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Toll-like receptors 3, 7 and 8 are upregulated in the placental caruncle and fetal spleen of *Neospora caninum* experimentally infected cattle

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HIGHLIGHTS

- TLR modulation by *Neospora caninum* in placental and fetal tissues was evaluated.
- Higher basal TLRs levels in fetal spleen compared with placental tissues.
- TLRs 3, 7 and 8 were upregulated mostly in caruncle from *N. caninum*-infected heifers.
- TLRs 3 and 8 were upregulated in fetal spleen from *N. caninum*-infected animals.

Abstract

Innate immune responses at the maternal-fetal interface are key in the pathogenesis of *Neospora caninum*, an obligate parasite that causes abortion in cattle. Herein, we determined the gene expression of endosomal Toll-like receptors (TLRs) in the placenta and fetuses from both non-infected pregnant heifers and pregnant heifers intravenously challenged with live tachyzoites of *N. caninum* on day 70 of gestation. On day 104 of pregnancy, mRNA expression of TLRs 3 and 8, as well as that of TLRs 7 and 9, was high in the spleen of fetuses from *N. caninum*-infected heifers. Gene expression levels of endosomal TLRs were also detectable in the placenta and the maternal caruncle from infected heifers, being TLRs 3, 7 and 8 particularly upregulated, mostly in the caruncle. Basal TLR levels were higher in fetal spleen than in placental tissues. This study provides novel information on how innate TLR responses are induced at the maternal-fetal interface of cattle in response to intracellular *N. caninum*.

Keywords: Toll-like receptor; *Neospora caninum*; placenta; fetus; cattle.

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Short Communication

Neospora caninum is an *Apicomplexa* protozoan which constitutes a major cause of bovine abortion and produces important economic losses mainly in the dairy cattle industry worldwide (Reichel et al., 2013). Innate immunity plays an important role in the pathogenesis of protozoan infections (Gazzinelli and Denkers, 2006). Activation of innate responses is essential for the establishment of adaptive T-helper 1 (Th1) cellular immunity, given that parasite control is mainly based on early IFN-γ production, dependent on lymphocyte priming by antigen-presenting cells and IL-12 (Innes, 2007). These events are triggered by recognition of conserved pathogen-associated molecular patterns, mostly by Toll-like receptors (TLRs) (Mineo et al., 2010). The activation of endosomal TLRs 3, 7, 8 and 9, which recognize microbial RNA and DNA, is of special interest in bovine neosporosis as TLRs recognize intracellular eukaryotic microbes (Gazzinelli and Denkers, 2006; Beiting et al., 2014). These innate mechanisms are particularly unexplored at the maternal-fetal interface during *N. caninum* infection in pregnant cattle. In this regard, an exacerbated immune response during pregnancy can potentially damage the placenta (Chaouat et al., 2002). Moreover, the immune response elicited by *N. caninum* has been indicated as responsible for placental damage leading to abortion (Maley et al., 2006). Thus, the pathogenesis of bovine abortion due to *N. caninum* is complex and involves immune-pathological mechanisms not completely understood. Particularly, understanding whether changes in the levels of TLR expression occur at the maternal-fetal interface and fetuses might contribute to elucidating the signaling pathways involved in the abortion induced by *N. caninum*. Thus, the aim of this study was to characterize the gene expression of endosomal TLRs at the maternal-fetal interface and fetal tissues from pregnant heifers experimentally challenged with *N. caninum*. The findings of this study will contribute to understanding

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how these pathways can be manipulated to control the congenital transmission of this parasite.

Ten 22-month-old Angus heifers, which belonged to a beef herd located at the National Institute of Agricultural Technology (INTA) Balcarce, Argentina, were used in this experiment. Blood samples from heifers were collected monthly for a year prior to mating and their seronegative status to *N. caninum* was confirmed using an indirect fluorescent antibody test (negative at 1:25) (Venturini et al., 1999). In addition, heifers were confirmed to be seronegative to *Toxoplasma gondii* by means of a microagglutination test (Desmonts and Remington, 1980), and to Bovine Viral Diarrhoea Virus and Bovine Herpesvirus by serum neutralization tests. The herd was also free of brucellosis and tuberculosis and a vaccination program against foot-and-mouth disease was routinely performed. Heifers were estrus-synchronized and then allocated with healthy Angus bulls for 7 days for natural mating. Transrectal ultrasonography was performed 35 days after mating to confirm pregnancy. Pregnant animals (n= 8) were intravenously challenged with 4.7×10^7 tachyzoites of the NC-1 strain of *N. caninum* on day 70 of gestation. A mock-infected group (control group; n= 2) of pregnant animals received PBS as placebo on day 70 of gestation. Fetal viability was weekly checked by ultrasonography from challenge until slaughter on day 104 of pregnancy. *Post-mortem* examination was carried out in all dams and fetuses. All animals used in this study were handled in strict accordance with the guidelines of good animal practice and the conditions defined by the Animal Ethics Committee (CICUAE) of INTA. Immediately after slaughter, the whole reproductive tract was removed from each heifer and examined following standard gross pathology procedures (Campero et al., 2003). From each animal, four placentomes (maternal and fetal placenta), full-thickness pieces of manually separated caruncle (maternal placenta), and fetal spleen

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samples were collected and stored at -80°C for TLR expression studies by real-time RT-PCR as previously described (Marin et al., 2016).

TLRs are known to be highly expressed by immune and non-immune cells at the human and murine maternal-fetal interface (Koga and Mor, 2008). Nevertheless, no studies have been performed to determine TLR expression in the bovine placenta. Therefore, in the present study, we first determined the basal relative expression of endosomal TLRs in the placenta, caruncle and fetal spleen of non-infected cattle. We found that mRNA levels of TLRs 3, 7, 8 and 9 were high in fetal spleen and lower but still detectable in the bovine placenta and caruncle (**Fig. 1**). The relative mRNA levels of these TLRs in placenta and caruncle tissues were similar (**Fig. 1**).

The role of endosomal TLRs in the pathogenesis of *N. caninum* during pregnancy is unknown. Thus, we determined the mRNA levels of TLRs 3, 7, 8 and 9 in the placenta, caruncle and fetal spleen from heifers challenged with *N. caninum*. We found that the expression of TLRs 3, 7 and 8 was significantly upregulated (between 4 and 8 fold) in the caruncle of *N. caninum*-infected heifers ($P < 0.05$) with respect to the mock-infected group (**Fig. 2**). In the placenta, TLRs 7 and 8 were slightly upregulated. However, endosomal TLR transcription levels were not significantly different in *N. caninum*-infected heifers ($P > 0.05$) (**Fig. 2**). Regarding fetal tissues, the mRNA levels of TLRs 3 and 8 were upregulated (2 to 3 fold) in the spleen of fetuses from *N. caninum*-infected animals and these differences were statistically significant ($P < 0.05$) with respect to the mock-infected group (**Fig. 2**).

Although *N. caninum* is a major causative agent of bovine abortion worldwide, the involvement of the innate immune responses in the placenta and fetus of infected cattle remains unexplored. Particularly, the role of endosomal TLRs in the pathogenesis of bovine neosporosis during pregnancy is unknown. The findings from this work indicate

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that TLRs are probably critical in the initiation of the host pro-inflammatory and adaptive response to *N. caninum*. Caruncle TLRs 3, 7, and 8 were significantly upregulated at gene level in *N. caninum*-infected heifers. Likewise, the levels of TLRs 3 and 8 were increased in the spleen of fetuses from *N. caninum*-infected dams. In agreement with these findings showing TLR expression changes during *N. caninum* infection and the involvement of TLR signaling, it has been previously demonstrated that TLRs and MyD88, the key adaptor protein for most TLRs, are crucial in the recognition of *N. caninum* in mice since Myd88 knock-out mice lack IL-12 and IFN-γ production in response to *N. caninum*, resulting in increased mortality (Mineo et al., 2009).

Few studies have been performed to address the expression of endosomal bovine TLRs during *N. caninum* infection (Bartley et al., 2013; Beiting et al., 2014). Bartley et al. (2013) demonstrated that TLR9 expression is upregulated in fetal spleens from subcutaneously *N. caninum*-inoculated cows on day 210 of gestation. The lack of TLR9 upregulation in the fetal spleens from *N. caninum*-inoculated heifers of this study at earlier stages of gestation is probably explained by the lack of complete maturity of the fetal immune system at the time of infection, as previously demonstrated during infection with other pathogens at similar stages of gestation (Osburn et al., 1982). It could be hypothesized that the expression of endosomal bovine TLRs in the fetal spleen may vary along gestation, even when considering infection by the same pathogen. In the present study, the levels of TLRs 3 and 8 were higher when the challenge was performed during early gestation. In contrast, as shown by Bartley et al. (2013), TLR9 levels are increased only when the challenge is performed during late gestation.

Even when parasites were probably actively replicating in fetal tissues 34 days post-infection (Hecker et al., 2013), the TLR expression levels in the fetuses were lower than

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in caruncles. Since the challenge was performed on day 70 of gestation, this finding may indicate a poor development of the innate immune mechanisms in the bovine fetuses. TLRs 3, 7 and 8 were expressed at higher levels in the caruncles although many immune-suppressive mediators are produced in the maternal-fetal interface. Indeed, *Neospora* infection triggers innate immune mechanisms, even under an immune-suppressive environment, like the one present in the bovine placenta.

The increased TLR expression in the caruncle compared with the whole placentome suggests that the initial recognition of the protozoa at placental level would occur in the maternal fraction. This study revealed that the single-stranded RNA-sensing TLRs 7 and 8 could be associated with the control of the intracellular infection by *N. caninum*. Activation of TLR7 has been shown to be involved in the development of host-protective Th1 responses to related intracellular protozoan parasites, such as *T. gondii*, *Trypanosoma cruzi* and *Leishmania* spp (Paun et al., 2011; Ghosh and Stumhofer, 2013). This work also confirms that TLR3, which is upregulated in infected caruncle and fetal spleen, is implicated in the host resistance to infection by *N. caninum*, as observed in macrophages from mice (Beiting et al., 2014). Likewise, infection of macrophages and transfection with *Neospora* RNA have been shown to elicit TLR3-dependent induction of type-I IFN responsive genes (Beiting et al., 2014).

At the maternal-fetal interface, human and murine TLRs are expressed not only by immune but also by non-immune cells, such as trophoblasts and decidual cells, with variations along pregnancy (Koga and Mor, 2008). The expression and distribution of TLRs in the placenta of cows have not been studied before. This study showed that the expression levels of endosomal TLRs were similar in the placenta and caruncle but higher in fetal spleen than in placental tissues. It is known that hyporesponsive mechanisms exist in the mother to tolerate the fetus, and this might be related to the

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findings in this study. In summary, this study provides novel information on how innate responses are induced at the maternal-fetal interface in response to *N. caninum* in cattle, characterized herein by the upregulation of key TLRs in the detection of intracellular parasites.

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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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References

Bartley, P.M., Katzer, F., Rocchi, M.S., Maley, S.W., Benavides, J., Nath, M., Pang, Y., Cantón, G., Thomson, J., Chianini, F., Innes, E.A., 2013. Development of maternal and foetal immune responses in cattle following experimental challenge with *Neospora caninum* at day 210 of gestation. Vet. Res. 3, 44–91.

Beiting, D.P., Peixoto, L., Akopyants, N.S., Beverley, S.M., Wherry, E.J., Christian, D.A., Hunter, C.A., Brodsky, I.E., Roos, D.S., 2014. Differential induction of TLR3-dependent innate immune signaling by closely related parasite species. PLoS One 9, e88398.

Campero, C.M., Moore, D.P., Odeón, A.C., Cipolla, A.L., Odriozola, E., 2003. Aetiology of bovine abortion in Argentina. Vet. Res. Commun. 27, 359–369.

Chaouat, G., Zourbas, S., Ostojic, S., Ostojic, S., Lappree-Delage, G., Dubanchet, S., Ledee, N., Martal, J., 2002. A brief review of recent data on some cytokine expressions at the materno–fetal interface which might challenge the classical Th1/Th2 dichotomy. J. Reprod. Immunol. 53, 241–256.

Desmonts, G., Remington, J.S., 1980. Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity andspecificity. J. Clin. Microbiol. 11, 568–652.

Gazzinelli, R.T., Denkers, E.Y., 2006. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nat. Rev. Immunol. 6, 895–906.

Ghosh, D., Stumhofer, J.S., 2013. Do you see what I see: Recognition of protozoan parasites by Toll-like receptors. Curr. Immunol. Rev. 9, 129–140.

Hecker, Y.P., Moore, D.P., Quattrocchi, V., Regidor-Cerrillo, J., Verna, A., Leunda, M.R., Morrell, E., Ortega-Mora, L.M., Zamorano, P., Venturini, M.C., Campero, C.M.,

ACCEPTED MANUSCRIPT

2013. Immune response and protection provided by live tachyzoites and native antigens from the NC-6 Argentina strain of *Neospora caninum* in pregnant heifers. Vet. Parasitol. 197, 436–446.

Innes, E.A., 2007. The host-parasite relationship in pregnant cattle infected with *Neospora caninum*. Parasitology 134, 1903–1910.

Koga, K., Mor, G., 2008. Expression and function of toll-like receptors at the maternal-fetal interface. Reprod. Sci. 15, 231–242.

Maley, S.W., Buxton, D., Macaldowie, C.N., Anderson, I.E., Wright, S.E., Bartley, P.M., Esteban-Redondo, I., Hamilton, C.M., Storset, A.K., Innes, E.A., 2006. Characterization of the immune response in the placenta of cattle experimentally infected with *Neospora caninum* in early gestation. J. Comp. Pathol. 135, 130–141.

Marin, M., Quintana, S., Leunda, M., Odeón, A., Pérez, S., 2016. Bovine alpha-herpesviruses distribution and toll-like receptors expression in the respiratory system of experimentally infected calves. Res. Vet. Sci. 105, 53–55.

Mineo, T.W., Benevides, L., Silva, N.M., Silva, J.S., 2009. Myeloid differentiation factor 88 is required for resistance to *Neospora caninum* infection. Vet. Res. 40, 32.

Mineo, T.W., Oliveira, C.J., Gutierrez, F.R., Silva, J.S., 2010. Recognition by Toll-like receptor 2 induces antigen-presenting cell activation and Th1 programming during infection by *Neospora caninum*. Immunol. Cell Biol. 88, 825–833.

Osburn, B.I., MacLachlan, N.J., Terrell, T.G., 1982. Ontogeny of the immune system. J. Am. Vet. Med. Assoc. 181, 1049–1052.

Paun, A., Bankoti, R., Joshi, T., Pitha, P.M., Stäger, S., 2011. Critical Role of IRF-5 in the Development of T helper 1 responses to *Leishmania donovani* infection. PLoS Pathog. 7, e1001246.

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Reichel, M.P., Ayanegui-Alcérreca, M.A., Gondim, L.F.P., Ellis, J.T., 2013. What is the global economic impact of *Neospora caninum* in cattle–the billion dollar question. Int. J. Parasitol. 43, 133–142.

Venturini, M.C., Venturini, L., Bacigalupe, D., Machuca, M., Echaide, I.,Basso, W., Unzaga, J.M., Di Lorenzo, C., Guglielmone, A., Jenkins, M.C.,Dubey, J.P., 1999. *Neospora caninum* infections in bovine fetuses and dairy cows with abortions in Argentina. Int. J. Parasitol. 29, 1705–1708.

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FIGURE CAPTIONS

Figure 1. Relative expression of endosomal bovine TLRs in the maternal-fetal interface and fetal tissues.

The results represent the mean fold change of TLR transcription levels in the maternal caruncle and fetal spleen of uninfected control cattle over levels detected in placental tissues, which were set to be 1. *: statistically significant differences (P <0.05).

Figure 2. Relative expression of TLR3 (2A) and TLRs 7-9 (2B, C and D) in the placenta, caruncle and fetal spleen of *Neospora caninum* experimentally infected cattle.

The results represent the mean fold change of TLR transcription levels in specific areas of the maternal-fetal interface or fetal spleen of infected animals over levels detected in tissue sections of uninfected animals, which served as the control group. C: control; Nc: *N. caninum*-infected; *: statistically significant differences (P <0.05) with respect to the uninfected control.

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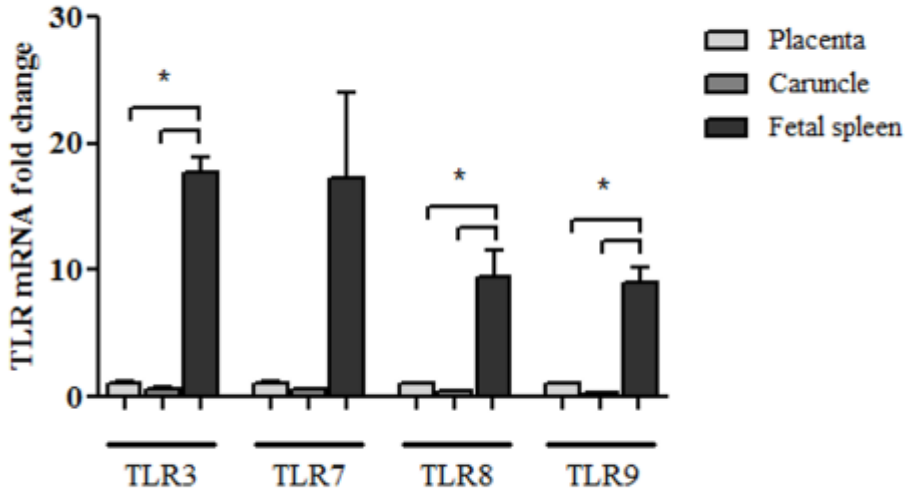

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