Experimental Parasitology 160 (2016) 1–10

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Full length article

Immunization with *Neospora caninum* profilin induces limited protection and a regulatory T-cell response in mice

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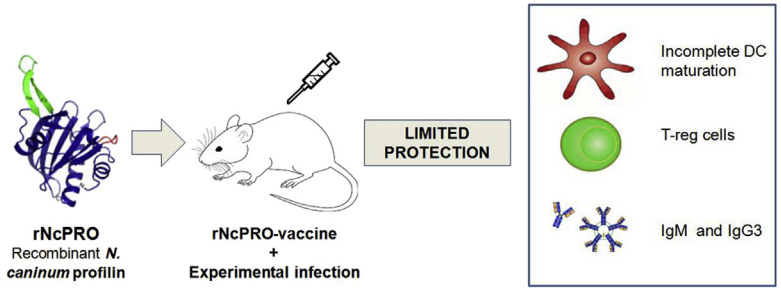
HIGHLIGHTS

- Apicomplexan profilins are proposed as efficient vaccine antigens.
- The immunogenicity of *Neospora caninum* profilin (NcPRO) is unknown.
- We characterized the immunogenicity and protection induced by *NcPRO* in mice.
- NcPro-specific IgM and IgG3 and regulatory T-cells were elicited after vaccination.
- NcPRO-vaccinated mice had a reduced cellular immune response to *N. caninum* antigens.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 May 2015
Received in revised form
23 August 2015
Accepted 29 October 2015
Available online 10 November 2015

Keywords:
Profilin
Neospora caninum
Dendritic cells
Vaccine
Regulatory response

ABSTRACT

Profilins are actin-binding proteins that regulate the polymerization of actin filaments. In apicomplexan parasites, they are essential for invasion. Profilins also trigger the immune response of the host by activating TLRs on dendritic cells (DCs), inducing the production of pro-inflammatory cytokines. In this study we characterized for the first time the immune response and protection elicited by a vaccine based on *Neospora caninum* profilin in mice. Groups of eight BALB/c mice received either two doses of a recombinant *N. caninum* profilin expressed in *Escherichia coli.* (rNcPRO) or PBS, both formulated with an aqueous soy-based adjuvant enriched in TLR-agonists. Specific anti-profilin antibodies were detected in rNcPRO-vaccinated animals, mainly IgM and IgG3, which were consumed after infection. Splenocytes from rNcPRO-immunized animals proliferated after an *in vitro* stimulation with rNcPRO before and after challenge. An impairment of the cellular response was observed in NcPRO vaccinated and infected mice following an *in vitro* stimulation with native antigens of *N. caninum*, related to an increase in the percentage of CD4+CD25+FoxP3+. Two out of five rNcPRO-vaccinated challenged mice were protected; they were negative for parasite DNA in the brain and showed no histopathological lesions, which were found in all PBS-vaccinated animals. As a whole, our results provide evidence of a regulatory response

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http://dx.doi.org/10.1016/j.exppara.2015.10.008

elicited by immunization with rNcPRO, and suggest a role of profilin in the modulation and/or evasion of immune responses against *N. caninum*.

1. Introduction

Neospora caninum is an obligate intracellular apicomplexan parasite, closely related to *Toxoplasma gondii*, which has emerged as a major cause of reproductive failure in cattle worldwide, with abortions and stillbirths caused by mid-gestational exposure or recrudescence of latent infections (Dubey and Schares, 2011; Innes et al., 2005). The parasite seriously impacts the economic performance of the dairy and beef industries (Reichel and Ellis, 2006) and there is no effective control method. Thus, the development of an effective and safe vaccine against *N. caninum* is of great importance (Dubey et al., 2007).

Because it is an intracellular parasite, the proteins involved in host cell invasion and intracellular development represent potential targets for vaccination (Hemphill et al., 2006). Host-cell invasion relies on changes in the actin cytoskeleton, especially at the apical end. As actin filaments are in low abundance, they depend on proteins governing actin dynamics, like formins and profilins (Gordon and Sibley, 2005). Profilins are small monomeric actin binding proteins located at the apical end of tachyzoites that play multiple roles in the regulation of actin polymerization (Skillman et al., 2012). It has been reported that *T. gondii* profilin (TgPRO), which is 89% homologous to *N. caninum* prolifin (NcPRO), is passively released by the parasites through an unknown mechanism (Plattner et al., 2008; Yarovinsky et al., 2005) and is needed for the invasion of host cells (Plattner et al., 2008).

Besides being essential for invasion, TgPRO binds TLR11 and TLR12 activating murine dendritic cells to release IL-12, which is involved in the production of IFN-γ via the MyD88-dependent pathway and in the differentiation of naïve T lymphocytes to Th1 phenotype (Koblansky et al., 2013; Raetz et al., 2013; Yarovinsky et al., 2005), an immune profile related to protection against *N. caninum* in some animal models (Mineo et al., 2009). Profilins from other apicomplexan parasites, including *Plasmodium falciparum*, *Cryptosporidium parvum* and *Eimeria tenella* also activate TLR11-dependent signaling, but to a lesser extent than TgPRO (Rosenberg et al., 2005; Yarovinsky et al., 2005). Jenkins et al. demonstrated in 2010 that a recombinant NcPRO expressed in bacteria stimulated the release of IFN-γ in cultured spleen cells, and was capable of eliciting systemic IFN-γ and IL-12 responses between 6 and 24 h after injection into BALB/c mice (Jenkins et al., 2010). This is the only report on the immune activity of this protein.

Profilins from *T. gondii* and *Eimeria tenella* have been evaluated as vaccine antigens. TgPRO formulated with oligomannose-coated liposomes induced high titers of IgG2a and IFN-γ in C57BL/6 after three immunizations, increasing the survival of infected mice and reducing the parasite burden in their brains (Tanaka et al., 2014). A protective effect of *E. tenella* profilin (EtPRO) against avian coccidiosis was observed after challenge infection in chickens when it was combined with potent adjuvants (Jang et al., 2013, Jang et al., 2011a, Jang et al., 2011b, Jang et al., 2011c). To our knowledge, there is no information available on the immunogenicity of NcPRO.

Here we characterize for the first time the immune response induced by a recombinant profilin (rNcPRO) formulated with a soy–lecithin based adjuvant enriched in TLR2 agonists (*Providean*-AVEC®, "AVEC", Tecnovax S.A., Buenos Aires, Argentina) that acts via the MyD88-dependent pathway, required for resistance to *N. caninum* infection (Mineo et al., 2009). Vaccinated BALB/c mice were challenged with tachyzoites of *N. caninum* Nc1 strain to assess the protective capacity of rNcPRO as vaccine antigen, evaluating protection to cerebral infection as described before (Collantes-Fernández et al., 2002; Mansilla et al., 2012). Immune responses elicited after vaccination and infection were characterized.

2. Methods

2.1. Cells and parasites

N. caninum tachyzoites (Nc1 strain) were cultured in VERO cells under previously standardized conditions (Moore et al., 2011). Briefly, tachyzoites were released by sequential passages of the cell monolayer through 21, 25 and 27 gauge needles, washed with sterile PBS and counted with a hemocytometer. They were used either to formulate the live inoculum (1×10^6 tachyzoites/100 μl) or to obtain the native antigen extract. Soluble extracts of native antigen (sNcAg) were prepared as described before (Mansilla et al., 2013, Mansilla et al., 2012; Moore et al., 2011). Proteins in the antigen preparations were quantified using a commercial kit (Micro BCA Pierce, Rockford, US).

2.2. Recombinant NcPRO

Total RNA from Nc1 *N. caninum* purified tachyzoites was obtained using Trizol® reagent (Invitrogen. NY, USA) following manufacturer's instructions. The cDNA coding for NcPRO was amplified by RT-PCR using a commercial kit (One Step RT-PCR, Qiagen, West Sussex, UK) and previously described primers (Jenkins et al., 2010) with EcoRI and XbaI restriction sites (forward and reverse, respectively) and an His-Tag. This fragment was cloned into a commercial vector (pCI–Neo, Promega, Maddison, WI, USA). The identity of the construct (pCI-NcPRO) was evaluated by restriction analysis and sequencing (not shown).

Recombinant profilin (rNcPRO) was expressed in *E. coli* (BL21plys strain) following the manufacturer's protocol. Inclusion bodies (IB-rNcPRO) were resuspended in 8 M Urea prior FPLC-IMAC purification using Zinc columns (GE Healthcare, Sweden). The refolding of the Urea-solubilized IB-rNcPRO was made by performing a decreasing gradient step of Urea (8 M–0 M) before gradient elution with Imidazole (50 mM – 500 mM). Purified-refolded rNcPRO was desalted and concentrated by ultrafiltration using a 3000Da cut-off Vivaspin 15™column (Sartorius-Stedim, Scotland). Its reactivity was evaluated by western blot, incubating the membranes with a monoclonal antibody anti-His (GE Healthcare) and with serum from an experimentally infected mouse (20) (not shown). The homology between the profilin expressed in bacteria (rNcPRO), different apicomplexan profilins (NcPRO-Nc1 strain, NcPRO-Liv strain, TgPRO, EtPRO) and others from non-related organisms (*Mus musculus* and *Bos taurus*) was assessed by an *in silico* analysis using Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal IW (http://www.expasy.org/genomics/sequence_alignment).

2.3. Activation of bone-marrow derived dendritic cells and cytokines profile

Bone-marrow derived dendritic cells (DCs) were differentiated *in vitro* from bone-marrow precursors of 8 week-old naïve BALB/c mice as previously reported (Mansilla et al., 2012). Cells were stimulated for 24 h with purified rNcPRO (1 μg/mL), sNcAg (1 μg/mL, (Mansilla et al., 2012), complete RPMI medium + PBS (mock negative control, Gibco®, Invitrogen) or LPS (10 ng/mL, Sigma Chemical Co., St. Louis, MO, US) as positive control. The up-regulation of CD11c and co-stimulatory molecules was determined evaluating median fluorescence intensity (MFI) and percentages of double-positive cells by flow cytometry using fluorochrome-labelled monoclonal antibodies: anti-CD11c (HL3); anti-CD40 (3/23 RUO); anti-CD80 (16-10A1); anti-CD86 (GL1) and anti MHC−II−IAD (AMS-32.1 RUO) (BD Pharmingen, Franklin Lakes. New Jersey. USA).

The concentration of IL-6, MCP-1 and TNF-α was determined in the supernatant of stimulated splenocytes by flow cytometry using CBA Mouse Inflammation Kit (BD Pharmingen).

2.4. Vaccine formulations

rNcPRO was diluted in PBS under sterile conditions. Each dose contained 2 μg of rNcPRO in a final volume of 100 μl formulated with an aqueous a soy−lecithin based adjuvant (30% volume) developed in our laboratory (*Providean*−AVEC®, "AVEC", Tecnovax S.A.) which proved to be effective in the BALC/c model (Mansilla et al., 2012).

2.5. Animal studies

Animal procedures were performed according to standard guidelines of humane care and treatment of animals from Laboratory Animal Welfare Act, within Tecnovax S.A. animal facility, supervised by the company's animal welfare committee. Mice were supervised by veterinarians on daily bases. Experiments were carried out with 22 female BALB/c mice (8−10 week old and18−20 g weight at the beginning of the experiment), that were randomly divided into 3 groups. Each animal received two immunizations (SC, in a final volume of 100 μl) at 0 and 14 days post vaccination (dpv) with rNcPRO + AVEC (n = 8) or PBS + AVEC (control animals, n = 8). A group of 6 animals was left as sentinel (non-vaccinated/non-infected), and three of those animals were infected. Bleedings were performed from the retro-orbital sinus at 0, 14, 21, 28 and 38 dpv and serum samples were conserved. At 38 dpv, three animals from the experimental groups were euthanized to assess the cellular response induced by vaccination. The rest of the animals (n = 5) from these groups were IP challenged with partially purified 1×10^6 tachyzoites (Nc1 strain) from VERO cells, as previously described in 100 μl of sterile PBS (Mansilla et al., 2012). We followed a published procedure (Mansilla et al., 2012). Briefly, whole blood samples (in 1% EDTA solution) at 4 and 21 days post infection (dpi). Samples were centrifuged and plasma and blood cells were stored at −20 °C to perform ELISA and DNA extraction, respectively. Animals were sacrificed on 21 dpi: and blood, spleen and whole brain were recovered. One brain hemisphere of each animal was fixed in 10% neutral formalin for histopathological analysis and the other half was processed for DNA extraction (Wizard® Genomic DNA Purification Kit, Promega) to determine cerebral infection. Splenocytes were purified for lymphoproliferation assay and CD4+/CD25+/FoxP3+ cells detection.

2.6. Humoral immune responses

NcPRO specific antibodies were assessed in an in house indirect ELISA, using 2 μg/mL of IB-rNcPRO in carbonate-bicarbonate buffer as capture antigen. Plates (Microlon™ HB, Grenier Bio One, Austria) were incubated ON at 4 °C and blocked with PBS-10% equine serum for 1 h at 37 °C. Pooled samples from each group were pre-incubated with an extract of non-induced BL21 bacteria for 20 min at room temperature and plated (1:50) in PBS-10% equine serum, and incubated for 1 h at 37 °C. An anti-mouse IgG(H + L):HRP conjugate was added (1:1000, Jackson ImmunoResearch, PA, USA) and incubated for 1 h at 37 °C. Then, a solution of ABTS substrate and H_2O_2 0.13% was incorporated and plates were incubated 20 min in a dark place. The reaction was stopped with NaF 0.5 M and plates were read at 405 nm.

For detecting anti NcPRO isotypes, we adapted the above protocol using biotinilated anti-mouse IgG1, IgG2a (1:1000, Sigma), IgG3 and IgM (1:750, Santa Cruz Biotech, TX, USA) after washing out serum samples, followed by Streptavidin:HRP (1:500, Jackson ImmunoResearch) for 20 min at room temperature.

Specific antibodies anti *N. caninum* were measured on sera from each vaccinated or non-vaccinated animal at 0 dpv, 4 and 21 dpi, using a commercial ELISA (ID Screen® *N. caninum* indirect Multi-species, ID Vet, Montpellier, France). Results are expressed as OD values ± SD.

2.7. Lymphoproliferation assay

Splenocytes were obtained at 38 dpv and 21 dpi. Suspensions containing 1×10^7 splenocytes were stained with CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) for standard proliferation assessment following the manufacturer's instructions. After staining, 2.5×10^5 cell/well (100 μl) were plated in 96 wells-plates and incubated with Pockeweed Mitogen (PWM, Sigma, 10 μg/mL), sNcAg, (1 μg/mL), rNcPRO (1 μg/mL) or PBS. Each sample was run by triplicate. Cell-antigen mixture was cultured for 5 days at 37 °C (5% CO_2). After incubation, cells were read by FACS. 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 ± 0.15 $\log_{10}$ either side of each cell division peak). The proliferation index was determined as the rate of proliferating cells in treated vs mock-treated cells, expressed as percentage ±SD (Lyons, 2000).

The supernatants of the different replicates of each treatment were pooled and the expression of IFN-γ, TNF-α and IL-10 was determined in the supernatant of cultured cells by flow cytometry using BD™ CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Pharmingen), following the manufacturer's instructions. Results were analyzed using FCAP Array Analysis Software (Soft Flow, Inc.), expressed in pg/ml.

2.8. Detection of CD4+CD25+FoxP3+ cells

The percentage of CD4+CD25+FoxP3+ cells was assessed in splenocytes. Briefly, 1×10^6 cells from each animal were fixed in Fix/Perm™ buffer (BD Pharmingen) and then permeabilized in Perm/Wash™ buffer (BD Pharmingen). Cells were run by triplicate and incubated with anti-FoxP3:APC (or its isotype control, BD Pharmingen) for 20 min at room temperature. The cells were then washed and incubated with anti-CD4: FITC and anti-CD25:PE (and their isotype controls, BD Pharmingen) for 30 min at 4 °C. Finally, cells were washed again, fixed in paraformaldehyde, resuspended in FACSflow™ (BD) and then analyzed by flow cytometry. Results are expressed as the percent of CD4+/CD25+/FoxP3+ cells ±SD.

2.9. Protection to cerebral infection

Brains were obtained from challenged mice at 21 dpi and divided in two pieces. One hemisphere was fixed in formalin and a histopathological analysis was performed on multiple sections of different regions of the brain, looking for lesions characteristic of or consistent with *N. caninum* infection (Collantes-Fernández et al., 2002; Mansilla et al., 2012). Genomic DNA was extracted from the other hemisphere of the brain using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega), to evaluate vaccine efficacy in controlling infection by nested PCR using primers already described (Buxton et al., 1998) and following a previously standardized protocol (Mansilla et al., 2012).

2.10. Statistical analysis

All values were compared using Student's t test or Mann–Whitney U (if normality failed). One way ANOVA followed by Tukey Test and Kruskal–Wallis nonparametric ANOVA, followed by Dunn's test, were used for multiple comparisons. Differences were considered significant with a 95% confidence interval. Statistical analysis was performed using SigmaStat 3.5 (Systat software).

3. Results

3.1. Cloning and expression of NcPRO

The ORF for NcPRO was obtained from total mRNA extracted from live tachyzoites as described in the Materials and Methods section (item 2.2). The identity of the cloned sequence was 99% homologous to that of the NcPRO-Liverpool strain (XM_003879549.1), and to the one described by Jenkins et al. (Nc1 strain, BK006901.1). We detected one mismatch that introduced a silent mutation (240C to T; aa Gly; Fig. 1) compared to the above sequences. Our sequence was 89% homologous to the TgPRO profilin mRNA sequence (accession AY937257.1), and had no similarities with sequences of mRNA of mice (31.6%) or bovine (36.2%) profilins (Fig. 1).

3.2. Effect of rNcPRO on bone-marrow derived DC maturation

N. caninum profilin was shown to induce the production of IFN-γ in mice splenocytes (Jenkins et al., 2010), suggesting that this protein can activate cells of the innate immune system. However, the direct effect of NcPRO on dendritic cells, which link the innate and adaptive immunity, has not been investigated. We then studied if rNcPRO can trigger the maturation of bone-marrow derived (CD11c+) dendritic cells (DCs).

DCs were incubated overnight with rNcPRO, sNcAg, LPS or PBS and the expression of MHC-II and co-stimulatory molecules CD40, CD80 and CD86 was assessed by flow-cytometry. The expression of CD40 was strongly up-regulated by all treatments with higher MFI values for sNcAg (133.13) than rNcPRO (49.04), except for PBS. Lower up-regulation of CD80 and CD86 was also observed for both *N. caninum* antigens. There was no evident effect on the expression of MHC–II–IAD (Fig. 2). A pro-inflammatory profile was observed in cells that were stimulated with rNcPRO, with high levels of TNF-α and IL-6 and a low production of MCP-1. The stimulation of those cells with sNcAg induced a similar milieu, but TNF-α was not detected (not shown). This data verified the biological functionality of rNcPRO.

3.3. Humoral immune responses to NcPRO

Groups of eight BALB/c mice received either two doses of a biologically active recombinant *N. caninum* profilin expressed in *E. coli.* (rNcPRO) or PBS formulated with an aqueous soy-based adjuvant enriched in TLR-agonists. Three animals were euthanized at 38 dpv to study cell mediated immunity and five mice from each group were experimentally challenged with live tachyzoites. Six animals were left untreated (sentinels) and three of them were challenged. The kinetics of specific antibodies anti-NcPRO were measured by ELISA before and after challenge.

An increase in total antibody levels was detected in animals immunized with rNcPRO + AVEC after the first dose, which were maintained over time. Anti-profilin antibodies were consumed after challenge, and levels were recovered later on (i.e. 21 dpi) without significant differences between them and naïve-infected animals or those that had been vaccinated with PBS + adjuvant (59 dpv; Fig. 3A).

We also characterized the humoral response elicited by vaccination in terms of the isotype of specific antibodies. IgM and IgG3 were the main isotypes induced in mice immunized with rNcPRO + AVEC (Fig. 3B and C). Low levels of IgG1 were detected after the second dose (Fig. 3D), while IgG2 levels were negligible. Control animals exhibited low levels of anti rNcPRO-IgG2a after challenge (not shown). The decrease in total anti-profilin antibodies after infection (Fig. 3A), correlated to a transient decline in IgM and IgG3 NcPRO-specific antibodies (Fig. 3B and C), and both total antibodies and isotypes recovered their pre-challenge levels at 21 dpi.

3.4. Cellular immune responses to NcPRO

Cellular mediated immune response induced by vaccination was assessed by lymphoproliferation at 38 dpv and 21 dpi, studying recall responses to sNcAg and rNcPRO. Cells from rNcPRO + AVEC vaccinated animals proliferated after an *in vitro* stimulation with rNcPRO at 38 dpv; with higher proliferation indexes (PI) compared to sNcAg-treated cells ($p < 0.01$). Control mice (vaccinated with PBS + AVEC) showed low PI (Mean range: 1.04–1.07), without significant differences between treatments (Fig. 4A). Significant proliferation levels were observed after challenge (21 dpi) in splenocytes from all infected animals (vaccinated and non-vaccinated) that were stimulated with rNcPRO or sNcAg, with higher PI compared to those observed when the same cells were stimulated with PBS (data not shown).

In order to compare the proliferative responses between groups, we determined the percentage of progenitor cells that proliferated after an *in vitro* stimulation with rNcPRO or sNcAg in mice vaccinated with rNcPRO + AVEC or PBS + AVEC compared to naïve (non-vaccinated, non-infected) animals at 21 dpi. After rNcPRO stimulation, an increase in the percentage of progenitor dividing cells was observed in rNcPRO + AVEC vaccinated animals, with significant differences compared to the other groups ($p < 0.01$, Fig. 4B). PBS + AVEC-vaccinated and infected mice exhibited significant differences in the percentage of dividing cells after an *in vitro* stimulation with sNcAg, when compared to naïve animals ($p < 0.01$). However, infected mice that received rNcPRO + AVEC vaccine, showed no proliferative anamnestic responses to sNcAg stimulation, as the percentages of dividing cells were similar to those of naïve (not-infected) animals ($p > 0.05$, Fig. 4C).

The concentration of IFN-γ, TNF-α and IL-10 was determined by flow cytometry in the supernatant of splenocytes of vaccinated animals (38 dpv and 21 dpi) stimulated with sNcAg. Animals vaccinated with PBS + AVEC had higher levels of IFN-γ and TNF-α after infection (21 dpi) compared to those measured after vaccination (38 dpv). The same cytokine profile was induced by rNcPRO vaccination at 38 dpv, with a marked increase after challenge (21 dpi, Fig. 4D and E). The splenocytes obtained at 38 dpv from

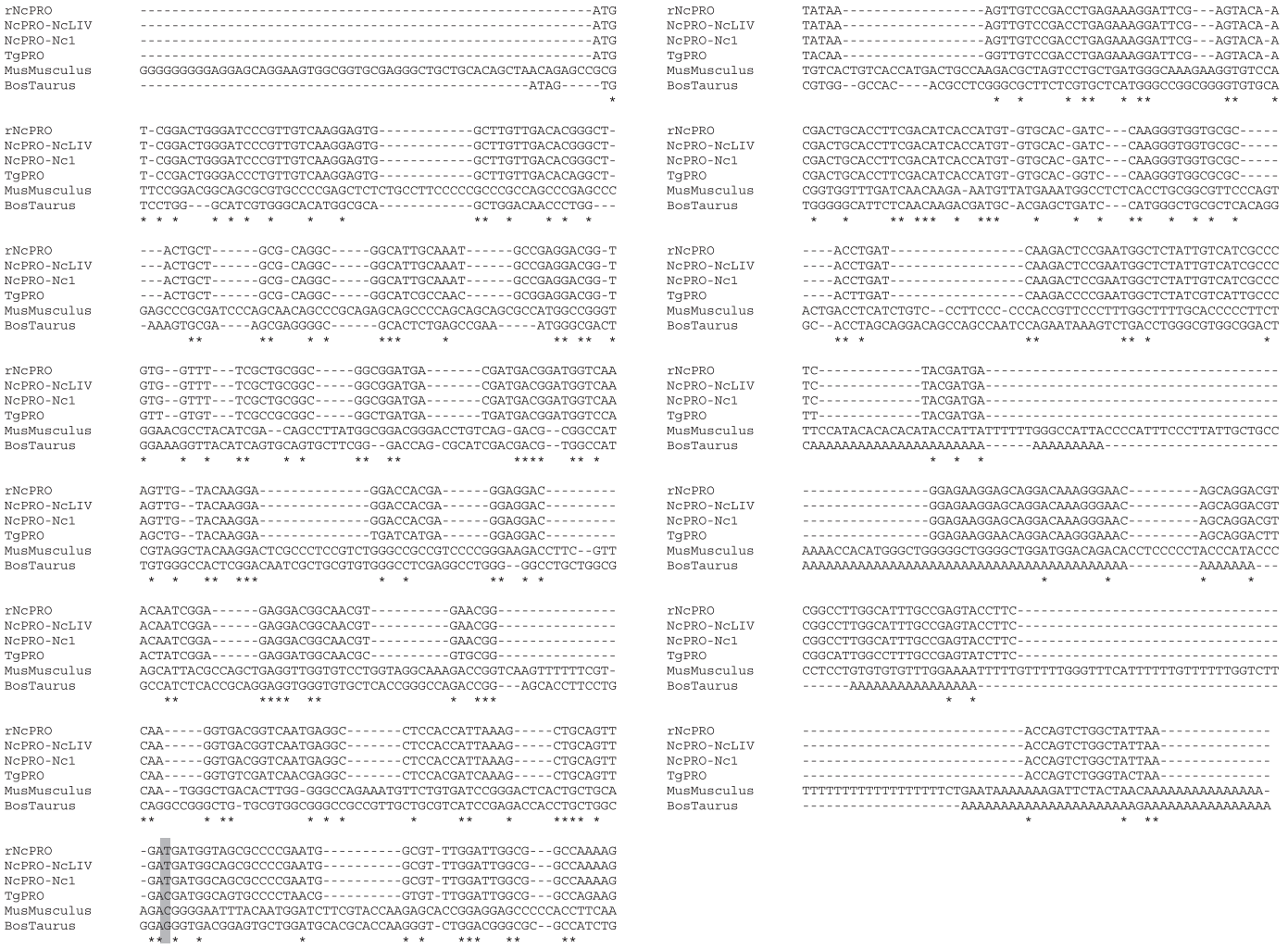

Fig. 1. Multiple sequence alignment. The homology between different profilins was assessed by an *in silico* analysis using Nucleotide and Clustal IW alignment. The silent mutation observed in rNcPRO (240C > T) is depicted with a grey square. rNcPRO: Recombinant *Neospora caninum* profilin used in this study; NcPRO-Liv: *Neospora caninum* profilin NcLiverpool strain; NcPRO-Nc1: *Neospora caninum* profilin Nc1 strain; TgPRO: *Toxoplasma gondii* profilin; EtPRO: *Eimeria tenella* profilin; Mus: *Mus musculus* profilin; Bos: *Bos taurus* profilin. * Conserved residue.

rNcPRO vaccinated animals incubated just with PBS yielded negligible IL10 levels but there was a basal expression of TNF-α and IFN-γ (data not shown), thus, we cannot assign specificity to the response observed after sNcAg stimulation for these cytokines. IL-10 was up-regulated by vaccination with rNcPRO at 38 dpv. In this group, IL-10 levels increased after infection (690.96 pg/mL at 38 dpv, 2893.79 pg/mL at 21 dpi; Fig. 4F). IL-10 levels within the detection limit (20 pg/mL) were measured in the PBS + AVEC vaccinated group at 38 dpv, that increased about ten times at 21 dpi (241.4 pg/mL).

Altogether, the above results suggested the possibility of a T-regulatory response induced by rNcPRO vaccination. Consequently, we searched for regulatory T-cells by assessing the percentage of CD4+CD25 + FoxP3+ cells in splenocytes of vaccinated and non-vaccinated mice at 38 dpv and also after challenge, at 21 dpi (Fig. 5). An increase in the percentage of CD4+CD25 + FoxP3+ cells was observed in PBS + AVEC vaccinated animals after infection (21 dpi), compared to 38 dpv, although differences were not significant ($p = 0.2568$). We did not observe differences in the number of these cells after vaccination with rNcPRO compared to PBS (38 dpv). After infection, mice immunized with rNcPRO + AVEC showed a significant increase in the percentage of regulatory T-cells, compared to PBS + AVEC ($p = 0.012$) and rNcPRO-vaccinated animals before challenge ($p = 0.014$; Fig. 5). This data suggest that vaccination with NcPRO primed a regulatory response, which was subsequently expanded by the infection.

3.5. Protection to cerebral infection

The protective capacity of the vaccine was evaluated by analyzing the presence of *N. caninum* DNA in the brains (by nested PCR) and searching for histopathological lesions compatible with *N. caninum* infection.

Five animals from each group were challenged with live tachyzoites. High levels of total antibodies anti- *N. caninum* (sNcAg) were observed in all vaccinated and non-vaccinated animals at 21 dpi, with OD values ranging between 0.64 and 1.10 (Fig. 6A). Antibody levels induced by rNcPRO immunized animals were not significantly different from those elicited by infection in PBS + AVEC vaccinated mice, thus indicating the efficacy of the challenge infection.

Animals vaccinated with PBS + AVEC or infected sentinels were positive for *N. caninum* DNA in brain tissue at 21 dpi (detected by nested-PCR) and had histopathological lesions compatible with

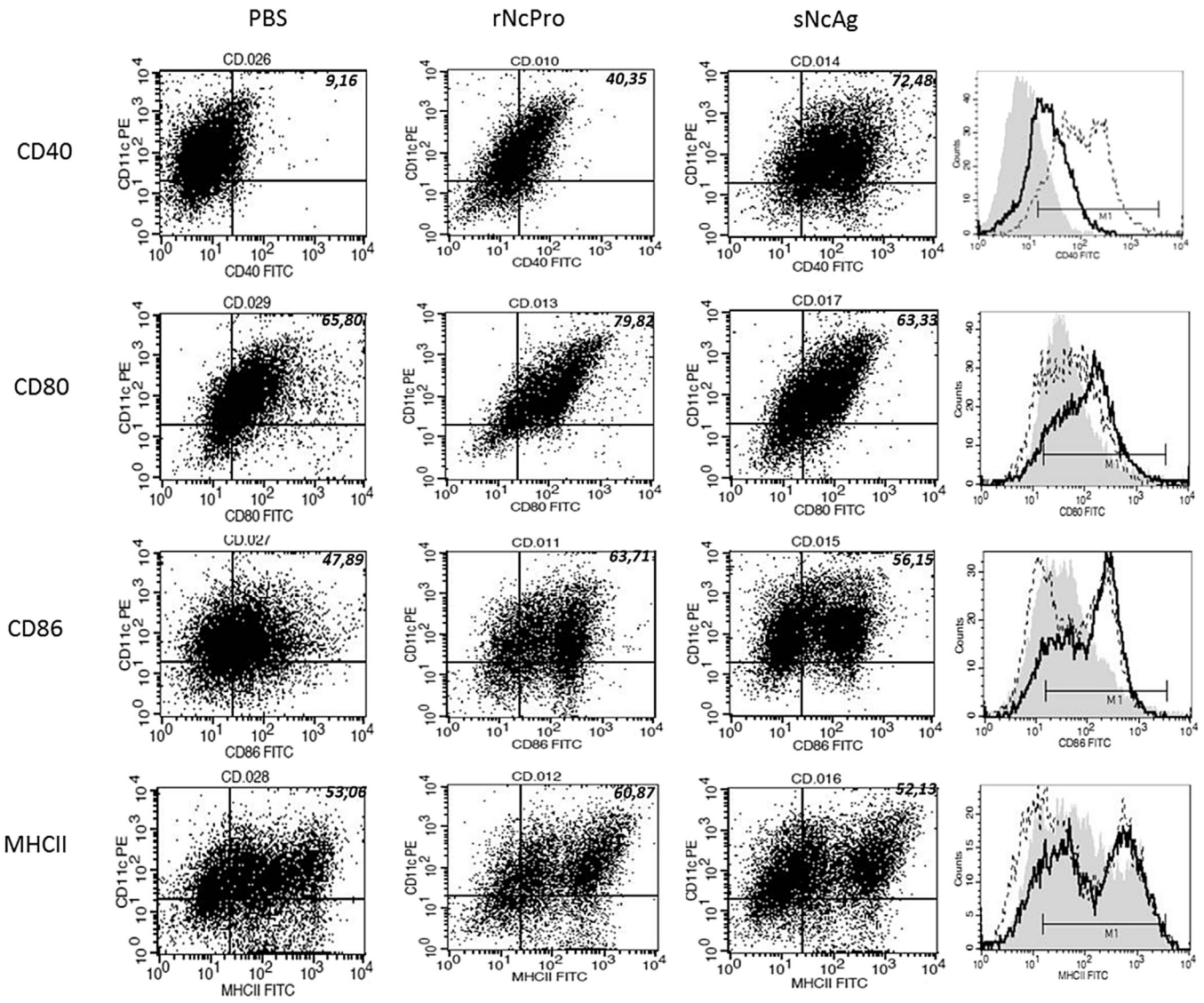

Fig. 2. Maturation of Dendritic Cells. The maturation of DCs induced by rNcPRO or sNcAg was assessed by flow cytometry. The expression of co-stimulatory molecules (CD40, CD80, CD86) and MHC-II was determined in CD11c + cells that were *in vitro* stimulated with both antigens. The percent of double positive cells and MFIs are depicted in the graph. Grey histograms correspond to non-stimulated DCs (PBS-treated); full black lines depict rNcPRO treatment and dotted black lines correspond to sNcAg stimulation.

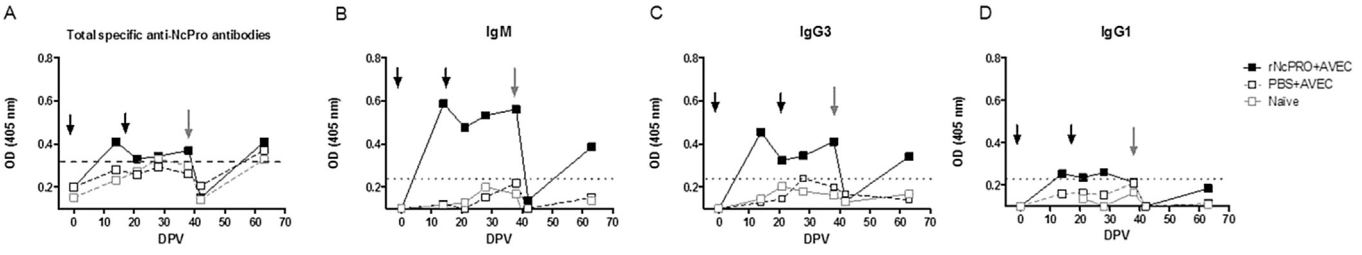

Fig. 3. Antibody response. The kinetics of specific antibodies anti-NcPRO was assessed by ELISA, in terms of total antibodies and isotypes induced after vaccination and infection in pooled serum samples. Black arrows indicate the immunizations and the grey arrows, the challenge infection. A) Total antibodies. B– C-D) IgM, IgG3 and IgG1, respectively. * Significant differences compared to values obtained at 0 dpv and 4 dpi ($p < 0.05$).

encephalitis caused by *N. caninum* (Fig. 6). Only 2 out of 5 mice vaccinated with rNcPRO + AVEC had no signs of encephalitis and were negative in nested PCR, thus indicating a partial protection induced by vaccination (data not shown).

4. Discussion

This is the first report describing the effect of *N. caninum* profilin on dendritic cells and the assessment of its immunogenicity. The vaccine containing a recombinant profilin (rNcPRO) was

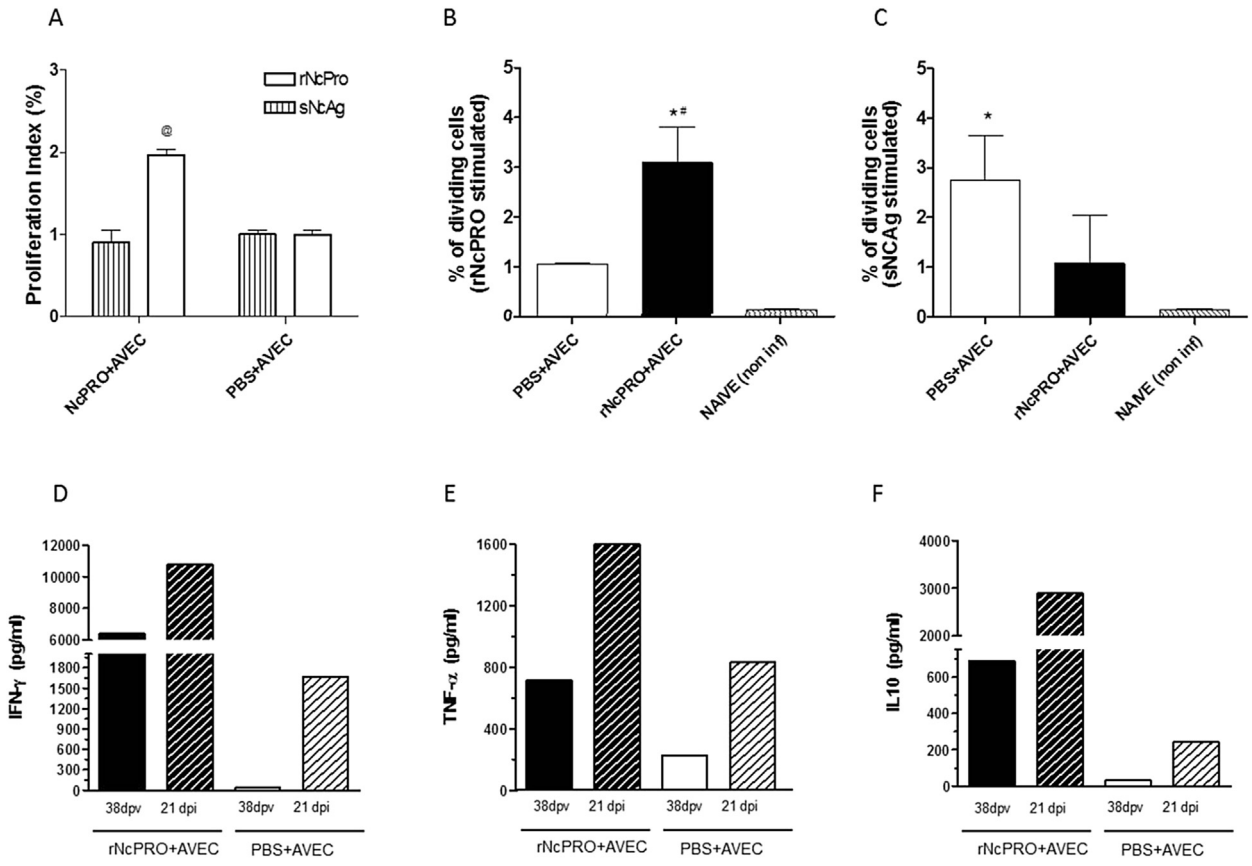

Fig. 4. Cell mediated Immune response. Cellular responses were tested by a lymphoproliferation assay performed at 38 dpv and 21 dpi. Cultures were run by triplicate (A) Percent of dividing cells in vaccinated (rNcPRO + AVEC) and control (PBS + AVEC) animals at 38 dpv (±SD), after an *in vitro* stimulation either with rNcPRO or sNcAg. (B) Percent of dividing cells at 21 dpi, after an *in vitro* stimulation with rNcPRO (±SD). (C) Percent of dividing splenocytes obtained at 21 dpi after stimulation with sNcAg (±SD). Experimental and control groups are compared to naïve animals (non-vaccinated, non-infected). The concentration of IFN-γ (D), TNF-α (E) and IL-10 (F) was determined by flow cytometry in the supernatant of splenocytes of vaccinated animals (38 dpv and 21 dpi) stimulated with sNcAg. @ Significant differences in cells from rNcPRO + AVEC vaccinated animals between sNcAg and rNcPRO *in vitro* stimulation at 38 dpv ($p < 0.01$). * Significant differences compared to naïve animals ($p < 0.01$). # Significant differences with PBS + AVEC vaccinated animals ($p < 0.01$).

formulated with an adjuvant containing TLR-2 agonists, which has already been used in mice and cattle (Mansilla et al., 2013, Mansilla et al., 2012, Mansilla et al., 2015); vaccines prepared with this particular adjuvant and low doses of sNcAg induced protection in BALB/c mice (Mansilla et al., 2012). The rationale was to favor the activation of MyD88 pathway in antigen presenting cells by tacking different TLRs, as MyD88 is required for resistance against *N. caninum* in mice (Mineo et al., 2009).

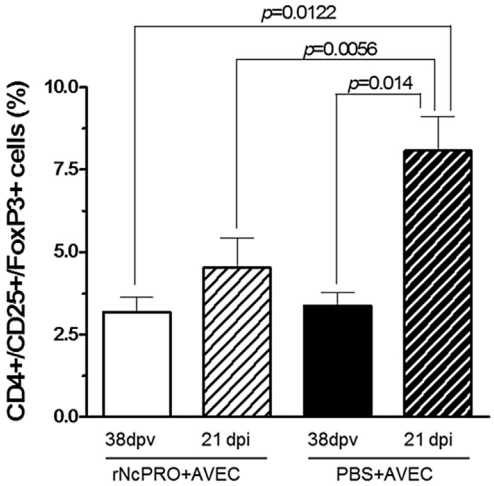

Fig. 5. Regulatory response. The presence of CD4+/CD25+/FoxP3+ cells was determined by flow cytometry in splenocytes of rNcPRO + AVEC and PBS + AVEC vaccinated animals at 38 dpv and after challenge (21 dpi). Results are expressed as mean percentage of CD4+/CD25+/FoxP3+ (±SD).

The effect of some apicomplexan profilins on DC has been studied regarding their capacity to induce the synthesis of pro-inflammatory cytokines. There was no data on the modulation exerted by an apicomplexan profilin on the expression of co-stimulatory molecules and MHC-II in DCs. Stimulation of DCs with rNcPRO and sNcAg up-regulated the expression of CD40, was less efficient activating CD80 and CD86 and did not modify the expression of MHC-II. Although, it is clear that the profilin itself has effect on DC maturation, it is incomplete. Partially matured DCs can preferentially activate natural regulatory T-cells, regardless of their low expression of CD80 and CD86 (Onishi et al., 2008; Takahashi et al., 1998; Thornton and Shevach, 1998), which can account for the presence of profilin-specific regulatory T-cells observed in NcPRO vaccinated and infected mice.

Regulatory responses have been described after infection with *N. caninum* (Teixeira et al., 2007) and other apicomplexan parasites like *T. gondii* in mice (Fenoy et al., 2009). We found that vaccination with rNcPRO + AVEC primed regulatory T-cells that were expanded after infection to higher levels than those found in non-vaccinated and infected mice. IL-10 was also up-regulated. Splenocytes from

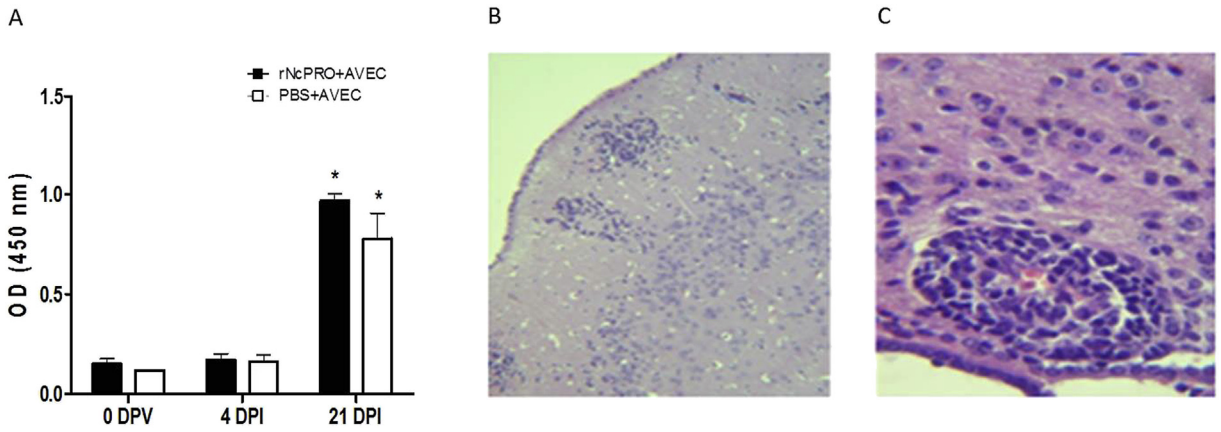

Fig. 6. Outcome of challenge infection. (A) Total anti-sNcAg antibodies were measured in individual serum samples from experimentally infected animals at 0 dpv, 4 and 21 dpi. All challenged animals, either vaccinated or naïve, elicited similar levels of anti-sNcAg antibodies after challenge. Results are expressed as OD values ± SD. (A) Total anti-sNcAg antibodies were measured in sera from all challenged animals at 0 dpv, 2 and 21 dpi. All infected animals, either vaccinated or naïve, elicited similar levels of anti-sNcAg antibodies after challenge. Different sections of brain tissue obtained at 21 dpi were submitted to a histopathological examination for lesions compatible with *N. caninum* infection. Pictures show representative lesions of PBS + AVEC immunized animals. A) Focal gliosis, B) Non suppurative perivascular cuffing.

animals vaccinated with rNcPRO + AVEC proliferated after incubation with rNcPRO but not with sNcAg at 38 dpv, which can be due to the presence of insufficient amounts of profilin in the lysate. However, after infection, proliferation induced by sNcAg was abrogated in rNcPRO-vaccinated mice. This data, together with the elevated count of CD4+CD25 + FoxP3+ cells can be an evidence of the activity of NcPRO-specific regulatory T-cells.

It is known that regulatory T-cells can provide protection against some parasitic infections (*Plasmodium* sp., *T. gondii*) (Rowe et al., 2012), limiting the damage produced by inflammation. However, this can also be a strategy to weaken the immune response against the parasite, enabling the infection to forge ahead. The role of profilin as an inducer of the innate immunity and at the same time, a promoter of a regulatory response reveals the importance of this protein in the immunopathogenesis of this parasite. These two activities may be linked by the effects of rNcPRO on DCs, which needs further studies.

It is interesting to note that, even when directing the immune response towards only one antigen (NcPRO) and without eliciting strong cell-mediated immunity against the parasite's antigens, some level of protection was obtained. The reduction in total antibody titers observed after infection coincided with a decrease, one to four days after infection, in the mean titers of rNcPRO-specific IgM and IgG3. This may indicate that, at least in some of the animals, circulating antibodies might have succeeded in clearing away the parasite administered by the IP route. To our knowledge, there is no previous data relating IgM and IgG3 with protection to *N. caninum* infection. It has been reported that *T. gondii* profilin is passively released by the parasites through an unknown mechanism (Plattner et al., 2008; Yarovinsky et al., 2005) and is needed for the invasion of host cells (Plattner et al., 2008); thus, capturing free profilin may reduce the parasites infection rate. This hypothesis, however, needs to be investigated.

NcPRO is highly homologous to TgPRO (89%) and both are less related to *Eimeria*'s profilin (EtPRO, 68% of homology). EtPRO has shown to be a successful vaccine antigen when applied with a potent adjuvant (Lee et al., 2010; Jang et al., 2011a; b; Lee et al., 2012; Jang et al., 2013). *Eimeria acervulina* profilin (EaPRO) was also proposed as an adjuvant itself (Hedhli et al., 2009). Our data suggest that *N. caninum* profilin may not be a promising candidate to be used as adjuvant or antigen. Even though some protection was observed, probably due to circulating antibodies, cell-mediated responses were not sufficiently tackled for targeting the intracellular stages of the parasite.

More studies are needed to understand the role of profilins in the modulation and/or evasion of immune responses against *N. caninum*. Many parasites' proteins related with cell-invasion seem to have evolved to be poorly immunogenic or even act as decoy antigens (Arnot, 2014; Malpede and Tolia, 2014). There are different strategies to enhance their immunogenicity, e.g. constructing chimeric proteins (Shi et al., 2007; Alaro et al., 2013), selecting strong adjuvants (Mansilla et al., 2012) or activating different TLRs (Grossmann et al., 2009) as we did here. Other approaches need to be evaluated and may be useful for modulating the immune response elicited by profilin-based vaccines.

5. Conclusions

We describe for the first time the immune responses elicited by immunization with rNcPRO, and its capacity to induce regulatory T cells in the mouse model. This study offers new insights and poses new questions on the actual mechanisms of the profilins in promoting protection or evading the immune response.

Conflicts of interest

AM and AF are employed by Tecnovax S.A., a private laboratory. The other authors declare that they have no competing interests.

Acknowledgments

The authors are very thankful to Mrs Mariela Gammella and Dr Wenzel Czepluch for their technical assistance with flow cytometry; also Bioch. María de los Ángeles Lavoria and Dr. Karina Trono for critical reading and editing of the manuscript.

This work was funded by Tecnovax SA, in collaboration with INTA and CONICET. Funding for developing the adjuvant and assessing vaccine formulations was from the National Agency for the Promotion of Science and Technology (ANPCyT) of Argentina through the PID 2008-11 awarded to AVC. DPM, MW and AVC are researchers of CONICET. FCM and MEQ are doctoral fellows of CONICET.

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