (iii) two functional HSV sequences (*ori* and *pac*) that support replication of the amplicon DNA and the packaging into HSV-1 particles.

The *msp5* open reading frame (Acc. num.: M93392.1) was PCR amplified from recombinant plasmid *pGEM/AmMSP5* using specific primers (sense: 5'-*GTCGAC*ATGAGAATTTTCAAGATTGT-3' and antisense: 5'-*TTCGAA*CTAAGAATTAAGCATGTGAC-3'). The resulting product was inserted into the *Sal*I and *Bst*BI restriction sites of $pHSV_S$, generating *pHSV/MSP5* amplicon plasmid. An amplicon vector expressing the EGFP gene, *pHSV-EGFP* (D'Antuono et al., 2010), was used as negative control in immunofluorescence assays and immunization experiments. *pHSV-EGFP* possesses a transcription unit composed by the HSV-1 IE4/5 promoter, the EGFP coding sequence, and the SV40 early region polyadenylation site.

2.3. Stocks of amplicon vectors

In order to generate stocks of amplicon vectors, the amplicon plasmid DNA was transfected into T75 flasks of 70% confluent Vero-7b cells, using Lipofectamine™ and Plus™ Reagent (Invitrogen), following manufacturer's instructions. Transfected cells were infected the next day with HSV-1-LaLΔJ helper virus, at a multiplicity of infection (MOI) of 0.3 PFU/cell in medium 199 (M199, Invitrogen) supplemented with 1% FBS. When total cytopathic effect was observed, cells were scraped off and centrifuged at 2000 × *g* 5 min at 4 °C and the pellets were resuspended in 400 μl of M199 1% FBS and subjected to three cycles of freezing/thawing. The suspended pellets were sonicated on ice for 10 s, and clarified at 3000 × *g*, 10 min at 4 °C. Released amplicon vectors were passaged once in Vero-7b cells, by adding HSV-1-LaLΔJ at an MOI of 0.1 helper virus per cell. Titers of helper virus in viral stocks were determined by plaque assay on Vero-7b cells (PFU/ml) and amplicon vectors titers were assessed on Gli36 cells by scoring the number of GFP positive cells under a fluorescence microscope, and expressed as Transducing Units per milliliter (TU/ml).

2.4. Immunofluorescence analysis

For immunofluorescence analysis, Vero or MA104 cells were seeded on round 12-mm cover glasses placed in 24-well plates at a density of 10^5 cells per well. After 24 h, the monolayers were washed with PBS, and infected at a MOI of 0.1 TU/cell with amplicon vectors diluted in DMEM. The particles were allowed to adsorb for 1 h at 37 °C, 5% CO_2, washed with PBS, overlaid with DMEM supplemented with 10% FBS, and incubated at 37 °C, 5% CO_2. At 24 h post infection (hpi), the cells were washed once with cold PBS, fixed with 3.7% paraformaldehyde in PBS at room temperature (RT) for 15 min, and neutralized for 5 min with 100 mM glycine in PBS. After being washed with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min at RT, and blocked with PBS supplemented with 3% bovine serum albumin (PBS-B) for 30 min at RT. Fixed cells were first incubated during 1 h in a humid chamber with the anti-MSP5 monoclonal antibody (MAb) ANAF16C1 (VMRD, Inc.) 2 μg/ml in PBS-B. After three washes with PBS, the cells were incubated, with a rabbit anti-mouse IgG Alexa Fluor 594-conjugated secondary antibody (Molecular Probes), diluted at 2 μg/ml in PBS-B buffer. All incubations were carried out at RT. Cell nuclei were stained with 300 nM DAPI (4',6'-diamidino-2-phenylindole; Invitrogen) in PBS for 5 min. The monolayers were washed three times with PBS, once with distilled water, and mounted using FluorSave™ Reagent (CalBiochem®) to preserve the fluorescence-labeled samples. Images were acquired using a Nikon Eclipse E600 microscope equipped with the Nikon DS-Fi1 camera. Images were processed with ImageJ software (Schneider et al., 2012).

2.5. Expression of recombinant MSP5 protein

Two different recombinant MSP5 proteins were produced for different purposes: MSP5-Maltose Binding Protein (MBP) fusion and MSP5-6xHis (H_6) tagged. To obtain the recombinant proteins, *msp5* open reading frame was amplified by PCR (sense: 5'-*GGATCC* ATGAGAATTTTCAAGATTGT-3' and antisense: 5'-*CTGCAG*CTAAGA ATTAAGCATGTGAC-3') and subcloned into the *BamH*I and *Pst*I sites of *pMAL-c2x* (New England Biolabs, Inc., Ipswich, MA, USA) or *pRSET* (Invitrogen) plasmids, respectively.

The recombinant plasmid *pMAL/MSP5* was used to transform *Escherichia coli* Top10 (Invitrogen), and the rMSP5-MBP fusion protein produced was purified by affinity chromatography on an amylose resin (New England Biolabs, Inc., MA, USA), as described elsewhere (Knowles et al., 1996). This protein was used for animal immunization, and a competitive inhibition enzyme-linked immunosorbent assay (cELISA).

For lymphoproliferation and total antibody ELISA assay, the recombinant MSP5 protein with a 6xHistidine tag was used (MSP5-H_6). For this purpose, the *pRSET/MSP5* plasmid was used to transform *E. coli* BL21(DE3)pLysS (Invitrogen Corporation), and the recombinant MSP5-H_6 protein produced was purified by nickel metal affinity chromatography. Briefly, 3 ml of an overnight culture was inoculated into 300 ml of LB medium with 100 μg of ampicillin and 34 μg of chloramphenicol per milliliter, and grown at 37 °C to 0.5 $OD600_{nm}$. At that point, the expression of rMSP5-H_6 was induced by the addition of 1 mM of isopropyl-β-D-thiogalactopyranoside. The bacteria were incubated at 37 °C for 3 additional hours, and harvested by centrifugation at 3500 × *g* for 20 min and the pellet was

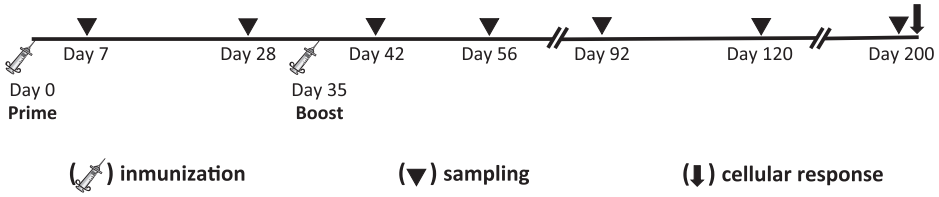

Fig. 1. Schematic representation of the experimental design. BALB/c mice (six animals per group) were immunized twice with 35 days interval using the different regimens shown in Table 1. At day 200 post-vaccination, mice were killed and splenocytes harvested for the analysis of cellular immune response (↓). Blood samples were collected at the indicated times (♦).

suspended in 30 ml of binding buffer (20 mM sodium phosphate buffer, 500 mM NaCl, 25 mM imidazole, pH = 7.8). Cells were disrupted by sonication in an ice-chilled water bath (2.5 min in 30-s pulse, 90 W), treated with DNAse (5 μg/ml) and RNAse (5 μg/ml) 20 min at 4 °C. Clarified cell extract was prepared by centrifugation at 12000 × *g*, 4 °C, and filtered through a 0.45 μm filter. The purification process was carried out in a Fast Performance Liquid Chromatography ÄKTA purifier system, using a 5 ml HisTrapFF column (GE Healthcare Bio-Sciences, Uppsala, Sweden), following an adapted protocol from a previous report (Zimmerman et al., 2006). Aliquots of isolated recombinant proteins were collected from the fractions across the major peak, purity assessed by SDS-PAGE, concentration by Bradford assay (Bio-Rad, Hercules, CA, USA), and protein identity was determined by Western Blot using a MAb anti-polyHistidine antibody (Sigma-Aldrich, St. Louis, MO, USA).

2.6. Experimental animals

Five-weeks-old BALB/c mice were obtained from the animal facilities of Pablo Cassará Laboratory, and maintained in animal Biosafety Level-2 facilities. Prior to immunization, mice were bled and sera were collected for subsequent ELISA. Five groups (G) of six mice were immunized according to the schedule described in Fig. 1. For immunizations using recombinant protein, mice received an intramuscular (IM) injection of 10 μg of rMSP5-MBP, formulated with Montanide™ISA50 (Seppic S. A., Paris, France), prepared according to the manufacturer's instructions. For immunizations using packaged amplicon particles, mice received 1×10^6 TU via the IM route (Table 1). Booster immunizations were administered 5 weeks after priming, and mice were sacrificed at 23 weeks following the boost. Blood samples were collected 1 week after each injection and at different time points during a period of 4 months (Table 1), and the obtained sera stored at –20 °C until required. The animals were maintained in accordance with federal guidelines and institutional policies.

2.7. Analysis of antibody responses

The humoral immune responses were evaluated by measuring total specific IgG by standard indirect ELISA using rMSP5 protein, and epitope specific IgG using cELISA, in plates coated with rMSP5-MBP fusion protein.

The cELISA with MSP5-MBP was modified from a previously described assay used to detect antibody against MSP5 in *A. marginale* infected cattle (Knowles et al., 1996). Briefly, purified rMSP5-MBP was serially diluted in coating buffer (0.05 M carbonate/bicarbonate, pH 9.6) and incubated in microtiter plates. The appropriate antigen dilution was determined through a checkerboard titration of rMSP5-MBP and MAb ANAF16C1. The mix, in PBS pH 7.2 containing 20 mM $MgCl_2$, was used to coat individual wells of flat-bottomed 96-well plates (Greiner-BioOne, Frickenhausen, Germany) for 1 h at 37 °C. Coated plates were blocked with 100 μl per well of coating buffer, supplemented with 10% of non-fat dry milk, for 1 h at 28 °C. Blocked plates were washed five times with PBS-T (0.01 M PBS containing 0.02% Tween 20). Mice sera were diluted 1:100 in PBS/0.075 M EDTA-EGTA (pH 6.3) with 10% non-fat milk and incubated and 100 μl samples were added to wells, incubated for 1 h at 28 °C, and washed five times with PBS-T. Horse-radish peroxidase-conjugated MAb ANAF16C1 was added at 0.2 μg per well, incubated for 1 h at 28 °C. Following washing, bound MAb was detected by addition of 100 μl of 3% H_2O_2/0.04 MABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] substrate, the reaction was terminated with 2N H_2SO_4, and optical density at 405 nm was recorded with an ELISA reader, after 30 min of reaction.

2.8. Lymphocyte proliferation assay

For proliferation assay of lymphocytes from immunized mice, spleen cells were harvested at sacrifice, cultured in 100 μl of RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, in 96-well plates (10^6 cells per well), and stimulated with rMSP5-H_6 (12.5 μg/ml) or ConA (2.5 μg/ml) for 3 days at 37 °C, in a 5% CO_2 incubator. The in vitro cell proliferation was measured by scintillation counting, when pulsed, during the last 20 h of the experiment, with 1 μCi of [*methyl*-^{3}H]-thymidine. Proliferative responses were calculated as the mean of triplicate wells and expressed as a Stimulation Index (SI) calculated as mean counts per min in the presence of the antigen/mean counts per min in the absence of antigen. The SI per group was expressed as the arithmetic mean ± standard error of the mean (SEM).

2.9. Statistical analysis

Data analyses and graphs were performed using GraphPad Prism v6.0 (GraphPad Software, La Jolla, CA, USA) or IBM SPSS Statistics software v17.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed for homogeneity of variances using Levene's test for equality, and for normal distribution using Shapiro–Wilk test. Mean group differences were analyzed using Tukey test *post hoc* the one-way analysis of variance. Data were expressed as mean ± SEM and differences were considered significant for $p < 0.05$.

Table 1
Mice immunization groups.

Groups ($n = 6$)	Prime (day 0)	Boost (day 35)	Adjuvant	Dose
1	rMSP5	rMSP5	ISA50*	10 μg/10 μg
2	rMSP5	HSV[MSP5]	ISA50/–	10 μg/10^6TU
3	HSV[MSP5]	rMSP5	–/ISA50	10^6TU/10 μg
4	HSV[MSP5]	HSV[MSP5]	–/–	10^6/10^6 TU
5	HSV[GFP]	HSV[GFP]	–/–	10^6/10^6 TU

rMSP5: recombinant MSP5-MBP fusion protein; HSV[MSP5]: herpesvirus amplicon vector expressing MSP5; HSV[GFP]: control herpesvirus amplicon vector expresing GFP; ISA50: Montanide™ ISA50; TU: Transducing Units.

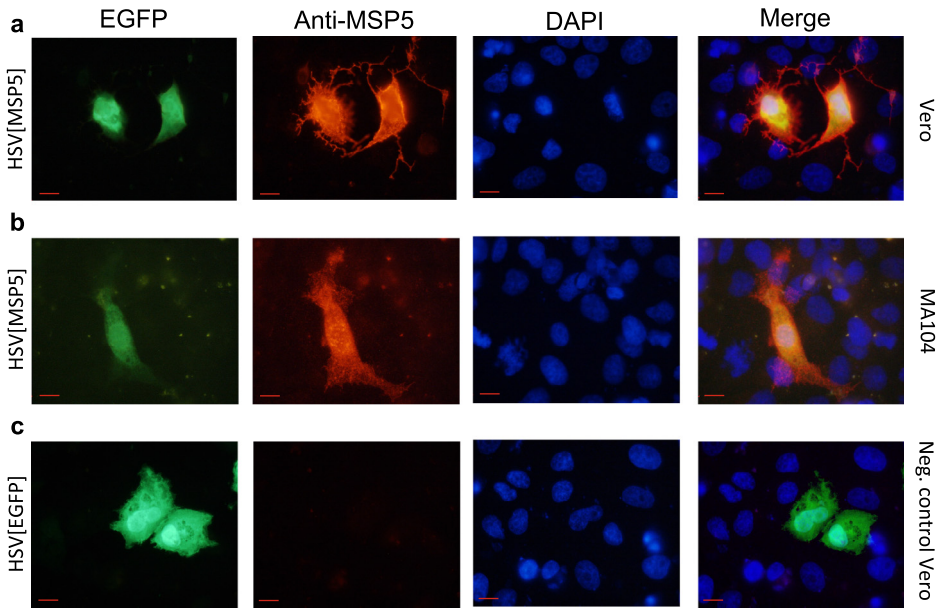

Fig. 2. Intracellular expression of *A. marginale* MSP5 and GFP proteins driven by HSV-1 derived amplicons. Vero and MA104 cells, were transduced with HSV[MSP5] (a) and (b), respectively, or HSV[GFP] amplicons (c), as indicated at the left of the panels. Different cell treatments are indicated at the top of the panel. Nuclei DNA was stained with DAPI. GFP fluorescence was observed under the microscope. Permeabilized cells were probed with MAb ANAF16C in order to detect expression of MSP5. The right panels are merged images and bars represent 10 μm.

3. Results

3.1. Characterization of amplicon vectors

Vero and MA104 cell monolayers infected with HSV[MSP5] were stained with the MAb ANAF16C1, after 24 hpi, and vector-mediated MSP5 expression was observed under a fluorescence microscopy (Fig. 2a and b, respectively). The infected cells showed a cell membrane pattern very different from control-infected cells (Fig. 2c), indicating that the MSP5 over expression would affect the cell morphology. EGFP expression was used to identify vector-infected cells. Cells infected with HSV[EGFP] did not show immunoreactivity with the MAb ANAF16C1 (Fig. 2c).

3.2. Expression of rMSP5-H_6 and rMBP-MSP5

The recombinant protein rMSP5-H_6 was obtained in bacterial cultures in both soluble and insoluble fractions, while rMBP-MSP5 was expressed mainly in the soluble fraction. Both recombinant proteins migrated in SDS-PAGE close to their expected molecular weight, 27 and 66 kDa, respectively (Fig. 3, panels a and b).

The chimeric proteins were purified by affinity chromatography with amylose resin (rMBP-MSP5, Fig. 3a) and nickel-sepharose resin (rMSP5-H_6, Fig. 3b), and the final yield was of 2.29 and 0.072 g/l, respectively. Endotoxins were removed as previously described (Zimmerman et al., 2006). The rMSP5-MBP protein was used for mice immunization (Groups 1–3, Table 1) and for antibody

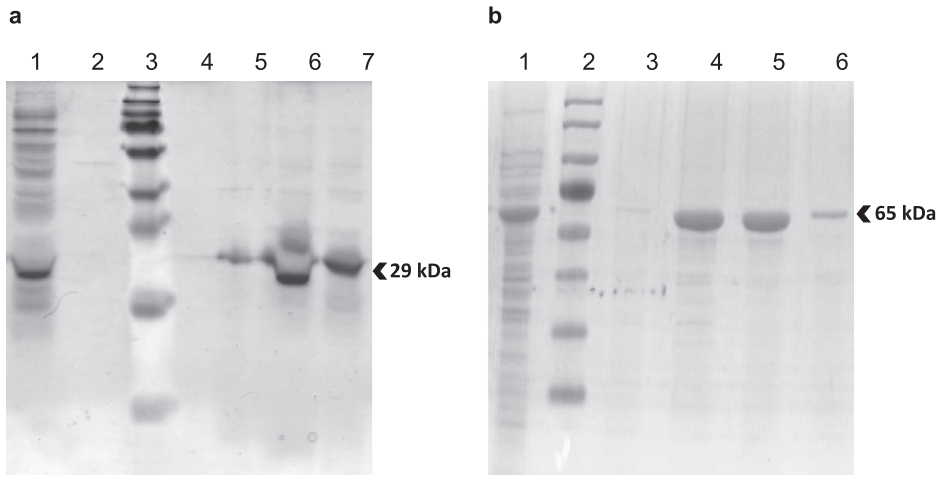

Fig. 3. Expression and purification of rMSP5-H_6 and rMSP5-MBP proteins. (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified rMSP5-H_6 protein expressed in bacteria. Lane 1, *E. coli* BL21(DE3)pLysS [pRSET/AmMSP5] induced culture, 4 h after induction; lane 2, flow through; lane 3, molecular weight markers (PAGE-Ruler, Fermentas); lane 4, column wash; lanes 5–6, eluted fractions 8, 11 and 12, respectively. (b) SDS-PAGE of purified rMSP5-MBP protein expressed in bacteria. Lane 1, *E. coli* BL21(DE3)pLysS [pMAL-c2X/AmMSP5] induced culture, 4 h after induction; lane 2, molecular weight markers (PAGE-Ruler, Fermentas); lane 3, column wash; lanes 4–7, eluted fractions 1–4, respectively. Arrowheads indicate recombinant rMSP5-H_6 and rMSP5-MBP proteins.

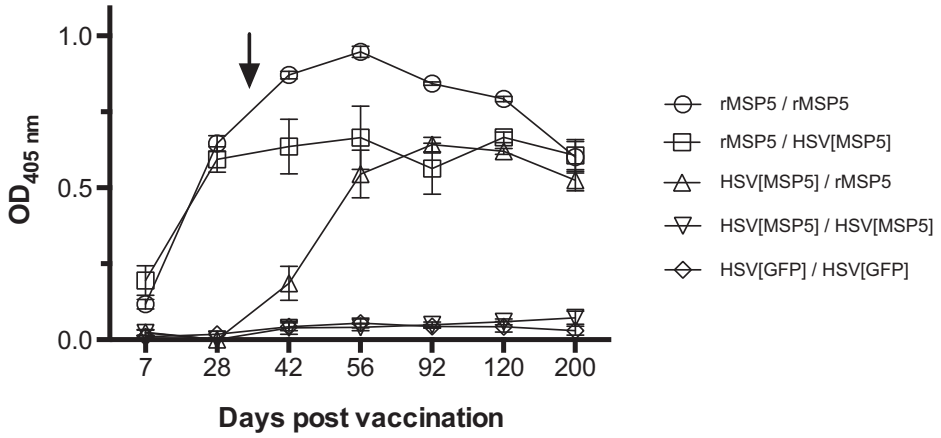

Fig. 4. Antibody responses elicited in mice after administration of HSV[MSP5] and/or rMSP5-MBP. Specific antibodies were measured by a competitive ELISA. Days post-vaccination are shown on the *X*-axis. Data represent the mean of OD_{405nm} reached in each group of mice at the different time points. Error bars show the standard errors of the mean. Groups: rMSP5/rMSP5 (○); rMSP5/HSV[MSP5] (□); HSV[MSP5]/rMSP5 (△); HSV[MSP5]/HSV[MSP5] (▽); HSV[GFP]/HSV[GFP] (◇).

quantification by ELISA, and rMSP5-H_6 protein was used in lymphoproliferation assays.

3.3. Antibody response to MSP5

Vaccination with 10 μg of oil adjuvanted rMBP-MSP5 rapidly raised a specific antibody response at 28 dpv (OD = 0.646). Following a boost with the same antigen at 35 dpv, it reached the maximum level at 56 dpv (OD = 0.947, Fig. 4). A booster immunization with 10^6 TU of HSV[MSP5] (Group 2, Table 1) did not increase the antibody levels, but titers were maintained up to 200 dpv (OD = 0.606). Priming with the HSV[MSP5] (Group 3) did not induce detectable antibody titers at 28 dpv, although a fast increase in specific antibodies was observed after boosting the animals with purified rMSP5 (OD = 0.525 at 200 dpv, Fig. 4). At the end of the experiment, 200 days after the prime inoculation, there were no significant differences in the levels of antibody titers elicited with two doses of purified protein, or a prime with the amplicon vector followed by a boost of protein ($p = 0.839$) or the reverse sequence regimen ($p = 0.999$, Groups 2 and 3). Homologous prime-and-boost with the amplicon vector alone (Group 4) did not elicit detectable antibodies and was indistinguishable from the negative control group (Group 5) inoculated with HSV[GFP] ($p = 0.502$).

3.4. Lymphoproliferation assays

Lymphoproliferation assays were carried out at 200 dpv to monitor the cellular response after stimulation of splenocytes cultures from mice of each group with purified rMSP5-H_6 or ConA. SI calculated from groups of mice that received one or two doses of amplicon vectors were higher than that achieved in the group vaccinated with two doses of purified protein (Fig. 5). The highest SI came out from the group immunized with a heterologous prime-boost schedule, which was primed with purified protein and followed by inoculation on 10^6 TU of the amplicon vector expressing MSP5 *in situ* ($p = 0.01$).

4. Discussion

It has been shown that protective immunity against *A. marginale* requires the presence of humoral and cellular responses, with antibodies directed to outer membrane epitopes, and a coordinated

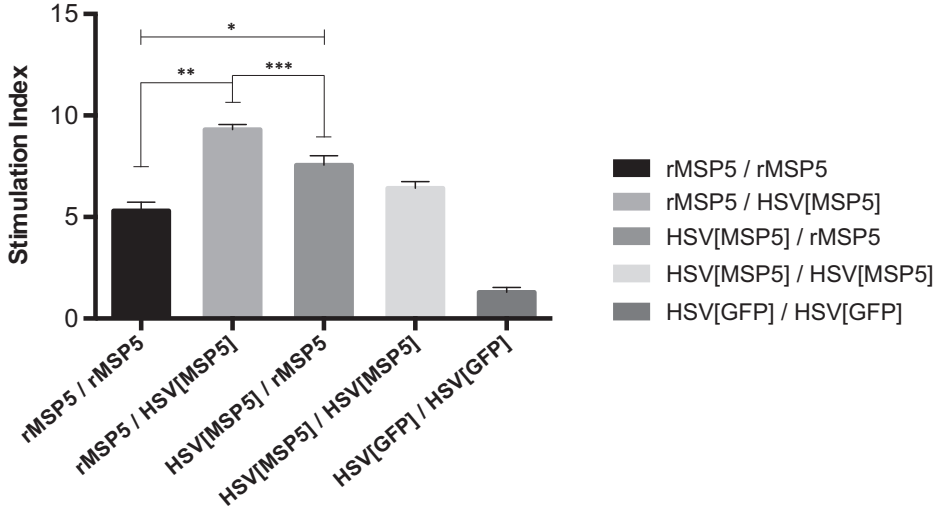

Fig. 5. Lymphoproliferation assays. Lymphocyte proliferative response, measured at 200 days post priming. SI, Stimulation Index. Data represent the mean of each group of mice. Error bars show the SD and asterisks shown above bars denote the relevant *p* values: * $p = 0.04$; ** $p = 0.01$; *** $p = 0.0006$.

activation of macrophages to enhance phagocytosis and killing (Brown et al., 1998). Regarding to the prevention of clinical disease, the *A. marginale spp. centrale* strain is currently used as a live vaccine, but it is not approved in the USA or the European Union because it is a blood-based vaccine (Hammac et al., 2013). This vaccine does not prevent infection, and persistently infected cattle are the major reservoir of *A. marginale* (Kocan et al., 2003).

In this work we evaluated a novel vaccination approach, studying the immune responses elicited by vaccines based on a herpesvirus derived amplicon vector expressing the major surface protein MSP5. The amplicon vectors were inoculated either alone or in combination with the recombinant protein expressed in bacteria, in a heterologous prime-and-boost immunization regimen.

The recombinant protein rMSP5 was highly immunogenic, as shown by prime-boost strategies with the homologous regimen rMSP5/rMSP5 (Group 1) or the combination rMSP5/HSV[MSP5] (Group 2) or HSV[MSP5]/rMSP5 (Group 3). In contrast, although HSV[MSP5] amplicons drove the *in situ* expression of MSP5, which was recognized by MAb ANAF16C1 in transduced Vero or MA104 cells (Fig. 2), the strategy involving prime-boost with HSV[MSP5] alone (Group 4) did not result in detectable antibodies measured by cELISA, and its profile was indistinguishable from that displayed by a negative control group (Group 5, $p = 0.501$).

Despite the different antibodies kinetics observed among groups 1, 2 and 3, at the end of the experience (200dpv) the antibody levels showed no significant differences among the three groups ($p = 0.824$, Fig. 4). A similar pattern was observed when total IgG against rMSP5 was measured (not shown).

Interestingly, the cellular immunity of animals that were subjected to heterologous vaccination regimens, and which included at least one inoculation with the amplicon particles (e.g., rMSP5 protein followed or preceded by HSV[MSP5]), was higher than in the animals treated with two doses of adjuvanted recombinant protein ($p = 0.01$ and $p = 0.04$, respectively). Of note, the group immunized only with amplicons (Group 4), which did not denote an increase in antibodies titers, showed significant cellular proliferation, at similar levels than the animals inoculated with adjuvanted protein ($p = 0.12$, Group 1). As the vaccine design strategies against anaplasmosis might take advantage of the simultaneous stimulation of humoral and cellular immune responses, this is an interesting result.

Groups 2 and 3, immunized using heterologous prime-boost regimens (protein and viral vectors) showed a long-term and balanced humoral and cellular response, almost independently of the immunogen used for the priming. These results are partially supported by a previous report in which, using one immunization with a HSV-1-based vector, elicited a long-term and protective immune response against the intracellular bacteria *Lysteria monocytogenes* (Lauterbach et al., 2004).

Protection in cattle immunized with purified MSPs was associated with a MSP-specific CD4+ T cells response, including the production of IFN-γ, TNF-α and the induction of specific antibodies promoting the opsonization of bacteria, stimulation of macrophages to perform phagocytosis, production of other cytokines and nitric oxide, which help eliminate intracellular bacteria (Brown et al., 1998; Palmer et al., 1999). Nevertheless, it has been reported that *A. marginale* specific CD4+ T cells primed during infection develop a poor memory response, probably preventing development of the proinflammatory mechanisms required for clearance of the pathogen (Han et al., 2010). During persistent infection, the adaptive immune response is able to control, but not to clear the infection. The inability to clear the infection is thought to be due to the generation of antigenically variant surface proteins, which escape detection and allow for a window of pathogen replication (Palmer et al., 2009). It has been hypothesized that different immunologic mechanisms control bacteremia in infected animals as compared with immunized animals, although the reason is still unknown, animals immunized with outer membrane proteins were better able to control bacteremia as compared with infected animals (Noh et al., 2010). Thus, in contrast to naturally acquired immunity, sterile immunity to *A. marginal*e can be induced by immunization with formulations of outer membrane proteins. The difference between the evasion of immunity resulting in persistent infection and the immunization-induced complete clearance is likely due to induction of antibody to conserved proteins that occurs following immunization, but does not occur during natural infection. Noh et al. (2010) studied several different epitopes of MSP2, which was a likely candidate for protection, concluding that protective antibodies were not directed against this outer protein. Nevertheless, it has been suggested that shifting the immune response toward other conserved epitopes that are poorly recognized during infection may be the key to effective vaccine development.

In this regard, conserved epitopes of MSP5 or other proteins might be properly exposed to the immune system through *in situ* expression driven by herpesvirus vectors, and therefore, future work using this system to express different *A. marginale* proteins may be of interest.

Moreover, the ability of the amplicon vectors to incorporate several copies of the same or different genes (Laimbacher and Fraefel, 2014) and the fact that they are barely affected by preexisting immunity (Brockman and Knipe, 2002), makes them promising tools to be explored. HSV-1 amplicons may accommodate multiple transcription units to co-express selected antigens (Bozac et al., 2006; D'Antuono et al., 2010; Laimbacher et al., 2012; Romanutti et al., 2013; Santos et al., 2006), such as other outer membrane proteins, multi-epitope sequences, cytokines or immunomodulatory molecules. The use of multi-antigenic vaccines may provide knowledge about the immunological synergies or interferences between different *A. marginale* antigens.

As it has previously reported using vaccines formulated with different antigens (Kano et al., 2008; Kawasaki et al., 2007; McGuire et al., 1994; Silvestre et al., 2014), we have shown as proof of concept, that the experimental vaccines based on *in situ* expression of antigens driven by HSV amplicon vectors were able to induce specific immune responses against *A. marginale* in a controlled in vivo experiment.

The evaluation of vaccines against anaplasmosis still relies on bovine tests, and no other animal model has been consistently established for this disease. Nevertheless, infection of BALB/c mice with *A. marginale* has been recently reported (Santos et al., 2013) and might prove to be a valuable tool to evaluate novel antigens. Further studies in the target host, or in an experimental animal model with correlation with protection in the host animal, will be necessary to assess the protective potential of these experimental vaccines.

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