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Research paper

# Immunization with inactivated antigens of *Neospora caninum* induces toll-like receptors 3, 7, 8 and 9 in maternal-fetal interface of infected pregnant heifers

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ABSTRACT

*Neospora caninum* is an obligate parasite and a major cause of abortion in cattle. Pregnancy failures appear to be associated with weak innate defences on the maternal-fetal interface during infection with *N. caninum*. Herein, we studied the gene expression of Toll-like receptors (TLRs) in pregnant heifers immunized with different vaccine formulations against *N. caninum* before mating and then challenged the heifers with live *N. caninum* on day 70 of gestation. TLR7 and TLR8 expression was upregulated in the placental caruncle of infected-pregnant heifers previously exposed to live *N. caninum* as immunogen. However, TLR7 and 8 expression in both placenta and caruncle as well as, TLR3 and 9 expression in caruncle were upregulated when heifers were previously immunized with inactivated soluble whole antigens and recombinant NcSAG1, NcHSP20 and NcGRA7 proteins. All dams were carrying viable fetuses when they were culled at day 104 of gestation. Upregulation of TLR7 and IFNγ expression was detected in fetal spleen when their mothers where previously vaccinated with soluble antigens and recombinant NcSAG1, NcHSP20 and NcGRA7 proteins. These studies demonstrate that soluble or recombinant NcSAG1, NcHSP20 and NcGRA7 antigens induce key TLRs expression at the maternal-fetal interface, probably triggering damaging inflammatory cellular immune responses associated with abortion. Previous infection with *N. caninum* seems to attenuate the innate immune response at the maternal-fetal interface, which could favour pregnancy maintenance and perpetuation of the disease. This finding represents novel information on how *N. caninum* vaccination and infection modulate TLRs expression at the placenta and fetal spleen, the possible role in the pregnancy outcomes and transplacental transmission of the protozoa.

## 1. Introduction

*Neospora caninum* is an Apicomplexan protozoan, closely related to *Toxoplasma gondii*, which infects a wide range of animal hosts including dogs and cattle (Dubey, 2003). Transplacental transmission is key in maintaining *N. caninum* infection in a bovine herd. Infection with *N. caninum* tachyzoites can result in fetal death or birth of persistently infected calves (Trees and Williams, 2005). Thus, neosporosis is a major cause of abortion in the cattle industry around the world (Dubey and Schares, 2011; Reichel et al., 2013). *N. caninum* is difficult to control as the parasite is capable of invading different host cells, may become latent and displays evolved mechanisms of immune modulation (Hemphill et al., 2006). Furthermore, no effective chemotherapeutic agent or vaccine has been developed to cure or prevent bovine neosporosis (Haddad et al., 2005; Dubey and Schares, 2011).

Innate responses are the first immune mechanisms activated in response to *N. caninum*. Pathogen recognition by Toll-like receptors (TLRs), the development of the T-helper 1 (Th1) immune response and early IFNγ production have shown to limit the dissemination of *N. caninum* (Innes, 2007; Mineo et al., 2010). Endosomal TLRs 3, 7, 8 and 9, sensors of intracellular microbial RNA and DNA (Mineo et al., 2010), have been described as key factors in *N. caninum* recognition (Bartley

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et al., 2013; Beiting et al., 2014; Koga and Mor, 2008; Marin et al., 2017). However, the inflammatory immune response is mostly attenuated in the placenta during pregnancy in order to prevent tissue damage and potentially, abortion (Raghupathy, 1997; Chaouat et al., 2002). The Th2 immune response is commonly detected in the placenta during fetal implantation and maintenance of early pregnancy. Furthermore, the development of a Th2 immune response in the maternal-fetal interface may contribute to the failure to control *N. caninum* infection during pregnancy (Chaouat et al., 2002; Innes et al., 2002). In this regard, we have recently reported increased expression of TLRs 3, 7 and 8 in fetal spleens and placental caruncles of heifers experimentally infected with *N. caninum* (Marin et al., 2017). However, whether immunization with antigens of *N. caninum* modulates TLRs on the maternal-fetal interface and prevents pregnancy losses remains unknown. Thus, the aim of this study was to define TLR expression in pregnant heifers immunized with different vaccine formulations against *N. caninum* before mating and then experimentally challenging the heifers with live *N. caninum* tachyzoites. The findings of this study will contribute to our understanding of the mechanisms of vaccine-induced immune responses, and therefore how these pathways can be manipulated in order to congenital transmission of this parasite.

## 2. Materials and methods

### 2.1. Parasite strains and vaccine formulations

Live tachyzoites of *N. caninum* NC-6 (Basso et al., 2001) and NC-1 (Dubey et al., 1988) strains were generously donated by Dr. Venturini (National University of La Plata, Argentina). NC-6 strain was used to inoculate naive heifers, intravenously (iv), in the jugular vein [$6.2 \times 10^7$ live tachyzoites in 2 mL of sterile phosphate-buffered saline (PBS) (pH 7.2)] (Live NC tachyzoites group). NC-6 strain was also used to extract soluble whole antigens (Soluble NC antigen group). Briefly, $1 \times 10^9$ NC-6 tachyzoites were purified using Sephadex columns (GE Healthcare, Little Chalfont, United Kingdom) and pelleted by centrifugation (1500 × g, 10 min). Parasite pellets were re-suspended in buffer [1 mL 10 mM Tris hydrochloride (pH 7.0) containing 2 mM of phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO, USA)], disrupted by three freeze-thaw cycles, and sonicated (6 × 30 s bursts on ice at maximum setting) (Sonifier 450, Branson Ultrasonic Co., USA). Protein content was determined using the Micro BCA protein assay method (Pierce, Rockford, USA) and the supernatant aliquoted and cryopreserved at −80 °C. Each soluble antigen vaccine dose was formulated as a mixture of soluble antigen (250 µL containing 500 µg/mL of protein) and immune stimulating complexes (ISCOMs; 200 µL containing 750 µg/mL; Abisco-300, ISCONOVA, Uppsala, Sweden). The ISCOMs were kindly provided by Dr. Morein (Uppsala University, Sweden).

Recombinant NcSAG1 (rNcSAG1), NcHSP20 (rNcHSP20) and NcGRA7 (rNcGRA7) proteins were cloned and purified from NC-1 strain (Hecker et al., 2014) (Recombinant NC antigen group). These proteins of *N. caninum* have shown to be highly immunogenic and indispensable for parasite replication (Hemphill et al., 2006; Huang et al., 2007; Cóceres et al., 2012). Moreover, they have been shown to be protective against *N. caninum* cerebral infection in mice (Cannas et al., 2003; Nishikawa et al., 2009). Each recombinant NC antigen vaccine was formulated as an equal mixture of NcSAG1, NcHSP20 and NcGRA7 recombinant proteins (30 µg of each protein; total 90 µg protein/dose) and ISCOMs (200 µL containing 750 µg/mL; Abisco-300, ISCONOVA, Uppsala, Sweden).

### 2.2. Animals and experimental design

All animals were handled in strict accordance with the guidelines of good animal practice and animal welfare defined by the Animal Ethics Committee (CICUAE) of INTA. This study was approved by the CICUAE.

Twenty-two 22-month-old Angus heifers, seronegative for *N. caninum*, *T. gondii*, Bovine Viral Diarrhoea Virus, Bovine Herpesvirus and free of brucellosis and tuberculosis were randomly divided into six groups. Heifers from Live NC tachyzoites group (n = 4) were inoculated iv once with live NC-6 tachyzoites 4 weeks before mating. Heifers from Soluble NC antigen and Recombinant NC antigen groups (n = 4 in each group) were injected subcutaneously (sc) with two doses 4 weeks and 1 week before mating. Two groups of heifers (n = 4 in each group) were sc immunized twice with sterile PBS (PBS group) or adjuvant (Adjuvant ISCOMs group), 4 weeks and 1 week before mating, as placebo. Heifers were then estrus synchronized with synthetic prostaglandins according to the manufacturer's instructions (D cloprostenol, Tecnofarm, Argentina) and naturally mated with four healthy Angus bulls over the course of seven days. Pregnancy was confirmed by transrectal ultrasonography 35 days after mating. Pregnant heifers were challenged iv with live NC-1 tachyzoites ($4.7 \times 10^7$ in 3 mL of PBS) at day 70 of gestation. Two non-vaccinated pregnant heifers received 2 mL of PBS iv at day 70 of gestation as placebo (Non-vaccine group). Fetal viability was checked by ultrasonography every week following challenge and until slaughter at an abattoir at day 104 of gestation.

### 2.3. Tissue collection

Immediately after slaughter, the whole reproductive tract was removed from each heifer and macroscopically examined. Four whole placentomes (maternal and fetal placenta), manually separated caruncles (maternal placenta), and samples of fetal spleen were collected from each heifer and stored at −80 °C for TLR expression studies.

### 2.4. RNA extraction, DNase treatment and reverse transcription

Total RNA was isolated from tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and digested with DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C to remove genomic DNA (gDNA). Quality and quantity of the resulting RNA were determined using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA). Complementary DNA (cDNA) was synthesised using a reaction mixture containing 1 µg of total RNA, random hexamers (12 ng/µl) (Promega, Madison, WI, USA) and Moloney murine leukaemia virus reverse transcriptase (10 U/µl) (Promega, Madison, WI, USA). Negative controls, omitting the RNA or the reverse transcriptase, were included.

### 2.5. Real-time RT-PCR

Real-time RT-PCR reactions for bovine TLRs (TLR3, TLR7, TLR8 and TLR9) and IFNγ were carried out using specific primers described by Marin et al. (2014) and Pérez (2006), respectively (Table 1). Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control (McGuire et al., 2004). The PCR reactions contained 800 nM specific forward and reverse primers, 1X PCR Master Mix (KAPA HRM FAST Master Mix, Biosystems, Woburn, USA) and 1 µL of cDNA sample in a final volume of 20 µL. The amplification and detection of the specific products were carried out in a Rotor Gene Q thermocycler (Qiagen, Hilden, Germany), with the following amplification conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 20 s at 95 °C and 60 s at 60 °C. After amplification, a melting curve analysis was performed, which resulted in a single product-specific melting curve. Samples were run in duplicate and negative controls for cDNA synthesis and PCR procedures were included. The amplification efficiency was determined for each gene using 10-fold dilutions of the cDNA. Housekeeping GAPDH expression levels remained constant in samples from all animals and a linear relationship between the amount of the template and Ct values was observed when the amplification efficiency for each gene was determined (data not shown). The results are reported as the mean fold change of TLR transcription levels in

**Table 1**
Sequences of primers for mRNA relative quantification by Real time RT-PCR.

| mRNA | Primer sense | Amplicon Size (base pairs) | 5′-3′sequences | Reference |
|---|---|---|---|---|
| GAPDH | F[a] | 112 | TTCTGGCAAAGTGGACATCGT | McGuire et al. (2004) |
| | R[b] | | CTTGACTGTGCCGTTGAACTTG | |
| TLR3 | F | 143 | CAGGTCAACAGTCCCGAA | Marin et al. (2014) |
| | R | | GCAGCACATTCCCCACAT | |
| TLR7 | F | 144 | TAAAACTCTGCCCTGTGATG | Marin et al. (2014) |
| | R | | CCTGCTATGTGGTTAATGGT | |
| TLR8 | F | 117 | TTATTGCAGAATGTAATGGTCG | Marin et al. (2014) |
| | R | | GAAAGGATTCATTCGTTACCC | |
| TLR9 | F | 113 | AACCTGCCCGCCAGACCCT | Marin et al. (2014) |
| | R | | GCCAGGGCCACTGCCAGTG | |
| IFNγ | F | 248 | ACTGCTCTGTGGGGCTTTTG | Pérez (2006) |
| | R | | CCAAA AAACAAACACATGTAGC | |

[a] Forward primer.
[b] Feverse primer.

tissues from vaccinated/infected heifers over the levels detected in non-vaccine group.

### 2.6. Statistical analysis

The relative gene expression analysis of the target genes was performed using the Relative Expression Software Tool (REST, Qiagen Inc., Valencia, CA, USA). The REST tool compares the expression of the target gene in a sample group relative to a control group with 2–16 data points, evaluating group differences for significance with a pair-wise fixed reallocation randomisation test (Pfaffl et al., 2002). The real-time RT-PCR efficiency for each gene was determined by a linear regression model according to the equation: $E = 10[-1/\text{slope}]$.

## 3. Results

### 3.1. TLR expression in placenta and caruncle

#### 3.1.1. TLR3

TLR3 expression was similar in the whole placentome and caruncles from Live NC tachyzoites, PBS, Adjuvant and Non-vaccine groups after challenge with live *N. caninum* during gestation ($P > 0.05$) (Fig. 1A). However, TLR3 expression in caruncles from heifers in the Soluble NC antigen and Recombinant NC antigen groups was significantly higher (7- and 17-fold, respectively) than expression in caruncles from heifers in the Non-vaccine group ($P < 0.05$) (Fig. 1A).

#### 3.1.2. TLR7

TLR7 expression was significantly increased in placentomes from heifers of Soluble NC antigen (2.2-fold) and Recombinant NC antigen (8.5-fold) groups compared with placentomes from heifers in other groups ($P < 0.05$) (Fig. 1B). In the caruncle, an augmented TLR7 expression was observed in all heifers challenged with *N. caninum* (Live NC tachyzoites, Soluble NC antigen, Recombinant NC antigen, PBS and Adjuvant groups) compared with the non-challenged heifers (Non-vaccine group) ($P < 0.05$). However, the highest levels of TLR7 expression were detected in heifers that were previously infected with *N. caninum* (24-fold) or immunized with soluble (16.8-fold) or recombinant surface protein (27.9-fold) antigens compared to expression levels in heifers of PBS and Adjuvant groups (Fig. 1B).

#### 3.1.3. TLR8

The relative expression level of TLR8 mRNA was significantly upregulated in the placentomes of pregnant heifers in the Soluble NC antigen group (5.7-fold) compared with the other groups ($P < 0.05$) (Fig. 1C). In the caruncle, all groups challenged with *N. caninum* (vaccinated or not) showed significant upregulation of TLR8 mRNA expression ($P < 0.05$). The highest levels of TLR8 expression were detected in heifers that received soluble (15-fold) and recombinant surface antigens (33.2-fold) (Fig. 1C).

#### 3.1.4. TLR9

Relative TLR9 mRNA levels were significantly lower ($P < 0.05$) in the placenta of heifers previously exposed to live tachyzoites and re-exposed to *N. caninum* (0.2-fold) compared with the non-vaccinated/non-infected heifers (Non-vaccine group) (Fig. 1D). In the caruncle, TLR9 transcript levels were upregulated in heifers in the Recombinant NC antigen group (7.6-fold) ($P < 0.05$) (Fig. 1D).

### 3.2. TLR and IFNγ expression in fetal tissue

All fetuses were viable and no gross lesions were observed at 104 days of gestation. The relative levels of TLR3, TLR8 and TLR9 tended to be higher in the spleens of fetuses from *N. caninum*-infected heifers compared with non-infected heifers (Non-vaccine group) but these differences were not significant ($P > 0.05$) (Fig. 2). Relative expression of TLR7 was significantly upregulated ($P < 0.05$) in the spleen of fetuses from heifers in the Soluble (5.9-fold) and Recombinant NC antigen (6.7-fold) groups. Levels of IFNγ expression were significantly higher ($P < 0.05$) in fetal spleens from *N. caninum*-challenged pregnant cattle that were immunized with soluble (5.7-fold) or recombinant surface (2.9-fold) antigens, PBS (6.8-fold) or ISCOMs adjuvant (7.1-fold) in comparison to the unvaccinated or uninfected group (Non-vaccine group).

## 4. Discussion

Despite *N. caninum* being a main cause of abortion in cattle and the congenital route being a major way of parasite transmission, the participation of TLR signalling triggered by *N. caninum* infection has not been elucidated. It is known that peritoneal macrophages and bone marrow-derived dendritic cells from mice exposed to *N. caninum*-soluble antigens presented upregulated expression of TLR2 (Mineo et al., 2010). Likewise, *N. caninum* RNA elicited TLR3-dependent responses in murine macrophages (Beiting et al., 2014). All these interactions, in turn, triggered the production of pro-inflammatory cytokines that may define parasite survival and pregnancy maintenance. It has been only recently observed that TLRs 3, 7 and 8 are responsive to *N. caninum* in the placental caruncle and fetus (Marin et al., 2017). In this study, TLR mRNA expression in pregnant cattle after immunization with different formulations of *N. caninum* vaccines was analyzed.

Our results showed that vaccination with soluble antigens and recombinant surface proteins promoted TLR7 and TLR8 expression in the placenta and caruncle of *N. caninum*-challenged pregnant heifers. These immunogens also increased TLR7 expression in the spleen of fetuses from infected-pregnant heifers. On the contrary, previous immunization

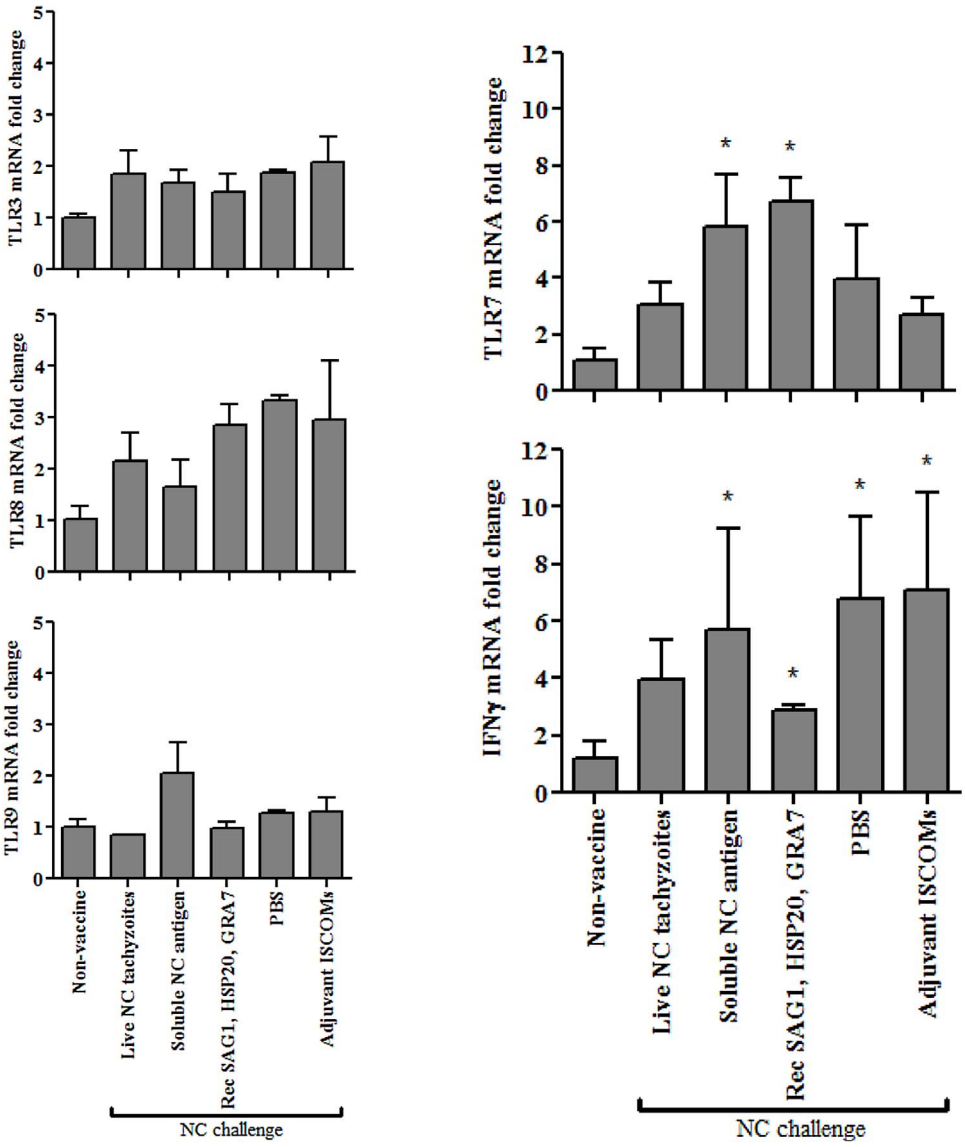


**Fig. 1.** Relative expression of TLR3 (1A) and TLRs 7–9 (3B, C and D) in the placenta and caruncle of pregnant heifers immunized with different vaccine formulations against *Neospora caninum* and challenged with *N. caninum* tachyzoites.
The results represent the mean fold change of TLR transcription levels in specific areas of the maternal-fetal interface of vaccinated/infected animals over levels detected in tissue sections of non-vaccinated/uninfected animals, which served as the control group. NC: *N. caninum*; Rec SAG1, HSP20, GRA7: recombinant *N. caninum* proteins; *: statistically significant differences ($P < 0.05$) with respect to the Non-vaccine group.

with live *N. caninum* and then challenge, showed a limited maternal TLR7 and 8 response in the caruncle. These findings could suggest that TLR7 and TLR8 expression is limited to the mother during natural infection but exposure to the inactivated antigens extends TLR7 and TLR8 to the fetus. TLR7 activation is required for the development of host-protective Th1 responses to other intracellular protozoan parasites (Paun et al., 2011; Ghosh and Stumhofer, 2013). Our findings demonstrated that expression of TLR7, and its closely related receptor TLR8, is induced by infection with *N. caninum* in the caruncle, as described previously (Marin et al., 2017). Moreover, we showed that previous infection with live tachyzoites specifically induced TLR7/8 response in the caruncle accompanied by a suppression of basal placental TLR9 response. Thus, stimulation of TLRs 7 and 8 in the caruncle by *N. caninum* may promote a Th1 response through stimulating the production of IL-12 and IFNγ. These TLR7/8 mechanisms seem to be key in inhibiting *N. caninum* replication by inducing a Th1 immune response. Our study suggests that previous infections with *N. caninum* might contribute to trigger such TLR response restricted to the maternal side of the placenta after challenge, preventing local inflammation and abortion. In this regard, heifers that were previously immunized with live *N. caninum* tachyzoites showed a lower inflammatory response and number of T cells in the placenta (Hecker et al., 2015). Furthermore, vertical transmission of *N. caninum* is mostly avoided when the dam has been infected before the pregnancy was established (Innes et al., 2001; Hecker et al., 2013).

Immunization with soluble and surface immunogens of *N. caninum* before mating also stimulated TLR7 and 8 expression in the fetal spleen and placenta, in agreement with the severe infiltration of different T cells populations in the placentomes after challenge (Hecker et al., 2015). Thus, inactivated antigens may trigger TLR7/8 and cellular immune responses, not only in the maternal side but also in the fetus. These TLR7/8-driven responses induced by *N. caninum* may also have adverse consequences, such as abortion, if damaging inflammation in the placenta occurs. In addition, vaccination with soluble or recombinant *N. caninum* antigens may not prevent vertical transmission of the parasite. Mice vaccinated with an inactivated vaccine of *N. caninum* excreted-secreted antigen induced a strong cellular immune response associated with high levels of IFNγ and inflammation, rendering mice more susceptible to parasite challenge (Ribeiro et al., 2009), which is in agreement with this finding. The fact that infection with *N. caninum* promoted IFNγ in the spleen of fetuses from either immunized or non-vaccinated heifers suggests fetal stress in primiparous *N. caninum* infection, and may predispose to fetal death under other inflammatory stimuli (e.g. TLR7/8 ligands). Likewise, first time *N. caninum* infections in cattle during mid and late gestation induced upregulated IFNγ mRNA and protein expression in the fetal spleen

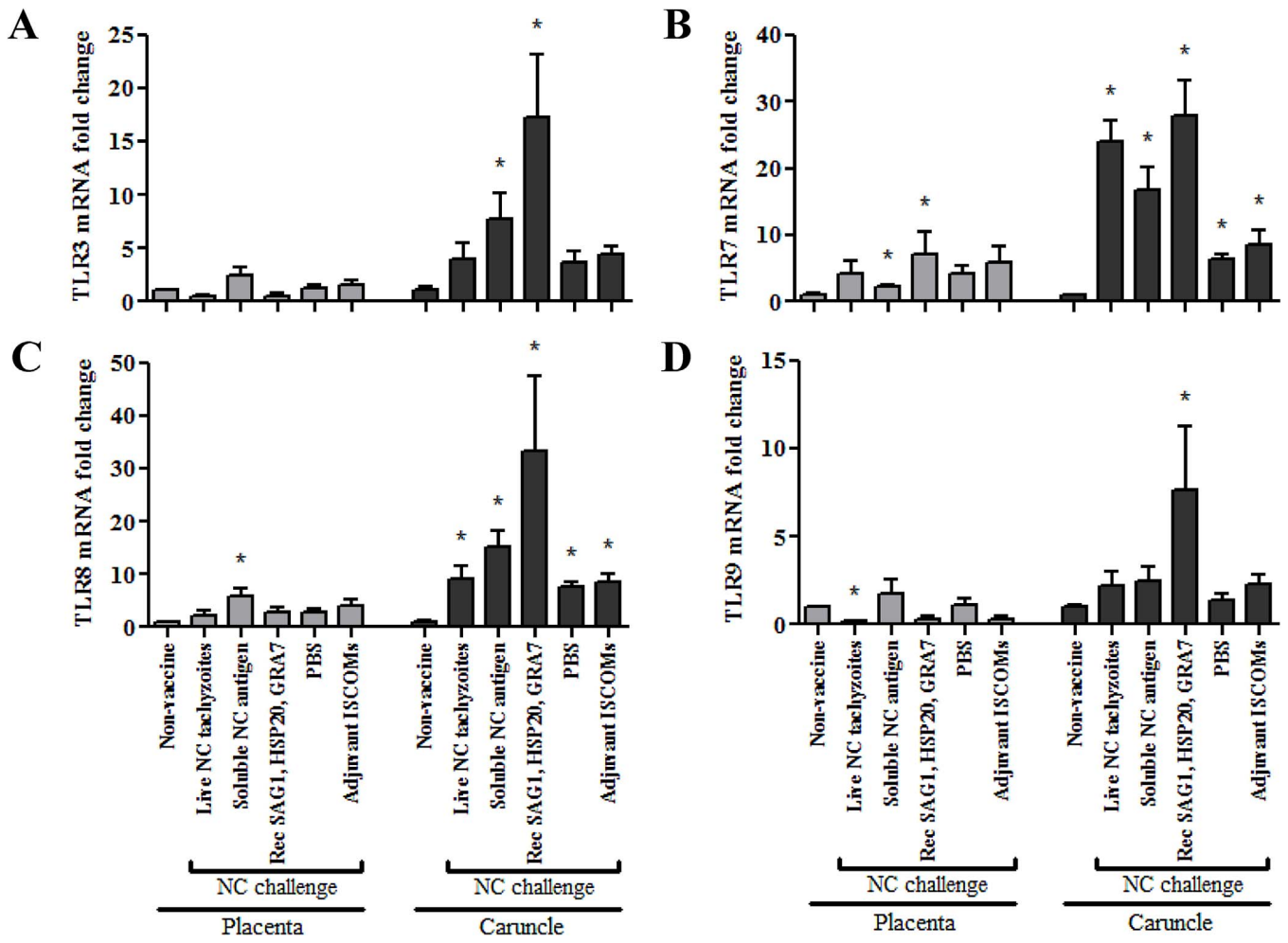


**Fig. 2.** Relative expression of endosomal TLRs and IFNγ in fetal tissues from animals immunized with different vaccine formulations against *Neospora caninum* and challenged with *N. caninum* tachyzoites.
The results represent the mean fold change of TLR and IFNγ transcription levels in spleen from fetuses of vaccinated/infected pregnant heifers over levels detected in tissue sections of non-vaccinated/uninfected animals, which served as the control group. NC: *N. caninum*; Rec SAG1, HSP20, GRA7: recombinant *N. caninum* proteins; *: statistically significant differences ($P < 0.05$) with respect to the Non-vaccine group.

(Bartley et al., 2013; Almeria et al., 2016). Interestingly, no IFNγ increase was detected in the fetuses when the dams were infected before mating. In accordance with these findings, previous exposure with live *N. caninum* tachyzoites before pregnancy prevented vertical transmission in the next gestation (Hecker et al., 2013). Thus, infection with *N. caninum* before pregnancy is established may promote a stronger protective immune response in the mother without affecting the fetus than the inactivated vaccines evaluated in this study.

Soluble and surface immunogens of *N. caninum* increased TLR3 and/or TLR9 expression in the maternal placenta of challenged-pregnant heifers. TLR3 has been implicated in murine macrophage resistance to *N. caninum* (Beiting et al., 2014). Therefore, soluble antigens, rNcSAG1, rNcHSP20 and/or rNcGRA7 proteins could exclusively promote TLR3 and TLR9 and provide some protection against *N. caninum*. However, the overall inflammation in the placenta and fetus may be deadly for the offspring. More specific *N. caninum* epitopes for TLR3 response, preventing other TLR ligands, could offer better vaccine alternatives.

In summary, this study provides novel information on how different vaccination approaches modulate the profile of TLR expression at the maternal-fetal placenta and in the spleen of the fetus. The tested soluble and surface antigens of *N. caninum* promoted the expression of TLR7/8 in the placenta and fetus that could trigger lethal responses for fetal viability. Previous exposure to live *N. caninum* induced similar TLR response, although restricted to the maternal side of the placenta which could indicate better protection against *N. caninum* congenital transmission and abortion.

## Conflict of interest statement

The authors declare no potential conflicts of interest with respect to the research, authorship, publication of this article and/or financial and personal relationships that could inappropriately influence this work.

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