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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 [6.2×10^7 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, 1×10^9 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 (4.7×10^7 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