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Cell mediated immune responses in the placenta following challenge of vaccinated pregnant heifers with *Neospora caninum*

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ABSTRACT

The aim of the present study was to investigate and correlate the cell-mediated immune response and pathological changes at the maternal-fetal interface of *Neospora*-challenged pregnant cattle previously immunized with live and inactivated experimental vaccines. Pregnant heifers naïve to *Neospora caninum* were divided in 5 groups of 4 animals, each one immunized before mating: Group A heifers were intravenously (iv) immunized with 6.25×10^7 live tachyzoites of the NC-6 strain; group B heifers were immunized twice subcutaneously (sc) 3 weeks apart with native antigen extract of the NC-6 strain formulated with ISCOMs; group C heifers were sc immunized twice 3 weeks apart with three recombinant proteins (rNcSAG1, rNcHSP20, rNcGRA7) of the NC-1 strain formulated with ISCOMs; group D heifers were sc injected with sterile phosphate-buffered saline (PBS) and group E heifers received sc ISCOM-matrix (ISCOMs without antigen). All groups were iv-challenged with 4.7×10^7 NC-1 tachyzoites at 70 days of gestation. Heifers were culled at day 104 of gestation and placentomes were examined to evaluate lesions and local cellular immune responses using histopathology, immunohistochemistry and real time-PCR. Immunohistochemistry was performed using bovine leucocyte specific antibodies. Cytokine expression and levels (IFN-γ, IL-4, IL-10, IL-12 and TNF-α) were measured using real-time reverse transcription-PCR and ELISA, respectively. Minimal inflammation was observed in group A placentomes; while placentomes from group B, C, D and E had moderate to severe infiltration with $CD3^+$, $CD4^+$, γδ-T cells, $CD8^+$ cells and macrophages being more numerous in groups B and E placentomes, when compared with groups C and D ($P < 0.001$). Cytokine levels were significantly increased in the caruncles of animals of groups B and C in comparison with the other animal groups ($P < 0.001$). The results from this study showed that the strongest cellular immune responses were observed in the placentomes of animals that were immunized with inactivated vaccines (groups B and C) and in the placentomes of animals that were sc-sham-inoculated (groups D and E). On the other hand, animals that were immunized with live tachyzoites showed a milder immune cell infiltration to the placenta possibly due to the existence of a protective systemic maternal immune response that helped to minimize *N. caninum* infection at the maternal-fetal interface.

1. Introduction

Neospora caninum is an obligate intracellular protozoan parasite recognized as a major cause of abortion and severe economic losses to the cattle industry worldwide (Trees et al., 1999; Larson et al., 2004; Dubey and Schares, 2011; Reichel et al., 2013). Transplacental transmission is a major feature of the *N. caninum* bovine infectious cycle and can occur following recrudescence of a persistent infection during pregnancy -endogenous transplacental transmission-, or after a new infection by ingestion of oocysts shed by the definitive host -exogenous transplacental transmission- (Trees and Williams, 2005). Both forms of transmission can result in fetal death or birth of clinically normal but persistently infected calves.

Similar to other intracellular parasitic infections, cell-mediated immunity (CMI) plays an important role in reducing the multipli-

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cation of *N. caninum* within the host. T-helper 1 (Th1) immune responses involve the production of different cytokines including interferon-gamma (IFN-γ), interleukin-12 (IL-12) and tumor necrosis factor (TNF) and are thought to be important in host protective immunity along with the production of immunoglobulin G_2 (IgG_2) (Staska et al., 2003; Innes, 2007). However, during pregnancy these inflammatory immune responses can potentially damage the placenta and induce abortion of the fetus (Raghupathy 1997; Chaouat et al., 2002). The natural immunomodulation that occurs during gestation, favouring a bias toward Th-2 type responses, may alter the host-pathogen interaction enabling the parasite to become more active in the host (Innes et al., 2002). This Th-2 immune response is associated with a successful fetal implantation and maintenance of early pregnancy with suppression of local inflammatory responses but it may result in a failure to control *N. caninum* infection by the pregnant host (Wegmann et al., 1993; Chaouat et al., 2002). The pathogenesis of bovine abortion due to *N. caninum* is complex and an immune-mediated pathology mechanism has been proposed (Quinn et al., 2002). It is postulated that infection is associated with placental damage that could disrupt the vascular supply of nutrients leading to fetal death (Maley et al., 2003; Macaldowie et al., 2004).

Experimental infections have shown that fetal death may occur when naïve dams were challenged with *N. caninum* tachyzoites at day 70 of gestation (Williams et al., 2000; Macaldowie et al., 2004; Regidor-Cerrillo et al., 2014). On the other hand, the challenge during mid or late gestation resulted in vertical transmission of the parasite, but not in fetal death (Maley et al., 2003; Almería et al., 2011; Benavides et al., 2012). Cantón et al. (2014a) reported that the immune infiltrate was milder in placental tissues following experimental infection in naïve cattle during late gestation in comparison with infection of cattle earlier in gestation. These findings could partially explain the milder clinical outcome, i.e., congenital transmission but no abortions.

Previous studies have observed clear differences in the severity of pathological changes in the bovine placenta, parasite loads and expression of both pro-inflammatory and anti-inflammatory regulatory cytokines depending on the timing of infection during gestation (Maley et al., 2003; Macaldowie et al., 2004; Almería et al., 2011; Benavides et al., 2012; Cantón et al., 2014a; Regidor-Cerrillo et al., 2014). However, it is still unclear if it is infection and intracellular multiplication of the parasite or the strong host inflammatory immune response to the parasite which causes placental damage and ultimately leads to fetal death. Rosbottom et al. (2008, 2011) detected the presence of $CD4^+$ and $CD8^+$ T cells, expression of Major Histocompatibility Complex (MHC) Class II antigens and the production of both pro- and anti-inflammatory cytokines in placentas from cattle experimentally and naturally infected with *N. caninum*. In one of these studies (Rosbottom et al., 2011); naturally infected pregnant cattle were killed at the time of recrudescence of infection and all fetuses were alive at the time of maternal euthanasia indicating that an anti-*N. caninum* immune response in the placenta is not necessarily detrimental to fetal survival and may contribute to the control of placental infection.

The generation of a protective vaccine against *N. caninum* abortion and vertical transmission is needed in order to help control bovine neosporosis. A vaccine that mimics the immune response generated naturally (Moore et al., 2005) or by inoculation with live parasites (Innes et al., 2001; Williams et al., 2007) may prevent *Neospora*-associated abortion. In a previous study we reported the effective protection against vertical transmission provided by the immunization of pregnant heifers with live tachyzoites from the NC-6 strain isolated in Argentina (Basso et al., 2001) and challenged with a different strain of *N. caninum* (Hecker et al., 2013, 2014). In this study we focus attention on characterizing the cell mediated immune responses in the placenta in groups of heifers given different vaccine preparations prior to mating and then challenged at day 70 of gestation with live *N.caninum* tachyzoites.

2. Materials and methods

2.1. Animals and experimental design

A full description of the animals and experimental design was previously published (Hecker et al., 2013, 2014). Briefly, twenty 22-month old Angus heifers seronegative for *N. caninum*, *Toxoplasma gondii*, Bovine Viral Diarrhoea Virus, Bovine Herpesvirus and Brucellosis and Tuberculosis free were divided randomly in five groups: group A heifers ($n = 4$) were immunized intravenously (iv) with 6.25×10^7 tachyzoites of NC-6 Argentina strain (Basso et al., 2001) in sterile phosphate-buffered saline (PBS) (pH 7.2) 4 weeks before mating; group B heifers ($n = 4$) were subcutaneously (sc) immunized with 2 doses of a vaccine designed with a NC-6 Argentina tachyzoites antigen extract and ISCOMs (Abisco-300, ISCONOVA, Uppsala, Sweden) 3 weeks apart (Hecker et al., 2013); group C heifers ($n = 4$) were sc-immunized with 2 doses of a vaccine designed with a mixture of rNcSAG1, rNcHSP20 and rNcGRA7 antigens of NC-1 (Dubey et al., 1988) and ISCOM-MATRIX 3 weeks apart (Hecker et al., 2014); group D ($n = 4$) and E ($n = 4$) heifers were sc-sham-inoculated twice (3 weeks apart) with sterile PBS and ISCOM-MATRIX alone, respectively (Hecker et al., 2013, 2014). Four weeks after the first immunization, all groups heifers were estrus synchronized and then allocated with 4 healthy Angus bulls for natural breeding over 7 days. Transrectal ultrasonography was performed 35 days after mating and pregnancy confirmed. Nineteen pregnant animals carrying single fetuses and one of the dams with twins (group C) were iv-challenged with 4.7×10^7 NC-1 (Dubey et al., 1988) tachyzoites at day 70 of gestation. Fetal viability was confirmed by ultrasonography every week following challenge and until slaughter in abattoir at 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 good animal practice and the conditions defined by the Animal Ethics Committee (CICUAE) at INTA.

2.2. Parasite strains

Live tachyzoites of the NC-6 Argentina strain were used to immunize heifers of group A and for the production of native antigens for the experimental vaccine inoculated in group B heifers as previously mentioned (Hecker et al., 2013).

The NC-1 strain of *N. caninum* was used for the cloning and purification of the recombinant proteins NcSAG1 (rNcSAG1), NcHSP20 (rNcHSP20) and NcGRA7 (rNcGRA7) (Wilkowsky et al., 2011) that were used to immunize group C heifers, as described by Hecker et al. (2014). All heifers were iv-challenged with 4.7×10^7 live NC-1 tachyzoites in a final volume of 3 ml of PBS (Hecker et al., 2013, 2014).

2.3. Vaccine formulations

Group A heifers were immunized with 6.25×10^7 NC-6 live tachyzoites in 2 ml PBS as previously described by Hecker et al. (2013).

The production of the native antigen extract used for the formulation of the experimental vaccine of group B was previously described by Hecker et al. (2013). Each vaccine dose of this group was formulated as a mixture containing 250 μl of native antigen extract (containing approximately 500 μg/ml) and 200 μl of ISCOMs (with approximately 750 μg/ml), kindly provided by Dr. Morein (ISCONOVA).

Recombinant protein production and vaccine preparation of group C was previously described by Hecker et al. (2014). Each vaccine dose was formulated as a mixture containing 30 μg of each protein and 200 μl of ISCOM-MATRIX (approximately 750 μg/dose), kindly provided by Dr. Morein, according to the manufacturer's instructions (Abisco-300, ISCONOVA).

2.4. Tissue collection

Immediately after slaughter, the whole reproductive tract was removed from each heifer and examined following standard gross pathology procedures (Campero et al., 2003). Four placentomes of each animal were immersed in zinc salts fixative (ZSF) (pH 7.0–7.4) for immunohistochemistry (IHC). After 3 days of fixation, tissues were processed and then embedded in paraffin-wax. Five-μm-thick serial sections were trimmed from each ZSF-paraffin-embedded placentome and were mounted on glass microscope slides (Superfrost® Plus, Thermo Scientific, Braunschweig, Germany) as previously described by Cantón et al. (2013, 2014a,b). Four placentomes from each animal were stored at −70 °C for the IFN-γ quantification by ELISA. Similarly, full-thickness pieces of manually separated caruncle (maternal placenta) were placed in a 5× volume of TRIzol Reagent (Life Technologies, Pasley, UK), lysed and kept at −80 °C for cytokine mRNA expression by real-time PCR.

2.5. Cell immunolabelling on placental tissue

Immunohistochemistry was performed using a panel of monoclonal antibodies (mAb) and the phenotypes of inflammatory infiltrate were characterized as previously reported by Cantón et al. (2013, 2014a,b). Briefly, IHC was performed using an EnVision+kit (Dako North America Inc, Carpinteria, USA). ZSF-fixed placental sections were incubated overnight with mAbs (diluted in TBS) that specifically recognize bovine leucocytes: MMIA (CD3 for total T cells, VMRD Inc, Washington, USA), CC30 (CD4 for T helper cells, AbD Serotec, Oxford, UK), CC58 (CD8 for cytotoxic T cells, AbD Serotec), IL-A29 ($\gamma\delta TCR^+$ for γδ-T cells, VMRD Inc), $NKp46^+$ (CD335 for Natural killer—NK—cells, AbD Serotec), and EBM11 (raised against $CD68^+$ for macrophages, Dako Cytomation, Glostrup, Denmark). Sections of ZSF-fixed bovine lymph nodes were used as positive control tissues. Tissue sections from the same placentomes without the addition of any mAb were used as negative controls for the technique.

Slides were blind-coded and examined for each inflammatory cell marker (listed above) as previously reported Cantón et al. (2013, 2014a,b). The scores from 0 to 4 were defined according to the extent of cellular infiltrate of the placentomes and whether there were associated pathological changes. Score 0: no infiltration of labeled cells or diffuse/rare infiltration of labeled cells that are not associated with pathological changes; Score 1: minimal/diffuse infiltration of labeled cells (in some cases forming small foci) associated with small necrotic areas; Score 2: mild infiltration and focal aggregation of labeled cells surrounding necrotic foci; Score 3: moderate infiltration and focal aggregation of labeled cells surrounding areas of necrosis; and Score 4: severe and large aggregation of positive cells surrounding areas of necrosis. The individual scores from 4 sampled placentomes were used to calculate a single mean score for each animal, similar to previous descriptions (Cantón et al., 2013, 2014a,b).

2.6. Cytokine mRNA expression

RNA extraction, reverse transcription and real-time PCR analysis of cytokine expression were performed as described by Regidor-Cerrillo et al. (2014). Briefly, 100 μg of placental tissue was homogenized into TRIzol Reagent (Life Technologies). Total RNA was extracted by a combined method based on the TRIzol Reagent (Life Technologies) and Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was carried out using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Paisley, UK).

Real-time PCR was performed using primers for bovine IFN-γ, IL-12p40, TNF-α, IL-4, IL-10 and the housekeeping gene β-actin as previously was reported (Regidor-Cerrillo et al., 2014). Reactions were performed in an ABI 7300 Real Time PCR System (Applied Biosystems), including 10 pmol of each primer and 5 μL of diluted cDNA samples with a final volume of 20 μL using Power SYBR® PCR Master Mix (Applied Biosystems, Foster City CA, USA). Quantification was realized using a standard curve of genomic DNA; the β-actin gene was used as housekeeping in order to normalize the PCR for the amount of RNA added to the reverse transcription reactions. Quantification of cytokine mRNA expression levels was carried out using the $2^{-\lambda Ct}$ method (Livak and Schmittgen, 2001). Caruncles of animals not infected with *N. caninum* were used as negative controls.

2.7. IFN-γ measurement by ELISA

To determinate whether changes in mRNA expression correlated with levels of protein, the levels of placental IFN-γ protein were measured by ELISA. Manually separated maternal caruncles were homogenized in CelLytic™ MT Cell Lysis Solution (Sigma, Saint Louis, USA), and the supernatants assayed using Bovine IFN-γ specific ELISA Assay Kit (AbD Serotec, Kidlington, Oxford, UK) as described by Rosbottom et al. (2008, 2011). In the present study we only analyzed IFN-γ protein in placentas from animals experimentally challenged.

2.8. Statistical analysis

Non-parametric two-tailed Mann–Whitney tests and Kruskal–Wallis test, followed by a Dunn's Multiple Range test for all pair-wise analyses were conducted on the pooled data to investigate statistically significant differences in the distribution of scores for each cell type and differences in cytokine expression and IFN-γ concentration among animals from different groups using GraphPad Prism version GraphPad Prism 5 v.5.01 (San Diego, CA, USA). Statistical significance was reached when $P \leq 0.05$.

3. Results

3.1. Necropsy and histopathological finding

The clinical and post mortem examination findings from dams and fetuses were described previously by Hecker et al. (2013, 2014). Briefly, no clinical signs were observed in any heifer throughout the study and there were no significant local reactions at the site of injection of the experimental vaccines. All fetuses were viable and no gross lesions were observed following slaughter and post mortem examination. There was variation between groups, where placentomes showed different lesions in terms of severity and characteristics of the inflammatory infiltrate. Minimal to mild inflammatory changes were observed microscopically in placentomes from group A. In group B and C, placentomes showed moderate to severe inflammatory lesions with moderate multifocal, mononuclear interstitial infiltration and occasional foci of necrosis. All placentomes of dams from group D and E showed moderate to severe necrotic and inflammatory lesions.

3.2. Identification of leucocytes in inflammatory infiltrate

3.2.1. CD3+ cells

Examples of infiltration scores of the different leucocytes present in placentomes in different experimental groups are shown in Fig. 1. Placentomes collected from group A heifers were mildly infiltrated with $CD3^+$ cells when compared with groups B, C and E placentomes ($P = 0.0012$) (Fig. 2). In group B and C placentomes, moderate numbers of $CD3^+$ cell aggregations were present in the caruncular base (Fig. 1a). Meanwhile, in group D and E placentomes, large numbers of $CD3^+$ cells were surrounding necrotic fetal villi in the maternal septa and within inflammatory aggregates in the base of caruncles (Fig. 1b).

3.2.2. CD4+ cells

Similar $CD4^+$ cell infiltration patterns to the $CD3^+$ cells previously described were recorded in groups A, B, C, D and E heifers. Statistically significant higher $CD4^+$ infiltration scores were recorded in group B in comparison with group A heifers ($P = 0.0285$), with group B showing greater infiltration of $CD4^+$ cells in the caruncular base. No significant score differences ($P > 0.05$) in $CD4^+$ infiltration were recorded among the other experimental groups (Fig. 2).

3.2.3. CD8+ cells

Small numbers of $CD8^+$ cells was observed in the placentomes of groups A and C heifers, mainly located in the maternal caruncular base. Significant higher $CD8^+$ infiltration scores were recorded in group B heifers when compared with group A and C ($P = 0.0036$). Moderate number of $CD8^+$ cells infiltrated the maternal-fetal interface of group D and E heifers (Fig. 1d) although no statistically significant $CD8^+$ infiltration score differences were recorded among the other experimental groups (Fig. 2).

3.2.4. γδTCR+ cells

$\gamma\delta TCR^+$ cell infiltration was minimal in the maternal caruncle base from all the experimental groups (Fig. 1e). Significant lower $\gamma\delta TCR^+$ cell infiltration score was recorded in group A heifers in comparison with the one recorded in group B animals ($P = 0.004$). No statistically significant $\gamma\delta TCR^+$ infiltration score differences were recorded among the other experimental groups (Fig. 2).

3.2.5. NKp46+ cells

Small cell numbers of $NKp46^+$ infiltrated the placentomes of the heifers of groups B, C, D and E. No $NKp46^+$ cells were observed in group A heifers; $NKp46^+$ infiltration scores were significantly higher in group B and E, in comparison with group A heifers ($P = 0.0139$) (Fig. 2).

3.2.6. CD68+ cells

Macrophages ($CD68^+$ cells) were infiltrating the placentomes of all groups of heifers. Larger numbers of $CD68^+$ cells (mild to moderate infiltration) were recorded in group B and E animals, in the caruncular base and between endometrial glands (Fig. 1f) in comparison with group D ($P = 0.0009$). No statistically significant differences were observed with $CD68^+$ infiltration score among the other experimental groups (Fig. 2).

3.3. Cytokine mRNA expression

3.3.1. IFN-γ

Significant higher IFN-γ expression were recorded in caruncles from group B, C, D and E heifers in comparison with negative control and group A animals ($P < 0.0001$). No differences were observed in animals of group A compared with negative controls ($P > 0.05$) (Fig. 3a).

3.3.2. IL-4

Statistically significant higher IL-4 expression were recorded in caruncles from groups B, C and E heifers when compared with negative control animals ($P < 0.0001$). Similarly, group A heifers expressed statistically significant lower IL-4 compared with groups B, C and E ($P < 0.0001$). There were no statistically significant differences among groups B, C and D ($P > 0.05$) (Fig. 3b).

3.3.3. IL-10

IL-10 expression was significantly higher in caruncles of groups B and C compared with the negative control heifers ($P < 0.0001$), . Otherwise, negative controls and groups A, D and E caruncles expressed statistically similar IL-10 levels ($P > 0.05$) (Fig. 3c).

3.3.4. IL-12p40

Significantly higher IL-12p40 expression was detected in the caruncles of groups B and C animals compared with negative control animals ($P < 0.001$) and when compared with groups A, D and E tissues ($P < 0.001$) (Fig. 3d).

3.3.5. TNF-α

Similarly to other cytokines, higher levels of TNF-α expression were observed in groups B and C placentas when compared with the negative controls, groups A, D and E animals ($P < 0.001$). No differences in the TNF-α expression were observed among the other groups ($P < 0.05$) (Fig. 3e).

3.4. IFN-γ concentration by ELISA

Even though the IFN-γ protein concentration was lower in the maternal caruncles of group A (0.15 ± 0.10 ng/mg total protein) when compared with those measured in groups B (0.30 ± 0.17), C (0.18 ± 0.10), D (0.20 ± 0.13) and E (0.25 ± 0.18) heifers, there were no significant differences between groups ($P < 0.05$).

4. Discussion

Differences in the cellular immune response at the maternal-fetal interface among experimentally immunized cattle challenged with *N. caninum* are presented in this study. Briefly, an up-regulation of the Th1 and Th2 cytokines were detected in the caruncles collected from heifers 30 days after challenge, particularly in those ones that received inactivated formulations (group B and C) or were sham-inoculated (groups D and E heifers). In contrast, the immune cell infiltrations as well as the cytokine mRNA expression pattern were significantly lower in placentomes from group A when compared with group B, C, D and E heifers. A possible explanation for this is that the group A animals were more effectively immunized with the live parasites and were able to better withstand the challenge. The superior protective immune response in the group A animals is likely to have decreased the dissemination of the parasite to the maternal-fetal interface after challenge and therefore vertical transmission to the fetus (Hecker et al., 2013). Previous studies (Innes et al., 2001; Williams et al., 2007; Weber et al., 2013; Hecker et al., 2013; Rojo-Montejo et al., 2013) have shown that an effective strategy to prevent vertical transmission is by inoculating live parasites before mating.

Another hypothesis about that the lower levels of cellular infiltration and cytokine expression in group A could be an earlier efficient immune response at the fetal-maternal interface in the group A heifers already solved at the time of culling. However, some kind of histopathological findings would be expected in these group A placentas.

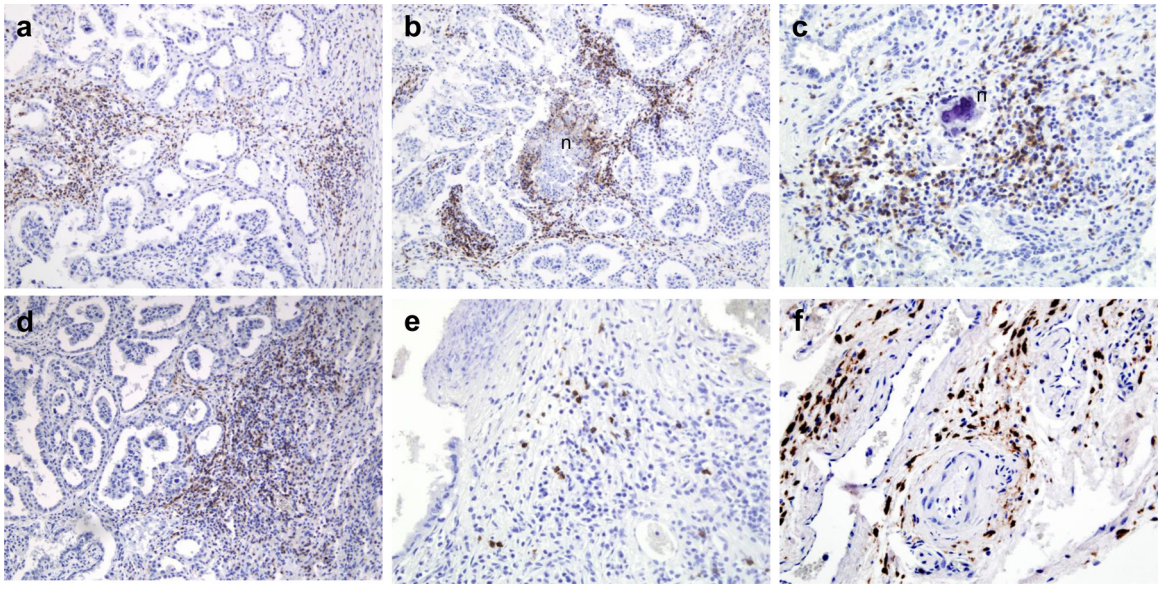

Fig. 1. Examples of immune cell infiltration in placentomes from different experimental groups. (a) Moderate infiltration of $CD3^+$ cells in the maternal caruncle collected from a group B heifer. Original magnification 100×. (b) Severe infiltration of $CD3^+$ cells in the maternal caruncle surrounding necrotic fetal villi (n) from a group D heifer. Original magnification 100×. (c) Moderate infiltration of $CD4^+$ cells surrounding a necrotic focus with calcification (n) in maternal caruncle of a group E heifer. Original magnification 200×. (d) Moderate aggregate of $CD8^+$ cells in connective tissue in the base of maternal caruncle from a group D heifers. Original magnification 100×. (e) Minimal infiltration of γδTCR$^+$ cells in the maternal caruncle from a group C heifer. Original magnification 200×. (f) Moderate infiltration of $CD68^+$ cells in the base of maternal caruncle of a group E heifer. Original magnification 200×. Counterstained with haematoxylin.

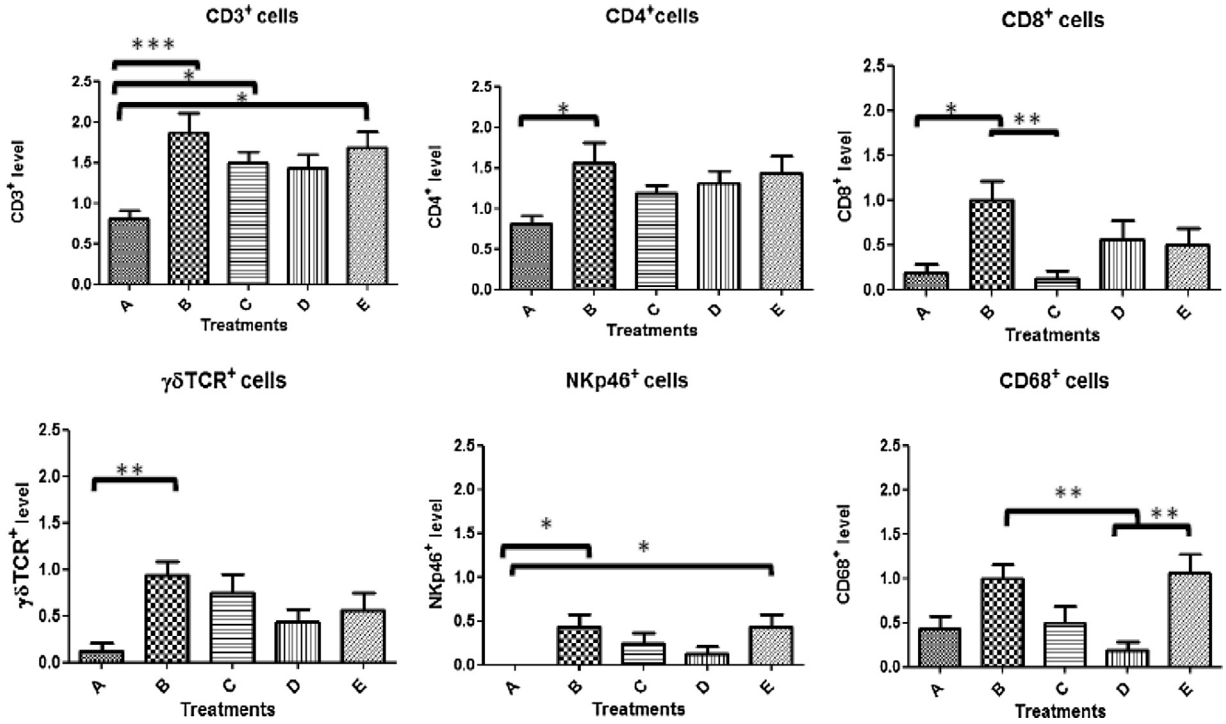

Fig. 2. Infiltration scores of the different phenotype of inflammatory cells on placentomes collected from different experimental groups. $CD3^+$ (total T cells), $CD4^+$ (T helper cells), $CD8^+$ (cytotoxic T cells), γδTCR$^+$ (γδ T cells), $NKp46^+$ (NK cells) and $CD68^+$ (macrophages). Numbers in the vertical axis represent mean scores of each group. Error bars indicate SEM. $^{**}P < 0.001$; $^{*}P < 0.05$.

Differences in of the immune cell phenotype in placentomes were observed between groups. Group A showed the lowest levels of T cells and these were associated with low levels of pathology (Hecker et al., 2013, 2014). Orozco et al. (2013) found scattered and low levels of $CD4^+$ and $CD8^+$ T cells in the uterus of pregnant cows naturally infected with *N. caninum* with an effective immune response. In contrast, high $CD3^+$, $CD4^+$ and γδ-T infiltration scores were recorded in groups B and C placentomes. Nevertheless, this immune cell infiltration was not able to prevent vertical transmission to their fetuses (Hecker et al., 2013, 2014). It is likely that both inactivated vaccines (group B and C) did not stimulate an effective maternal immune response able to prevent the parasite invading the placenta after challenge. Previous studies showed that experimental vaccination with inactivated antigens of *N. can-*

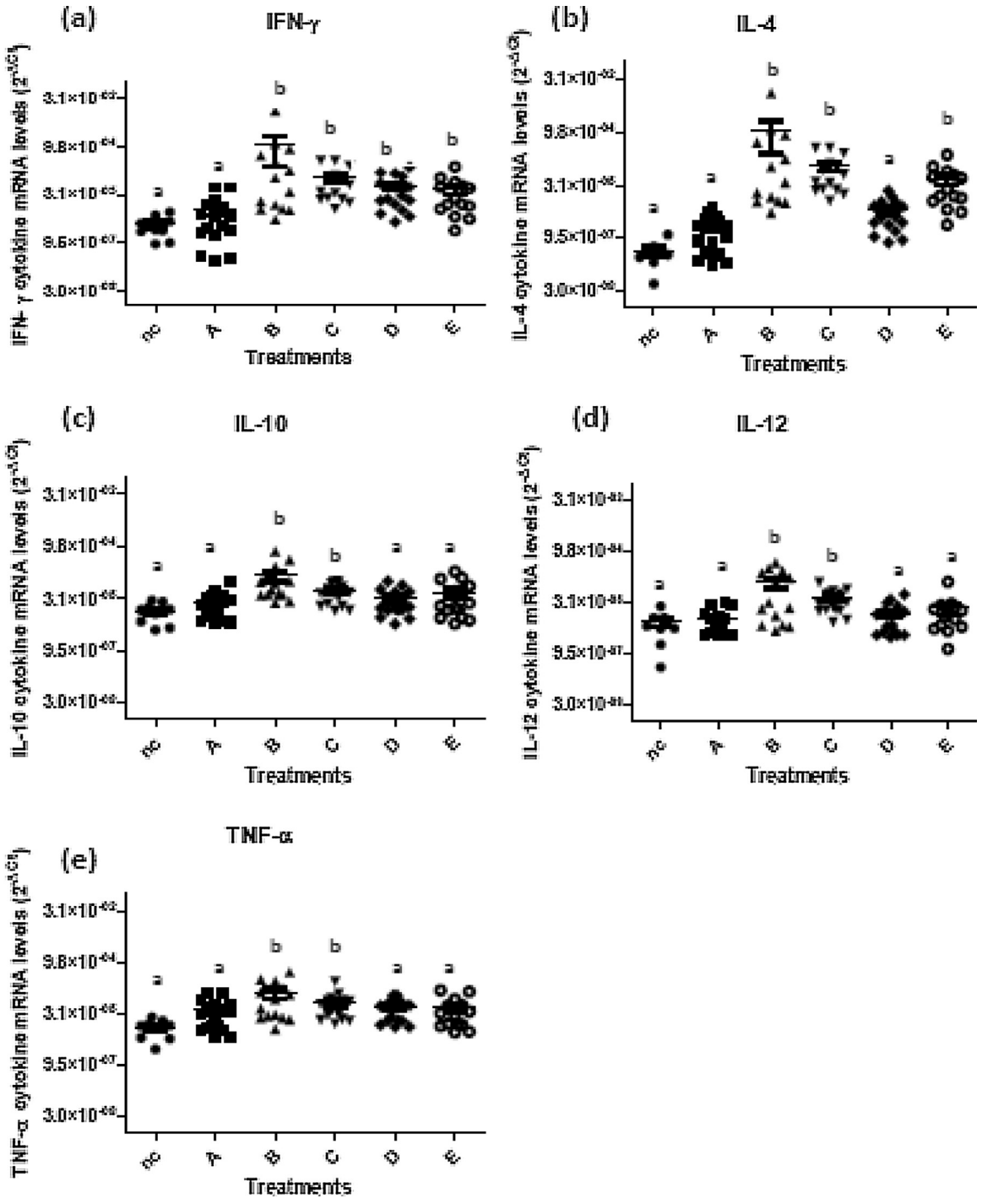

Fig. 3. Cytokine expression profiles in the placenta. Scatter-plot graphs of relative cytokine expression levels in caruncles from heifers of different experimental groups. Horizontal lines represent median values for each group. Lowercase letters indicate significant differences between different groups. nc: negative control (caruncles of healthy pregnant animals).

inum tachyzoites were immunogenic, but did not protect against transplacental infection and vertical transmission after experimental challenge in pregnant heifers (Andrianarivo et al., 2000; Williams et al., 2007).

In the present study, the high $CD4^+$ cell infiltration in placental samples from groups B, C, D and E is likely to be associated with the pathological changes found in those animals. Cantón et al. (2014a) suggested a positive association between high number of T lymphocytes (CD3+, CD4+, CD8+ and γδ) in the placental infiltrate and occurrence of abortion. In the present study all dams were carrying viable fetuses (Hecker et al., 2013, 2014) although vertical transmission was observed in groups B, C, D and E (Hecker et al., 2013, 2014), despite the short time between experimental challenge and slaughter. Those animals, that showed moderate to severe lesions in their placentas, also had significantly higher numbers of placental T cells suggesting that those fetuses would have not been born alive.

Mean $CD8^+$ T cells scores in the placental infiltrate were lower than those for γδ-T cells and T helper cells. Interestingly, group B placentomes were infiltrated with higher numbers of $CD8^+$ cells

when compared with group C. In relation to these results, we previously reported that the assessment of the ratio of $CD4^+/CD8^+$ versus fetal lesion score (Hecker et al., 2013). We observed that those animals vaccinated with native antigen extract plus ISCOMs had more fetal lesions and reduced ratio of $CD4^+/CD8^+$, with a high level of $CD8^+$ T cells, compared to those animals receiving live tachyzoites who had fewer fetal lesions and a high ratio $CD4^+/CD8^+$ (Hecker et al., 2013).

Significant differences were detected between the γδ-T cell scores in the placental infiltrate of group A when compared with groups B, C, D and E heifers, and the levels of these cells were lower than other T cells. Maley et al. (2006) reported that the initial lesions in the placentomes after *N. caninum* infection were associated with low numbers of γδ-T cells while moderate infiltration of this cell subset was observed later. The role of γδ-T cells against different infectious pathogens is still not completely understood. They constitute up to 50% of all T cells in the peripheral blood and lymphoid organs of young cattle and sheep (Hein and Mackay, 1991) but their role in combating *N. caninum* infections is not yet known (Maley et al., 2006). Cantón et al. (2013) showed that a mild γδ-T cell infiltration was detected after *N. caninum* inoculation of pregnant cattle during late gestation supporting the hypothesis that, during pregnancy, an anti-*Neospora* maternal immune response at a later stage is less harmful than one that occurs in early gestation.

The role of NK cells in pregnant cattle and in the host response to *N. caninum* is not known. Maley et al. (2006) showed that NKp46 cells were identified in relatively low numbers in placenta of dams carrying viable fetuses 2 weeks after inoculation with *N. caninum*. In the present work the degree of infiltration of NK cells was significantly lower in group A than in groups B, C, D and E and lower than other types of immune cells. A possible explanation of this fact could be that the low levels of NK cells would be related with the gap between the challenge and the slaughter where adaptive T-cell responses had already been established.

The results of the phenotypic analysis of the immune cell infiltrate in the placentas of the present study have shown that, although a differential infiltration pattern was observed between experimental groups, significantly higher macrophage infiltration scores were recorded in placentomes from groups B and E. It has been reported that macrophages are not only involved in antiparasitic activity but also play a key role in the tissue repair process (Leibovich and Ross, 1975). Considering that the placentomes of animals of these groups showed severe inflammatory and necrotic lesions (Hecker et al., 2013, 2014), the presence of $CD68^+$ cells could be in response to tissue damage.

The protective immune response against *N. caninum* is dominated by the production of Th1 cytokines (Khan et al., 1997; Baszler et al., 1999), but in pregnant cattle, such responses can potentially provoke rejection or abortion of the fetus (Quinn et al., 2002; Rosbottom et al., 2008; Almería et al., 2010; Cantón et al., 2014c; Regidor-Cerrillo et al., 2014). In the present study we found lower levels of IFN-γ (mRNA expression and protein), IL-4, IL-10, IL-12p40 and TNF-α mRNA in group A placentomes when compared with other experimental groups. These findings are consistent with other results indicating that the immune response at the maternal-fetal interface was also moderate in this group. A more effective protective immune response was induced in dams that received live tachyzoites limiting dissemination of *N. caninum* to the placenta. Regidor-Cerrillo et al. (2014) mentioned a clear influence of parasite load on cytokine levels. Although in the present study the numbers of parasites were not quantified, moderate lesions and low detection of *N. caninum* by PCR was described in group A (Hecker et al., 2013), possibly indicating low parasite load and consequently a mild immune response in the maternal-fetal interface.

A mixed Th1 (IFN-γ, IL-12p40 and TNF-α), Th2 (IL-4) and regulatory (IL-10) cytokine expression was detected in group B, C, D and E heifers, as previously described in *N. caninum*-infected caruncles (Rosbottom et al., 2008; Almería et al., 2011; Regidor-Cerrillo et al., 2014). The highest levels of IFN-γ and IL-4 expression were detected in groups B and C. However, no differences were found in the expression of IFN-γ between groups B and C. This suggests that the inactivated vaccine products had failed to induce effective protective immunity in the dams and thus the live *Neospora* challenge invaded and infected the placental tissues causing infiltration of immune cells trying to combat the infection.

In conclusion, the results from this study showed that, following a challenge with live *N. caninum* tachyzoites, the strongest cellular immune responses were observed in the placentomes of animals that had been immunized with inactivated vaccines (groups B and C) or sham-inoculated (groups D and E) in comparison with those immunized with live tachyzoites (group A) following challenge, probably due to the induction of an early effective protective systemic immune response that helped to minimize *N. caninum* infection at maternal-fetal interface.

Conflict of interest

There are no financial or personal relationships with other people or organizations that could inappropriately influence this work.

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References

Almería, S., Araujo, R., Tuo, W., Lopez-Gatius, F., Dubey, J.P., Gasbarre, L.C., 2010. Fetal death in cows experimentally infected with *Neospora caninum* at 110 days of gestation. Vet. Parasitol. 169, 304–311.

Almería, S., Araujo, R.N., Darwich, L., Dubey, J.P., Gasbarre, L.C., 2011. Cytokine gene expression at the maternal-fetal interface after experimental *Neospora caninum* infection of heifers at 110 days of gestation. Parasite Immunol. 33, 517–523.

Andrianarivo, A.G., Rowe, J.D., Barr, B.C., Anderson, M.L., Packham, A.E., Sverlow, K.W., Choromanski, L., Loui, C., Grace, A., Conrad, P.A., 2000. A POLYGEN-adjuvanted killed *Neospora caninum* tachyzoite preparation failed to prevent fetal infection in pregnant cattle following iv/im experimental tachyzoite challenge. Int. J. Parasitol. 30, 985–990.

Basso, W., Venturini, L., Venturini, M.C., Hill, D.E., Kwok, O.C.H., Shen, S.K., Dubey, J.P., 2001. First isolation of *Neospora caninum* from the feces of a naturally infected dog. J. Parasitol. 87, 612–618.

Baszler, T.V., Long, M.T., McElwain, T.F., Mathison, B.A., 1999. Interferon-gamma and interleukin-12 mediate protection to acute *Neospora caninum* infection in BALB ♀ mice. Int. J. Parasitol. 29, 1635–1646.

Benavides, J., Katzer, F., Maley, S.W., Bartley, P.M., Cantón, G.J., Palarea, J., Pang, Y., Burrells, A., Rocchi, M., Chianini, F., Innes, E.A., 2012. High rate of transplacental transmission of *Neospora caninum* following experimental challenge at late gestation. Vet. Res. 43, 83.

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.

Cantón, G.J., Katzer, F., Benavides-Silván, J., Maley, S.W., Palarea-Albaladejo, J., Pang, Y., Smith, S., Bartley, P.M., Rocchi, M., Innes, E.A., Chianini, F., 2013. Phenotypic characterisation of the cellular immune infiltrate in placentas of cattle following experimental inoculation with *Neospora caninum* in late gestation. Vet. Res. 44, 60.

Cantón, G.C., Katzer, F., Maley, S.W., Bartley, P.M., Benavides-Silván, J., Palarea-Albaladejo, J., Pang, Y., Smith, S.H., Rocchi, M.S., Buxton, D., Innes, E.A., Chianini, F., 2014a. Inflammatory infiltration into placentas of *Neospora caninum* challenged cattle correlates with clinical outcome of pregnancy. Vet. Res. 45, 11.

Cantón, G.C., Konrad, J.L., Moore, D.P., Caspe, S.G., Palarea-Albaladejo, J., Campero, C.M., Chianini, F., 2014b. Characterization of immune cell infiltration in the placentome of water buffaloes (*Bubalus bubalis*) infected with *Neospora caninum* during pregnancy. J. Comp. Path. 150, 463–468.

Cantón, G., Katzer, F., Maley, S.W., Bartley, P.M., Benavides-Silván, J., Palarea-Albaladejo, J., Pang, Y., Smith, S.H., Rocchi, M., Buxton, D., Innes, E.A., Chianini, F., 2014c. Cytokine expression in the placenta of cattle following

inoculation with *Neospora caninum*. Vet. Immunol. Immunopathol. 161 (1–2), 77–89.
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.
Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 193, 1259–1263.
Dubey, J.P., Schares, G., 2011. Neosporosis in animals-The last five years. Vet. Parasitol 180, 90–108.
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., 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.
Hecker, Y.P., Cóceres, V., Wilkowsky, S.E., Jaramillo Ortiz, J.M., Morrell, E., Verna, A., Ganuza, A., Cano, D.B., Lischinsky, L., Ángel, S.O., Zamorano, P., Odeón, A.C., Leunda, M.R., Campero, C.M., Morein, B., Moore, D.P., 2014. A *Neospora caninum* vaccine using recombinant proteins fails to prevent fetal infection in pregnant cattle after experimental challenge. Vet. Immunol. Immunopathol. 162, 142–153.
Hein, W.R., Mackay, C.R., 1991. Prominence of γδ T-cells in the ruminant immune system. Immunol. Today 12, 30–34.
Innes, E.A., Wright, S.E., Maley, S., Rae, A., Schock, A., Kirvar, E., Bartley, P., Hamilton, C., Carey, I.M., Buxton, D., 2001. Protection against vertical transmission in bovine neosporosis. Int. J. Parasitol. 31, 1523–1534.
Innes, E.A., Andrianarivo, A.G., Björkman, C., Williams, D.J.L., Conrad, P.A., 2002. Immune responses to *Neospora caninum* and prospects for vaccination. Trends Parasitol. 18, 497–504.
Innes, E.A., 2007. The host-parasite relationship in pregnant cattle infected with *Neospora caninum*. Parasitology 134, 1903–1910.
Khan, I.A., Schwartzman, J.D., Fonseka, S., Kasper, L.H., 1997. *Neospora caninum*: role for immune cytokines in host immunity. Exp. Parasitol. 85, 24–34.
Larson, R.L., Hardin, D.K., Pierce, V.L., 2004. Economic considerations for diagnostic and control options for *Neospora caninum*-induced abortions in endemically infected herds of beef cattle. J. Am. Vet. Med. Assoc. 224, 1597–1604.
Leibovich, S.J., Ross, R., 1975. The role of the macrophage in wound repair: a study with hydrocortisone and antimacrophage serum. Am. J. Pathol. 78, 71–100.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.
Macaldowie, C.N., Maley, S.W., Wright, S.E., Bartley, P.M., Esteban-Redondo, I., Buxton, D., Innes, E.A., 2004. Placental pathology associated with fetal death in cattle inoculated with *Neospora caninum* by two different routes in early pregnancy. J. Comp. Pathol. 131, 142–156.
Maley, S.W., Buxton, D., Rae, A.G., Wright, S.E., Schock, A., Bartley, P.M., Esteban-Redondo, I., Swales, C., Hamilton, C.M., Sales, J., Innes, E.A., 2003. The pathogenesis of Neosporosis in pregnant cattle: inoculation at mid-gestation. J. Comp. Pathol. 129, 186–195.
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.
Moore, D.P., Leunda, M.R., Zamorano, P.I., Odeón, A.C., Romera, S.A., Cano, A., de Yaniz, G., Venturini, M.C., Campero, C.M., 2005. Immune response to *Neospora caninum* in naturally infected heifers and heifers vaccinated with inactivated antigen during the second trimester of gestation. Vet. Parasitol. 130, 29–39.
Orozco, M.A., Morales, E., Salmerón, F., 2013. Characterization of the inflammatory response in the uteri of cows infected naturally by *Neospora caninum*. J. Comp. Pathol. 148, 148–156.
Quinn, H.E., Ellis, J.T., Smith, N.C., 2002. Neospora caninum: a cause of immune-mediated failure of pregnancy? Trends Parasitol. 18, 391–394.
Raghupathy, R., 1997. Th-1 type immunity is incompatible with successful pregnancy. Immunol. Today 18, 478–482.
Regidor-Cerrillo, J., Arranz-Solís, D., Benavides, J., Gómez-Bautista, M., Castro-Hermida, J.A., Mezo, M., Pérez, V., Ortega-Mora, L.M., González-Warleta, M., 2014. *Neospora caninum* infection during early pregnancy in cattle: how the isolate influences infection dynamics, clinical outcome and peripheral and local immune responses. Vet. Res. 45, 10.
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.
Rojo-Montejo, S., Collantes-Fernández, E., Pérez-Zaballos, F., Rodríguez-Marcos, S., Blanco-Murcia, J., Rodríguez-Bertos, A., Prenafeta, A., Ortega-Mora, L.M., 2013. Effect of vaccination of cattle with the low virulence Nc-Spain 1H isolate of *Neospora caninum* against a heterologous challenge in early and mid-gestation. Vet. Res. 44, 106.
Rosbottom, A., Gibney, E.H., Guy, C.S., Kipar, A., Smith, R.F., Kaiser, P., Trees, A.J., Williams, D.J.L., 2008. Upregulation of cytokines is detected in the placentas of cattle infected with *Neospora caninum* and is more marked early in gestation when fetal death is observed. Infect. Immun. 76, 2352–2361.
Rosbottom, A., Gibney, H., Kaiser, P., Hartley, C., Smith, R.F., Robinson, R., Kipar, A., Williams, D.J.L., 2011. Up regulation of the maternal immune response in the placenta of cattle naturally infected with *Neospora caninum*. PLoS One 6, e15799.
Staska, L.M., McGuire, T.C., Davies, C.J., Lewin, H.A., Baszler, T.V., 2003. *Neospora caninum*-infected cattle develop parasite-specific CD4+ cytotoxic T lymphocytes. Infect. Immun. 71, 3272–3279.
Trees, A.J., Davison, H.C., Innes, E.A., Wastling, J.M., 1999. Towards evaluating the economic impact of bovine neosporosis. Int. J. Parasitol. 29, 1195–1200.
Trees, A.J., Williams, D.J.L., 2005. Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. Trends Parasitol. 2, 558–561.
Weber, F.H., Jackson, J.A., Sobecki, B., Choromansky, L., Olsen, M., Meinert, T., Frank, R., Reichel, M.P., Ellis, J.T., 2013. On the efficacy and safety of vaccination with live tachyzoites of *N: caninum* for prevention of *Neospora*-Associated fetal loss in cattle. Clin. Vaccine Immunol. 20, 99–105.
Wegmann, T.G., Lin, H., Guilbert, L., Mossman, T.R., 1993. Bidirectional cytokines interactions in the materno-fetal relationship: is successful pregnancy a Th2 phenomenon? Immunol. Today 14, 353–356.
Wilkowsky, S.E., Bareiro, G.G., Mon, M.L., Moore, D.P., Caspe, G., Campero, C., Fort, M., Romano, M.I., 2011. An applied printing immunoassay with recombinant NcSAG1 for detection of antibodies to *Neospora caninum* in cattle. J. Vet. Diagn. Invest. 23, 971–976.
Williams, D.J., Guy, C.S., McGarry, J.W., Guy, F., Tasker, L., Smith, R.F., MacEachern, K., Cripps, P.J., Kelly, D.F., Trees, A.J., 2000. *Neospora caninum*-associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines fetal survival. Parasitology 121, 347–358.
Williams, D.J., Guy, C.S., Smith, R.F., Ellis, J., Björkman, C., Reichel, M.P., Trees, A.J., 2007. Immunization of cattle with live tachyzoites of *Neospora caninum* confers protection against fetal death. Infect. Immun. 75, 1343–1348.