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Short communication

Cytokine gene expression in aborting and non-aborting dams and in their foetuses after experimental infection with *Neospora caninum* at 110 days of gestation

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ABSTRACT

Neospora caninum is a major cause of abortion in cattle. However, it is not known why not all infected animals abort. In this study, Th1 (IFN-γ), Th2 (IL4) and T reg (IL-10) cytokine gene expression was examined by real time PCR using the TaqMan approach in all of these dams and their foetuses after experimental infection with the isolate Nc-Spain7 at 110 days of pregnancy and euthanasia 6 weeks after infection. In prior published work, foetal death was observed in three of six infected dams and transplacental infection in all the 6 infected foetuses. In the spleen of the dams, IL-4 expression was down-regulated in dams with aborted/non viable foetuses compared to both uninfected dams (controls, n = 3) and infected dams with live fetuses at euthanasia. In the lymph nodes draining the placenta, up-regulated expression of IL-4 was observed in infected dams with live foetuses compared to control dams. In the placenta, infected dams with live foetuses had significantly up-regulated IFN-γ in both caruncle and cotyledon and up-regulated IL-10 in cotyledon compared to control dams. Infected live foetuses showed up-regulated expression of IFN-γ and IL-10 in foetal spleen, and showed downregulated expression of IL-4 in the thymus compared to control uninfected foetuses. Expression of any cytokine in the thymus was significantly lower compared to the levels observed in foetal spleen. The results indicate an up-regulated expression of Th1, Th2 and Treg in infected dams with live foetuses and in their foetuses. On the other hand, down-regulation of Th2 immune responses and Treg cytokines were observed in infected dams which had aborted or had non-viable foetuses at euthanasia, suggesting an immunological recovery of cytokine gene expression levels in dams a few weeks after an abortion occurred.

Neospora caninum is an obligate intracellular parasite considered a very important cause of abortion in cattle worldwide (Almería and López-Gatius, 2013). The precise causes of foetal or placental damage are not well-known and the reasons why some animals abort and other do not remain unclear (Dubey et al., 2006).

Th1 cytokines such as IFN-γ inhibit the multiplication of *N. caninum* tachyzoites inside the cells and has been linked to protection against *N. caninum*-associated abortion in cattle (Almería et al., 2012). However, pro-inflammatory responses, effective against *N. caninum*, when excessive will likely result in foetal or placental damage (Almería et al., 2010).

In prior work, we standardized the infection model as an intravenous dose of 10^7 *N. caninum* tachyzoites for pregnant cows at day 110 of gestation using two different strains (Nc-Illinois in Almería et al., 2010 and Nc-Spain-7 in Almería et al., 2016). Animals were euthanized 6 weeks after infection. Foetal death was observed in some dams in both experiments (Almería et al., 2010, 2016), as occurs in field conditions. A protective immune response against abortion could not definitively be associated with IFN-γ levels alone, but significantly lower IFN-γ/IL-4 ratios were observed in the dams with live foetuses (Darwich et al., 2016), highlighting the importance of the Th1/Th2 balance in the course of *N. caninum* protection against abortion in cattle. In our experience, IFN-γ may be detected with a greater sensitivity when the cytokine is determined

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at the gene expression level (Almería et al., 2012). The present study analyzed Th1 (IFN-γ), Th2 (IL4) and T reg (IL-10) cytokine gene expression in immune tissues in infected dams with aborted-non viable foetuses and in dams with live foetuses at euthanasia compared to uninfected dams (controls) to try to establish the immune responses taking place in relation to protection against *N. caninum* abortion. Cytokine gene expression was also determined in foetal tissues and at the materno-foetal interface (caruncle and cotyledon) in infected dams and their live foetuses and control uninfected dams and their live foetuses.

The animals used and the infection protocol have been described elsewhere (Almería et al., 2016). Briefly, nine 14–16 month-old Holstein-Friesian heifers seronegative for *N. caninum* (CIVTEST, Girona, Spain) were artificially inseminated. Pregnancy was confirmed by ultrasonography 30, 45, 90 and 110 days after insemination. On Day 110 of gestation, six of the heifers were intravenously (IV) inoculated with 10^7 culture-derived tachyzoites of the *N. caninum* isolate Nc-Spain7. These six animals were euthanized around Day 152 of gestation. The three remaining heifers were kept as un-inoculated controls and were euthanized at the same time as the inoculated dams. Around Day 152 of gestation (Day 42 after infection) all animals were necropsied. Portions of foetal tissues were aseptically obtained and DNA was extracted.

The procedures used in the present study were approved by the Ethics Committee on Animal Experimentation of the Universitat Autonoma de Barcelona (UAB) (license number CEEAH.1426-08/02/2012) and of the Universitat de Lleida (license number CEEA.06-01/12). Animals were handled in accordance with good animal practices and the strict conditions defined by the Animal Ethics Committee at UAB and CReSA, Spain. Every effort was made to minimize suffering.

The lymphatic vessels draining the uterus, the internal iliac lymph nodes, named here as uterine lymph nodes (UTLN) and the spleen were collected from the heifers and spleen and thymus were collected from the foetuses for isolation of mononuclear cells as described by Almería et al. (2014). In addition, three placentomes –the cranial, medial and caudal placenta– were recovered from each animal. Both the maternal side of the placenta (caruncle) and the foetal side of the placenta (cotyledon) were careful separated manually from each placentome. Tissues were frozen and homogenized in liquid nitrogen and kept in Trizol at −80 °C. Craneal, medial and caudal placental samples collected from each animal were combined and used for RNA extraction to determine cytokine genes in maternal (caruncle) and foetal placenta (cotyledon).

Total RNA was extracted with phenol-cloroform. Samples were treated with DNAse in the presence of RNAse inhibitors for elimination of contaminating genomic DNA and RNA concentrations were determined and RNA integrity was checked. Complementary DNA was synthesized from 2 μg of total RNA and random primers with the High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's recommendations.

Messenger RNA expression was determined by real time RT-PCR following the Taqman approach in an ABI PRISM TM 7700 sequence detector (PE Applied Biosystem, Foster city, CA, USA). Probes and primers for bIL4 and bIFN-γ and bIL-10 have been described in Almería et al. (2003). Primers were designed to span an intron to avoid genomic contamination. Probes and primer pairs were used to quantify GAPDH RNA as the endogenous housekeeping control gene described by Leutenegger et al. (2000).

Probe and primer concentrations for the analyzed cytokines were determined and PCR amplifications were performed as in Almería et al. (2014). Endogenous GAPDH housekeeping expression was used to normalize levels of cytokine gene expression (Almería et al., 2012). For relative quantitation of gene expression, the comparative threshold cycle (CT) method (ABI PRISM7700 sequence detection system, user bulletin #2) was used as described in Almería et al. (2003).

Caruncle and cotyledon samples from the same dams, and thymus and foetal spleen samples from the same foetuses were analysed in the same run to be able to compare their expression levels.

Comparisons between groups (control, infected with live foetuses and infected with aborted/dead foetuses) were tested by a one-way ANOVA test. When statistically significant differences were found Bonferroniís test was applied to examine all possible pairwise comparisons. Comparisons between two groups (infected with live foetuses versus control uninfected; comparison of caruncle and cotyledon samples; foetal spleen and thymus samples) were performed by Student's *t*-test. All analyses were done using the SPSS computer package, version 17.0 (SPSS Inc., Chicago, IL). Differences were significant when $p \leq 0.05$.

The present study was designed on the basis of the results described in Almería et al. (2016). In this previous study, of six heifers infected as described here three suffered foetal mortality. All experimentally infected heifers were seropositive to *N. caninum* at euthanasia, and transplacental infection have already taken place in their foetuses. Control uninfected foetuses did not show antibodies and *N. caninum* DNA was not detected in any of their tissues (Almería et al., 2016).

In the spleen, lower IL-4 mRNA expression was observed between infected dams with aborted foetuses versus infected dams with live foetuses ($p = 0.019$) and uninfected dams (controls) ($p = 0.025$, one-way ANOVA, Bonferroni-multiple comparison test) (Fig. 1A). The expression of IL-4 was on average 6-fold lower (range 3.4–9.4-folds) in infected dams with aborted foetuses compared to infected dams with live foetuses and an average 2.9-fold lower (range 1.3–6.7-folds) in dams with aborted foetuses versus uninfected dams (controls). No significant differences were observed for IFN-γ and IL-10 ($p = 0.09$ and $p = 0.72$, respectively, one-way ANOVA, Bonferroni test) (Fig. 1A).

In UTLN, significant differences among groups were observed for IL-4 ($p = 0.045$; one-way ANOVA, Bonferroni test) (Fig. 1B). Compared to control uninfected dams, IL-4 expression was significantly up-regulated in infected dams with live foetuses by 7.3-fold (range 4.7–14.3-fold) ($p = 0.05$, one-way ANOVA, Bonferroni test). No significant differences were observed for IFN-γ and IL-10 ($p = 0.10$ and 0.09, respectively; one-way ANOVA, Bonferroni test) (Fig. 1B).

In the cotyledon (foetal placenta), expression levels of IFN-γ and IL-4 were undetectable in control uninfected heifers and low gene expression levels were also observed for IL-10. When normalized levels were compared, significantly increased expression of IFN-γ ($p = 0.01$; Student's *t*-test) was observed in cotyledon from infected dams with live foetuses versus uninfected controls. IFN-γ mRNA levels were upregulated 71.2-fold in the foetal placenta from infected dams with live foetuses compared to cotyledon from uninfected dams (range 30–120-fold) (Fig. 2). IL-10 mRNA was significantly up-regulated in infected animals by 39.2-fold in the infected group compared to the control uninfected group (range 13.4–136.5) ($p = 0.028$, Student's *t*-test). Very low expression levels for IL-4 were observed in both groups (average 3.4-fold up-regulation in infected dams with live foetuses; range 1.4–6.9 fold) and no significant differences were observed between groups ($p = 0.25$, Student's *t*-test). Significant differences between groups were observed in IFN-γ/IL4 ratios on the foetal placenta with the lowest ratios observed in infected foetuses ($p = 0.005$, Student's *t*-test).

In the caruncle, significantly up-regulated IFN-γ expression ($p = 0.03$) was observed in infected dams with live foetuses when compared to control uninfected heifers (Figs. 2 and 3). The expression of IFN-γ was on average 25.2-fold higher in infected dams with live foetuses compared to uninfected control foetuses (range