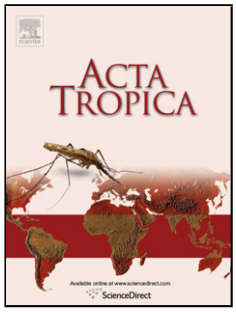

Title: The potential of a DIVA-like recombinant vaccine composed by rNcSAG1 and rAtHsp81.2 against vertical transmission in a mouse model of congenital neosporosis

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The potential of a DIVA-like recombinant vaccine composed by rNcSAG1 and rAtHsp81.2 against vertical transmission in a mouse model of congenital neosporosis

Running title: A DIVA-like vaccine against neosporosis

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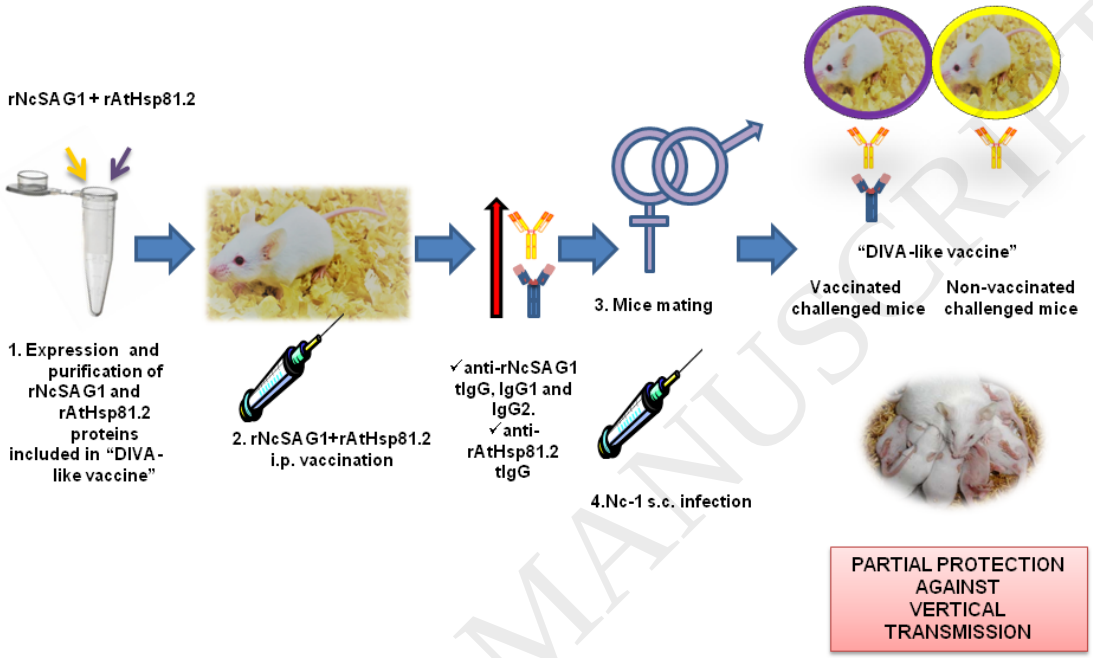
Graphical abstract



HIGHLIGHTS

- No vaccine is available against *Neospora caninum* .
- We demonstrated the DIVA-like character of rNcSAG1+rAtHsp81.2 vaccine in mice.
- rNcSAG1+rAtHsp81.2 immunization elicited high tIgG, IgG1 and IgG2a titers.
- rNcSAG1+rAtHsp81.2 conferred partial protection against vertical transmission.
- rNcSAG1+rAtHsp81.2 significantly increased pups median survival time.

ABSTRACT

Neospora caninum is the etiological agent of neosporosis, a worldwide infectious disease recognized as the major cause of abortions and reproductive failures in livestock, responsible for significant economic losses in cattle industries. Currently, there are not cost-effective control options for this pathology, and the development of a vaccine involving new and integrated approaches is highly recommended. In this study, we evaluated the immunogenic and protective efficacy, as well as the potential DIVA (Differentiation of Infected from Vaccinated Animals) character of a recombinant subunit vaccine composed by the major surface antigen from *N. caninum* (NcSAG1) and the carrier/adjuvant heat shock protein 81.2 from *Arabidopsis thaliana* (AtHsp81.2) in a mouse model of congenital neosporosis. BALB/c female mice were intraperitoneal (i.p.) immunized with a mixture of equimolar quantities of rNcSAG1 and rAtHSP81.2 or each protein alone (rNcSAG1 or rAtHsp81.2). The vaccine containing a mixture of rNcSAG1 and rAtHsp81.2 significantly enhanced the production of specific anti-rNcSAG1 total IgG (tIgG), IgG1 and IgG2a antibodies in immunized mice when compared to control groups (non-vaccinated and rAtHsp81.2 immunized mice) as well as to the group of mice immunized only with the antigen (rNcSAG1). In addition, partial protection against vertical transmission and improvement of the offspring survival time was observed in this group. On the other hand, rAtHsp81.2 induced the production of specific anti-rAtHsp81.2 tIgG, allowing us to differentiate vaccinated from infected mice. Despite further experiments have to be made in cattle to test the capability of this vaccine formulation to differentiate vaccinated from infected animals in the field, our results suggest that the formulation composed by rNcSAG1 and rAtHsp81.2 could serve as a basis for the development of a new vaccine approach against bovine neosporosis.

Keywords: *Neospora caninum*, NcSAG1, plant Hsp90, DIVA-like vaccine, vertical transmission.

Introduction

Neospora caninum is an apicomplexan parasite and the causative agent of neosporosis, a worldwide distributed infectious disease (Dubey et al., 2007), which can infect a wide range of mammalian hosts (Donahoe et al., 2015). Neosporosis is regarded as the major infectious cause of abortions and reproductive failures in cattle (Reichel et al., 2007), leading to global estimated losses in cattle industries exceeding US $1.300 million per annum (Reichel et al., 2013). Despite the economic impact related to bovine neosporosis, there is no available treatment for this pathology. In addition, the potential development of chemotherapeutics and the implementation of their use would not be adequate, since they probably generate unacceptable residues in milk and meat that would avoid commercialization and consumption (Dubey and Schares, 2011). Although many control strategies have been proposed, the development of a prophylactic vaccine results the most appropriate one (Horcajo et al., 2016; Reichel and Ellis, 2009). Vaccines based on *N. caninum*-naturally attenuated strains have demonstrated to be useful in preventing abortions (Rojo-Montejo et al., 2013; Williams et al., 2007), however, none have proven to completely prevent transplacental transmission (Reichel et al., 2015). In addition, live vaccine approaches have been associated with several disadvantages, including questionable safety due to the risk of virulence reversion (Innes et al., 2011), costly production and distribution channels, and latency in the intermediate host (McAllister 2014). In contrast, recombinant subunit vaccines offer safer alternatives (Innes et al., 2011) and may provide the best long-term sustainable solution. However, recombinant antigens are often less immunogenic than live vaccines and require the addition of adjuvants to enhance the magnitude of the adaptive immune

response or to modulate it to produce the most effective form of immunity against each pathogen (Pérez et al., 2008; Petrovsky and Aguilar, 2004). In fact, Pinheiro et al., (2018) have demonstrated that vaccine formulations including recombinant NcSRS2 of *N. caninum* and adjuvanted with water-in-oil, alum hydroxide, or xanthan gum in BALB/c mice, depicted completely different humoral and cellular immune responses dynamics depending on the adjuvant used, confirming that the choice of adjuvant might play an important role in vaccine response. Although several different adjuvants have been included in subunit vaccines against *N. caninum*, none of them have proven to stimulate proper immune responses and immunoprotection (Innes and Vermeulen, 2006), thus search for more appropriate and effective adjuvants is still one of the main challenges in this research area. Moreover, there is an increasing interest in new and integrated approaches, including the development of marker vaccines, which allow the differentiation of infected from vaccinated animals (DIVA) by serological techniques (Marugán-Hernandez, 2017).

A promising alternative in adjuvant research is the use of Heat Shock Proteins (Hsps). Several Hsps from different organisms have been reported as strong immune-modulators (Tsan and Gao, 2009). In addition, different studies have demonstrated that the immunization of mice with Hsp-peptide/protein complex (naturally or artificially reconstituted) or Hsp-antigen fusion proteins, in the absence of exogenous adjuvants elicited strong antigen-specific immune response (Echeverría et al., 2006; Rico et al., 1999; Srivastava 2002; Valentinis et al., 2008). Moreover, in a recent study of our group, we have demonstrated that plant cytosolic Hsp90 isoforms from *Arabidopsis thaliana* (AtHsp81.2) and *Nicotiana benthamiana* (NbHsp90.3) expressed in *E. coli* are B cells mitogens (Corigliano et al., 2011). Also, in a follow-up study, we have shown that when recombinant NbHsp90.3 was incorporated as adjuvant in a vaccine

formulation, it elicited a strong immune response against a reporter antigen (Corigliano et al., 2013).

Several marker vaccines and compliant diagnostic tests for veterinary use have been successfully applied in different pathologies including blue tongue virus, bovine tuberculosis, foot-and-mouth disease, highly pathogenic avian influenza and classical swine fever (Anderson et al., 2014; Pasick 2004; Uttenthal et al., 2010; Vannie et al., 2007). However, no veterinary DIVA vaccine against parasite infections has been developed yet. The aim of the present report is to verify the capability of differentiate infected from vaccinated animals, as well as, to evaluate the immunogenic capacity and the protective efficacy of a recombinant subunit vaccine composed by one of the best characterized antigen from *N. caninum*, the major surface antigen (NcSAG1) (Hemphill et al., 1997), and the carrier/adjuvant heat shock protein 81.2 from *Arabidopsis thaliana* (AtHsp81.2) in a mouse model of congenital neosporosis.

2. Material and Methods

2.1. Neospora caninum tachyzoite culture and parasite purification

N. caninum tachyzoites of the Nc-1 strain (gently provided by Dr. M. Cecilia Venturini) were maintained by serial passages in Vero cells or HFF (Hemphill 1996). Parasites were harvested as described previously by Hemphill (1996). The eluted parasites were centrifuged and resuspended in cold DMEM medium, and counted in a Neubauer chamber. For the infection challenge, tachyzoites were prepared following standard procedures (Collantes-Fernández et al., 2004) and resuspended in 200 μl of PBS (2 x 10^6 Nc-1 tachyzoites per mouse) to use immediately (Collantes-Fernández et al., 2004; Jiménez-Ruiz et al., 2012).

2.2. Cloning, expression and purification of recombinant proteins

Extraction of genomic DNA (gDNA) from *N. caninum* tachyzoites was carried out using Qiagen DNA extraction kit under the manufacturer's conditions (Qiagen, Hilden, Germany). The gDNA obtained was used as a template for PCR reactions for gene cloning and expression analysis. NcSAG1 sequence was obtained from the Genbank database (accession number: AF132217.1) and used to design primers to amplify the encoding region of the mature NcSAG1$_{61\text{-}298}$ protein. The peptide signal (aa$_1$ to aa$_{60}$) and the hydrophobic C terminal region (aa$_{298}$ to aa$_{319}$) were removed in order to improve protein stability in bacteria heterologous system. The forward primer used was 5´-C ACC ATG AAC CAC ATC ACG CTC AAG -3', and the reverse primer was 5'-AAG CTT TCA ACC AAC ATT TTC AGC CGA CGA C -3'. In addition, 4 bases were added in the N-terminus for directional cloning in the pET200/D-TOPO® vector (Invitrogen, CA, USA) as well as a *Hind*III restriction site in the C-terminus (underlined). The amplified product was sequenced to confirm the presence of the insert containing the nucleotide fragment corresponding to NcSAG1.

The pET200/D-TOPO®-NcSAG1 plasmid was used to transform *Escherichia coli* BL21 Star™ (DE3) competent cells. Bacteria were grown in Luria–Bertani (LB) medium supplemented with 100 μg/ml Kanamycin at 37°C up to a cell density of 0.5 (O.D. =600 nm). Protein expression was induced by isopropyl-b-D-thiogalactoside (IPTG) to a final concentration of 1 mM for 4h. Cells were harvested by centrifugation and stored at -20°C until use.

All purification procedures were carried out as previously described (Corigliano et al., 2011). The soluble recombinant NcSAG1 and *Arabidopsis thaliana* Hsp81.2 (Corigliano et al., 2011) proteins (rNcSAG1 and rAtHsp81.2, respectively) were

purified under non-denaturing conditions using a nitrilotracetic acid-Ni^{2+} column (Qiagen, Hilden, Germany) as previously described by Corigliano et al., (2011). The recombinant proteins were separated by SDS-PAGE (15%) using the Mini-Protean system III (Bio-Rad, CA, USA). After electrophoresis, proteins were stained with Coomassie Brilliant Blue. PageRuler™ Prestained Protein Ladder (Fermentas, MA, USA) was used as molecular marker. Stained bands were excised and subjected to sequencing by MALDI-TOF-TOF spectrometer, Ultraflex II (Bruker), in the mass spectrometry facility CEQUIBIEM, Argentina. To eliminate endotoxins, rNcSAG1 and rAtHsp81.2 were passed through a polymyxin B-agarose column following the manufacturer's instructions (SIGMA, MO, USA). The concentration of lipopolysaccharide (LPS) in free-LPS recombinant proteins was measured using the HEK-Blue™ LPS Detection Kit (InvivoGen, CA, USA) as described previously (Corigliano et al., 2016). LPS contamination in either protein was lower than 1 ng/ml.

2.3. Production of polyclonal antisera

Antisera against rNcSAG1 and rAtHsp81.2 were produced in mice, by the administration of one intraperitoneal (i.p.) dose of 60 μg of the purified recombinant proteins emulsified in Freund's Complete Adjuvant (FCA) (1:1 v/v, SIGMA, MO, USA) followed by the i.p. administration of a booster dose of 30 μg of the recombinant proteins emulsified in Incomplete Freund's Adjuvant (IFA) 2 weeks apart. The kinetics of development of specific antibody responses upon immunization of mice with rNcSAG1 and rAtHsp81.2 were studied by indirect ELISA tests (Cooper and Paterson, 2009).

2.4. Western blotting

Western blotting was performed as previously described (Corigliano et al., 2013). Briefly, recombinant proteins previously separated by SDS-PAGE 15%, were transferred onto PVDF membranes (GE Healthcare, Buckinghamshire, UK) using an Electro transfer Unit (Bio-Rad). Firstly, the membranes were incubated with mouse anti-6XHIS monoclonal antibody (1:1,000, Cell Signaling Technology Inc., MA, USA), sera from mice experimentally infected with *N. caninum* 2 x 10^6 tachyzoites from Nc-1 strain (dilution, 1:500), mouse anti-rNcSAG1 polyclonal antibody (1:500) or mouse anti-rAtHsp81.2 polyclonal antibody (1:500) (Corigliano et al., 2011), as primary antibodies. Later, the membranes were incubated with alkaline phosphatase conjugated-goat polyclonal anti-mouse IgG (complete molecule) secondary antibody (1:5,000, SIGMA, MO, USA). After washing, the reaction was developed by the addition of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Promega, WI, USA) substrate. PageRuler™ Prestained Protein Ladder (Fermentas, MA, USA) was used as molecular marker.

2.5. Immunization protocol, mice mating and parameters analyzed

All procedures requiring animals were performed in agreement with institutional guidelines and were approved by the Independent Ethics Committee for the Care and Use of Experimental Animals of National University of General San Martin (C.I.C.U.A.E., IIB-UNSAM), and approved and conducted in accordance with the guidelines established by the National University of General San Martin (SC055) and the National Research Agency (PICT 2012).

Eight-week-old female BALB/c mice were purchased from the Bioterium of the Faculty of Exact and Natural Sciences of the University of Buenos Aires (FCEyN-UBA, Argentina), housed under controlled temperature (22ºC) and illumination (14:10h L:D

cycle; lights on at 05:00 hours) and allowed free access to rat chow (Purina, Buenos Aires, Argentina) and water. Mice were randomly allocated into 4 groups and were vaccinated two times by i.p. injection at 2 week intervals (0 and 15 days post first immunization, -d.p.i.-, respectively) (Debache el al., 2009, Monney et al., 2012) with approximately equimolar doses of each recombinant protein as follows (per mouse and injection, Table 1); Group 1 (G1, non-vaccinated negative control): 200 µl of sterile PBS, Group 2 (G2, adjuvant control): 30 µg of rAtHsp81.2 in 200 µl of sterile PBS; Group 3 (G3): 10 µg of rNcSAG1 in 200 µl of sterile PBS, Group 4 (G4): 10 µg of rNcSAG1+30 µg of rAtHsp81.2 in 200 µl of sterile PBS

. Sixty d.p.i., vaccinated and non-vaccinated virgin female mice were paired with 8–12-week-old BALB/c males for 96 hours. The day of appearance of a vaginal plug was considered as day 0.5 of pregnancy. Pregnant and non-pregnant mice were challenged on day 7.5 of pregnancy or 71 d.p.i., respectively (0 days post challenge -d.p.c.-), with 2 x 10^6 freshly purified *N. caninum* Nc-1 tachyzoites in 200 µl of sterile PBS by subcutaneous (s.c.) injection, (Debache et al., 2009; López-Pérez et al., 2006). A subgroup of non-vaccinated dams and a subgroup non-vaccinated non-pregnant female mice remained unchallenged (G1 dams/ G1 non-pregnant female mice, non-vaccinated negative controls), whereas another subgroup of non-vaccinated dams and a subgroup of non-vaccinated non-pregnant mice were challenged (G5 dams/ G5 non-pregnant female mice, non-vaccinated positive controls). Dams delivered on day 21.5$\pm$1 of pregnancy (p.d.) and were allowed to nurture their offspring normally.

All female mice were bled at 0, 15, 30 and 60 d.p.i (pre-challenge humoral determinations). After mating and challenge, pregnant and non-pregnant mice from each experimental group were bled at 7 d.p.c. (acute phase of infection). Dams and non-pregnant mice were euthanized at 35 d.p.c (chronic phase of infection) by the use of a

CO_2 chamber. Blood was collected from each animal when euthanized by cardiac puncture and serum separated and frozen at -80◦C until used. Brains from dams were collected and immediately frozen in liquid nitrogen and stored at -80◦C. Pups were evaluated from birth to 30 days post-partum (d.p.p.). Data on fertility rate, litter size, hebdomadal mortality, neonatal and prepuberal mortality and morbidity signs were collected during this time. Congenital neosporosis mouse model was repeated three times. For one experiment, all surviving pups were euthanized on 18 d.p.p. and tissue collected to evaluate parasitaemia by qPCR. Experimental protocols and data sampling are summarized in Figure 1.

2.6. Analysis of humoral immune response

Humoral immune response was analyzed in pregnant and non-pregnant female mice. Antibody titers and isotype profile anti-rNcSAG1 were determined in unchallenged virgin female mice on 0, 15, 30 and 60 d.p.i. In order to attain a comparative analysis of IgG profiles, serum samples from virgin female mice were used at 1:1,000 dilution and were evaluated at 60 d.p.i (pre-challenge determinations). IgGs levels anti-rNcSAG1 were also evaluated in dams and non-pregnant mice at the acute phase of infection (7 d.p.c.) and at the chronic phase of infection (35 d.p.c.) after experimental challenge. In this sense, serum levels of tIgG, IgG1 and IgG2a anti-rNcSAG1 were measured by ELISA as previously described (Del L Yácono et al., 2012). In addition, tIgG titers against rAtHsp81.2 were determined in unchallenged virgin female mice on 0, 15, 30 and 60 d.p.i. and tIgG antibody levels were also evaluated and compared in sera from unchallenged non-vaccinated virgin female mice (G1) (60 d.p.i.), challenged non-vaccinated dams (G5, 35 d.p.c), challenged vaccinated dams (G4, 35 d.p.c.) and their offspring (G5 and G4, 30 d.p.p. pups, respectively). Briefly, 96-well microtiter plates

(Immuno Plate Maxisorp; Nunc, Rochester, NY, USA) were coated overnight at 4 °C with 5 μg/ml of rNcSAG1 or rAtHsp81.2. Goat anti-mouse IgG-horseradish peroxidase conjugate (1:10,000; SIGMA, MO, USA) was used as secondary antibody, and rat anti-mouse IgG1 or IgG2a-horseradish peroxidase conjugates (1:3,000; BD Biosciences, CA, USA) were used for isotype analysis. Immune complexes were revealed with tetramethylbenzidine chromogen (TMB, One-Step; Invitrogen, Carlsbad, CA, USA), and optical density was read at 630 nm with an automatic ELISA reader (Synergy H1, Bio-Tek, VT, USA). Serial dilutions of sera were carried out to determine the titer, which was defined as the highest serum dilution that gave a value above the absorbance value for pre-immune sera plus two standard deviations (cut off). Pre-immune sera included in the different assays displayed values lower than the cut-off.

2.7. Analysis of cellular immune response

Splenocytes from the spleens of mice sacrificed 60 d.p.i. (5 mice/group) were aseptically removed. The preparation of splenocyte suspensions was carried out as previously described (Del L. Yácono et al., 2012). Splenocytes cultures (1.25×10^6 cells/well) in RPMI medium (SIGMA) were stimulated with rNcSAG1 (20 μg/mL). Supernatants were harvested at 48 hs (IFN-γ) or 72 h (IL-4, IL-5 and IL-10) and cytokine levels were determined by capture ELISA commercial kits (Pharmingen; BD Biosciences, Sandiego, CA). At least two independent ELISAs were performed for each sample.

2.8. Detection and quantification of parasite load by qPCR

Genomic DNA was extracted using Accuprep genomic DNA (gDNA) extraction kit (Bioneer, Korea) from 1 x 10^7 tachyzoites from *N. caninum* Nc-1 strain or 25 mg of

mice brain tissue following the manufacturer's instructions. Serial dilutions of *N. caninum* gDNA were done in a background of 100 ng of gDNA from uninfected mouse brain tissue. The parasite load in mice brain tissue and host DNA were quantified by real-time PCR using the DNA-binding SYBR Green Master Mix (Invitrogen, CA, USA) as previously described by Pinitkiatisakul et al., (2007). Briefly, *N. caninum* gDNA was detected using primers designed to amplify a 328 bp fragment from the Nc5 sequence (GenBank accession no.X84238), Forward primer Np6: 5′-CAGTCAACCTACGTCTTCT -3′ and reverse primer Np21: 5′-GTGCGTCCAATCCTGTAAC -3'). For the quantification of host DNA and to correct the presence of potential PCR-inhibiting compounds in the DNA samples, a 71 bp fragment from the rRNA S28 from *Mus musculus* (GenBank accession no. X00525) was amplified by using the following primers: Forward 5′-TGCCATGGTAATCCTGCTCA-3′, Reverse 5′CCTCAGCCAAGCACATACACC-3′. Amplification conditions were as previously described (Pinitkiatisakul et al., 2007). Amplification, data acquisition, and data analysis were carried out in an Mx3005P qPCR System using the MxPro qPCR Software 4.0 (Stratagene, CA, USA). Results were expressed as pg of parasite DNA/100 ng host DNA using absolute quantification based on standard curves of tachyzoite DNA and host tissue DNA.

2.9. Statistical analysis

Data analysis was conducted following the recommendation given by Morrison (2002) and extensively used by other researchers on the field (Jiménez-Ruiz et al., 2012). Optical density values obtained from ELISA analysis and parasite load in brain tissue from dams and pups were compared between groups by one-way or two-way ANOVA, followed by Tukey's multiple comparison tests or by Dunnet's multiple

comparison tests. Reproductive parameters, mortality and *N. caninum* infection/vertical transmission (fertility rates, proportion of dead pups and proportion of infected animals) were organized in contingency tables. The chi-squared test was performed when comparing three or more groups. $P < 0.05$ was considered as statistically significant. The portion of all surviving pups was estimated by The Kaplan–Meier survival method. The Gehan-Breslow-Wilcoxon statistical test was achieved to compare survival curves between the different groups. Statistical analyses were carried out using GraphPad Prism 7 Software (GraphPad, CA, USA)

3. Results

3.1. Recombinant protein expression

rNcSAG1 and rAtHsp81.2 were expressed and purified, yielding proteins with apparent molecular weights of approximately 34 and 90 kDa, respectively (Fig. 2). The identity of rNcSAG1 and rAtHsp81.2 were confirmed by mass spectrometry (data not shown). Some additional bands were also observed, and the mass spectrometry analysis confirmed that they were proteolytic products of their respective N-termini (Figs. 2A and 2C). In addition, rNcSAG1 was recognized by monoclonal anti-6XHIS and polyclonal anti-rNcSAG1 antibodies and by sera from mice experimentally infected with *N. caninum* (Fig. 2B) and rAtHsp81.2 was recognized by anti-rAtHsp81.2 polyclonal antibody (Fig. 2D).

3.2. Humoral immune responses against rNcSAG1 and rAtHsp81.2 prior to challenge

Figure 3A shows that G3 and G4 mice depicted high serum levels of anti-rNcSAG1 specific tIgG from 15 d.p.i. However, G4 mice showed the highest titers of

tIgG anti-rNcSAG1 from 30 d.p.i. (titer: 128,000) until the end of the evaluation period (titer: 4,000). In fact, anti-rNcSAG1 tIgG levels were significantly different between these two groups at 60 d.p.i. (Fig. 3C), confirming the immune modulator properties of rAtHsp81.2. Since the proposed adjuvant, rAtHsp81.2, is a recombinant protein, we decided to evaluate the production of anti-rAtHsp81.2 antibodies. Figure 3B shows specific anti-rAtHsp81.2 tIgG titers produced by mice from the different experimental groups.G4 and G2 mice produced high titers of anti-rAtHsp81.2 tIgG from 15 to 60 d.p.i. (titers: 62,000; Fig. 3B). To discard that these specific anti-rAtHsp81.2 antibodies present in sera from immunized mice could cross-react with mouse Hsp90s, we evaluated whether anti-rAtHsp81.2 mouse antibodies recognize mouse Hsp90s by Western blot (Fig. 3D). Interestingly, no band was revealed when sera from mice immunized with rAtHsp81.2 was used as primary antibody against liver or spleen extracts (Fig. 3D), demonstrating that antibodies against rAtHsp81.2 did not cross-react with mouse Hsp90s.

The profile of humoral immune response was determined by the presence of IgG1 and IgG2a against rNcSAG1 in sera from immunized mice (Fig. 3A). Although both vaccine formulations containing rNcSAG1 (G3 and G4) elicited the production of IgG1 and IgG2a from 15 to 60 d.p.i., G4 mice showed higher titers of both isotypes (IgG1: 128,000 , IgG2a: 32,000) 60 d.p.i. than G3 mice (IgG1: 32,000; IgG2a: 8,000). In addition, anti-rNcSAG1 IgG1 and IgG2a levels from G4 mice were significantly higher than those of G3 mice ($P < 0.001$ for each IgG comparison between G4 and G3 group) (Fig. 3C).

3.3. Humoral immune response against rNcSAG1 post-challenge

We analyzed the immune response in pregnant and non-pregnant vaccinated female mice after the experimental infection with *N. caninum* both, at the acute phase of infection, 14.5 days of pregnancy and 78 d.p.i., respectively (Fig. 1) and at the chronic phase of infection, 22 d.p.p. and 106 d.p.i., respectively (Fig. 1). As expected, during the acute phase of infection the tIgG, IgG1 and IgG2a levels detected in sera from pregnant and non-pregnant immunized female mice showed that pregnancy influenced the immune response in all groups (tIgG: $F_{(4, 25)}$= 28.11; P < 0.0001; IgG1: $F_{(4, 16)}$ = 5.988; P < 0.005; IgG2a: $F_{(4, 23)}$ = 4.602; P < 0.05) (Fig. 4A). In addition, tIgG levels of G3 and G4 showed significantly higher values than challenged mice from G2 and G5, both for pregnant and non-pregnant females (Fig. 4A). Similarly, at the acute phase of infection, pregnant and non-pregnant female mice from G3 and G4 showed significantly higher levels of IgG1 and IgG2a anti-rNcSAG1 than mice from challenged control groups (G2 and G5) (Fig. 4A).

The "pregnant condition" showed no effect on tIgG, IgG1 and IgG2a levels detected in sera at chronic phase of infection (analyzed by two-way ANOVA; P>0.05 for each IgG analyzed). Mice from groups that were immunized with rNcSAG1 alone or in combination with rAtHsp81.2 (G3 and G4, respectively) elicited significantly higher levels of tIgG, IgG1 and IgG2a anti-rNcSAG1 than challenged controls (G2 and G5, respectively; Fig. 4B). In addition, data presented here indicate that after the infection with *N. caninum*, the immune profile of vaccinated and challenged mice is preferentially pro-inflammatory (Th1 profile) since higher levels of IgG2a than IgG1 were obtained both at the acute (Fig. 4A) and at the chronic phase of infection (Fig. 4B). These results were similar for dams and non-pregnant female mice.

3.4. The "DIVA-like" character of the vaccine formulation containing rNcSAG1 and rAtHsp81.2

The "DIVA" character of a vaccine formulation refers to its capability in differentiating vaccinated from infected animals (Uttenthal et al., 2010). Considering that the adjuvant rAtHsp82.1 is a recombinant protein, we could hypothesize that immunized animals can produce specific anti-rAtHsp81.2 antibodies, allowing us to differentiate immunized from infected animals. In fact, serum samples from vaccinated challenged G4 dams as well as their pups showed increased specific tIgG anti-rAtHsp81.2 values compared to those from non-vaccinated unchallenged G1 virgin females (Fig. 5)($P < 0.0001$, One-way ANOVA followed by Tukey's multiple comparison's post-test), also confirming maternal antibody transfer. Additionally, neither non-vaccinated challenged G5 dams nor their offspring (G5 pups) showed specific tIgG anti-rAtHsp81.2 over the cut off value (Fig 5). Interestingly, the inclusion of rAtHsp81.2 in the vaccine formulation allowed us to differentiate vaccinated from infected mice.

3.5. Cellular-mediated immune response

Cytokine levels were assessed in culture supernatants after *ex-vivo* rNcSAG1 stimulation of splenocytes from vaccinated mice. Although mice from all groups showed detectable levels of IFN-γ, no significant differences were found when compared to G1 (Fig. S.1A). Also, splenocytes from mice in all groups, including G1, produced similar levels of IL-4 (Fig. S.1B) and IL-5 (Fig. S.1C) after rNcSAG1 stimulation. No detectable levels of IL-10 were found in any immunized group.

3.6. Efficacy of vaccine formulation: reproductive parameters and offspring survival rate

None of the infected non-pregnant female mice or the infected dams showed any clinical sign of neosporosis during the experiments. Table 2 shows the results of fertility rate, number of pups born, hebdomadal mortality (48 h p.p.), cumulated neonatal and prepuberal mortality (18 d.p.p. and 30 d.p.p., respectively). The fertility rate was not affected by any of the formulations administered in the immunization protocol, since no differences were found among groups ($P > 0.05$, $X^2 = 17.00$). On the contrary, hebdomadal mortality, either analyzed by pups or litters, showed significant differences between groups ($P < 0.0001$, $X^2 = 32.52$ and $P < 0.0004$, $X^2 = 20.32$, respectively). Interestingly, G4 showed the lowest hebdomadal mortality among infected groups (10%), whereas G2, G3 and G5 showed hebdomadal mortalities between 35.9% and 51.5%. Similar results were obtained when hebdomadal mortality was analyzed by litter (Table 2). Regarding cumulated neonatal mortality, either analyzed by pups or litters, it also varied significantly among groups ($P < 0.0001$, $X^2 = 45.15$ and $P< 0.0001$, $X^2 = 37.34$, respectively). As determined for hebdomadal mortality, G4 showed also the lowest mortality rate among infected groups (31.2%) when analyzed by pups. Concerning cumulated prepuberal mortality, it was significantly different among groups when studied by pups ($P < 0.0001$, $X^2 =47.21$) and again, G4 showed the lowest mortality rate among infected groups (59.1 % vs. 72.8% - 90%). However, no differences were found between infected groups when analyzed by litters (100% each group). The offspring of unchallenged non-vaccinated G1 mice did not show hebdomadal, neonatal or prepuberal mortality.

The Kaplan-Meier survival curves for the follow up period showed a clear decrease in the number of G4 dead pups compared to the other challenged groups (Fig. 6), which

was significantly different from the survival curve from challenged non-vaccinated G5 mice ($P < 0.0037$, Gehan-Breslow-Wilcoxon test).

3.8. Infection and parasite load in dams and vertical transmission

In order to determine the immunoprotective efficacy of the vaccine formulation in dams, we analyzed the presence of Nc-1 gDNA in brain tissue at the chronic phase of infection. As shown in Table 3, *N. caninum* was detected in dams from all challenged groups, with frequencies varying from 40% to 100%. G3 and G4 dams showed lower percentages of infection (40% and 50%. respectively,Table 3), as well as significantly lower parasite loads in brain tissue compared with those of G5 dams (Fig. 7A, $P<0.005$, one-way ANOVA followed by Dunnet's multiple comparison tests).

Considering the Kaplan-Meier survival curves for pups from previous experiments, we decided to evaluate infection and parasite load in brains at 18 d.p.p. (Fig. 6 and Table 2). The frequency of vertical transmission evaluated by detection of *N. caninum* gDNA in brain tissue from pups showed no significant differences between challenged groups (Table 3, $P > 0.05$, $X^2 = 3{,}992$). However, it is noteworthy that offspring from G4 dams showed the lowest frequency of infection compared with the rest of challenged groups (50%, Table 3), in accordance with the lowest percentage of neonatal cumulative mortality previously shown (31.2 % at 18 d.p.p., Table 2). In those pups where *N. caninum* gDNA was detected, no statistical differences were found in parasite load between challenged groups (Fig.7B, $P>0.05$, one-way ANOVA).

4. Discussion

Neosporosis occurs worldwide and is considered the major infective cause of reproductive failures in cattle (Dubey and Schares 2011; Dubey et al., 2007). However,

there is no available vaccine or treatment for this pathology. In the last few years, the application of innovative vaccine technologies against neosporosis including the development of efficient adjuvants and marker vaccines has been highly recommended (Horcajo et al., 2016; Marugán-Hernandez, 2017).

It has been previously proposed that a suitable antigen candidate for a recombinant vaccine against *N. caninum* should be exposed at the parasite or host cell surface, either constitutively or stage-specifically (Hemphill et al., 2013). These include mostly immune-dominant antigens functionally involved in tachyzoite-host cell interactions, such as the immune-dominant surface protein, NcSAG1 (Hemphill et al., 2006). Based on their promising characteristics, NcSAG1 has been expressed in different systems (Cannas et al., 2003; Yoshimoto et al., 2015) and has proven to be highly immunogenic both in the mouse model (Cannas et al., 2003; Nishikawa et al., 2001; Yoshimoto et al., 2015) and in the target species, *Bos taurus* (Hecker et al., 2014). However, disparate results have been obtained related to immunoprotection, probably depending on the animal model, the infection protocol and the adjuvants included in vaccine formulations (Cannas et al., 2003; Hecker et al., 2014; Nishikawa et al., 2001; Yoshimoto et al., 2015). In fact, it has been recently reported that vaccine formulations against *N. caninum* composed by the same antigen depicted clearly different immune profiles depending on the companion adjuvant (Pinheiro et al., 2018).

In this study, we evaluated the DIVA-like character of a recombinant vaccine which includes one of the best characterized antigens from *N. caninum*, rNcSAG1, and as potential adjuvant and marker protein, rAtHsp81.2, in a mouse model of congenital neosporosis. On the present study, rNcSAG1 was successfully expressed and purified as a (His)6-fusion protein in *E. coli*, and it was recognized by sera from *N. caninum*-infected mice, confirming its antigenicity, as it was expected based on previous reports

(Cannas et al., 2003; Hecker et al., 2014; Howe et al., 1998; Nishikawa et al., 2001). The immunization of mice with rNcSAG1 evoked the production of high titers of specific tIgG, IgG1 and IgG2a antibodies, which was enhanced when rNcSAG1 was administered in combination with rAtHsp81.2, demonstrating the adjuvant character of this protein. The immunization of mice with rNcSAG1 + rAtHsp81.2 not only elicited a strong and prolonged specific anti-rNcSAG1 antibody response, but also triggered the production of anti-rAtHsp81.2 specific antibodies, demonstrating the potential of this formulation as a DIVA-like vaccine. Although the vaccine only conferred partial protection against vertical transmission, it improved median survival time of offspring.

It is generally accepted that an appropriate immune response against *N. caninum* is primarily dependent on the cellular immunity mediated by both CD4+ and CD8+ T cells and their ability to secrete cytokines such as IFN-γ (Horcajo et al., 2016). In the present study, non significant differences were found among groups when stimulated with rNcSAG1 protein, neither in the IFN- γ nor in the IL-4 levels. In agreement with these results, Marugán-Hernandez et al., (2011) have reported that a transgenic *N. caninum* strain constitutively expressing the bradyzoite NcSAG4 protein conferred significant levels of protection against vertical transmission when used as live vaccines in mice without enhancing the cellular immune response. Therefore, another parameter used to monitor the infection, which is generally associated with the Th1/Th2-type immune response modulation is the ratio of IgG1/IgG2a subclasses (Marugán-Hernandez et al., 2011). Our results showed that both IgG1 and IgG2a specific antibodies were obtained. However, an increased IgG1/IgG2a ratio, generally associated with a Th2 immune response (Raghupathy 1997), was biased during the pre-challenged phase in G3 as well as in G4. Although a correlation between the Th2-immune profile depicted by G3 and G4 vaccinated mice and the BALB/c strain of mice used in the present study must not

be discarded (Charles et al., 1999), it was surely not the only factor that influenced the profile of this immunological response. In fact, we have previously reported that plant Hsp90 incorporated as adjuvant in a recombinant vaccine triggered the generation of a Th1 response in the same mice strain (Corigliano et al., 2013). This apparently controversial result could be explained by the fact that, in that report, pHsp90 was fused to the reporter-protein (Corigliano et al., 2013), whereas in the present study the vaccine formulation was a mixture of both proteins. In fact, despite several peptide-Hsp fusion proteins have demonstrated to elicit a strong Th1 immune response without further adjuvants (Chitradevi et al., 2016; Corigliano et al., 2013; Rico et al., 1999; Shokouhi et al., 2018, Sánchez-López et al., 2019), unrelated results have been obtained when mixtures of Hsp/antigen were administered (Blachere et al., 1997; Holakuyee et al., 2012; Labrador-Garrido et al., 2014). In fact, Buriani et al., (2011), demonstrated that the immune response elicited by pHsp70 depended on the charged peptide.

Conversely to that observed prior to challenge, in vaccinated G3 and G4 mice, the ratio IgG1/IgG2a anti-rNcSAG1 in pregnant as well as in non-pregnant mice decreased considerably after challenge, which indicates a shift from an IgG1-dominated to a mixed IgG2a/IgG1 profile, both during the acute and the chronic phase of infection. Our results are in agreement with those reported by Ellis et al., (2008), who found that *N. caninum* recombinant proteins induced a stronger IgG1 response before a challenge was given, which changed to a dominant IgG2a response after challenge. In fact, dams from G3 and G4 showed lower percentages of cerebral infection (40% and 50%. respectively), as well as lower values of parasite load in brain tissue. Since no significant differences were found in the cellular immune response of vaccinated mice compared to controls, we suggest that immune protection against *N. caninum* infection observed in dams from both G3 and G4 could be associated with the post-challenge shift from a Th2 to a mixed

Th1/Th2-type immune response, as previously seen by Monney et al., (2011), as well as, with the ability of anti-rNcSAG1 specific antibodies to inhibit the process of invasion of the parasite onto the host cell, at least partially, as it has also been previously demonstrated *in vitro* (Cannas et al., 2003; Howe et al., 1998; Nishikawa et al., 2001).

Regarding vertical transmission, data presented here show that administration of the subunit vaccine formulation containing rNcSAG1 + rAtHsp81.2 conferred partial protection against congenital neosporosis. In fact, pups born from G4 dams showed the lowest hebdomadal, neonatal and prepuberal mortality rates between infected groups. This result was also reflected on the Kaplan-Meier analysis, since G4 survival curve was significantly different from the survival curve from non-vaccinated infected mice (G5). These results are in agreement with previous reports studying the protective efficacy of subunit vaccines in the pregnant mouse model, in which the best results showed only partial protection, either using only one recombinant antigen, e.g., NcSRS2 (Haldorson et al., 2005), recNcROP2 (Debache et al., 2009) or the combination of various recombinant antigens, e.g. NcROP40 + rNcROP2, rNcROP40, rNcROP2, rNcGRA7 and rNcNTPase (Pastor-Fernández et al., 2015) and recNcROP2/NcMIC1/NcMIC3 (Debache et al., 2009). Moreover, among the large number of experimental vaccines developed and evaluated in animal models [for details see the reviews (Hemphill et al., 2013; Horcajo et al., 2016; Reichel and Ellis 2009)], only few formulations, mostly based on live vaccines (Ellis et al., 2008; Marugán-Hernández et al., 2011; Miller et al., 2005) or killed parasite lysates (Liddell et al., 1999) conferred considerably high levels of protection against vertical transmission. In the present study, we were not able to elucidate the mechanisms by which the vaccine formulation containing rNcSAG1 and rAtHsp81.2 elicited protection against vertical transmission, since a similar immune profile was obtained in dams from the group vaccinated only with rNcSAG1 (increased

IgG2a specific antibodies). However, it is feasible that maternal antibody transfer (through the placentae and/ or maternal milk), evidenced in our study by the presence of anti-rAtHsp81.2 tIgG in offspring from G4 vaccinated dams, could have had some beneficial effect in pup survival, since higher titers of rNcSAG1 antibodies in dams from this experimental group are concurrent with increased survival rate, particularly during lactation period (0-18 d.p.p).

Whether a pro-inflamatory Th1/IgG2- or a Th2/IgG1-biased immune response by immunization protects against vertical transmission is not clear (Aguado-Martínez et al., 2017). It is noteworthy that differences found in protection against vertical transmission in the pregnant mouse model between live and subunit vaccines would not depend on the immune profile elicited, since highly protective live formulations such as Nc-Nowra tachyzoites and live transgenic Nc-1 SAG4c1.1-parasites evoked IgG2a increased levels compared to the amount of IgG1 and IgG1 antibody levels consistently higher than IgG2a levels, respectively (Marugán-Hernández et al., 2011; Miller et al., 2005). A similar picture is observed when the immune profile was determined in subunit vaccines immunized pregnant mice after challenge (Debache et al., 2009; Haldorson et al., 2005; Monney et al., 2013).

In the last few years, several researchers have stated that marker vaccines and DIVA-complaint test (differentiate infected from vaccinated animals) are particularly advantageous in cases where the infection and illness of animals could not be completely avoided by vaccination and control and eradication programs must combine both, vaccination and management of affected cattle (Henderson 2005). Our results showed that, concomitantly with the ability of rAtHsp81.2 to boost the production of anti-rNcSAG1 IgGs, mice vaccinated with rNcSAG1 and rAtHsp81.2 or only with rAtHsp81.2 produced high titers of tIgG anti-AtHsp81.2. Moreover, mice from G4,

evaluated both pre- and post-challenge elicited high specific tIgG anti- rAtHsp81.2 levels (expressed as O.D. values), whereas sera from non-vaccinated challenged mice (G5) depicted similar O.D. values than sera from non-vaccinated unchallenged mice (G1). Although further experiments have to be made in cattle in order to evaluate the capability of rAtHsp81.2 to differentiate infected from vaccinated animals in the field, data obtained in mice are promising.

In this scenario, where only live vaccines have proven to confer high levels of protection against vertical transmission of neosporosis, but without exclusion of the associated risk of reversion to virulence, we propose that the search for potential antigens must continue and new formulations should integrate the "DIVA-vaccine approach", containing not only the combination of different antigens and improved adjuvants, but also enhancers of the immune response (e.g. fusion to Toll like receptor ligands and other "danger signals").

CONCLUDING REMARKS

To our knowledge, this is the first study that evaluates a potential DIVA-like vaccine formulation against *N. caninum* in a well-established pregnant mouse model. The proposed adjuvant and marker protein, rAtHsp81.2 enhanced the production of anti-rNcSAG1 antibodies and allowed us to differentiate vaccinated from infected mice, suggesting a promising role for this protein in future DIVA-like vaccine designs. In addition, the vaccine formulation consisting of rNcSAG1 and rAtHsp81.2 conferred partial protection against vertical transmission and increased pup median survival time.

CONFLICT OF INTEREST

There is no conflict of interest.

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Figure legends

Figure 1. Schematic representation of the experimental design over time.

Exceptions on the day of euthanasia from virgin female mice to evaluate cellular immune response and on the day of euthanasia of pups to determine parasite burden (Experiment 3 of the congenital neosporosis mouse model) are marked and clearly defined. Abbreviations; d.p.i.: days post-first immunization; p.d.: pregnancy date; d.p.p.: days post-partum; I.P.; intraperitoneal; S.C.; subcutaneous; CEL.; cellular; EXP.; experiment.

Figure 1

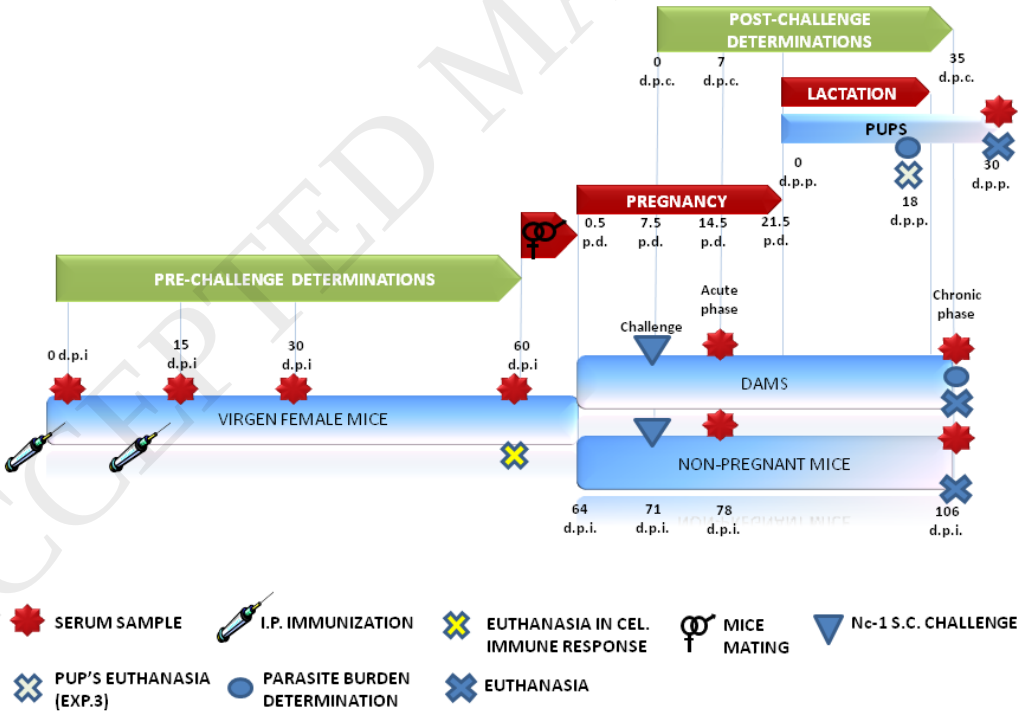

Figure 2. **Analysis of the expression of recombinant proteins included in the vaccine formulations.** The expression and purification of recombinant proteins were checked by Coomassie Brilliant Blue stained polyacrylamide gel and immunoblotting. (A) Expression and purification of *Neospora caninum* SAG1 protein (rNcSAG1). MW: molecular weight marker; Line NI: lysate of *E. coli* BL21 Star^{TM} (DE3) without induction; Line I: lysate of *E. coli* BL21 Star^{TM} (DE3) after IPTG induction; Line E: elute after Ni^{2+} affinity purification; (B) Western blot from rNcSAG1 elute after Ni+ affinity purification using as primary antibody : Line 1) anti 6XHIS monoclonal antibody (1:1,000); Line 2) sera from mice experimentally infected with *N. caninum* (1:500) or Line 3) mouse polyclonal anti-rNcSAG1 antibody (1:500). Arrow indicates the band corresponding to the whole protein identified by MALDI-TOF-TOF, whereas asterisks indicate the bands corresponding to proteolytic products (C) Expression and purification of recombinant *Arabidopsis thaliana* Hsp81.2 (rAtHsp81.2). MW: molecular weight marker; Line NI: lysate of *E. coli* Rosetta (DE3) without induction; Line I: lysate of *E. coli* Rosetta (DE3) after IPTG induction; Line E: elute after Ni+ affinity purification; (D): western blot from rAtHsp81.2 elute after Ni+ affinity purification using a mouse polyclonal anti-rAtHsp81.2 antibody (1:500). Arrow indicates the band corresponding to the whole protein identified by MALDI-TOF-TOF, whereas asterisks indicate the bands corresponding to proteolytic products

Figure 2

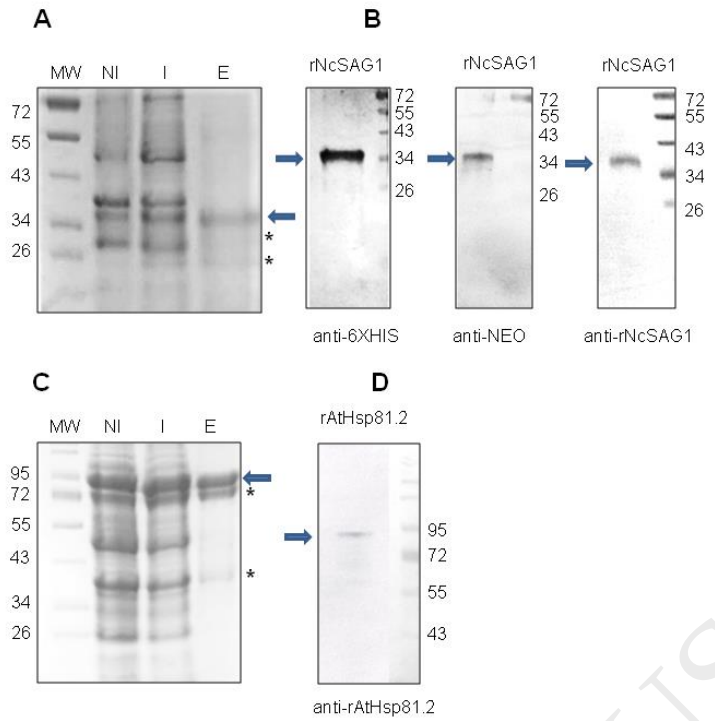

Figure 3. **Humoral immune response against rNcSAG1 and rAtHsp81.2 prior to challenge: (A)** Titers of total Immunoglobulin G (tIgG), IgG1 and IgG2a anti-rNcSAG1. **(B)** Titers of tIgG anti-rAtHsp81.2. Serum samples were obtained from 8 weeks-old BALB/c female mice (n=10/group) which were intraperitoneally immunized as described in the experimental protocol for the following groups: G1, G2, G3 and G4. Sera from mice from each group were pooled for each time point (0, 15, 30 and 60 d.p.i.) and serial dilutions were analyzed by ELISA. Results are expressed as endpoint titers, and the cut-off value was defined as the highest serum dilution that gave a value above the absorbance value for pre-immune sera plus two standard deviations. Two independent ELISA were assayed for each pooled serum samples and the analyses were performed over three independent experiments. **(C)** ELISA detection of rNcSAG1-specific tIgG, IgG1 and IgG2a antibodies. Sera were collected from immunized mice (n=10/group) from the experimental groups G1, G2, G3 and G4, 60 d.p.i., diluted

1:1,000 and assayed for the presence of specific antibodies. Results are shown as the mean O.D ±S.E.M (Abs 630nm).Statistical analyses were performed by one-way ANOVA test using Tukey's Multiple Comparison post-test: a vs. b=P>0.05; a vs. c, d= P<0.001; b vs. c, d= P<0.001; and c vs. d= P<0.001. Two independent ELISA were assayed for each serum sample over three independent experiments. **(D)** Reactivity of naïve mouse tissue samples against anti-rAtHsp81.2 antibodies. Liver and spleen total protein extracts (100 μg) were separated on SDS-12% PAGE gel, transfer to PVDF membranes and incubated with mouse monoclonal anti-human Hsp90 (1:500) or sera from G4 mice (1:500). Phosphatase alkaline conjugated goat anti-mouse tIgG (1:5,000) was used as secondary antibody. The reaction was developed by the addition of NBT/BCIP substrate. Pre-stained proteins were included in Western blots as protein weight marker (WM). rAtHSP81.2 protein was used as positive control (+).

Figure 3

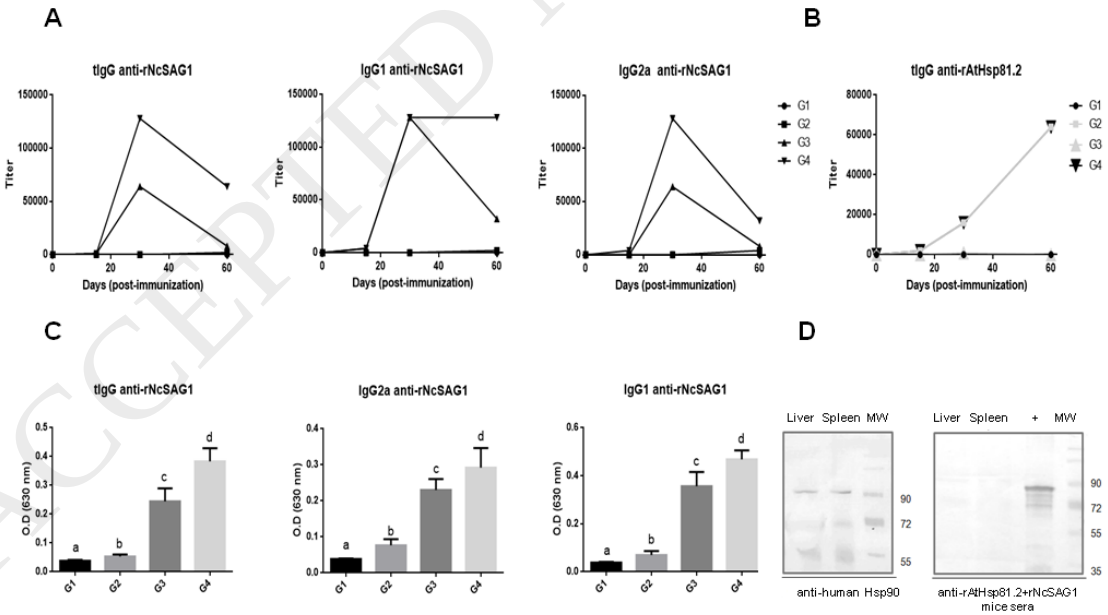

Figure 4. **Humoral immune response against rNcSAG1 post-challenge: ELISA detection of rNcSAG1-specific tIgG, IgG1 IgG2a antibodies (A)** at the acute phase of infection (7 d.p.c.) and **(B)** at the chronic phase of infection (35 d.p.c) in diluted sera (1:300) from G1, G2, G3, G4 and G5 non-pregnant female mice and pregnant female mice. Results are shown as the mean O.D +S.E.M (Abs 630nm). Statistical analyses were performed by two-way ANOVA test and differences within rows in non-pregnant (lowercase letters) and pregnant (capital letters) female mice (experimental groups) were analyzed by Tukey's Multiple Comparison post-test, $P<0.05$ was considered statistically significant. Different letters indicate significant differences between groups. Two independent ELISA were assayed, and the analyses were performed over three independent experiments.

Figure 4

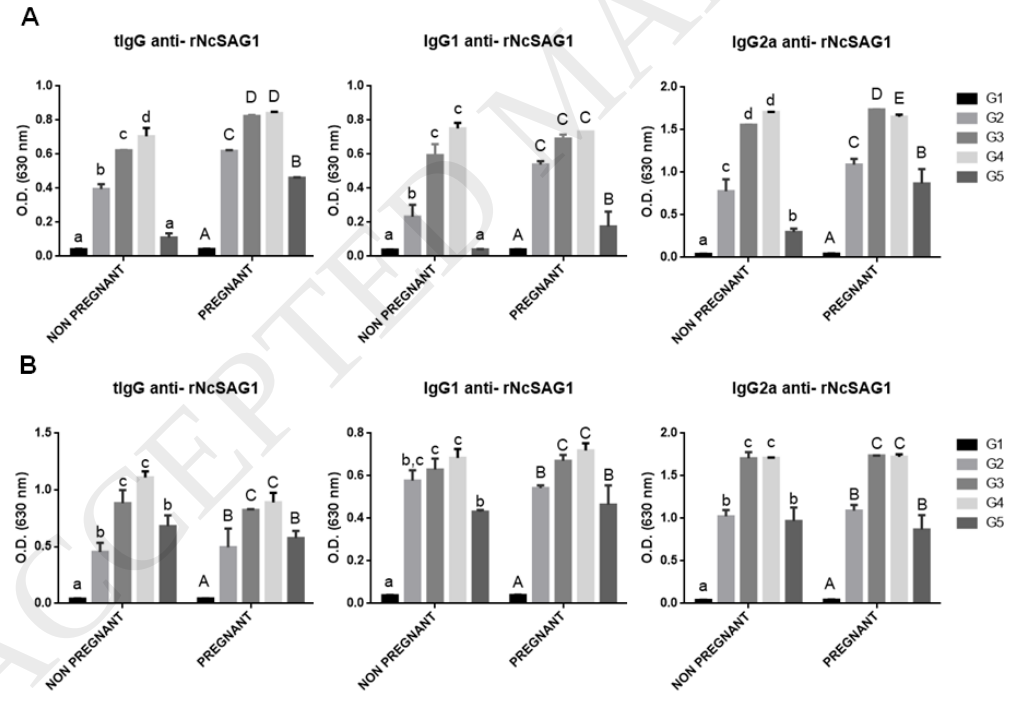

Figure 5. **Differentiation between vaccinated and infected animals: DIVA character of rNcSAG1 + rAtHsp81.2 vaccine formulation. ELISA detection of**

rAtHsp81.2-specific tIgG in diluted sera (1:1,000) from: unchallenged non-vaccinated female mice, 60 d.p.i. (G1 virgin female mice, n=10), challenged non-vaccinated dams, 35 d.p.c. (G5 dams, n=10) and their offspring (G5 pups, n=8), challenged vaccinated dams, 35 d.p.c (G4 dams, n=10) and their offspring (G4 pups, n=8). Results are shown as the mean O.D +S.E.M (Abs 630nm). Statistical analyses were performed by one-way ANOVA test and differences between groups were analyzed by Tukey's Multiple Comparison post-test: $P<0.05$ was considered statistically significant. Different letters indicate significant differences between groups. Two independent ELISA were performed.

Figure 5

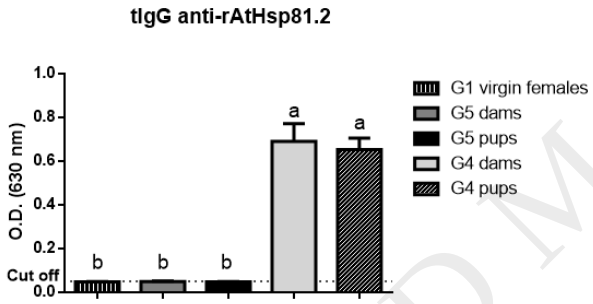

Figure 6. Kaplan–Meier survival curves for pups born from dams from the congenital neosporosis mouse model, corresponding to the experimental groups G1, G2, G3, G4 and G5. The curves represent percent survival as the proportion of all individuals surviving over a period of 30 d.p.p. Vertical steps downward correspond to d.p.p. when

at least one death was observed. Percent survival among mice from G4 was statistically greater than that among mice from G5 (P<0.0037, Gehan-Breslow-Wilcoxon test). Analyses were performed over three independent experiments.

Figure 6

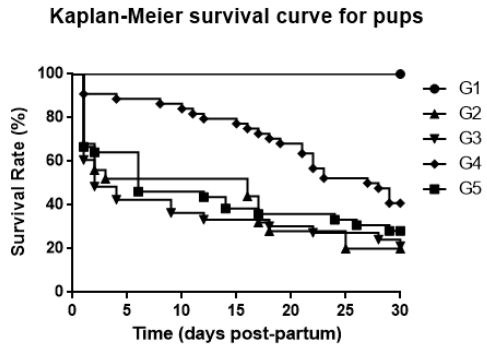

Figure 7. Cerebral parasite burden assessed by quantitative real-time PCR in A) dams from G1, G2, G3, G4 and G5 at 35 d.p.c. and B) their pups at 18 d.p.p.. Results are shown as pg of parasite genomic DNA detected in 100 ng of host genomic DNA extracted from brain tissue (mean per group) + S.E.M (Abs 630nm). Statistical analyses were performed by one-way ANOVA test and differences between groups were analyzed by Dunnet's Multiple Comparison post-test. Only positive samples from challenged groups were considered for the analyses. **P<0.005 compared to G5.

Figure 7

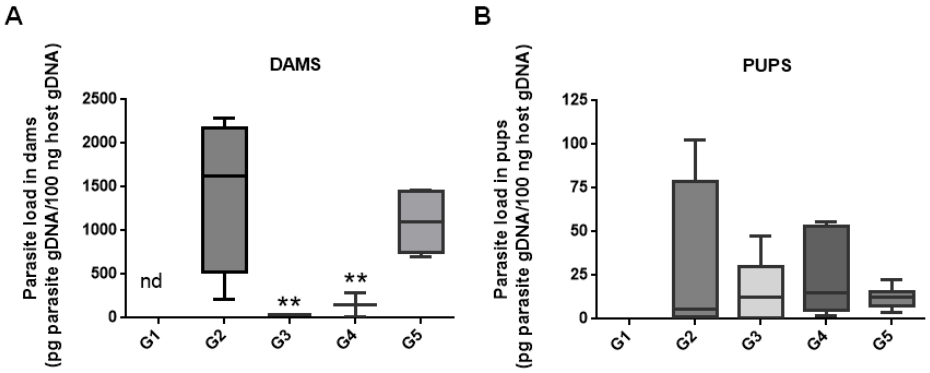

Table 1. Groups of mice employed in the experimental protocols.

Group	Immunization	Challenge
G1 (C-, non-vaccinated)	200 µl PBS	-
G2 (Adjuvant control)	30 µg of rAtHsp81.2 in 200 µl of sterile PBS	2 x 10^6 Nc-1 tachyzoites
G3	10 µg of rNcSAG1 in 200 µl of sterile PBS	2 x 10^6 Nc-1 tachyzoites
G4	10 µg of rNcSAG1 + 30 µg of rAtHsp81.2 in 200 µl of sterile PBS	2 x 10^6 Nc-1 tachyzoites
G5 (C+, non-vaccinated)	200 µl PBS	2 x 10^6 Nc-1 tachyzoites

Table 2. Fertility rates of dams and number of born pups, hebdomadal, neonatal and prepuberal mortality of pups.

Group	Fertility rate (a)	**Born pups (b)**	Hebdomadal mortality per pups (c)	per litter (d)	Neonatal mortality (18 d.p.p.) per pups (e)	per litter (f)	Prepuberal mortality (30 d.p.p.) per pups (g)	per litter (h)
G1	10/22 (45.5)	25/10	0/25 (0)	0/10 (0)	0/25 (0)	0/10 (0)	0/25 (0)	0/10 (0)
G2	6/15 (40)	25/6	12/25 (48)	5/6 (83.3)	18/25 (72.2)	6/6 (100)	20/25 (80)	6/6 (100)
G3	8/16 (50)	33/8	17/33 (51.5)	7/8 (87.5)	23/33 (70)	8/8 (100)	25/33 (78.8)	8/8 (100)
G4	8/16 (50)	44/8	4/44 (10)	4/8 (50)	13/44 (31.2)	8/8 (100)	26/44 (59.1)	8/8 (100)
G5	10/16 (62.5)	39/10	14/39 (35.9)	8/10 (80)	25/39 (64.1)	9/10 (90)	28/39 (72.8)	10/10 (100)

d.p.p: days post-partum.

a. Number of pregnant female mice/ total number of female mice paired with males.

b. Total number of pups born (alive or dead)/ total no. of dams from each experimental group.

c. No. of pups that died during the first 48 hours post-partum / total no. of pups born in the group (percentage).

d. No. of litters with at least one pup that died during the first 48 hours post-partum / total no. of litters in the group (percentage).

e. No. of pups that died during the first 18 days post-partum /no. of pups born in the group (percentage).

f. No. of litters with at least one pup that died during the first 18 days post-partum /no. of litters in the group (percentage).

g. No. of pups that died during the first 30 days post-partum /no. of pups born in the group (percentage).

h. No. of litters with at least one pup that died during the first 30 days post-partum /no. of litters in the group (percentage).

Table 3. Infection and vertical transmission.

Group	Nc1+ dams/ total dams (a)	Nc1+ pups/total pups (b)
G1	0/6 (0)	0/11 (0)
G2	5/5 (100)	4/5 (80)
G3	2/5 (40)	5/6 (83)
G4	3/6 (50)	8/16 (50)
G5	5/7 (71.4)	8/10 (80)

Parasite detection in brain tissue from dams and pups correspond to samples from the same experiment in order to correlate results from infection in pregnant mice and their offspring.

a. Number of dams tested positive for *N. caninum* gDNA in brain (35 d.p.c.) / total number of challenged dams (35 d.p.c.) (percentage)

b. Number of pups (18 d.p.p.) tested positive for *N. caninum* gDNA in brain / total number of pups (18 d.p.p.) (percentage).