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Dose-dependent immunogenicity of a soluble *Neospora caninum* tachyzoite-extract vaccine formulated with a soy lecithin/ β -glucan adjuvant in cattle

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ABSTRACT

Mice immunized with a soluble extract of *Neospora caninum* tachyzoites (sNcAg) formulated with Providean-AVEC[®], an aqueous soy-based adjuvant, are fully protected from *N. caninum* multiplication. Here we evaluated the dose-dependent immunogenicity of this vaccine formulation in cattle. Cattle ($N = 3$ per group) were immunized with two applications (30 days apart) of formulations containing Providean-AVEC[®] and different payloads of sNcAg (100, 50 and 10 μ g), that were five to fifty times lower than the only reported study using this same antigen in cattle. Kinetics and magnitude of the vaccine-induced immune responses were dose-dependent. Cattle immunized with 100 μ g-sNcAg elicited high-avidity specific antibodies 3 weeks after the primary vaccination while those that received 50 μ g of antigen had maximum levels of specific high-avidity antibodies 5 days after the day 30 boost. Vaccination with 10 μ g of sNcAg induced comparable antibody responses after 2 weeks post re-vaccination. IgG1 was the predominant isotype in all vaccinated animals. Maximum systemic IFN- γ levels were measured in cattle immunized with 50 and 100 μ g-sNcAg (14 ± 2.8 ng/ml). CD4⁺-T cells from vaccinated animals proliferated after sNcAg stimulation *in vitro*, producing IFN- γ . Recall IFN- γ responses mediated by CD4⁺-T cells were detected up to 140 days post vaccination. Formulations containing Providean-AVEC[®] and 50 μ g of sNcAg stimulated broad cellular and humoral immune responses against *N. caninum* in cattle. The profile and magnitude of the immune response elicited by this vaccine can be modified by the antigen-dose and vaccination schedule. This is the first dose-response study performed in cattle using sNcAg as antigen.

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1. Introduction

The intracellular protozoan parasite *Neospora caninum* is a cyst-forming coccidian recognized worldwide as an important cause of reproductive failure in

cattle (Dubey, 2003). Neosporosis is generally latent and asymptomatic in non-pregnant cattle, although the consequences of infection in a pregnant cow can be abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf (Innes et al., 2002). Currently, there is no effective method for controlling neosporosis, except for intensive farm management to reduce the probability of infection. Therefore, the development of an effective and safe vaccine against *N. caninum* is of great importance due to the significance of the economic losses in the dairy and beef industries (Dubey et al., 2007).

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There is accumulating evidence that cattle previously exposed to *N. caninum* are less likely to abort than those undergoing a primary infection (Innes et al., 2002). Vaccination of dams with live parasites protected against fetal death by inducing strong cellular and IFN- γ responses (Williams et al., 2007). However, the use of live vaccines can have some disadvantages including reversion to high virulence creating sources of persistent infections, risk of transmission of prion diseases and high costs for industrial production. Although there was evidence of efficacy using a commercial vaccine based on a crude lysate of tachyzoites (Romero et al., 2004) a recent clinical trial concluded that vaccination after conception prevented 61% abortions in one of five herds and that vaccination may have increased the risk of early embryonic death (Weston et al., 2012). Fractions of *N. caninum* soluble native antigens (sNcAg) constitute a promising candidate antigen for vaccine development (Alvarez Garcia et al., 2006; Baszler et al., 2000; Mansilla et al., 2012; Moore et al., 2011; Otsuki et al., 2013), as it does not require inactivation treatments that could modify and ruin antigenic determinants. Proteins present in the soluble-fraction have been identified as stimulators of CD4⁺-T cell responses (Rocchi et al., 2011; Tuo et al., 2005) and dendritic cell maturation, promoting the acquired immunity, which could not be achieved using inactivated parasites (Mansilla et al., 2012).

Several studies performed in mice demonstrate the need for careful adjuvant selection to enhance the immunogenicity of killed parasites (Ribeiro et al., 2009; Rojo-Montejo et al., 2011), crude whole tachyzoites extract (Williams et al., 2007) or the soluble parasite fraction as vaccine antigen (Mansilla et al., 2012). In this regard, upon screening for aqueous-based adjuvants in a mouse model, comparing ISCOM matrix, Aluminum hydroxide and a vegetable-based adjuvant developed in our laboratory (Providean-AVEC®), we showed that low sNcAg payloads (i.e. 0.4 μ g/dose) formulated with Providean-AVEC® adjuvant were protective in the BALB/c model, while other adjuvants required 10 times more antigen to induce protection (Mansilla et al., 2012). Despite all these advances in using mice as a strategic screen for vaccine efficacy, one ultimately needs to do cattle trials with putative vaccine candidates.

Antigen payload has been shown to influence both the type of immune response and the production of cytokines (Hosken et al., 1995). In a previous study we compared two sNcAg doses in mice and evaluated protection against brain infection (Mansilla et al., 2012) but the evaluation of sNcAg dose–response has not been addressed in cattle. Only one high payload vaccine containing 500 μ g of sNcAg has been tested in this model (Moore et al., 2011). This amount of antigen thwarts the feasibility of producing a sNcAg-based commercially available vaccine against this parasite.

In the present study, we evaluated the immunogenicity of three different sNcAg antigen payloads formulated with Providean-AVEC® in naïve cattle by analyzing the cell-mediated immunity induced by the formulations and by screening for the production of high-avidity antibodies. To our knowledge, sNcAg dose–response kinetics in cattle has never been evaluated. We are interested in examining the possibility of inducing protective immunity in ruminants

under experimental conditions to further investigate the protective effect of vaccination against abortion caused by *N. caninum*.

2. Materials and methods

2.1. Parasite and antigen preparation

N. caninum tachyzoites (Nc1 isolate) were cultured in Vero cells under conditions previously standardized (Collantes-Fernandez et al., 2002). Soluble extracts of native antigen were prepared as described before (Moore et al., 2011). Briefly, tachyzoites (2×10^9) were partially purified using sephadex columns (Sephadex™ G-25 Medium, GE Healthcare, Sweden). After centrifugation, the pellet fraction was disrupted by ultrasonic treatment in an ice-bath, and centrifuged at $10,000 \times g$ for 30 min at 4°C. Soluble antigens (sNcAg) were recovered from the supernatant. Proteins in the antigen preparations were quantified by using a commercial protein assay method (Micro BCA Pierce, Rockford, USA). Production yielded 3.0 μ g of soluble native protein per 1×10^6 tachyzoites.

2.2. Vaccines

All vaccines contained 0 (control), 10, 50 or 100 μ g of sNcAg antigen per dose, formulated with 30% Providean-AVEC® (Tecnovax S.A., Buenos Aires, Argentina) (Mansilla et al., 2012). Each 2 ml dose was injected subcutaneously (sc) on the neck (the area in front of the shoulder) using a 21-gauge needle.

2.3. Animal use and vaccination schedule

Twelve 2-year-old Aberdeen Angus heifers, free of several diseases (brucellosis, tuberculosis, campylobacteriosis and trichomonosis) were involved in the study. These heifers were seronegative for *N. caninum* by indirect fluorescent antibody test ($<1:25$ serum dilution). They were in good body condition and had adequate genital development at the start of the trial. Routine clinical examination and vaccination with inactivated vaccines against Bovine Viral Diarrhea Virus (BVDV) and Bovine Herpesvirus-1 (BHV-1) (Tecnovax S.A.) were performed twice prior to the start of the study in 21 days interval. Animals were randomly sorted into four groups. Each group of 3 animals received two applications of the corresponding experimental vaccines at day 0 and 30 days post vaccination (dpv). Reactions at injection sites were evaluated visually and by palpation on daily bases along the week following each immunization. Serum samples were obtained a week before the experiment, the day of the first vaccination (0 dpv) and at regular intervals thereafter up to 130 days after the first vaccine dose (21, 35, 42, 50, 57, 85 and 130 dpv). Whole blood samples for assessment of cell-mediated immune responses were taken at day 0 and 21 dpv, as well as three time points after the boost at 42, 50 and 140 dpv.

2.4. Indirect fluorescent antibody test (IFAT)

IFAT was performed as previously described (Venturini et al., 1999) using a fluorescein isothiocyanated (FITC)-labeled affinity-purified rabbit anti-bovine IgG antibody (Sigma, St. Louis, USA). *N. caninum* specific antibodies were measured in serum samples, tested in serial two-fold dilutions from 1.25 to endpoint titer. Positive and negative control sera were used (Venturini et al., 1999). Slides were examined with an epifluorescence microscope (Olympus BX51, Olympus Inc., Tokyo, Japan). Antibody titers were expressed as the reciprocal of the highest serum dilution that showed complete peripheral fluorescence of tachyzoites (Pare et al., 1995).

2.5. ELISA tests for serological assessments

Serum samples from immunized and control cattle were analyzed for specific Antibodies by ELISA using the ID Screen® *N. caninum* indirect ELISA (ID-VET, Montpellier, France) following the manufacturer's protocol. Cut-off value was calculated to be 30% with respect to the kit's positive control by evaluating 25 negative (IFAT titer < 1:25) and 10 positive bovine serum samples (IFAT titer > 1:200).

Isotype ELISA was assessed on the same platform revealing with horseradish peroxidase-conjugated sheep anti-bovine IgG1 or IgG2 (1:1000, AbD Serotec, Oxford, UK).

For avidity studies serum samples were run in duplicate wells and paired wells were washed with regular washing buffer (ID Screen® *N. caninum* indirect ELISA) or with 6 M urea-PBS (Franco Mahecha et al., 2011; Lavoria et al., 2012). The ELISA then followed the manufacturer's protocol. Percentage of residual reactivity after the urea-wash treatment was calculated and expressed as avidity index "AI%" (Lavoria et al., 2012).

2.6. IFN- γ ELISA

Whole blood cultures were performed using 1.5 ml aliquots from each animal in 24-well sterile cell culture plates. Replicate wells were stimulated with sNcAg at a final concentration of 10 μ g/mL, and Pokeweed mitogen (PWM, 10 μ g/mL; Sigma) or phosphate buffer saline (PBS) as controls. Plates were incubated for 16 h at 37 °C with 5% CO₂ and then centrifuged for plasma collection. Stimulated plasma samples were stored at –20 °C until processing. IFN- γ present in stimulated plasma was detected using a commercial Bovine Interferon Gamma ELISA KIT (AbD Serotec, Oxford, UK) performed according to manufacturer's specifications. A standard curve was generated with known concentrations of recombinant bovine IFN- γ (AbD Serotec, Oxford, UK) in a range from 50 ng/ml to 0.025 ng/ml, and was included in each trial. Results from each plasma sNcAg assessment were considered to be valid when PBS stimulation rendered less than 1.45 ng/ml and PWM, above 3.2 ng/ml.

2.7. Flow-cytometric analyses

Peripheral blood mononuclear cells (PBMC) were purified using Histopaque 1083 (Sigma) and frozen in liquid nitrogen until evaluation. All antibodies for flow-cytometry were purchased from AbD Serotec (Oxford, UK).

For the lymphoproliferation assay 2×10^7 defrosted PBMC from each animal were recovered by overnight culture and stained with CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, NY, US) for standard proliferation assessment following the manufacturer's instructions. After staining, 2.5×10^5 cell/well (100 μ l) were plated in 96-well plates and incubated with PWM (10 μ g/ml), sNcAg (1 μ g/ml) or PBS in a 96-well cell in triplicates. Cell-antigen mixture was cultured for 5 days at 37 °C, 5% CO₂. After incubation the entire cell population was analyzed on a flow cytometer (BD Biosciences FACSCalibur™ flow cytometer; BD CellQuest Pro Software). The percentage of dividing cells was calculated by setting markers over peaks on histograms using the mean undivided cell CFSE fluorescence, minus auto fluorescence (boundaries were $\pm 0.15 \log_{10}$ either side of each cell division peak).

Cells (5×10^5) were also stained with anti bovine CD4-RPE (IgG2a, Isotype control: IgG2a-RPE), CD8-Alexa 647 (IgG2a, Isotype control: IgG2a-Alexa 647) and WC1-FITC (IgG2a, Isotype control: IgG2a-FITC) to identify which cell population underwent anamnestic responses.

PBMC were stained for intra-cellular IFN- γ after 1 h of *in vitro* stimulation with the different antigens, following a standard protocol. Briefly, 1×10^6 stimulated cells from individual animals were treated with Brefeldin A (Sigma) and incubated for 3 more hours. Then, anti CD4-FITC (IgG2a, Isotype control: IgG2a-FITC) and anti CD8-RPE (IgG2a, Isotype control: IgG2a RPE) were added, incubated for 30 min and followed by a cytofix-cytoperm™ (BD Pharmingen, Biosciences, New Jersey, U.S.) treatment and additional anti-IFN- γ Alexa 647 staining (IgG1, Isotype control: IgG1-Alexa 647).

2.8. Cytokine qRT-PCR

Total messenger RNA was extracted from PBMC of vaccinated animals after being stimulated for 2 h with 1 μ g sNcAg, PWM (10 μ g/ml) or PBS, using an RNeasy Mini Kit (Qiagen, West Sussex, UK) according to the manufacturer's guidelines. RNA was treated with 1 U of RQ1 RNase-free DNase (Promega, Madison, WI, US) following the manufacturer's specifications. Purified RNA was quantified using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, US). Reverse transcription was performed with oligo (dt) primers (Biodynamics SRL, Bs. As., Argentina), 1 μ l of 25 mM of dNTPs (Promega) and 200 ng of RNA in DEPC water. MMLV reverse transcriptase (Promega) and RNasin ribonuclease inhibitor (Promega) were used for reverse transcription at 42 °C for 60 min. Primers for bovine GAPDH, IL-4, TNF- α and IL-10 were described previously (Seo et al., 2007). Real-time PCR was assessed with an ABI Prism 7500 real-time PCR system (PE Applied Biosystems, Foster City, CA, US). Each reaction contained 12.5 μ l of Mezccla Real® (Biodynamics), 0.5 μ l of ROX reference dye (Invitrogen), the corresponding primer set

and 2 μ l of cDNA in a 25 μ l total volume mix. PCR conditions were: 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s; 60 °C for 1 min after. Relative Expression levels of each cytokine were calculated by the comparative CT method (Livak and Schmittgen, 2001). The amounts of targets were expressed as an n-fold change relative to the calibrator and $2^{-\Delta\Delta CT}$, normalized to GAPDH.

2.9. Statistical analysis

Comparisons between two groups were performed using Student's *t* test or Mann–Whitney *U* (if normality failed). Serum antibody responses (IFAT and isotype titers) and IFN- γ responses were analyzed using one-way repeated measures analysis of variance (ANOVA) with treatment as the grouping factor and time as the repeated measures factor. *Post hoc* Tukey's pairwise comparisons were performed when significant differences between treatment groups were detected. Differences with $p < 0.05$ were considered significant at the 95% confidence interval. Statistical analysis was performed using Sigma Stat 3.5 (Systat software).

3. Results

3.1. Reactions to the vaccine formulation

Local inflammatory reactions at injection sites were monitored visually and by palpation. No reactions were recorded in any of the heifers. No indurations or adverse effects were observed.

3.2. Serum antibody responses

Neospora-specific antibodies were followed by taking serum samples after the first vaccine-dose (21 dpv) and also at different time points after the 30 dpv boost. The presence of specific antibodies was determined by indirect ELISA and IFAT (Fig. 1).

The first observation was that the kinetics of induction of specific antibodies was related to the vaccine payload. Heifers vaccinated with 100 μ g of sNcAg responded earlier than those immunized with 50 and 10 μ g of antigen, with IFAT titers over control-group values (Fig. 1A). Peak antibody levels were obtained at 35 dpv for the three

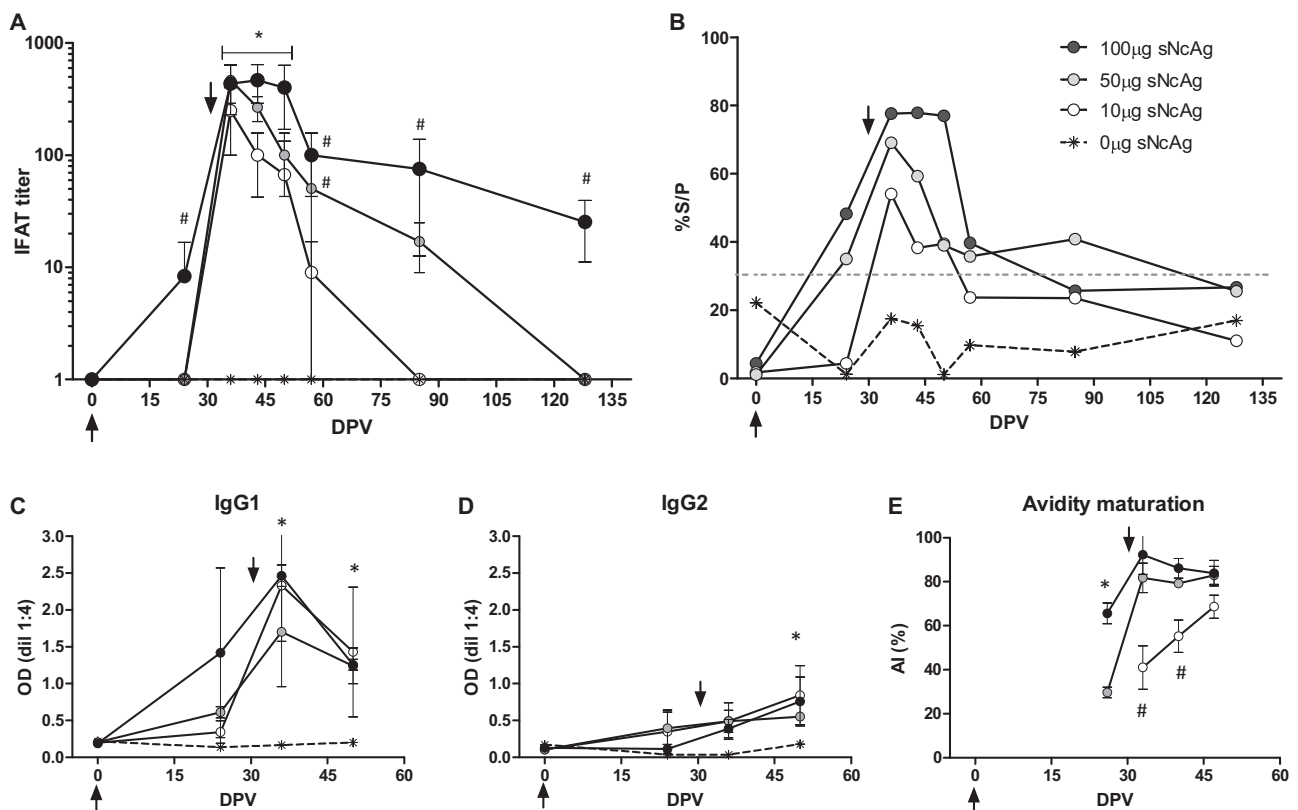


Fig. 1. Time course serum antibody response of cattle immunized with different sNcAg payloads formulated with *Providean-AVEC*[®]. The primary immunization was administered at day 0 followed by a booster immunization at 30 dpv (indicated by arrows). (A) Mean antibody titers \pm standard deviation (SD) measured by indirect immunofluorescent assay (IFAT), showing significantly higher titers ($*p < 0.05$) in vaccinated animals from days 35 to 60 post-vaccination compared with control-immunized animals (0 μ g sNcAg/*Providean-AVEC*[®]). Significant differences compared to 10 μ g sNcAg-vaccinated calves are also indicated (#). (B) Pooled-serum samples were assessed in a commercial ELISA test. Percent reactivity relative to the kit positive control is depicted. The dotted line indicates the assay's cut-off value. Mean \pm SD anti *N. caninum* IgG1 (C) and IgG2 (D) serum titers measured from day 0 to 50 dpv, to correspond with the peak in detectable antibody peak. Values that are significantly different from the control-immunized animals are indicated ($*p < 0.05$). (E) Avidity maturation of anti *N. caninum* specific IgG corresponding with the peak in detectable antibody. Data are expressed as mean avidity indexes (%) \pm SD. This corresponds to residual reactivity of diluted sera incubated in the presence of a chaotropic agent to remove low-strength binding antibodies. *Significantly different from the 10 μ g-sNcAg vaccine value ($p < 0.05$). #Significantly lower than the other groups ($p < 0.05$).

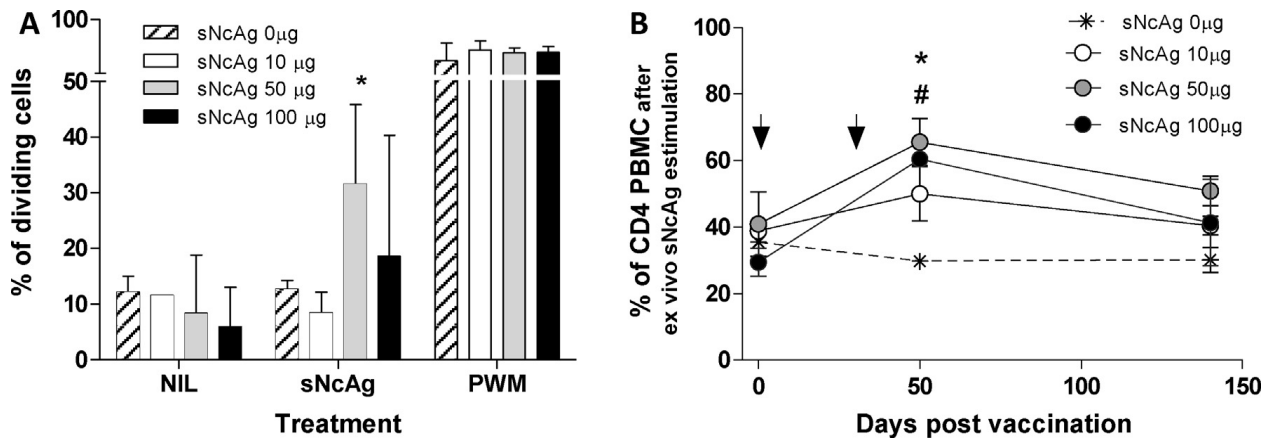


Fig. 2. (A) Proliferative response of peripheral blood mononuclear cells obtained at 50 dpv, stimulated with 1.0 µg/ml PWM, 1.0 µg/ml of sNcAg, or 1.0 µg/ml of Vero cell lysate (NIL) in cattle immunized with 0, 10, 50 or 100 µg of sNcAg/*Providean-AVEC*[®], as indicated. Percentage of dividing cells was calculated by using the mean undivided cell CFSE fluorescence at each division. *Significantly different from control vaccinated animals (group 0 µg sNcAg; $p < 0.05$). (B) Percentage of CD4⁺-T cells in sNcAg stimulated PBMC quantified by flow cytometry at 0, 50 and 140 dpv for the different vaccines. *Percentage of CD4⁺-T cells was significantly higher than control-vaccinated animals at the same time point ($p < 0.05$). #Differences were significant with respect to values measured at 0 dpv ($p < 0.05$).

vaccines, 5 days after the vaccine boost was administered. Antibody levels were maintained but did not increase after the boost when using the highest antigen payload (100 µg). Nevertheless, differences between three sNcAg-vaccinated groups were not significant from 35 to 60 dpv. Antibodies measured by ELISA (Fig. 1B) showed similar kinetics except that both 50 and 100 µg-sNcAg induced antibodies over the cut-off level after the first immunization (21 dpv).

Antibody levels declined earlier when lower amounts of antigen were used. Antibodies from cattle vaccinated with 50 and 100 µg persisted over control values (Fig. 1A) or ELISA cut-off levels (Fig. 1B) up to 2 months after the first vaccination, while the lower dose rendered undetectable responses about 1 month after the boost. Animals immunized with adjuvant alone (control group) did not elicit *N. caninum* specific antibodies throughout the study.

Isotypes of specific IgG were analyzed up to 50 dpv, corresponding to the peak in total antibody. Kinetics of specific IgG1 (Fig. 1C) were similar to total IgG curves at the same time points, while IgG2 levels were low throughout the study, and only a slight increase was observed at 50 dpv (significantly different from control-vaccinated animals, $p < 0.05$).

Avidity of specific antibodies was determined along the time points of high antibody levels, at 21, 35, 42 and 50 dpv (Fig. 1E). After the first dose, antibody avidity was about $65.65 \pm 4.73\%$ for animals vaccinated with 100 µg-sNcAg and $29.68 \pm 2.37\%$ for those immunized with 50 µg. Differences between these groups were significant at this time point ($p < 0.05$). Avidity increased after the boost reaching similar levels for both vaccines: AI = $82.93 \pm 4.14\%$ and $86.13 \pm 5.78\%$ for the 50 and 100 µg sNcAg-dose, respectively, that were significantly different to avidity values induced by the 10 µg-sNcAg vaccine at 35 dpv. Avidity of antibodies from cattle immunized with 10 µg-sNcAg increased after the boost, reaching similar levels as the other vaccines at 50 dpv (AI = $66.69 \pm 5.23\%$).

3.3. Lymphoproliferation

Proliferative responses were studied by stimulating PBMC from control and sNcAg vaccinated animals with sNcAg, VERO cell-lysate (NIL) or PWM (cell-viability control) and measuring dividing cells using CFSE staining and flow-cytometry analysis. Proliferative responses were observed at 21, 50 and 140 dpv, however responses significantly different to cells from control-vaccinated animals were induced only at 50 dpv ($p < 0.05$) (Fig. 2A). At this time point, PBMC from animals vaccinated with 50 and 100 µg of sNcAg proliferated upon contact with *N. caninum* antigens to levels significantly superior than NIL treatment and pre-immune samples ($p < 0.05$). These proliferating cells were mostly CD4⁺-T cells (Fig. 2B) as the number of total systemic CD4⁺-T cells increased at 50 dpv. This increase was significant ($p < 0.05$) in PBMC from 50 and 100 µg-sNcAg vaccinated animals. This particular population proliferated after incubation with sNcAg as no significant changes in

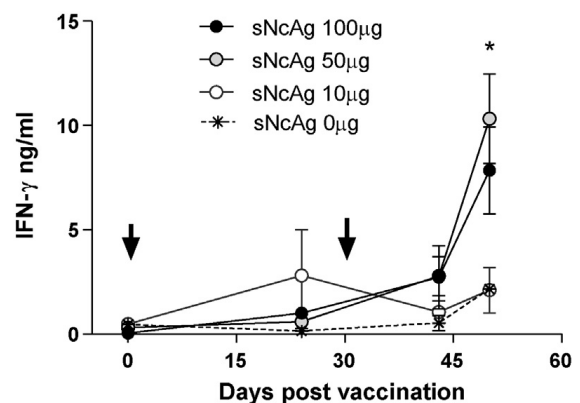


Fig. 3. IFN-γ responses (mean ± SD) measured by ELISA in sNcAg (10 µg/ml) stimulated plasma from cattle immunized with sNcAg/*Providean-AVEC*[®]. Arrows indicate the time of each vaccination. *Values in 50 and 100 µg-sNcAg groups were significantly different from negative control ($p < 0.05$).

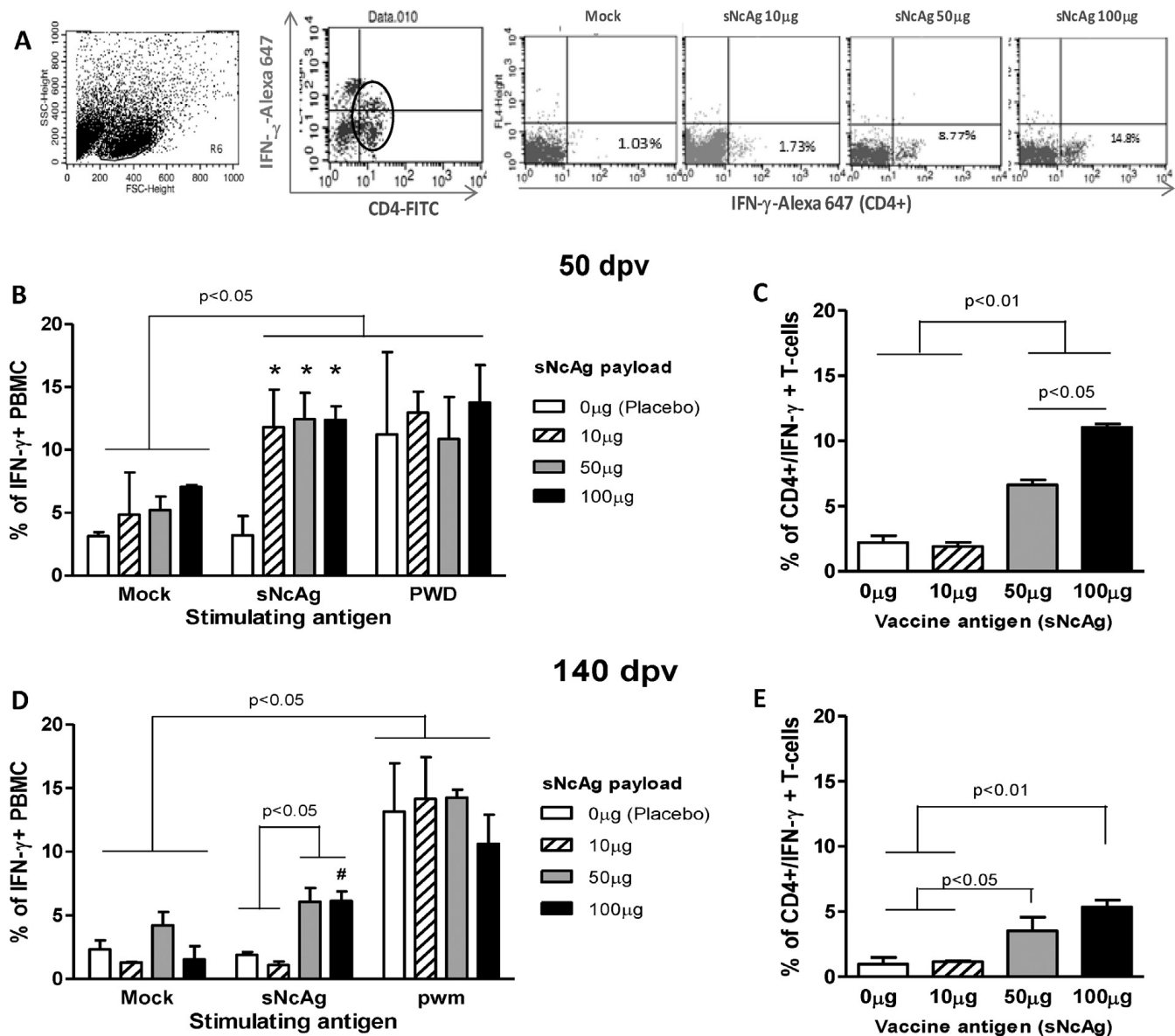


Fig. 4. Identification of PBMC and CD4⁺ T cells producing IFN-γ (mean ± SD values from triplicate experiments) upon stimulation with sNcAg and Pockweed mitogen (PWM), assessed at 50 and 140 dpv, as indicated. (A) Representative assessment of the evaluation of CD4⁺/IFN-γ⁺ T-cells in stimulated PBMC. Cells were double-stained for intracellular IFN-γ with Alexa 467 conjugated anti IFN-γ and for surface expression of CD4 marker using a FITC-conjugated anti CD4 antibody. Double positive cells were gated and analyzed separately. Percentage of PBMC at 50 dpv (B and C) and 140 dpv (D and E) producing IFN-γ after incubation with PWM, sNcAg or mock treatment as indicated. *Values significantly different from control, 0 μg sNcAg group ($p < 0.05$). #Significantly higher than mock treated cells ($p < 0.05$).

the total number of CD8⁺ or γδ-WC1⁺ T cells were found in blood samples (data not shown).

3.4. Cytokines induced by vaccination

Systemic IFN-γ responses were analyzed by culturing whole blood of vaccinated animals taken at 0, 21, 42, 50 and 140 dpv, with sNcAg (Fig. 3). After the first vaccination IFN-γ responses were undetectable in all animals except one immunized with 10 μg of sNcAg. Twenty days after the boost (50 dpv), cattle vaccinated with 50 and 100 μg-sNcAg induced high IFN-γ responses that were not detected at 140 dpv (data not shown). Cattle vaccinated with 10 μg sNcAg elicited low amounts of IFN-γ.

Using intracellular staining with specific antibodies, we analyzed a number of PBMC expressing this cytokine. CD4⁺ T cells were selected since they proliferated at 50 dpv (Fig. 4). The number of total PBMC expressing IFN-γ did not differ between the sNcAg-vaccines and was, in all cases, superior to mock-treated cells at 50 dpv (Fig. 4B). However, the number of IFN-γ⁺/PBMC decreased at 140 dpv for the lower-dose vaccinated animals; there was a higher percentage of IFN-γ⁺/PBMC compared to mock in those vaccinated with 100 μg-sNcAg (Fig. 4D).

The percentage of CD4⁺/IFN-γ⁺ T cells, however, was significantly higher at 50 dpv in the groups immunized with 50 and 100 μg of sNcAg relative to control-vaccinated animals ($p < 0.01$) (Fig. 4C). The number of CD4⁺/IFN-γ⁺ T cells between these two groups was also significantly

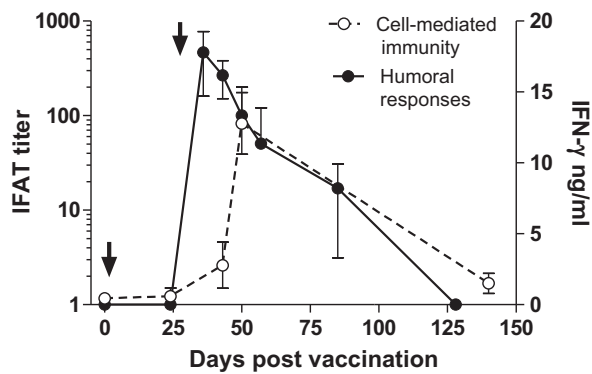


Fig. 5. Time course of humoral and cellular systemic adaptive responses in cattle after immunization with a vaccine formulated with a 50 µg sNcAg/Provident-AVEC® formulation. Each immunization is indicated with an arrow. Humoral responses: mean IFAT titers \pm SD values (left axis). Cellular immune responses: IFN- γ concentration in stimulated plasma (mean \pm SD values, right axis).

different ($p < 0.05$), indicating that the stimulation of IFN- γ producing cells was related to the vaccine-antigen payload. The higher number of CD4 $^{+}$ /IFN- γ T-cells with respect to the control group were maintained in 50 and 100 µg sNcAg-vaccinated cattle at 140 dpv, but the significance of this difference diminished in the 50 µg-sNcAg vaccinated animals ($p < 0.05$, Fig. 4E; $p < 0.01$, Fig. 4C).

Vaccination with 10 µg of sNcAg/Provident-AVEC® did not increase the number of CD4 $^{+}$ /IFN- γ T-cells. However, significant numbers of IFN- γ $^{+}$ /PBMCs were detected ($p < 0.05$, Fig. 4B), showing that this vaccine probably stimulated innate-IFN- γ producing immune cells rather than priming specific T-cell responses.

Cytokines produced by PBMC of vaccinated animals were measured upon stimulation with sNcAg comparing the amount of expressed mRNA related to control PBMC cells. Samples were obtained at 50 dpv, when peak IFN- γ responses were elicited. The three antigen payloads up-regulated the expression of TNF- α and IL-4; while no changes were observed for IL-10 expression (data not shown).

3.5. Time course of specific immune responses after immunization with sNcAg/Provident-AVEC®

In order to visualize altogether the time-course of humoral and cellular responses in cattle immunized with 50 µg of sNcAg/Provident-AVEC®, mean systemic anti *N. caninum* responses (tittered by IFAT) together with IFN- γ anamnestic responses, measured in sNcAg *in vitro* stimulated plasma, were depicted (Fig. 5). This antigen payload was selected based on immunogenicity and feasibility for cost-effective high-scale vaccine production and formulation. The profile was similar for the higher vaccine dose except that antibody titers were maintained within the same levels for 1 month after the boost.

Onset of humoral responses was established 5 days after the boost, while the development of *N. caninum*-specific IFN- γ response was delayed in 1 week respect to the humoral response. Mean titers of *N. caninum*-specific antibodies were maintained over background levels after

the boost (90 dpv) while IFN- γ levels also declined by 140 dpv.

4. Discussion

Our results depict an important issue for designing a *N. caninum* vaccine based on sNcAg: the tight relationship between vaccine antigen payload and immune responses in cattle. We showed that the potential of applying a sNcAg vaccine relies on the careful selection of an efficient adjuvant to formulate an adequate, commercially reasonable amount of sNcAg, enough to elicit humoral and IFN- γ responses in the natural host. In a previous publication we screened for the antigen *in vitro*, then for the adjuvant in the BALB/c mouse model of brain infection. The current study evaluated the selected candidate in a dose–response trial in cattle with the goal of identifying the formulation required to eventually assess its efficacy in protection against abortion. We designed the pre-clinical studies based on the Russell and Burch's 3Rs concept (Refine-Reduce-Replace animals) (Russell, 1959) focused on reducing and refining animal experiments.

The use of sNcAg as effective vaccine antigen is supported by several studies performed by our group and others (Alvarez Garcia et al., 2006; Baszler et al., 2000; Mansilla et al., 2012; Moore et al., 2011). Soluble antigens of *N. caninum* tachyzoites can activate CD4 $^{+}$ T-cells (Rocchi et al., 2011), which are thought to be important in protecting cattle against this parasite's infection (Rocchi et al., 2011; Staska et al., 2003). The immunogenicity of sNcAg depends on the adjuvant used to formulate the vaccine in mice (Mansilla et al., 2012; Ribeiro et al., 2009). In cattle, sNcAg had only been tested in a high payload vaccine (500 µg of antigen) formulated with ISCOM matrix, and no differences in the duration of the parasitemia was observed when compared with calves receiving a vaccine based on live parasites previously to the challenge (Moore et al., 2011). In mice, we were able to lower the amount of sNcAg by 10-fold when using Provident-AVEC® instead of Aluminum hydroxide or ISCOM matrix and still obtain full protection (Mansilla et al., 2012). In cattle, the highest dose we evaluated in this study was 5 times lower than the one that has been published (Moore et al., 2011), obtaining high levels of specific, high-avidity antibodies as well as CD4 $^{+}$ T cells producing IFN- γ at levels comparable to those obtained for PWM-stimulated PBMC.

The administration of sNcAg/Provident-AVEC® vaccines induced antibody responses that were dose-dependent. Cattle immunized with higher payloads elicited specific antibodies faster and antibody levels lasted longer. The magnitude of the antibody response is difficult to compare between studies of different laboratories because of protocol variability. The highest IFAT antibody titer induced in calves immunized with 500 µg sNcAg formulated with ISCOMS was 500 (Moore et al., 2011) while pregnant heifers inoculated with 1×10^8 live tachyzoites by the intravenous route reached a maximum IFAT-titer of 160 (de Yaniz et al., 2007). Both measurements were performed in the same laboratory than this study, with the same protocol. Here we found IFAT titers from 400 to 500 for the three formulations after the boost. Detectable specific antibodies were found

over 60 dpv while IFAT-titers over negative control levels were maintained for at least 4 months post-vaccination in those calves immunized with 100 µg of sNcAg/Providean-AVEC®.

A dose-dependent profile was observed for the kinetics of avidity maturation of specific antibodies. All vaccine formulations elicited high avidity antibodies after two applications. IgG avidity of anti *N. caninum* antibodies has been intensely studied for diagnostic purposes and found to be a useful tool for discriminating between recent and long-standing infection (Aguado-Martinez et al., 2005; Bjorkman et al., 1999; Maley et al., 2001). The relevance that high avidity antibodies may have in protection against abortion was explored in one study and concluded that lower avidity antibodies were observed in aborting cows when compared to animals without abortion problems; indicating that low avidity antibodies can be an indicator for increased abortion risk (Sager et al., 2003). The relationship between vaccination and avidity of the induced antibodies has been analyzed in cattle immunized with live parasite and whole crude tachyzoite extract vaccines, with no clear relationship between avidity index and protection (Williams et al., 2007). The presence of low-avidity antibodies in T-dependent responses denotes a poor induction of T-helper cell immunity. In our study, the magnitude of both humoral responses (specific IgG avidity) as well as cell-mediated immunity (proliferation of CD4⁺ T-cells) were dose-dependent. We presume that high-avidity antibodies may facilitate antibody-mediated immunity mechanisms, such as opsono-phagocytosis. To the best of our knowledge, this is the first report evaluating the avidity of specific IgG elicited in cattle after vaccination with *N. caninum* soluble antigens.

IgG1 was the major isotype induced in all sNcAg/Providean-AVEC® vaccinated cattle. The role of the different isotypes in protection against abortion in cattle is still unclear. Almería et al. (2009) showed that an immune response, in which IgG2 antibodies prevail, could be protective against *N. caninum* abortion, but only in the presence of IFN-γ production. Another study found differences in humoral mechanisms against *N. caninum* infection and abortion depending on the breed, which indirectly could be an indication of differences in cellular immune mechanisms (Santolaria et al., 2011).

Analysis of the isotype of specific antibodies in three infected heifers revealed a predominant IgG1 response in one heifer and a predominant IgG2 response in the other two (Moore et al., 2005). Similar titers of IgG1 and IgG2 occurred in heifers immunized with a killed whole *N. caninum* tachyzoites preparation in oil adjuvant (45 mg of protein/dose) at weeks 13, 15 and 17 of gestation (Moore et al., 2005). Currently, the *in vitro* functionality of bovine IgG1 and IgG2 has not been completely discerned (Pastoret, 1998) to explain differential clearance mechanisms associated with the IgG subclass. The fact that IgG1 is the main antibody found in mucosal surfaces as well as in colostrum and milk (MacLachlan and Dubovi, 2010; Pastoret, 1998) may indicate a differential role of this isotype in antibody-mediated effector mechanisms. The possibility of inducing IgE and the role to this isotype in mediating the killing of

intra-cellular parasites, as described for *Toxoplasma gondii* (Vouldoukis et al., 2011), is another issue to pursue.

Time-course analysis of antibody response show that mean titers of *N. caninum*-specific antibodies in 50 and 100 µg sNcAg-vaccinated animals were maintained over background levels (control group) for about 2 months after the boost while IFN-γ levels peaked at 50 dpv and decayed by 140 dpv (Fig. 5). At 50 dpv, there was a large percentage of CD4⁺-T cells producing IFN-γ in those animals immunized with 50 and 100 µg of sNcAg (Fig. 4C). At 140 dpv the number of CD4⁺/IFN-γ⁺ T-cells decreased but were still significant (Fig. 4E), indicating that the administration of 50 or 100 µg of sNcAg/Providean-AVEC® vaccine in a two-vaccination schedule primed T-cells for defense upon re-exposure to tachyzoite antigens (*i.e.* during infection).

We characterized the immune response elicited by commercially-feasible antigen payloads of sNcAg formulated with Providean-AVEC®, an adjuvant pre-selected to enhance cell-mediated immunity. This is the first dose-response study performed in cattle using sNcAg as antigen. Our results show that a vaccine containing 50 µg of sNcAg is sufficient to induce cell-mediated and humoral (high avidity) immune response against *N. caninum* in cattle. We demonstrated that the profile and magnitude of the cellular responses can be tailored with the antigen-dose and vaccination schedule, allowing the modulation of immune responses that could eventually be detrimental for pregnancy. The time-course of the induced immune responses will be applied to design a suitable immunization schedule to assess the efficacy of the vaccine formulation in the prevention of abortion. Further studies are also needed to identify the specific immunogens in the sNcAg preparation that are responsible for the protective effect, if any, in order to develop a recombinant vaccine.

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