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ORIGINAL ARTICLE

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Fusion of foreign T-cell epitopes and addition of TLR agonists enhance immunity against *Neospora caninum* profilin in cattle

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Summary

We demonstrated recently that immunization with recombinant *Neospora caninum* profilin (rNcPRO) induces limited protection and a regulatory T-cell response in mice. The aim of this study was to evaluate the immune response elicited by rNcPRO in cattle and assess a strategy to enhance its immunogenicity, combining the addition of T-cell epitopes and immune modulators. We developed a chimeric recombinant profilin fused to functional T-cell epitopes present in the N-terminal sequence of vesicular stomatitis virus (VSV) glycoprotein G (rNcPRO/G). Groups of three cattle were immunized with two doses (2 weeks apart) of rNcPRO or rNcPRO/G formulated with alum hydroxide or a nanoparticulated soya-based adjuvant enriched with Toll-like receptor (TLR) 2 and TLR9 agonists, aimed to tackle the MyD88 pathway (AVEC*plus*). rNcPRO induced only a primary immune response (IgM mediated), while antibodies in rNcPRO/G-vaccinated animals switched to IgG1 after the booster. The vaccine formulated with rNcPRO/G and AVEC*plus* improved the production of systemic IFN-γ and induced long-term recall B-cell responses. Overall, our study provides data supporting the use of T-cell epitopes from VSV glycoprotein G and TLR agonists to enhance and modulate immunity to peptide antigens in bovines, particularly when using small proteins from parasites for which immune responses are usually feeble.

KEYWORDS

cattle, *Neospora caninum*, profilin, recombinant vaccines

1 | INTRODUCTION

Neospora caninum is an apicomplexan parasite that causes one of the main reproductive diseases in cattle, which results in significant impact to producers of both meat and dairy industries, associated with economic losses related to abortion, premature culling and reduced milk yields.[1–3] Although many studies have been published since its discovery in 1988,[4] neosporosis is still a major problem, as there is not an efficacious control method. In this context, the development of an effective vaccine is paramount.

Many proteins involved in parasite's motility and host invasion are crucial for its intracellular development,[5] and some of them have been evaluated as potential targets for vaccination.[6] Profilins are small actin-binding proteins localized at the apical end of tachyzoites that regulate the polymerization and depolymerization of actin filaments, leading to their motility and allowing the assembly of different proteins from micronemes and rhoptries that mediate the invasion of the host cells.[5] Apart from being crucial for invasion, it was reported that *Toxoplasma gondii* profilin (TgPRO) can bind Toll-like receptor (TLR) 11 and TLR12 on dendritic cells, activating the production of pro-inflammatory cytokines. It was reported though that only TLR11 can recruit MyD88 to initiate MyD88-dependent signalling events downstream of profilin recognition.[7] Once inside the host cells, TgPRO is mainly dispensable for parasite replication or egress from the infected cells.[8] Thus, the innate immune system detects an early event that is associated with parasite invasion and motility. Interestingly, the dendritic cell (DC)-mediated recognition of TgPRO occurs before direct interaction with the parasite, using the "*detection at a distance*" strategy [9] as TLR11

recognizes the profilin, which is released by the parasites through an unknown mechanism.[8,10,11]

Profilins from *T. gondii* and *Eimeria acervulina* induced certain level of protection when used as vaccine antigens in mice and chicken, respectively, dependent on the vaccination schedule and the use of strong adjuvants.[12–18] *Neospora caninum* profilin (NcPRO), first characterized in 2010, is 89% homologous to TgPRO and can induce the production of IFN-γ after injection in mice, both in vivo and in vitro.[19] However, when we evaluated the efficacy of a vaccine based on a recombinant NcPRO (rNcPRO) in a mouse model, partial protection was observed, associated with the presence of antigen-specific $CD25^+$/$CD4^+$/$FoxP3^+$ T cells.[20]

To our knowledge, there is no available information on the antigenicity of NcPRO in bovines. The objective of this study was to evaluate the immune response elicited by rNcPRO in cattle and assess a strategy to enhance its immunogenicity. For that purpose, we fused NcPRO with the N-terminal portion of the vesicular stomatitis virus glycoprotein G (VSV-G) sequence, providing functional bovine T-cell epitopes. We have demonstrated before that the association of short peptides to N-terminal sequences of VSV-G can enhance T-cell responses to the third party antigen in bovines.[21] The aim of this approach was to activate naïve T cells, which are difficult to tackle due to the highly variable bovine major histocompatibility complex (BoLA).[22,23]

Tackling different TLRs is another strategy to improve vaccine efficacy.[24–26] TLR agonists are recognized by innate immune receptors expressed by DCs while particulate adjuvants, such as mineral salts and oil-in-water emulsions, do not activate DCs directly, and their mechanism of action is poorly characterized.[24] In farm animals, the presence of TLR ligands as adjuvants in conjunction with a vaccine can increase the efficacy and response to the immunization with a particular antigen.[27] The adaptor molecule MyD88, which is downstream of many TLRs and IL-1 receptor, is essential for host resistance to *T. gondii* [28] and *N. caninum*.[29] Thus, we hypothesized that tackling this pathway will enhance immunogenicity against rNcPRO. We improved the formulation of *Providean*-AVEC® adjuvant [30] that already provides TLR2 agonists by the addition of TLR9 agonists, creating "AVEC*plus*" adjuvant.

2 | MATERIALS AND METHODS

2.1 | Vaccine antigens

Cloning, expression and purification of rNcPRO have already been described.[20] *E. coli*-expressed protein was obtained from inclusion bodies (IB-rNcPRO) and purified by affinity chromatography following standard protocols.

The sequence encoding for NcPRO was previously cloned into a pCI-Neo vector (Promega, Madison, WI, USA)[20] and fused downstream the 212 amino acid of VSV-G, which was already cloned in pCDNA3.1 (VSV-GΔ212 [21]). A new forward primer with NcoI restriction site (5′-GTGACCATGGACTGGGATCCCGTTGTCAAG-3′) and a reverse primer with an XbaI site (5′-GCTCTAGATCACTAATAGCCAGACTGGTGAAGG-3′) were designed. The DNA template was pCI-NcPRO.[20] PCR was developed as follows: 4 minutes to 94°C, 35 cycles of 1 minutes at 94°C, 1 minutes to 50°C and 1 minutes to 72°C. PCR product was checked in agarose gel 1.2% and digested with EcoRI and NcoI. To release the VSV-GΔ212, pcDNA-VSV-G was digested using EcoRI and NcoI and cloned in pCI-NcPRO. Enzymes were purchased from Promega. The construct was digested again with EcoRI and NcoI and checked in an agarose gel 1.2%.

Recombinant profilin (rNcPRO) was expressed in *E. coli* (BL21plys strain) following a conventional protocol. The recombinant protein was obtained by adding IPTG 1 mol/L to the culture of BL21plys. Inclusion bodies (IB-rNcPRO/G) were purified following conventional protocols,[20] and rNcPRO was purified by Ni^{2+} affinity chromatography and used for vaccine formulations and in vitro assays.

2.2 | Vaccines

Vaccines (2 mL per dose) were formulated with 100 μg of recombinant Ni^{2+}-purified antigens rNcPRO [20] or rNcPRO/G, and two different adjuvants. One formulation used "AVEC*plus*". This adjuvant is based on *Providean*-AVEC®, a nanoparticulated soya-based adjuvant that contains TLR2 agonists (developed in our laboratory and transferred to Tecnovax S.A. Buenos Aires, Argentina) which was enriched with pcDNA-LacZ (kindly provided by Dr. Grigera, ICT-Milstein) that has CpGs motives that provide additional TLR9 agonists (20 μg per dose). The adjuvant was used at 30% volume (0.6 mL per dose). AVEC*plus* was also mixed with PBS alone to immunize the control group. The other adjuvant was aluminium hydroxide ("ALUM", Rehydragel HPA. Chemtrade, Toronto, Ontario, Canada, Reheis®, New York, USA) and was used at 4%, as previously reported.[31]

2.3 | Animal studies

Fourteen female adult heifers, seronegative for *N. caninum* (assessed by ELISA using a commercial kit purchased from ID Vet, Montpellier, France) were used in this study. They were in good body condition, free of BVDV and had adequate genital development at the beginning of the trial. The animals used in this experiment were traced, kept in a fenced area, with sentinels around to detect any introduction of *N. caninum*. Each group was immunized with two doses of the following formulations at day 0 and 21 post-vaccination (dpv): 100 μg of rNcPRO+AVEC*plus* (Group 1, n=3), 100 μg of rNcPRO+ALUM (Group 2, n=3), 100 μg of rNcPRO/G+AVEC*plus* (Group 3, n=3), 100 μg of rNcPRO/G+ALUM (Group 4, n=3) or PBS+AVEC*plus* (Group 5, n=2). Animals were boosted with the same vaccines at 240 dpv to measure the anamnestic immune response. Each 2 mL dose was injected subcutaneously (sc) on the neck using a 21-gauge needle. Reactions at injection sites were evaluated visually and by palpation during a week following each immunization. Serum samples were obtained at 0, 14, 21 28, 42, 60 and 100 dpv. Whole-blood samples were obtained at 60 and 247 dpv. All experiments were completed following biosecurity and animal welfare internal and federal regulations, and according to protocols 05/2010, 25/2013 and 20/2014 approved by the Institutional Committee for the Use and Care of Experimental Animals (CICUAE), CICVyA, INTA.

2.4 | ELISA tests for serological assessments

Kinetics of specific antibodies anti-NcPRO was tested in an *in-house* ELISA. Plates (Greiner Bio One, Austria) were coated with 2 µg per well of rNcPRO in carbonate–bicarbonate buffer as capture antigen. Samples were diluted 1:50 in PBS 10% equine serum, added to the plate and incubated for 1 hour at 37°C. The presence of profilin-specific antibodies was revealed using an antibovine IgG(H+L):HRP conjugate (1:1000, Jackson ImmunoResearch. West Grove, Pennsylvania, USA) followed by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) substrate (ABTS) – H_2O_2 0.13%. Plates were incubated for 20 minutes at room temperature in a dark place. The reaction was stopped with NaF 1 mol/L, and the plates were read at 405 nm.

The avidity of those antibodies was determined by incubating plates with 100 µL per well of 6 mol/L urea–PBS for 20 minutes after the first washing step.[32,33] The ELISA then followed the protocol previously described. Percentage of residual reactivity after the urea-wash treatment was calculated and expressed as avidity index "AI%".[33]

Immunoglobulin isotypes were detected using the protocol described above, but revealing with sheep antibovine IgG1 (1:750), IgG2a (1:750) and IgM (1:5000) conjugated to peroxidase (Serotec, Oxford, UK).

2.5 | ELISPOT of antibody-secreting cells

MultiScreen-HA 96-well nitrocellulose plates (EMD Millipore, Billerica, Massachusetts, USA) were coated with 20 µg/mL of rNcPRO in PBS, washed three times with PBS and blocked with 50 µg per well of PBS milk 4% for 2 hours at room temperature. Wells coated with PBS were left as negative controls. Purified peripheral blood mononuclear cells (PBMCs) obtained at 247 dpv were suspended in 150 µL complete RPMI medium to a final concentration of 2.5×10^5 to 5×10^5; cells were seeded in the plates by triplicate and incubated for 36 hours at 37°C and with 5% CO_2. Antibody-secreting cell (ASC) spots were revealed with antibovine IgG:HRP (1:1000, Jackson Immuno Research), incubated for 1 hour at room temperature and washed five times with PBS. True Blue (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, Maryland, USA) was used as substrate (50 µL per well). Spot-forming cells (SFC) were counted manually under stereomicroscope. Results are expressed as mean number of SFC (IgG-ASC) per 5×10^5 splenocytes from replicate cultures, after subtracting the number of SFC from wells form mock-treated cells.

2.6 | IFN-γ ELISA

Whole-blood samples were obtained from each animal at 60 dpv. Aliquots (1 mL each) were in vitro stimulated with rNcPRO (200 ng/mL), pokeweed mitogen (PWM, 20 µg/mL, Sigma, St. Louis, MO, USA) or PBS (positive and negative control, respectively) using 24-well plates for 16 hours at 37°C with 5% CO_2. Samples from nonimmunized animals were also ex vivo stimulated with rNcPRO/G (200 ng/mL). Plasma samples were collected by centrifugation. IFN-γ present in stimulated plasma was measured using a commercial Bovine Interferon Gamma ELISA Kit (ID Vet) performed according to manufacturer's specifications.

2.7 | Statistical analysis

All values were compared using Mann-Whitney *U*-test. For IFN-γ results, Welch's correction was used. Differences were considered significant with a 95% confidence interval. Statistical analysis was performed using SigmaStat. Systat Software Inc. San Jose, California, USA.

3 | RESULTS

3.1 | Antibody responses

3.1.1 | Total antibodies

Recombinant NcPRO alone or fused to the N-terminal end of VSV-G was formulated with AVEC*plus*, that provides TLR2 and TLR9 agonists, which signal through the MyD88-dependent pathway stimulating the IL12-induced IFN-γ production, a profile required for resistance to *N. caninum* infection.[34–36] To verify the modulation of the immune response induced by AVEC*plus*, we also formulated the antigens with aluminium hydroxide (ALUM), known to promote a Th2-biased immune response. Animals received two vaccinations, 3 weeks apart, and serum samples were obtained at different time points after the first and second vaccine dose.

The kinetics of total specific serum antibodies anti-NcPRO was determined by ELISA. NcPRO-based vaccines induced low levels of specific antibodies, barely over the cut-off value (Fig. 1a). Antibodies induced by rNcPRO+AVEC*plus* were not significantly different to the control group at all time points ($P>.05$), while antibody levels in the NcPRO+ALUM-vaccinated animals showed significant differences to those induced by PBS+AVEC*plus* at 42 and 60 dpv ($P<.05$).

The addition of T-cell epitopes to the antigen induced a more pronounced antibody response, with overall higher IgG levels than those of rNcPRO alone, despite of the adjuvant (Fig. 1a,b). The formulation containing rNcPRO/G+ALUM elicited antibody levels superior to the cut-off value after the first vaccination (14 dpv, $P<.05$) which declined at 21 dpv. After the booster, serum antibody levels against rNcPRO were recovered. Differences were statistically significant to the control group in cattle immunized with rNcPRO/G with either adjuvant at 28, 42 and 60 dpv ($P<.05$; Fig. 1b).

Avidity indexes (AI) of anti-NcPRO antibodies were measured at 42 dpv. All vaccinated animals elicited high-avidity antibodies. Although mean AI was higher when rNcPRO/G was combined with ALUM (Fig. 1c), differences were not statistically significant due to the high variability in the AI values.

Specific antibody levels decreased at 100 dpv to values similar to the cut-off of the assay (Fig. 1a,b). To assess whether vaccination had induced memory B cells, animals were boosted at 240 dpv and the presence of specific anti-rNcPRO antibodies secreting plasmocytes

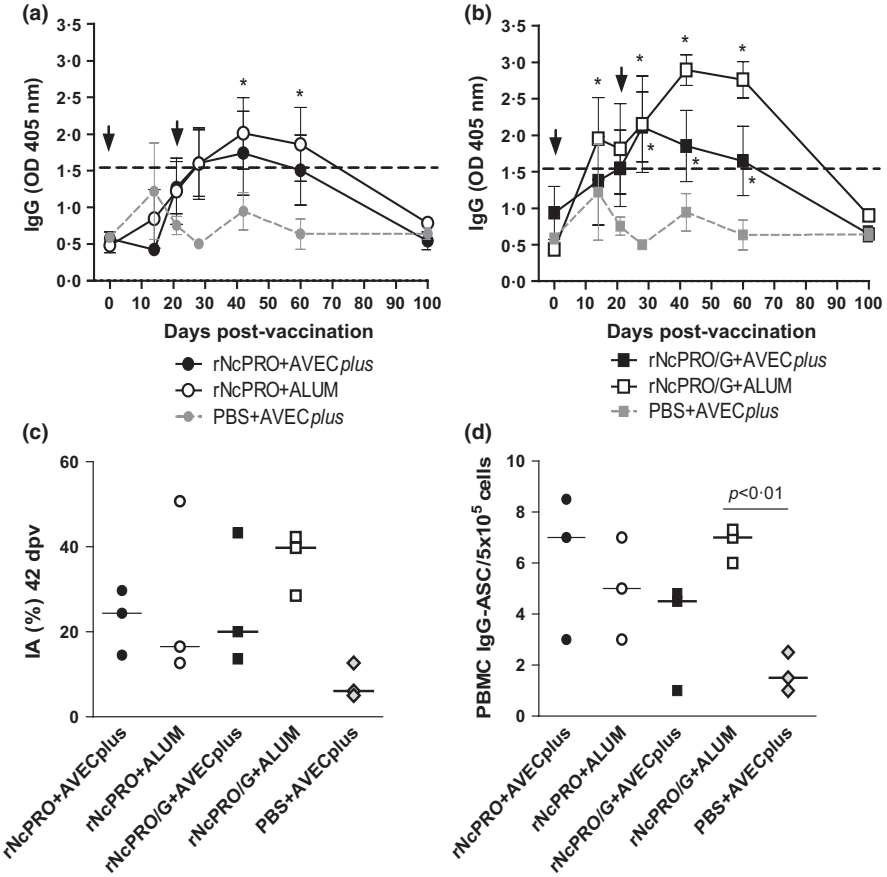

FIGURE 1 The kinetics of specific antibodies anti-NcPRO (mean ±SD) in animals immunized with NcPRO (a) or NcPRO/G (b) vaccines was determined by ELISA. Black arrows indicate each immunization. The horizontal dotted line depicts the cut-off value of the ELISA test. (c) Avidity of specific antibodies (Avidity Index, AI%,) against rNcPRO was determined by ELISA at 42 dpv. Data are depicted individually, and median values are shown for each group (horizontal lines). (d) The number of spot-forming cells (SFC) depicting memory IgG-secreting cells (ASC) specific anti-NcPRO was determined by ELISPOT in PBMCs in vitro stimulated with rNcPRO at 247 dpv (7 days post-boost). Individual values and median SFC/5.10^5 PBMC (horizontal lines) are shown. *Significant differences compared to PBS-vaccinated animals (P<.05)

was evaluated at 247 dpv (7 days post-boost). PBMCs were obtained from each individual animal and were ex vivo stimulated with purified rNcPRO. The presence of anti-rNcPRO-specific (ASC) was assessed by ELISPOT. NcPRO-specific IgG-ASC were detected in all vaccinated animals, with higher counts in those immunized with rNcPRO/G+ALUM (P<.01, Fig. 1d).

3.1.2 | Isotypes

The immunoglobulin isotypes induced by vaccination and their kinetics were also assessed by ELISA (Fig. 2). Animals immunized with rNcPRO formulated either with AVEC*plus* or ALUM exhibited a more delayed kinetics of IgM than those immunized with rNcPRO/G (Fig. 2a,b). Significant differences were observed at 14, 42 and 60 dpv induced by rNcPRO+ALUM compared to PBS+AVEC-vaccinated animals (P<.05; Fig. 2a). Serum IgM values over the cut-off were also measured in animals immunized with rNcPRO+AVEC*plus* at 28 dpv. Differences, however, were not statistically significant due to the small number of samples. High IgM levels were found 14 days after the first vaccination in those animals immunized with rNcPRO/G+AVEC*plus* (P<.05; Fig. 2b), which declined thereafter and increased after the booster at 42 dpv, although they were variable between animals. IgM levels were variable at all time points in cattle vaccinated with rNcPRO/G+ALUM (Fig. 2b).

No evidence of isotype switching was found in serum samples from cattle vaccinated with rNcPRO with either adjuvant. IgG1 levels were not different from control group along the experiment (Fig. 2c). On the other hand, rNcPRO/G-vaccinated animals switched to serum rNcPRO-specific IgG1 after the booster (Fig 2d). IgG1 levels in rNcPRO/G+ALUM-vaccinated animals were significantly higher compared to those induced by rNcPRO/G+AVEC*plus* or PBS+AVEC*plus* formulations at 42 and 60 dpv. We did not find rNcPRO-specific IgG2 in sera from animals immunized with any vaccine formulation (data not shown).

3.2 | Cell-mediated immunity

The production of IFN-γ was measured at 60 dpv by ELISA using a commercial kit on plasma samples collected from blood that was in vitro stimulated with rNcPRO. Viability was controlled by treating PBMC with pokeweed mitogen as described before,[37,38] and background values were subtracted from cells that were stimulated with PBS. Cells from vaccinated animals treated with PBS only (Fig. 3) and those from nonimmunized animals incubated either with rNcPRO or rNcPRO/G (data not shown) did not produce measurable INF-γ levels. Higher levels of IFN-γ were observed in animals immunized with formulations containing AVEC*plus*, either with rNcPRO or rNcPRO/G; however, results obtained from rNcPRO+AVEC*plus* were scattered. IFN-γ levels measured in stimulated plasma of rNcPRO/G+AVEC*plus*-vaccinated animals were significantly higher than those of PBS+adjuvant-vaccinated animals (P<.05; Fig. 3).

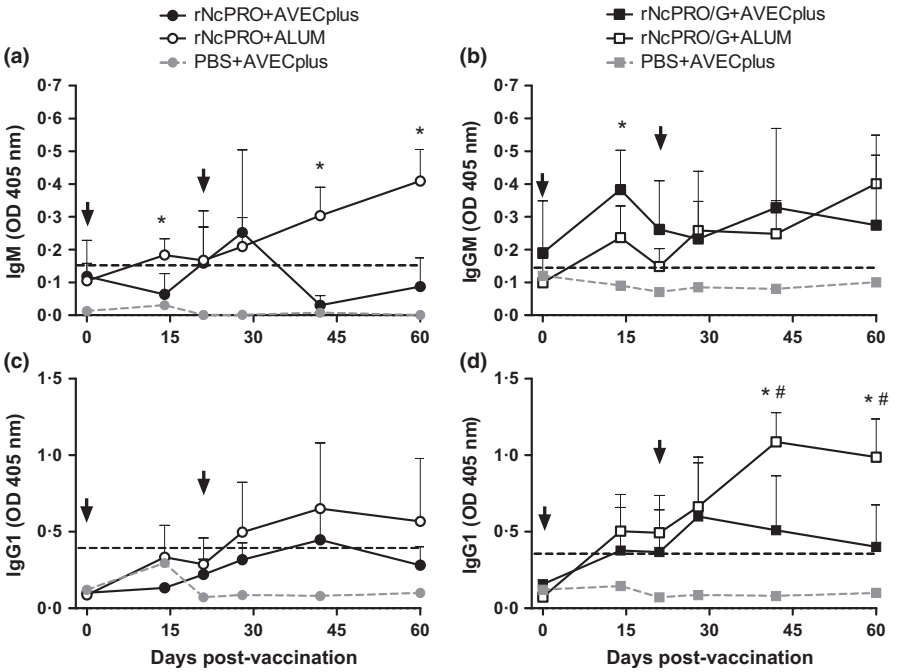

FIGURE 2 Kinetics of IgM (upper panel) and IgG1 (lower panel) anti-NcPRO induced by vaccines containing rNcPRO (a,c) or rNcPRO/G (b,d) with the indicated adjuvants were determined by ELISA (mean +SD). Black arrows indicate each immunization. The horizontal dotted line depicts the cut-off value of each ELISA test. *Significant differences compared to PBS-vaccinated animals ($P<.05$). #Significant differences between animals immunized with vaccines formulated with ALUM or AVEC*plus*

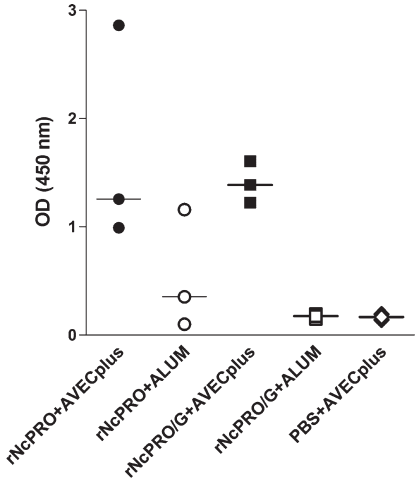

FIGURE 3 Individual IFN-γ responses were measured by ELISA in rNcPRO-stimulated plasma from cattle immunized with the different vaccines, as indicated. Blood samples were taken at 60 dpv. Median values are shown with horizontal lines. *Significant differences compared to PBS+AVEC*plus*-vaccinated animals ($P<.05$)

4 | DISCUSSION

There are no vaccines currently available to control the major parasitic diseases, although in many cases, there is evidence of acquired immunity and resistance to reinfection. Here, we show that the poor immunogenicity of *N. caninum* profilin described in the mouse model[20] can be counteracted with a chimeric recombinant antigen containing T-cell epitopes of another nonrelated protein formulated with an adjuvant that enables the arrival of intact antigen to the draining lymph nodes and provides TLR agonists that signal through the MyD88 pathway.

The fusion of a VSV-G T-cell epitope, capable of activating an adequate cellular-mediated immunity,[39] constituted an effective strategy to improve the humoral and cellular immune response against NcPRO in cattle. Animals immunized with rNcPRO/G elicited high-avidity specific antibodies, mainly IgG1, and developed memory B cells. The same epitope had been successfully applied to improve the immune response induced by a recombinant vaccine against FMDV.[21] Moreover, memory B cells were detected more than 8 months post-vaccination, which highlights the efficient priming of the acquired immune response. Conversely, when rNcPRO (nonchimeric) antigen was used, only IgM was induced after each vaccination and no IgG switch was observed.

The mechanisms underlying the higher immunogenicity of the chimeric antigen may respond to the presence of VSV T-cell epitopes that can magnify the response against NcPRO by bystander effect. Antigen presentation of this chimera to naïve T cells by professional APC will lead to primed T cells that will collaborate with B cells that can present the same T-cell epitope and produce, in rejoinder, IgG against *N. caninum* profilin. Bystander activation of T cells has been best described for CD8 T cells.[40] CD4 T cells, however, also undergo bystander activation, although the signals inducing this antigen-nonspecific stimulation of CD4 T cells are less well known.[41] This crosstalk between a stimulated CD4 memory T-cell response and CD4 T cells activated by an unrelated antigen was previously reported in mice, as memory CD4 T cells against tetanus toxoid influenced the immune response of CD4 TCR transgenic T cells specific for an unrelated OVA peptide.[42] Also, in a murine model of stromal keratitis, it was reported that non-virus-specific CD4 T cells could become activated by an inflammatory milieu consisting of enhanced accessory molecules and pro-inflammatory cytokines in the cornea.[43]

The adjuvant used to formulate the vaccines modified the magnitude of the antibody response. Higher levels of total antibodies were

measured in serum from cattle immunized with formulations containing ALUM. However, we did not find major differences in terms of the IgG isotype profile, as none of the vaccines elicited IgG2. In a previous study, we showed that a vaccine formulated with a similar adjuvant (AVEC®) and a protein lysate from *N. caninum* also induced IgG1 in cattle.[38] This adjuvant-induced modulation of the immune response was also reported by Jones et al.[44] who described an IgG1-restricted antibody response against bovine tuberculosis induced by vaccines formulated with TLR4 and TLR7/8 agonists. It has been suggested before that high levels of high-avidity IgG1 and IFN-γ may be related to protection.[45] Although in cattle the Th1-2 profile based on IgG isotypes is less clear than in other species,[46] the presence of IFN-γ and IgG1 may be indicative of a Th1 profile.[46,47]

IFN-γ has both immunostimulatory and immunomodulatory properties, and its production is known to play a more significant role than cytolytic activity towards *N. caninum*-infected cells.[48] Although this is a pilot study due to the small number of animals included in each experimental group, our results suggest that the addition of different TLR agonists that activate the MyD88-dependent pathway improved the immune response elicited by vaccination. Vaccine formulations containing TLR agonists (those included in AVEC*plus*) induced the higher levels of systemic IFN-γ at 60 dpv. Even though in some animals immunized with ALUM, IFN-γ was detected, these levels were higher and more consistent in those that received AVEC*plus* formulations. The mechanisms underlying the role of TLR2 and 9 in the induction of acquired immunity in cattle have not been described, but given to the fact that TLRs are conserved, we can infer that they may behave in a similar way than the TLRs from other species.[49]

The use of a TLR agonist-enriched adjuvant will create a cytokine milieu that may favour the production of IFN-γ. Although the exact mechanisms involved in naturally acquired immunity remain poorly defined, it is feasible to think that the first line of defence is antibody-producing B cells that target newly inoculated tachyzoites. Several studies also assign an essential role of IFN-γ in controlling parasitemia.[36,50,51] In this scenario, a vaccine eliciting high-avidity antibodies and circulating IFN-γ may be adequate to block *N. caninum* infection.

5 | CONCLUSION

Here, we demonstrated that the fusion of functional T-cell epitopes to a poorly immunogenic protein, combined with the use of strong adjuvants containing TLR agonists, enhances its immunogenicity, modulating the profile of the immune response induced. Currently and to our knowledge, there are only two studies assessing the immunogenicity of recombinant or chimeric parasite vaccines against bovine neosporosis.[52,53] For *N. caninum*, usually live and attenuated vaccines [54] or vaccines based on tachyzoite lysates [38] have been evaluated in cattle. Our study constitutes, as a whole, a relevant contribution to the future development of recombinant vaccines against *N. caninum* in the natural host and provides useful strategies to improve the immunogenicity of recombinant proteins used as vaccine antigens in cattle.

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DISCLOSURES

None.

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