THE B SUBUNIT OF *Escherichia coli* TOXIN LT FUSED WITH TRUNCATED rROP2 PROTEIN OF *Neospora caninum* INDUCES MIXED Th2/Th17 IMMUNE RESPONSE IN MICE

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ABSTRACT

Neospora caninum ROP2 protein is a promising vaccine antigen candidate for controlling neosporosis. In this study, we evaluated the immune response against a chimera formed by *Escherichia coli* heat labile B portion of the toxin (LTB) and its association with a truncated *N. caninum* ROP2 in a murine model. The recombinant proteins were expressed in *E. coli*, maintaining antigenic epitopes that were recognized by specific antibodies. Antigenicity was verified by western blot using serum from naturally infected cattle, and by ELISA using serum from mice infected experimentally with *N. caninum*. Immunogenicity was evaluated by total serum IgG, IgG1 and IgG2a isotypes in mice vaccinated with the experimental vaccines. Splenocyte transcription of the cytokines IL-10, IL-12, IL-17A and IFN-y were analyzed by qPCR. The chimeric construction rLTB/tROP2 modulated a significant increase ($p<0.05$) in total antibody titer against rROP2 by day 14 post inoculation, whereas the rROP2 induced higher ($p<0.05$) total IgG after the vaccine boost until the end of the experiment. Splenocytes from both groups stimulated with the rROP2 induced significant ($p<0.05$) higher IL-10 (18-27 folds), and IL-12 (7-12 folds) transcription, with a significant ($p<0.0001$) fold transcription increase for IL-17 (40-3000 folds). When stimulated with rLTB/tROP2, a significant ($p<0.05$) higher IL-10 (46-76 folds), IL-12 (4-25 folds) and IL-17 (40-400 folds) transcription were observed. Our results exhibit evidence that these recombinant proteins were able to modulate a mixed Th2/Th17 immune response, suggesting they may be a promising vaccine to be used for the control of *N. caninum*.

Keywords: Chimera. Rhoptry. Protozoan.

INTRODUCTION

The Apicomplexa protozoan *Neospora caninum* is reported to be the main cause of abortion in cattle, causing significant economic losses to the world's cattle livestock (REICHEL et al., 2013). It is also capable of infecting a wide variety of domesticated and wild hosts (DUBEY et al., 2007). Most recombinant subunit vaccine candidates against *N. caninum* evaluated to date have been prioritizing tachyzoite antigens involved in adhesion and host cell invasion processes (HEMPHILL et al., 2016).

The rhoptry proteins of *N. caninum*, especially the ROP2 protein, have demonstrated significant protection against experimental infection in mice (DEBACHE et al., 2008, 2009, 2010). Up to the moment, the efficacy conferred by *N. caninum* subunit vaccines in murine models is based on either Th1-directed immune response alone (BASZLER et al., 1999; KHAN et al., 1997), or a mixed Th1/Th2 responses (DEBACHE et al., 2008; MONNEY et al., 2011).

An important role for Th17 cells during *N. caninum* infection in the generation of a protective antibody response has been suggested, thereby IL-17 cytokine may provide protection against *N. caninum* infection (FLYNN; MARSHALL, 2011). Thus, a vaccine that is able to promote a Th-17 response might be beneficial for the *N. caninum* protection (PECKHAM et al., 2015). An ideal vaccine response to control *N. caninum* should inhibit horizontal and vertical transmission as well as allow the maintenance of pregnancy.

The use of adjuvant molecules to potentiate and modulate immune vaccine response represents an important to improve its efficacy (MBOW et al., 2010). The use of the B subunit from the heat labile enterotoxin of *Escherichia coli* (LTB) as immune modulator may also be a promising alternative to improve vaccine efficacy and achieve the desired outcomes of a *N. caninum* vaccine (DA HORA et al., 2011). It has been reported that the interaction between LTB and the cell receptor (e.g. GM1) activates $CD4^+$ T cells and B cells, enhancing antigen presentation by facilitating antigen uptake by dendritic cell (HAAN; VERWEIJ, 1998).

The aim of this study was to evaluate immune responses of chimeric form of truncated *N. caninum* rROP2 associated with *E. coli* LTB protein, in a murine model.

MATERIAL AND METHODS

Production of recombinant proteins

The nucleotide sequence of the ROP2 gene used in the construction of the proteins of this study was based on the sequence deposited on GenBank under the number HM587954 (MONNEY et al., 2011). Specific primers were constructed for amplification of a part of the truncate ROP2 nucleotide (tROP2) sequence referring to amino acids 191 to 240 and cloning into the pAE and pAE/LTB vector (RAMOS et al., 2004). The PCR products were inserted into the restriction sites of the *Xho*I and *Eco*RI enzymes so that the frame allowed expression of the products with a 6-histidine tag (Figure 1). The cloning products were also sequenced (ACTGene Analysis). For expression of the recombinant proteins, *E. coli* strain BL21 (DE3) (Invitrogen) was used. Induction was performed with 1 mM IPTG for 4 h at 37 ^{o}C. Bacteria were recovered by centrifugation, sonicated, and eluted in 8 M urea. Solubilized recombinant proteins were purified using the ÄKTA Primer (GE Healthcare) system. Desalting of the samples was carried out in a Desalting 5 ml HisTrap column (GE Healthcare), and concentration using dialysis membranes (cut-off 14 kDa, INLAB). Subsequently, the proteins were analyzed using SDS-PAGE. The concentration of the purified protein was determined using the commercial BCA Protein Assay kit (GE Healthcare). Purified protein aliquots were stored at -70 °C until use.

Western blot

The purified proteins were subjected to SDS-PAGE and electro-transferred to a nitrocellulose membrane (GE Healthcare). The membrane was incubated with blocking solution (PBS-T with 5% milk powder) for 1 h at 37 ^{o}C. Afterwards, the membrane was cropped, and each strip was incubated separately for 1 hour at 37 ^{o}C with one of the following primary antibodies: anti-histidine (1:5,000); anti-cholera toxin (CT) (1:5,000); or bovine serum positive for neosporosis (1:400) previously confirmed by indirect immunofluorescence reaction (IFAT). They were then used at the same dilution (1:5,000), as secondary: anti-mouse; anti-rabbit; and anti-bovine antibodies, respectively. The reactions were revealed with a solution containing DAB (0.6 mg diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-

HCl pH 8.0 and 15 µl hydrogen peroxide). *E. coli* BL21 (DE3) a non-transformed strain was used as negative control, and rLTB (FISCHER et al., 2010) was used as positive control.

Indirect enzyme-linked immunosorbent assays

The evaluation of total serum IgG antibodies against rROP2 and rLTB/tROP2 an indirect enzyme-linked immunosorbent assay (ELISA) was performed following Dummer et al. (2014) with modifications. Briefly, the plates (Polysorp Surface, Nunc, Sigma-Aldrich, St. Louis, MO, USA) were sensitized with 100 µl of a suspension containing 0.1 mg/ml recombinant proteins, or a *N. caninum* tachyzoites (1×10^6) suspension lysed, both diluted in carbonate-bicarbonate at pH 9.6 at 4 ^{o}C for 18 h. The plates were washed three times with phosphate buffer solution at pH 7.6 (PBS) containing 0.5% Tween 20 (PBS-T). Sera diluted in PBS-T 1/100 were added to the wells in duplicate (100 µl/well) and incubated at 37 ^{o}C for 60 min. The plates were washed three times with PBS-T. Then, 50 µl of peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts A/S), diluted 1/4,000 in PBS-T was added to each well, and the plates were incubated at 37 ^{o}C for 60 min. Immediately afterward, the plates were washed five times with PBS-T, and then 50 µl of o-phenylenediamine (OPD, Sigma Aldrich) chromogenic substrate was added, and the mixture was allowed to react in the dark for 15 min at room temperature, then a 100 µl stop solution (sulfuric acid 3%) was added. Absorbance readings were conducted using a microplate reader MR 700 (Dynatech Microplate Reader) at 492 nm, and the results are expressed as the total IgG increase.

The IgG1 and IgG2a isotypes were evaluated by ELISA using pooled sera (ROOS et al., 2012). Briefly, the plates were coated as described for the rROP2 and rLTB/tROP2 ELISA above. Fifty microliters (50 µl/well) of pooled sera diluted 1/200 in PBS-T was added to the wells; and the plates were incubated at 37 ^{o}C for 60 min. After this period, the plates were washed three times with PBS-T, and after adding 50 µl/well of anti-mouse IgG1 isotype antibody (Sigma Aldrich) diluted 1:5,000 in PBS, they were incubated at 37 ^{o}C for 60 min. The same protocol was performed using anti-mouse IgG2 isotype antibody.

Vaccination

A total of 24 Balb/c isogenic female mice (weighing 16–21 g) were randomly divided into four groups of 6 animals. The mice were inoculated by intramuscular injection of 0.2 ml

vaccine formulated with recombinant proteins. The vaccines were formulated using 25 µg of protein/dose in PBS adsorbed in 10% aluminum hydroxide ($Al(OH)_3$; Sigma Aldrich) as an adjuvant. Mice in group 1 were inoculated with a vaccine formulated with rROP2, group 2 with rLTB/tROP2, group 3 were inoculated with 0.9% saline solution plus 10% aluminum hydroxide, and group 4 were inoculated, intraperitoneally, with 1×10^6 *N. caninum* tachyzoites. The animals were inoculated on day 0 and received a booster on day 14 of the experiment. Blood samples were collected by the submandibular puncture on days 0, 14, 28, 35, 42 and 60. After collection, serum was separated, labeled, and stored at -20 ^oC until analysis.

All protocols were reviewed and approved by the Ethics Committee on Animal Experimentation (CEEA Nº 9651) of the Federal University of Pelotas (UFPEL). The UFPEL - CEEA agreement is approved by the Brazilian National Council for Animal Experimentation Control (CONCEA).

***Ex-vivo* spleen stimulation and cytokine transcripts**

The mice in the experimental groups were euthanized on day 60 of the experiment, and their spleen were collected. The spleen cells (2×10^6) were cultured in 1 ml RPMI 1640 (Gibco, Thermo Scientific) containing 10% fetal bovine serum (Gibco, Thermo Scientific), antibiotic and antifungal agents (penicillin 10,000 IU/ml, streptomycin 10 mg/ml and amphotericin B 25 mg/ml) (Gibco, Thermo Scientific) in 24-well plates (Kasvi, São José do Pinhais, PR, Brazil) and incubated for 24 h at 37 ^oC in 5% CO_2 atmosphere. Culture medium was replaced after 24 h and the cells were stimulated with 10 µg of rROP2, 10 µg of rLTB/ROP2, 2.5 µg of concanavalin A (ConA, Sigma Aldrich), or with RPMI 1640 and incubated for approximately 18 h under same conditions. ConA and RPMI were used as a control for cell stimulation. After incubation, the supernatant was discarded, the cells collected in TRIzol® reagent (Thermo Scientific) and total RNA extracted by the TRIzol method according to the manufacturer's instructions.

The total RNA from the splenocytes was extracted, and cDNA was synthesized using the High Capacity cDNA Kit (Thermo Scientific) and then stored at -20 ^oC. The gene expression levels were determined by quantitative real-time polymerase chain reaction (qPCR). The relative

amount of mRNA for each gene was determined by the comparative threshold cycle ($^{(\Delta\Delta}$CT) method. Beta-actin and GAPDH genes were used as endogenous reference controls, but subsequently, β-actin was selected as the internal reference standard based on its efficiency (M-value of 1.8 and 1.98 for GAPDH and β-actin, respectively). The primer sequences used for amplifying the IL-12p40, IL-17A, IL-10, IFN-y, β-actin and GAPDH genes, as well as qPCR conditions for the latter two have been described previously (CARDONA et al., 2003; DUMMER et al., 2014).

Statistics

The ELISA values of the IgG, isotypes (IgG1, IgG2a) and cytokine increase results were analyzed by two-way ANOVA, and the Bonferroni post-test. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Expression of proteins

Analysis of the DNA fragment for the partial gene of the ROP2 protein cloned into the pAE vector presented 100% identity and 100% coverage (sequence deposited in GenBank; number HM587954). The proteins were expressed as inclusion bodies, yielding ~ 125 mg/l for rROP2, and ~ 25 mg/l for rLTB/tROP2 (Figure 1).

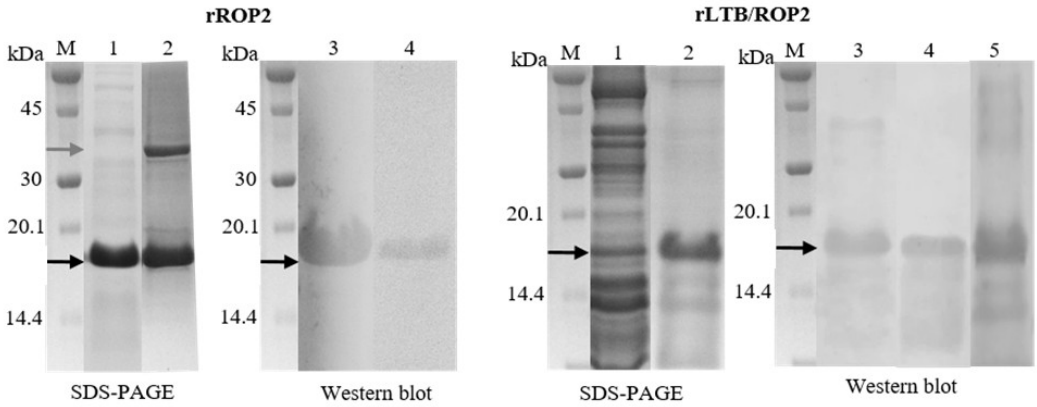

Figure 1 - SDS-PAGE and western blot analyses of rROP2 and rLTB/tROP2. Lane M, Molecular weight marker; lane 1 non-purified rROP2; lane 2 purified rROP2. Western blot, lane 3 probed with anti-His; lane 4 probed with anti-*N. caninum* antibodies from *N. caninum* infected bovine. rLTB/tROP2 SDS-PAGE, lane M, Molecular weight marker; lane 1 non-purified rLTB/tROP2; lane 2 purified rLTB/tROP2. rLTB/tROP2 western blot, lane 3 probed with anti-His; lane 4 probed with anti-*N. caninum* antibodies from *N. caninum* infected bovine serum; and lane 5 probed with anti-CT.

Antigenicity

In the western blotting analyses, the rROP2 and tROP2 portion of the chimera were recognized by sera from cattle naturally infected with *N. caninum* (Figure 1, lane 4) suggesting that the expressed proteins maintained its epitopes similar to native ROP2 in *N. caninum*. For the rLTB/tROP2 chimera, using anti-CT antibodies (Sigma Aldrich), the LTB portion was also recognized (Figure 1, lane 5). The rROP2 and rLTB/tROP2 induced the production of antibodies against epitopes similar to the native ROP2 protein present in tachyzoites as demonstrated by ELISA (Figure 2C).

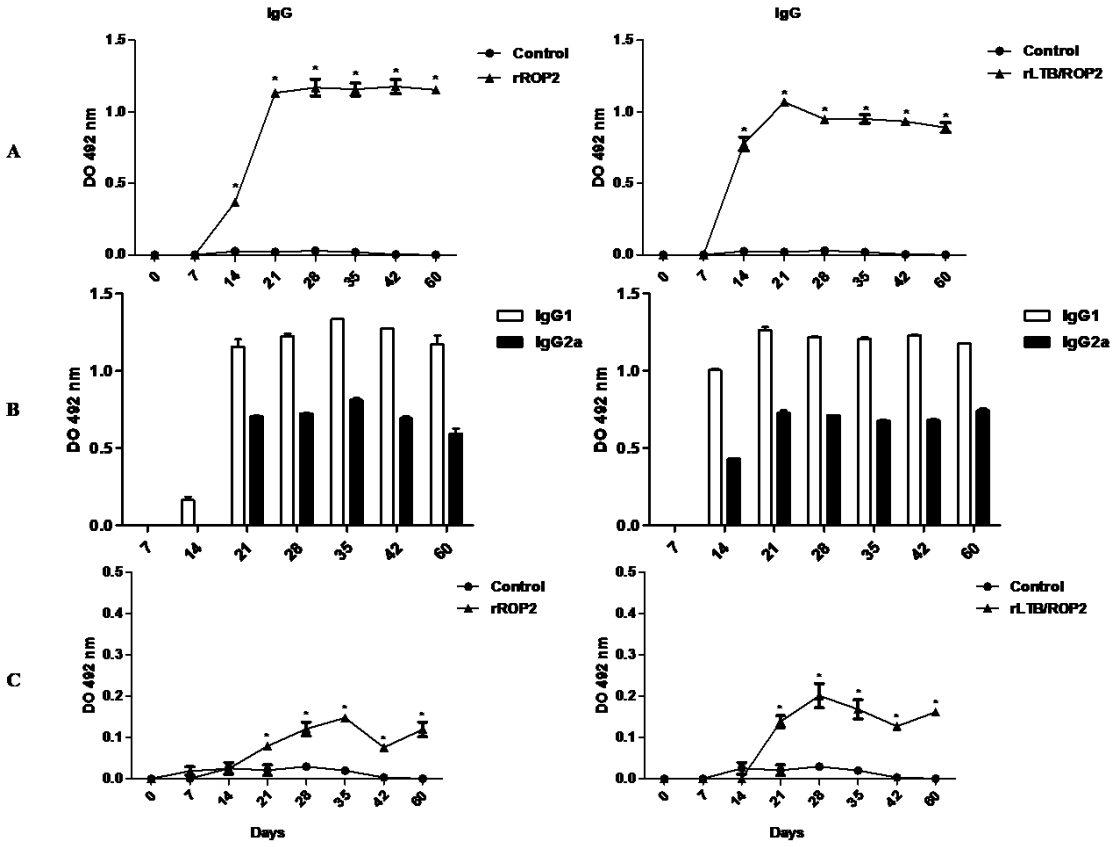

Figure 2 - IgG dynamics. The data represent the mean ELISA values (± standard error) of total serum IgG from mice inoculated with rROP2 or rLTB/tROP2. (A) Total IgG dynamic. (B) IgG1 e IgG2a dynamics. (C) Total IgG dynamic against lysed *N. caninum* antigen. The statistical analysis was performed by two-way ANOVA followed by the Bonferroni post-test. Asterisks (*) indicate significant difference ($p<0.05$) between the mice inoculation with rROP2 or rLTB/tROP2 (inoculated days 0 e 14) and the control groups ($p<0.001$) on days 14, 28, 35, 42 and 60.

Immunogenicity

Both proteins were able to induce IgG specific to rROP2 as well as for tachyzoites, and the production of antibodies was significantly higher ($p<0.05$) at 14 days post inoculation (PI). At 14 days the chimera had a significant ($p<0.05$) increase of total IgG comparing with the rROP2 and control groups. The boost induced a significant ($p<0.05$) increase of total IgG, being stable until the 60th day of the experiment (Figure 2A). There were significant ($p<0.05$) production of total IgG, with the rLTB/tROP2 at day 14th PI, and significant ($p<0.05$) increase for the isotypes IgG1 and IgG2a, as compared to rROP2 group (Figure 2B). Interesting, from the 21th days PI until the end of the experiment, the group rROP2 had significant ($p<0.05$) higher IgG titer than the chimera, but no isotopes difference (IgG1 and IgG2a), titers and ratio (Figure 2 A and B). The control group did not react against the recombinant antigen.

Ex-vivo splenocytes transcripts of IL-10, IL-12, IL-17A and IFN-y

The splenocytes cytokine transcription levels were assessed by qPCR on day 60 of the experiment (Figure 3). Splenocytes stimulated with rROP2 induced significant ($p<0.001$) IL-10 transcription levels in both vaccinated, and in the infected groups. An 18-fold increase was observed in splenocytes from the rROP2 vaccinated, a 20-fold increase was observed in the rLTB/tROP2 vaccinated group, and 27-fold increase was observed in the infected mice. When the splenocytes were stimulated with rLTB/tROP2, a significant ($p<0.05$) increase in IL-10 transcription occured comparing with the rROP2 stimulation in all groups, with 60, 46 and 76-folds increase for rROP2, rLTB/tROP2 and infected, respectively. For IL-12 splenocytes transcription, the stimulation with rROP2 was able to induce a 9-fold increase in the rROP2 vaccinated group, 7-fold increase in the rLTB/tROP2 vaccinated group, and 12-fold increase in infected mice. When the stimulus was done by the rLTB/tROP2 in the splenocytes from rLTB/tROP2 and rROP2 vaccinated groups, a 4 and 5-folds increase were observed, respectively, and a 25 folds increase in the infected group. The IL-17 transcription in the splenocytes from rROP2 vaccinated mice stimulated with rROP2 induced a significant ($p<0.001$) 170-fold increase comparing with the control group. Surprisingly, there was a significant ($p<0.001$) 3000-fold increase of IL-17 transcription level in the splenocytes from the rLTB/tROP2 vaccinated mice when stimulated with rROP2, and a 379-fold increase in the

splenocytes from the infected group. When the stimuli were done by the rLTB/tROP2 protein, a significant ($p<0.001$) 400-fold increase occurred in the splenocytes from the LTB/tROP2 vaccinated, a 40-fold increase in the rROP2 vaccinated group, and in the infected group a 102-fold increase. No splenocytes mRNA transcription was observed for IFN-y independent of stimuli (rROP2 or rLTB/tROP2) and groups (Figure 3), only with the concavaline A stimulus (data not shown).

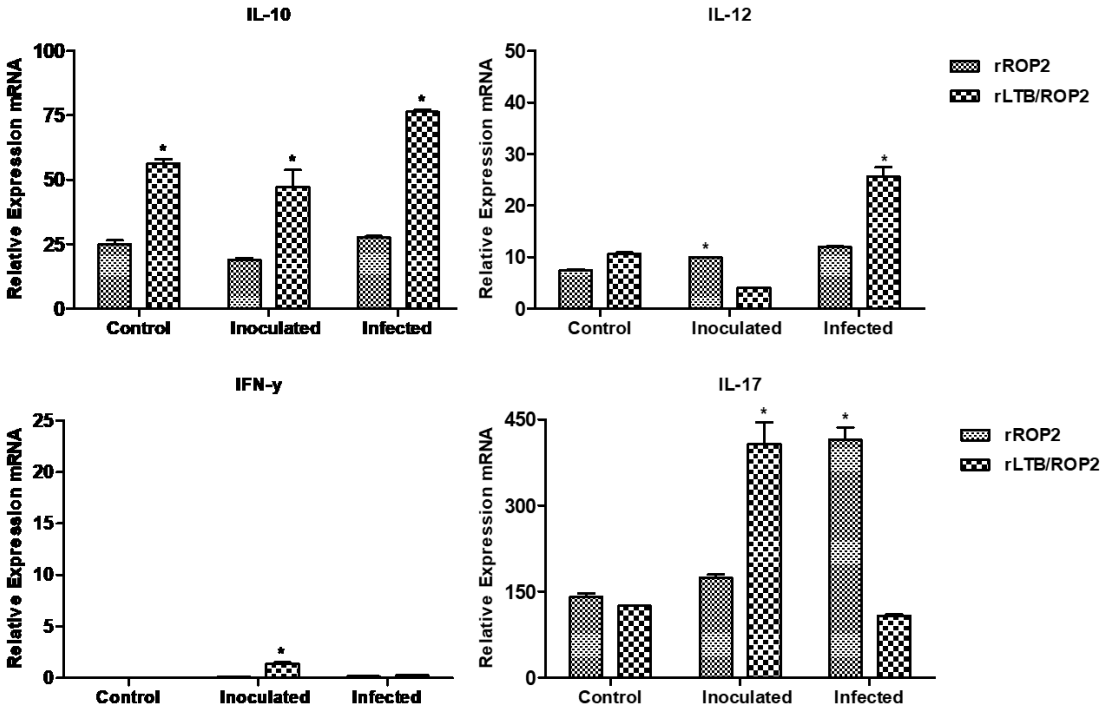

Figure 3 - Ex-vivo cytokine mRNA transcription. Splenocytes cells of the control, vaccinated and infected group were stimulated *in vitro* with rLTB/tROP2 and rROP2 for 18 h. Total RNA was extracted, and the correspondent cDNA subjected to qPCR. The fold changes for IL-12; IFN-y; IL-10; IL-17 were calculated from the threshold cycle (Ct) values normalized to Ct values obtained from non-inoculated mice. The results were expressed as mean ± S.E.M. and statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparisons test to investigate statistical differences obtained from repeated measures, (*) indicate significant difference ($p<0.05$).

Vaccines need be designed based on the protective immune response established between host/pathogen interactions. Thus, antigen selection and the incorporation of a suitable adjuvant into a vaccine should achieve immune response towards a type of immunity that effectively protects the host. The successive natural infections of *N. caninum* in cattle are able to confer an immunological memory that drastically reduces the chances of abortion

(DUBEY et al., 2007), suggesting that vaccination may be used as a tool to control *N. caninum* infection.

To induce specific and effective antibody production against a pathogen, recombinant vaccine antigens need to preserve epitopes with identity similar to the native protein. The loss of original conformation among *N. caninum* epitopes has been reported as a possible cause of low protection in murine challenge models (SRINIVASAN et al., 2007). The use of LTB as adjuvant in subunit vaccines has been an efficient option to overcome the poor immunogenicity of many recombinant subunit vaccines (WILSON-WELDER et al., 2009). In addition, LTB has shown that it is capable of enhancing the associated cytokine response of Th1, Th2 and Th17 when fused with different antigens (ZHOU et al., 2009).

This study demonstrated that the antibodies produced against the recombinant antigens, rLTB/tROP2 and rROP2, recognized the native protein, ROP2, in the tachyzoite lysates (Figure 2), as well as the LTB by anti-CT-antibodies, confirming that these constructs maintained epitopes similar to those of the native proteins. The chimeric construction (rLTB/tROP2) induced production of specific antibody and was able to generate a significantly ($p<0.05$) early response in total specific rROP2 IgG production (Figure 2A), and of IgG1 and IgG2a isotypes as well (Figure 2B). Previous studies suggested that IgG appearance can be mediated by antigen presentation, increased signaling mediated by LTB portion in the chimeric protein, and stimulation of B cells proliferation and differentiation to faster IgG switch and production (DA HORA et al., 2011; MUDRAK; KUEHN, 2010). Serum IgG production in experiments with mice demonstrated the good effect of rLTB on co-administered or fusion antigens, confirming efficacy as a systemic adjuvant (GRASSMANN et al., 2012). Our data are consistent with previous studies (GRASSMANN et al., 2012; LIU et al., 2011; ZHOU et al., 2009), which highlight the efficacy and advantages of chimeras composed of LTB in experimental vaccines.

The proteins, chimeric form or rROP2 alone, were able to induce a significant ($p<0.05$) IgG1 production compared with IgG2a (Figure 2). These results suggested that both recombinant proteins modulated an immune response towards Th2. However, aluminium hydroxide was used as adjuvant in the vaccine and it is well known that alum-based adjuvant, in mice,

polarise to a Th2 response by inhibiting IL-12 from DCs (COFFMAN et al., 2010; LI et al., 2007; MORI et al., 2012). So, the Th2 profile observed might be an association of antigen-adjuvant, or an independent role mediated by the antigen or by the adjuvant. Considering that both recombinant antigens were able to induce splenic IL-12 transcription in the infected mice (Figure 3), the adjuvant may have played a role in the IL-12 transcription, consequently in the IgG profile. However, this hypothesis was out of the study scope.

IL-10 plays an important role in controlling inflammatory processes as well as in the priming and proliferation of B-lymphocytes (OUYANG et al., 2011). Thus, its transcription levels observed may suggest a possible role of IL-10 in the immune modulation of IgG1 in the vaccinated groups, since this cytokine may have an antagonist effect on IFN-γ (FIORENTINO, 1989) (Figure 2). A similar result, towards Th2 response, was reported using ROP2 as an experimental vaccine antigen (DEBACHE et al., 2008). Also, previous studies with chimeras composed of ROP2 demonstrated a significant protects against acute neosporosis and limits cerebral parasite load in the mouse model for *N. caninum* infection protection, which also was attributed to the strong stimulation of IgG1 production (MONNEY et al., 2011). The protection of infection *N. caninum* was assigned by the presence of high specific IgG1 titers against ROP2; emphasizing that Th2 response plays an important role in preventing neosporosis. Studies using different *N. caninum* recombinant antigen, such as microneme protein NcMIC3, showed that protection against *N. caninum* brain infection in a murine model was associated with IgG1 antibody response (CANNAS et al., 2003). A immune response towards a Th2, as the one observed in this study, has been also reported by others, suggesting that a Th2 response is important for protection against *N. caninum* during pregnancy (DEBACHE et al., 2009; INNES; VERMEULEN, 2006). For protection against fetal infection and consequently abortion, a Th2-type immune response is ideal, since it is physiologically modulated, and has been shown that can control proliferation and spread of *N. caninum* during gestation (INNES, 2007).

IL-17 is not a potent inducer of inflammation on its own (GAFFEN, 2009), and its effects during inflammation derive from its ability to recruit immune cells through the expression of chemokines and the expression of cytokine receptors (ZRIOUAL et al., 2009). Studies indicate

that IL-17 is associated with the triggering of a Th2 response (ZRIOUAL et al., 2009). The interaction between IL-17 and the Th2 response is different according to time and the types of cells involved (KINYANJUI et al., 2013). Neutrophil activated by IL-17 initiate events that may lead to a Th2-type response, which in turn suppresses excessive polymorph nuclear cells recruitment (ALLEN et al., 2015). Studies have shown that models of mice with IL-17 deficiency have reduced levels of Th2-type cytokines (NAKAJIMA et al., 2014). The IL-17 producing cells can also express IL-10, and IL-12, although the expression of each cytokine can be regulated separately (DIXON et al., 2016; GUO et al., 2009).

In this study, we observed a significant increase in the *ex-vivo* splenic transcription of IL-17A in all groups stimulated with rLTB/tROP2 or rROP2. Enrichment of IL-17-producing T lymphocytes has been reported in epithelial barriers (ROMAGNOLI et al., 2016), where populations of γδ T lymphocytes expressing IL-17 are located playing an important role in the protection against pathogen invasion (ZHANG et al., 2012). Through activation of the IL-17 receptor, a cascade process results in increasing retention of B lymphocytes at germinal centers, and enhancement of antigen affinity of secreted antibodies (ROMAGNOLI et al., 2016; ZHANG et al., 2012). There are some studies that reported the importance of the IL-17 in parasites infections; *Trypanosoma cruzi* infection seems to be regulated by IL-17 (GUEDES et al., 2010), and the modulation of IL-17 response is important for the control of *Leishmania braziliensis* and *L. donovani* infection (NOVOA et al., 2011; PITTA et al., 2009). In cattle, the IL-17 produced by δγ T cells plays a significant role protecting against *N. caninum* infection, and it was also observed that the ability of macrophages to restrict parasitic replication during infection was associated with IL-17 production (FLYNN; MARSHALL, 2011). Considering that IL-17 may act controlling the initial infection, and bovine Th17 cells may be activated by the vaccination, the expression of this cytokine may be desirable. Since the Th17 response can activate the innate and acquired immunity against *N. caninum* infection, and an immune response towards a Th2 response, it may provide protection for the horizontal and vertical transmission of *N. caninum* (BEAGLEY; GOCKEL, 2003).Thus, a vaccine inducing a mixed Th2/Th17 response might be ideal for *N. caninum* control.

The results obtained in this study are promising since the vaccinal antigens tested were able to promote an immune response towards a Th2 profile, in agreement with immunity

mediated through Th2 modulation and also to induce a significant IL-17A transcription, which has been suggested to be important for controlling *N. caninum* infection (FLYNN; MARSHALL, 2011; KHAN et al., 1997; MONNEY et al., 2011; PECKHAM et al., 2015). On other hand, there are some limitations to our study. First, the cytokine results are based only in splenic transcription and not by protein quantification. However, there is a large body of evidence suggesting that there is a good correlation between the level of mRNA and its corresponding protein (DU et al., 2014; LINE et al., 2013). A second limitation is that we did not performed *N. caninum* challenge in the vaccinated experimental groups, however we were able to characterize the immune response induced by the antigens evaluated.

CONCLUSION

The vaccination with the chimeric form of rROP2 associated with LTB induced an immune response with a Th2/Th17 profile. These results bring a new perspective for the developing of an effective vaccine to prevent neosporosis.

A SUBUNIDADE B DA TOXINA LT de *Escherichia coli* FUSIONADA COM PROTEÍNA rROP2 TRUNCADA DE *Neospora caninum* INDUZ RESPOSTA IMUNE Th2/Th17 MISTA EM CAMUNDONGOS

RESUMO

A proteína ROP2 de *Neospora caninum* é uma candidata promissora de antígenos vacinais para o controle da neosporose. Neste estudo, avaliamos a resposta imune contra uma quimera formada pela porção B da toxina (LTB) de *Escherichia coli* termolábil e sua associação com ROP2 de *N. caninum* truncada em modelo murino. As proteínas recombinantes foram expressas em *E. coli*, mantendo epítopos antigênicos que foram reconhecidos por anticorpos específicos. A antigenicidade foi verificada por *western blot* utilizando soro de bovino naturalmente infectado e por ELISA utilizando soro de camundongos infectados experimentalmente com *N. caninum*. A imunogenicidade foi avaliada por isotipos séricos totais de IgG, IgG1 e IgG2a em camundongos vacinados com as vacinas experimentais. A transcrição das citocinas IL-10, IL-12, IL-17A e IFN-γ, de esplenócitos, foi analisada por qPCR. A construção quimérica rLTB/tROP2 modulou um aumento significativo ($p<0,05$) no título total de anticorpos contra rROP2 no dia 14 após a inoculação, enquanto a rROP2 induziu maior IgG total ($p<0,05$) após o reforço da vacina até

o final do experimento. Esplenócitos de ambos os grupos estimulados com a rROP2 induziu transcrição significativa (p<0,05) de IL-10 (18-27 vezes) e IL-12 (7-12 vezes), com um aumento significativo (p<0,0001) da transcrição para IL-17 (40-3000 vezes). Quando estimulados com rLTB/tROP2, observou-se uma transcrição significativa (p<0,05) de IL-10 (46-76 vezes), IL-12 (4-25 vezes) e IL-17 (40-400 vezes). Nossos resultados mostram evidências de que as proteínas recombinantes testadas foram capazes de modular uma resposta imune mista Th2/Th17, sugerindo que elas podem ser uma vacina promissora a ser utilizada para o controle de *N. caninum*.

Palavras-chave: Quimera. Roptrias. Protozoário.

LA SUBUNIDAD B DE *Escherichia coli* TOXIN LT FUSIONADA CON PROTEÍNA rROP2 TRUNCADA DE *Neospora caninum* INDUCE UNA RESPUESTA INMUNE MIXTA Th2/Th17 EN RATONES

RESUMEN

La proteína ROP2 de *Neospora caninum* es un candidato prometedor como antígeno de una vacuna para el control de la neosporosis. En este estudio, evaluamos la respuesta inmune contra una quimera formada por la porción B de la toxina termolábil (LTB) de *Escherichia coli* y su asociación con *N. caninum* ROP2 truncado en un modelo murino. Las proteínas recombinantes se expresaron en *E. coli*, manteniendo los epítopos antigénicos que fueron reconocidos por anticuerpos específicos. La antigenicidad se verificó mediante *western blot* utilizando suero bovino infectado naturalmente y mediante ELISA utilizando suero de ratones infectados experimentalmente con *N. caninum*. La inmunogenicidad se evaluó mediante los isotipos séricos totales de IgG, IgG1 e IgG2a en ratones vacunados con vacunas experimentales. La transcripción de las citocinas IL-10, IL-12, IL-17A e IFN-y, de esplenocitos, se analizó mediante qPCR. La construcción quimérica rLTB/tROP2 moduló un aumento significativo (p<0,05) en el título total de anticuerpos contra rROP2 el día 14 después de la inoculación, mientras que rROP2 indujo una IgG total más alta (p<0,05) después de la vacuna de refuerzo hasta el final del experimento. Los esplenocitos de ambos grupos estimulados con rROP2 indujeron una transcripción significativa (p<0,05) de IL-10 (18-27 veces) e IL-12 (7-12 veces), con un aumento significativo (p<0,0001) de la transcripción de IL-17 (40-3000 veces). Cuando se estimuló con rLTB/tROP2, se observó una transcripción significativa (p<0,05) de IL-10 (46-76 veces), IL-12 (4-25 veces) e IL-17 (40-400 veces). Nuestros resultados muestran evidencia de que las proteínas recombinantes probadas fueron capaces de modular una respuesta inmune mixta Th2/Th17, lo que sugiere que pueden ser una vacuna prometedora para el control de *N. caninum*.

Palabras clave: Quimera. Rhoptry. Protozoario.

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