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Immunogenicity of Hypothetical Highly Conserved Proteins as Novel Antigens in *Anaplasma marginale*

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Abstract *Anaplasma marginale* is a tick-transmitted Gram-negative intraerythrocytic bacterium and the etiological agent of bovine Anaplasmosis. Even though considerable research efforts have been undertaken, Anaplasmosis vaccine development remains a challenging field. Outer-membrane-specific antigens responsible for the ability of more complex immunogens could have a significant role in the protective response. Thus, the identification of outer-membrane antigens represents a major goal in the development of bacterial vaccines. Considering that 40 % of the annotated proteins in *A. marginale* remain as hypothetical, we selected three candidate antigens, AM1108, AM127, and AM216 based on experimental evidence, in silico structure prediction of β-barrel outer membrane, and orthology clustering. Sequence alignment and analysis demonstrated a high degree of conservation for the three proteins between the isolates from Argentina compared to the American strains. We confirmed the transcription of the three genes in the intraerythrocytic stage. AM1108 and AM216 recombinant proteins elicited specific T-cell response proliferation and a significant rise in TNF-α and IFN-γ transcript levels, respectively. Only AM1108 was able to be recognized by specific antibodies from infected bovines. This study allowed the identification of new candidate components of the outer-membrane fraction of *A. marginale*. Further studies will be required to analyze their potential as effective antigens for being included in rational vaccine strategies.

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Introduction

Intracellular rickettsial pathogens of the *Anaplasmataceae* and *Rickettsiae* families within the order Rickettsiales are main agents of tick-borne emerging diseases in humans and animals. *Anaplasma marginale*, a gram-negative intraerythrocytic bacterium, is one of the tick-transmitted etiological agents of the Anaplasmosis disease in cattle which causes dramatic weight lost, anemia, and often death during acute infection [16].

Anaplasma marginale invades and replicates in red blood cells during acute infection and can reach very high levels of erythrocyte infection. The acute infection in healthy animals usually resolves. However, this infection fails to be completely eliminated; which results in persistent infections that can be maintained over the entire life with fluctuating bacteremia levels [24]. The bacterium does not replicate in cells expressing MHC molecules. Besides, the available immunological data support that the *A. marginale* protective immunity mechanisms involve CD4+T cells response and INFγ production for the activation of macrophages which collaborate with B-cells in the induction of isotype switching to IgG2. The opsonization of bacteria or infected erythrocytes by activated macrophages enhanced phagocytosis along with cytokine and nitric oxide production; which leads to an incomplete intracellular bacteria clearence. In addition, high titers of fluctuating IgG1 and IgG2 antibodies directed to immunodominant major surface

protein (MSP) were identified during the course of infection [11, 24].

The immunization with outer-membrane (OM)-enriched fractions of *A. marginale* has been shown to induce efficient protection against bacteremia and disease after experimental challenges [34, 40]. Mostly, the identification of immunogenic proteins has been focused on the six well-defined *A. marginale* major surface proteins (MSPs) [1, 6, 19, 38]. Up to date, vaccine trials using purified MSPs have conferred a variable range of protective responses. For instance, some MSPs act as immunodominant proteins, eliciting high IgG antibody responses. However, these studies have failed in demonstrating a clear association between the magnitude of the antibody response and a full control of infection or bacteremia levels [35]. Moreover, during persistent infections, immune response evasion mechanisms driven by antigenic variation of MSP-2 and MSP-3 impaired the protective capacity of these antigens [18, 19, 31]. Therefore, it has been postulated that conserved specific antigens play a critical role on contributing to the ability of complex immunogens to induce protection. For this reason, efforts have been concentrated on the study of these antigens [2, 3, 14, 15, 28, 29, 37].

Surface-expressed proteins are consistent candidates for vaccine development and suitable targets to either induce protective immunity in the mammalian host or to prevent colonization of host cells and the tick vector. Genome-wide studies have identified outer-membrane proteins (OMPs) broadly conserved among rickettsial pathogens [37]. Several studies on intracellular pathogens attempt to explore putative exposed proteins to contribute to the description of the surface proteome in *A. marginale* as well as other related intracellular pathogens, such as *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *Neorickettsia sennetsu* [21–23]; which will open a new spectrum of potential candidates derived from massive identification studies [32, 43]. Particularly, the *A. marginale* genome revealed 160 coding sequences containing transmembrane domains [9]. In this regard, the transmembrane β-barrel (TMBB) is the prevailing architecture of the membrane-spanning proteins found in the outer membranes of Gram-negative bacteria [46]. Genetic variability is relevant in the evaluation of candidates for the development of vaccines, as it can result in significant protein polymorphism and could impair cross-protection between isolates [39] and with *Anaplasma centrale* vaccine strain.

Considering OMPs are suitable targets to induce protective immunity, the present study focuses on the identification of novel putative membrane proteins of *A. marginale* to assess the conservation from local isolates compared with other known sequenced isolates and test their capability of inducing B- and T-lymphocyte responses. All three orthologs of the genes are present in related intracellular pathogens and the proteins they encode are interesting candidates to be considered as potential antigens for vaccine rational design.

Materials and Methods

Animals Used in this Study

We evaluated four one-year-old bovines: 3 naturally infected Angus raised in a farm located at Mercedes locality, Corrientes Province (Argentina) and a naïve Angus (non-infected) from the National Institute of Agricultural Technology (INTA) at Castelar, Buenos Aires Province. The naturally infected animals were assigned as follows: N° 99 (acute phase of infection), N° 282, and N° 285 (chronic phase of infection). Additionally, we used an Angus animal N° 640 during a experimentally infected assay with *A. marginale* str. Salta [42] for the immunoblot and real-time q-PCR analysis. In all cases, whole blood was collected in a heparinized microtube and DNA was subsequently extracted for PCR assays, as previously described [25]. Specific primers of *msp5* from *A. marginale* str. Florida (GenBank accession M93392) were used to detect infection with *A. marginale* by heminested PCR (hnPCR) of *msp5* gene, as previously described [45]. The experiments carried out in bovines reported in this manuscript were conducted following the Guide for the care and use of animals-INTA (Approved by resolution CICVyA No. 14/07) and internationally recognized guidelines of "Care and Use of Experimental Animals" as Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd edition, 2010.

In Silico Analysis and Comparative Genomics

AM1108, AM127, and AM216 were amplified from genomic DNA of *A. marginale* local isolates (*A. marginale* str. Salta and *A. marginale* str. Mercedes) using specific primers based on *A. marginale* str. St Maries (Table S2). PCR amplicons were obtained with an automatic sequencer (ABI 3130, Applied Biosystems), assembled, and the consensus sequences were generated (Vector NTIv9). The obtained sequences for AM1108, AM127, and AM216 orthologs in both local strains were submitted to Genbank as follows: *A. marginale* str. Salta: KF053047, KF053049, and KF053051; *A. marginale* str. Mercedes: KF053048, KF053050, and KF053052. The gene localization in the genomic context and the predicted operons were obtained from the Database of prOkaryotic OpeRons [30] (Figure S3). For similarity searches and ortholog conservation analyses, BLASTP (*e*-value $= 10^{-5}$; Query coverage >70 %) was applied. For sequence functional annotation, the gene ontology database Blast2GO tool [13] was used. The percentage of identity

between the predicted amino acid sequences of the different strains were performed using ClustalW with MEGA 4 program [27, 44] compared to orthologs from *A. marginale* str. St Maries (Genbank CP000030), *A. marginale* str. Florida (Genbank CP001079), and *A. centrale* str. Israel (Genbank CP001759). Transmembrane β-strands and their topology with respect to the outer-membrane lipid bilayer were predicted using the posterior decoding method available at the PRED-TMBB [5]. PSORTb [20] was used for the protein subcellular localization analysis.

RNA Extraction and Transcription of Candidate Antigens in *A. marginale*

Total RNA from *A. marginale* str. Salta [36] was extracted from 2 ml of whole blood of the experimentally infected bovine N° 640 using RNeasy RNA extraction kit (Qiagen) and the concentration and quality were subsequently determined with a nanodrop RNA/DNA calculator (NanoDrop Technologies). The cDNA synthesis for reverse transcription PCR (RT-PCR) was performed as previously described [36]. Specific primers were designed for this study (Table S1).

Amplification, Cloning, and Sequencing

For recombinant protein production, AM1108, AM127, and AM216 gene sequences were amplified from genomic DNA of the *A. marginale* str. St. Maries. The gene sequences of AM1108, AM127, and AM216 were split into two fragments (N-terminal and C-terminal) excluding the predicted signal peptide in order to improve recombinant protein production. Specific primers (Table S3) were designed for the amplification of these sequences. Six recombinant fragments were obtained: AM1108 N-term, AM1108 (complete open-reading frame), AM127 N-term, AM127 C-term, AM216 N-term, and AM216 C-term (Fig. S1). PCR products were cloned into pTOPO2.1 or pGEMT vectors (Invitrogen, USA) and then subcloned in pRSET or pBAD-Thio-TOPO expression vectors (Invitrogen, USA). Positive clones were sequenced with an automatic sequencer (ABI 3130, Applied Biosystems) to verify proper fusion frame and to discard nucleotide changes that could lead to frameshifting.

Expression of Recombinant Proteins in *E. coli* and Purification

Expression vectors containing each insert (AM1108 N-term, AM1108, AM127 N-term, AM127 C-term, AM216 N-term, or AM216 C-term) as 6xHis-tagged fusion were transformed in *Escherichia coli* BL21 and XLBlue strains (with pRSET or pBAD-Thio-TOPO, respectively). *E. coli* transformed cells were grown in LB broth at 37 °C and induced with 1 mM of IPTG or with a final concentration of 0.02 % of arabinose. Cells were harvested by centrifugation after 4-h induction. Then, pellets were resuspended in lysis buffer and disrupted by sonication under 8 M urea solution and protease inhibitors (Roche). Recombinant proteins were purified by anti-HIS immunoaffinity chromatography (ProBond Resin, Invitrogen) using a Ni^{2+}-charged column under denaturing conditions and further elution using 125 mM to 1 M imidazole. Recombinant proteins were dialyzed overnight at 4 °C to diminish urea concentrations against a sterile phosphate-buffered saline solution (sterile PBS, pH 7.0). Purified recombinant proteins of the appropriate molecular weight were either tested in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Lymphocyte Proliferation Assays

For T-cell response evaluation, proliferation assays were performed using peripheral blood mononuclear cells (PBMC) purified from the three naturally infected animals (N° 99, 282, and 285) and the non-infected animal (naïve). Blood samples extracted with heparin were collected and PBMC were purified using Histopaq (Sigma). Then, PBMC were cultured at 2.5×10^5 cells per well in 96-well plates at a final volume of 200 µl with RPMI medium supplemented with bovine fetal serum 10 % and incubated at 37 °C, 5 %CO_2. PBMC-specific proliferation was assayed in quadruplicate wells with 100 µl culture media containing 1 µg/µl of recombinant proteins incubated for 4 or 6 days. Unspecific stimulation with Concanavalin A (ConA, 10 µg/ml) was assessed to confirm viability of PBMC. PBMC incubated with a non-related protein (BMFP) of *Brucella melitensis biovar abortus 2308* were also analyzed as a negative control. After incubation, PBMC were radiolabeled for 18 h with 1 µCi of (methyl-3H) thymidine (Perkin Elmer), subsequently harvested onto glass filters and the amount of incorporated label was measured by liquid scintillation counting (Wallac, Winspectral). Proliferative responses are expressed as the stimulation index (SI) calculated as the mean count per minute (cpm) of cell cultures with the tested antigen/mean cpm of cells cultured in RPMI medium. Statistical analysis was determined by one-way ANOVA and with Bonferroni's Multiple Comparison Test. Significance was retained when $P < 0.05$.

Immunoblotting

The recombinant proteins were subjected to 12 % SDS-PAGE and transferred to nitrocellulose membranes

(Bio-Rad). The membranes were blocked with 5 % (m/v) non-fat dry powder milk at 4 °C overnight and incubated either with: serum from *A. marginale*-infected animal N° 640, serum from animal N° 640 previous to infection, serum from calves pre-immunized and immunized with *A. marginale* OM protein (provided by Dr. G. Palmer) [28] or the specific monoclonal antibody MAb ANAF16 against MSP-5 membrane protein. Blots were incubated for 2 h at room temperature, washed in PBS 1X, and subsequently incubated with the secondary antibodies: rabbit anti-bovine IgG Alkaline Phosphatase (Sigma) or goat anti-mouse Alkaline Phosphatase (Applied Biosystems) according to the case. The membranes were developed with BCIP/NBT solution (Promega).

Cytokine mRNA Analysis from PBMC

PBMC were extracted from the experimentally infected bovine (N° 640), as described above. A total of 1×10^6 cells were incubated in duplicates with 1 µg/µl of recombinant proteins AM1108-ORF and AM216 C-term for 24 h. Total RNA was extracted using an RNeasy RNA extraction kit (Qiagen). Quality and quantity of total RNA were estimated by UV spectrophotometry (Nanodrop) and electrophoresis on 0.8 % agarose gel. DNA-free RNA (1 µg) was mixed with 1 µl (50 ng) of random primers (Invitrogen) at a final volume of 20 µl and reverse transcribed to total cDNA with SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. One microliter of the cDNA was used as a template for each real-time quantitative PCR (qPCR) reaction. Primers were designed using Primer Express Software 2.0 (Applied Biosystems) (Table S4). Tenfold serial dilutions of the cDNA were used to construct the standard curve and calculate the efficiency for each set of primers. Assays with a linear regression R value of >0.99 were considered acceptable. The qPCR reactions were performed in duplicate using SYBR green QuantiTec Mastermix (Qiagen) and standard cycling conditions (Applied Biosystem 7000). All qPCR data were analyzed by the 2^{-ddCt} (exponential transformation, *ddCt* package) with efficiency correction using REST beta 9 software [41]. For each animal, PBMC incubated with RPMI medium without the recombinant proteins served as a control for calibration condition and *pol II* was used as the reference gene. Differences in the mRNA transcription levels between groups were evaluated by a non-parametric analysis performing a Pair Wise Fixed Reallocation Randomisation Test.

Results

Among the 949 annotated proteins in *A. marginale* str. St. Maries genome [9], 383 (40 %) remain as hypothetical proteins. The selection of hypothetical proteins for further characterization was based on the combination of experimental evidence using a pPhoA library of *A. marginale* for the selection of sequence coding for exported products (manuscript in preparation), in silico structure prediction, and orthology clustering. For AM1108, the multipass transmembrane β-prediction using PRED-TMBB showed significant predictions for a β-barrel OMPs (Fig. 1a). The conserved domain analysis identified a Pentapeptide Repeat domain (IPR001646; PF00805) strongly conserved within the Anaplasmataceae family and in all orders of the

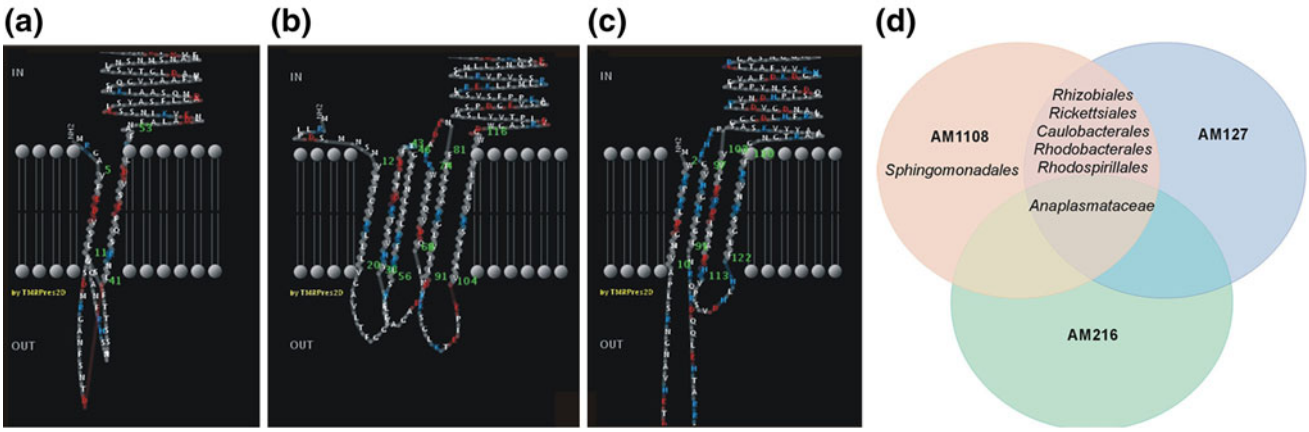

Fig. 1 Predicted two-dimensional structure of *A. marginale* hypothetical proteins **a** AM1108, **b** AM 127, and **c** AM 216 with respect to the outer-membrane lipid bilayer predicted using the Posterior Decoding method available in PRED-TMBB: graphical representations of the predicted topology [5]. The discrimination scores were 2.941, 2.955, and 2.959, respectively; a score lower than the threshold value of 2.965 is considered as a significant prediction value of β-barrel OMPs. **d** Ortholog genes for AM1108, AM127, and AM216 were identified in the different orders within the class

α-proteobacteria class (Table 1, Fig. 1d). For AM127, PRED-TMBB showed values that were statistically significant for β-barrel OMP prediction (Fig. 1b) and assigned a conserved domain of Acetamidase Formamidase conserved in almost all members of the class (Table 1; Fig. 1d). For AM216, this analysis also predicted a β-barrel protein (Fig. 1c). However, no conserved domains were identified for this protein, which is only conserved in *Anaplasma* spp. and *Ehrlichia* spp. (Table 1; Fig. 1d).

In order to determine the sequence similarity among the selected candidates between the American isolates (*A. marginale* str. St. Maries: AM1108, AM127, and AM216; *A. marginale* str. Florida: AMF_837, AMF_093, and AMF_157) and the local isolates from Argentina, we sequenced the target genes from two different isolates coming from separate geographical regions (for: *A. marginale* str. Salta: KF053047, KF053049, and KF053051, respectively; *A. marginale* str. Mercedes: KF053048, KF053050, and KF053052, respectively). Sequence analysis showed a high degree of conservation between the amino acid sequences from the local isolates, the American *A. marginale* strains, and the vaccine isolate *A. centrale str. Israel* (Table S7).

To confirm the transcription of the three candidate genes, total RNA was obtained from the local isolate *A. marginale* str. Salta and assessed by RT-PCR assays. Results were positive for the three genes in both organisms (Fig. 2). *A. marginale* str. St. Maries AM1108 N-term, AM1108 ORF, AM127 N-term, AM127 C-term, AM216 N-term, and AM216 C-term recombinant proteins were obtained (Fig. 3) for further characterization.

The infection with *A. marginale* was confirmed in the three naturally infected animals (N° 99, 282, and 285) (Fig. S2). To analyze if these candidate proteins are able to elicit immunological responses, we performed proliferation assays with PBMC extracted from naturally infected animals and the non-infected naïve bovine (Fig. 4). AM1108-

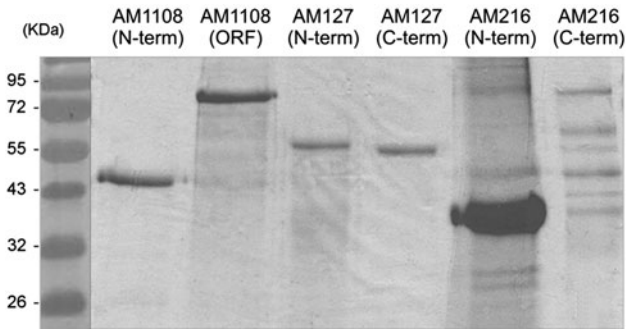

Fig. 3 Expression of recombinant proteins. Purified recombinant proteins of the expected molecular weight were tested in a SDS-PAGE stained with Coomassie brilliant blue. AM216 C-term degradation was observed even though protease inhibitors were added

Table 1 In silico analysis on selected candidates

Genome ID	Annotation	Predicted genome CDS		Multipass transmembrane β-barrel (PRED-TMBB)	Protein localization (PSORT)	Conserved domains		
		bp	Aa			Pfam	Blast2GO	Uniprot
AM1108	Hypothetical protein	2094	698	2	Non-Cytoplasmic	Pentapeptide repeat	Pentapeptide repeat	Pentapeptide repeat
AM127	Hypothetical protein	3000	999	6	OMP	ND	Acetamidase formamidase family	ND
AM216	Hypothetical protein	2530	842	4	Unknown	ND	ND	ND

CDS coding sequence, *ND* not detectable, *bp* base pairs, *aa* aminoacids

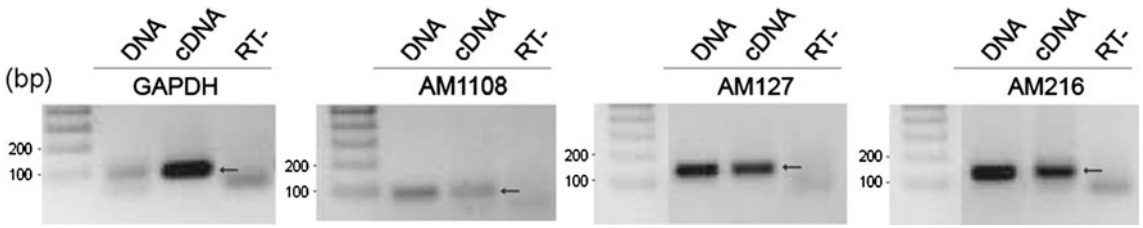

Fig. 2 Transcription analysis of candidate genes. *Black arrows* indicate bands corresponding to the expected amplicon size for AM1108 (101 bp), AM127 (108 bp), and AM216 (102 bp). GADPH gene was used as a housekeeping gene. Bands below 100 bp in the RT (−) *lanes* correspond to primer dimers. (RT−) corresponds to negative controls without adding RT enzyme

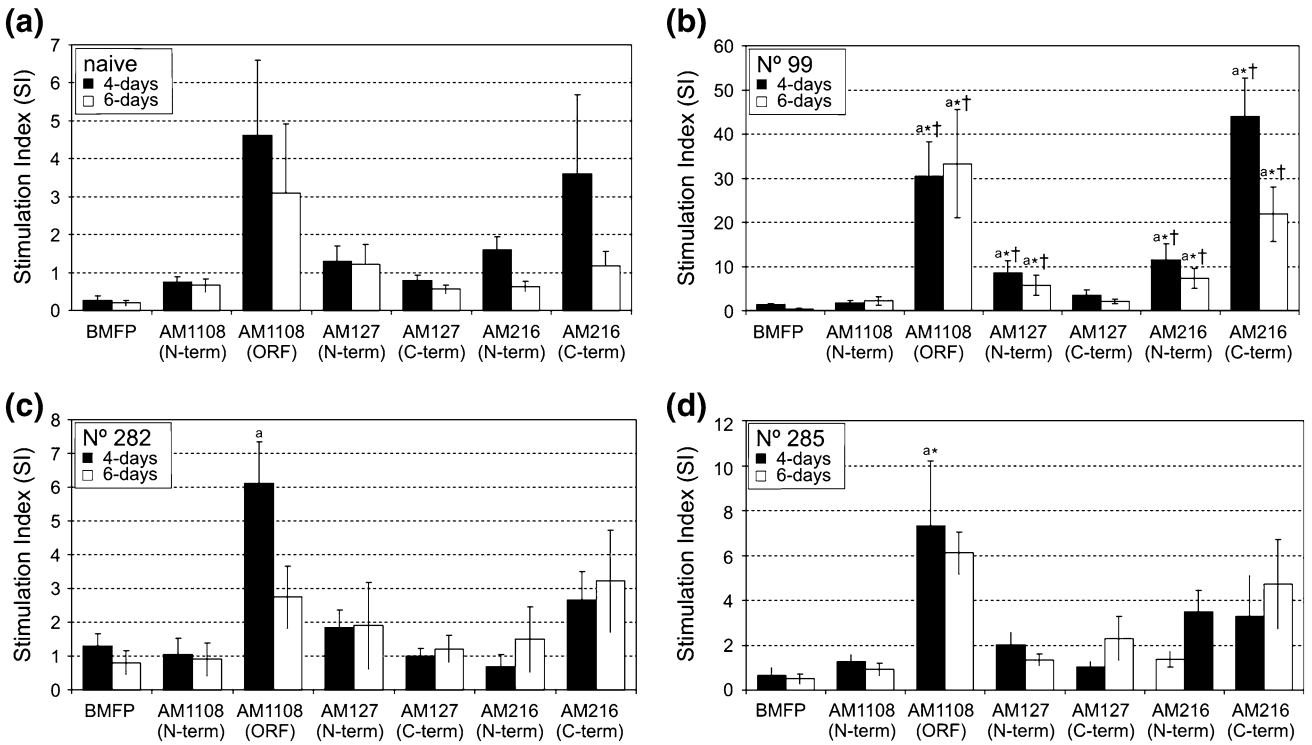

Fig. 4 PBMC lymphoproliferation assays. PBMC from infected and naïve animals were incubated for 4 days (*black bars*), or 6 days (*white bars*) with recombinant proteins or at control conditions: **a** Naïve, **b** N° 99, **c** N° 282, and **d** N° 285. Results of each *panel* are representative of three independent experiments, where the same trend was observed for the recombinant proteins for each calf. Statistical significance of a response compared to SI = 3 (**a**), to RPMI medium (*Asterisk)* and to naïve animal (†) are indicated ($P < 0.001$)

ORF induced a significant proliferative response of PBMC obtained from all infected cattle (Fig. 4b–d). AM216 C-term also showed a strong response when incubated with PBMC from the bovine N° 99 (Fig. 4b). Although AM216 N-term and AM127 N-term also induced a specific response for this same animal, the proliferation response was weaker (Fig. 4b). The SI was much higher in the acutely infected animal (N° 99) compared with the chronic-infected bovines (N° 282 and N° 285). The same trend was observed for the proliferation assays incubated for 4 or 6 days. PBMC from all animals failed to respond significantly to the unrelated *Brucella abortus* recombinant protein BMFP (Fig. 4a). Furthermore, no response was observed with any of the recombinant antigens tested for the naïve non-infected animal (Fig. 4a). PBMC from all animals showed a strong unspecific response to ConA indicating viability during the proliferation assay (Table S5).

We also evaluated humoral response against recombinant antigens using sera from bovines immunized with an OM-enriched fraction and from experimentally infected cattle (N° 640). Both sera recognized AM1108; which confirms the presence of specific antibodies against this protein (Fig. 5a). No detectable levels of specific antibodies were observed for the other tested recombinant proteins (data not shown). The lack of a detectable antigen–antibody interaction could be attributed to the absence of conformational epitopes in the recombinant versions of these proteins or to low undetectable antibody titers. Another possible explanation could be that these antibodies were present in a different time point in which this antigen was evaluated. Positive reactivity to MSP-5 was also analyzed as a positive control of the sera used for the assays (Fig. 5b).

For the evaluation of the cytokine response to the candidate recombinant antigens of *A. marginale*, the levels of the mRNA transcripts for IL-2, IL-10, IL-12p35, TNF-α, and IFN-γ were measured by q-PCR, These measurements were assessed in three independent experiments. The q-PCR assays were performed on PBMC extracted from blood samples collected from the experimentally infected animal N° 640 during the acute stage of infection, after stimulation with the proteins under study. TNF-α and IFN-γ were significantly upregulated by AM1108 and AM216 C-term, respectively ($P < 0.05$) (Table S6).

Discussion

Even though considerable research efforts have been undertaken, Anaplasmosis vaccine development remains a challenging field. In Argentina, vaccination with the less

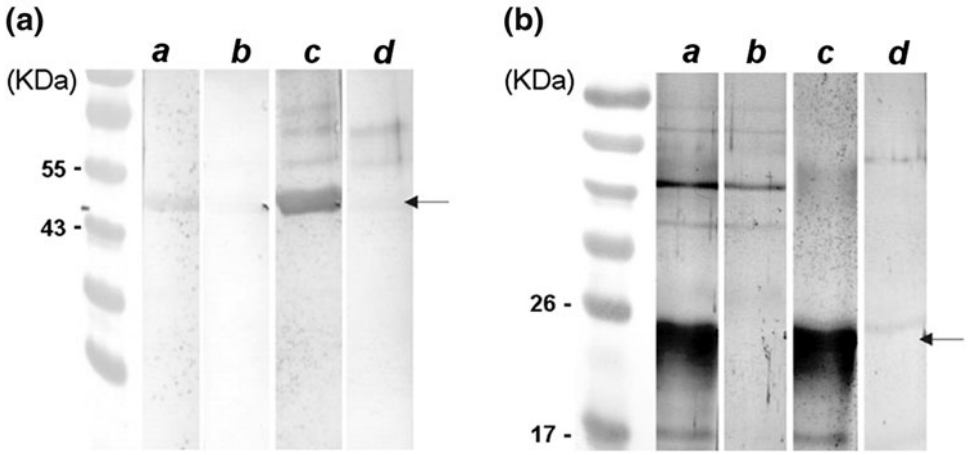

Fig. 5 Western blotting. **a** AM1108 ORF and **b** Msp-5, as a positive control, were probed with serum from positive and negative reference animals. *a* Sera from calves immunized with *A. marginale* outer-membrane proteins; *b* Sera from pre-immunized calves as a negative control; *c* Sera from an *A. marginale*-infected animal N°640; *d* Sera from the animal N°640 previous to infection. *Black arrows* indicate bands corresponding to the expected molecular size for recombinant proteins

virulent strain *A. marginale* subspecies *centrale* is the currently available protection strategy [4]. This live vaccine has been also largely used in Australia, South Africa, Uruguay, and Israel. However, breaks in vaccine protection [8] and the risk of disease induced by live vaccine strains themselves have prompted the search for better control alternatives. In this regard, immunization with outer membranes can induce sterile protective immunity against challenge with *A. marginale* [12]. Nevertheless, only partial immunity against heterologous challenge has been achieved. In addition, it has been shown that antibodies induced by *A. marginale* subspecies *centrale* live vaccine bound to OMPs that overlapped with OMPs that were immunogenic in animals vaccinated with inactivated vaccines and subsequently protected against bacteremia and disease. These identified OMPs are absent in the most immunodominant antigens (MSP-2 and MSP-3), supporting the hypothesis that protective immunity against *A. marginale* is associated to subdominant antigens [29]. In addition, these antigens appear to be less variable; which makes them good targets to be included in a broadly protective vaccine and their identification represents a major goal in the development of bacterial vaccines.

Ortholog proteins of AM1108 were identified in almost all organisms from the order Rickettsiales and several studies in related pathogens described them as surface-exposed proteins in *A. phagocytophilum* (APH_1170) [21], *E. chaffeensis* (ECH_1083) [22], and *N. sennetsu* (NSE_0725) [23]. Furthermore, AM1108 is highly conserved (>99 % identity) at the amino acid level among the distinct strains (Table S7) and is also conserved in the *A. centrale* str. Israel genome with 73 % of identity. In this study we showed that the recombinant AM1108 protein elicits lymphocyte proliferation and a rise in TNF-α mRNA levels. Furthermore this protein was recognized by sera either from animals immunized with OM-enriched fractions or from infected cattle. These results are consistent with the expression of this protein during infection, the expected cellular location and its potential to be considered as a vaccine candidate. Pentapeptide repeat domains were first identified in many cyanobacterial proteins and later on they were also found in bacterial and plant proteins [26]. The function of these repeats is still unknown and their predicted structure is a beta-helix [7]. Moreover, AM1108 and AM127 were described as part of the cluster three of orthologs conserved across all representative obligate and facultative intracellular pathogens and endosymbionts [17].

Recently, proteomic and immunologic studies using sera from cattle immunized with OM-enriched fractions have identified 24 surface-expressed OMPs including the AM127 hypothetical protein [29, 37]. In this study, AM127 was predicted to have six membrane β-strands suggesting a β-barrel outer membrane; which supports previous experimental evidence [29]. In addition, AM127 orthologs are present in almost all members of the α-proteobacteria class, absolutely conserved within *A. marginale* strains and also in the *A. centrale* (with 86 % identity). We further confirmed the transcription of the AM127 gene, at least in the intraerythrocytic stage. Interestingly, AM127 is the only one of the three analyzed genes that participate in an operon (code 84921) with *rnhA* gene encoding for RnhA ribonuclease H protein (COG0328) (Figure S3). AM127 was annotated as a hypothetical protein and lacked of associated information in the genome project [9]. However, Blast2GO annotation matched with an acetamidase formamidase domain which was also identified in the ortholog protein of *A. phagocytophilum* (APH_0110). The PBMC assays showed a significant stimulation using AM127 N-term. However, no IgGs-specific antigen recognition was detected in Western blot assays.

AM216 protein belongs to a cluster retained only in *Anaplasma* spp. and *Ehrlichia* spp. without orthologs in other families of the class. However, none of them was identified by other studies and no information associated

with conserved domains has been reported. Although bioinformatic analysis of the subcellular localization predicted AM216 as an OMP, no evidence of specific recognition of sera from infected or immunized animals have been detected in the study. The results of PBMC stimulation with AM216 elicited a significant proliferative response with concomitant increased levels of IFN-γ mRNA, one of the cytokines described as relevant in the course of *A. marginale* infection (Table S6) [11].

As *A. marginale* does not replicate within cells that express MHC molecules, current hypotheses of protective immunity are focused on CD4+ T cell activation and B-cell isotype switching to high-affinity IgG isotypes. These antibodies promote opsonization of bacteria and, therefore, INF-γ secretion. Both processes play a key role in the activation of macrophages to produce TNF-α and nitric oxide (NO) and also activate IgG2 production by B-cells [10, 11, 33]. AM1108 and AM216 C-term induced TNF-α and IFN-γ transcription, respectively. In addition, both recombinant proteins elicited significant lymphoproliferation. Besides, AM1108 was recognized by specific antibodies from bovines with acute anaplasmosis. Therefore, AM1108 and AM216 C-term could be involved in immune response pathways of the acute phase and could be posed to be important during the bacteremia control. Further immunological characterization must be carried out in order to demonstrate the role of both candidates in the protection of persistently infected cattle.

Taken together, bioinformatic and experimental evidence in this work allowed the identification of new putative OM proteins of *A. marginale* with the ability to elicit B- and T-cell responses. Immunological specific response represents an indirect evidence of protein expression along the time course of the infection. Further studies are required to confirm the surface location and the potential of the selected candidates as effective antigens for being included in rational vaccine strategies.

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