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Phenotypic characterization of immune cells in fetal tissues of cattle immunized and challenged with *Neospora caninum*

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ABSTRACT

The purpose of this work was to characterize the cellular phenotype in inflammatory infiltrates of fetal tissues from pregnant heifers immunized and experimentally challenged with *Neospora caninum*. Fetuses from 20 heifers separated into 5 groups were obtained. The experiment was designed as follow: Group A, heifers inoculated intravenously with live tachyzoites of Argentine strain NC-6 (n = 4); Group B heifers inoculated subcutaneously with soluble native antigen from the same strain formulated with immune stimulant complexes (ISCOMs) (n = 4); Group C heifers inoculated with recombinant proteins, rNcSAG1, rNcHSP20, rNcGRA7 formulated with ISCOMs (n = 4), Group D heifers inoculated subcutaneously with sterile phosphate buffered solution (n = 4) and Group E heifers inoculated subcutaneously with antigen-free ISCOMs (n = 4). Experimental challenge was performed at 70 days of gestation and all heifers were euthanized 34 days later. Fetal tissues were taken for histological studies. Inflammatory lesions were observed in brain and lung, and immunhistochemistry was used to identify $CD3^+$, $CD20^+$ and MHC II^+ cells. The majority of the cells that infiltrate and circumscribe the lesions in the brain and lung tissue expressed MHC II antigen; varying between 70–90% of the total cellular infiltrate. $CD3^+$ cells were also present within the lesions, contributing to up to 30% of the inflammatory cells. $CD20^+$ cells appeared as a marginal group, in some cases, with a range between 10 and 25%. As expected, the immunolabeling of MHC II^+ and $CD3^+$ cells in fetal tissues was associated with fetal infection with *N. caninum*. There were statistically significant differences in the distribution and population of the inflammatory infiltrate in relation to the immunogenic treatment and the type of tissue, with inflammatory cells being markedly less extensive fetuses from group A (dams previously exposed to *N. caninum*) and in brain tissue. This work showed that *Neospora*-infection induced MHC II^+ and $CD3^+$ cells in bovine fetuses from dams receiving experimental vaccines.

1. Introduction

Neosporosis is an infectious disease caused by *Neospora caninum*, an apicomplexan intracellular parasite that causes abortion and severe economic losses in the cattle industry worldwide (Dubey et al., 2017). *N. caninum* has a heteroxene biological cycle where dogs (McAllister et al., 1998) and other canids are its definitive hosts and bovines and domestic species are its intermediate hosts (Dubey et al., 2017). Unfortunately there is no vaccine or drug to prevent or control abortions due to *N. caninum* in cattle (Dubey et al., 2017).

Because *N. caninum* is one of the most well adapted parasite to its hosts, histopathological analysis of fetal tissues is an important tool for diagnosis of *N. caninum* abortion in cattle (Pescador et al., 2007; Dubey et al., 2017). With rates of vertical transmission higher than 80%, the parasite is efficiently passed from the dam to its progeny, and the severity of fetal inflammatory lesions must be carefully evaluated in order to determinate whether or not these are compatible with life (Dubey et al., 2017). Fetal lesions due to *N.caninum* are well described and include non-suppurative necrotic encephalitis, non-suppurative periportal hepatitis, interstitial pneumonia, and necrotic myocarditis and myositis have been largely described (Barr et al., 1991; Campero et al., 2003; Pescador et al., 2007; Dubey et al., 2017). However the

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phenotypic characterization of inflammatory infiltrates associated with these lesions in bovine fetal tissues has not been published to date.

Abortion due *N. caninum* depends on several immune and immunopathological processes in the dam, the placenta and the fetus (Innes et al., 2002). The bovine immune response to this pathogen in the dam and at the materno-fetal interphase have been studied (Andrianarivo et al., 2001; Benavides et al., 2012, Cantón et al., 2014), and the fetal immune response associated with *N. caninum* infections has partially described. This work reports the cellular phenotype of inflammatory infiltrates in brain and lung from bovine fetuses from previously exposed, vaccinated and control heifers experimentally challenged with *N. caninum*.

2. Material and methods

2.1. Animals and experimental design

Fetal tissue samples were obtained from a previous study, and a full description of the animals and experimental design is published elsewhere (Hecker et al., 2013, 2014, 2015). 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 the Instituto Nacional de Tecnología Agropecuaria (INTA), Balcarce, Argentina.

Briefly, twenty 22-month old Angus heifers seronegative for *N. caninum* were divided randomly in five groups as follow: Group A) heifers (n = 4) previously inoculated intravenously (iv) with 6.25×10^7 live tachyzoites of *N. caninum* NC-6 Argentina strain (Basso et al. 2001) in sterile phosphate buffered solution (PBS), 4 weeks before mating; Group B) heifers (n = 4) subcutaneously (sc) immunized with 2 doses of a vaccine formulated with an *N. caninum* tachyzoite antigen extract of NC-6 Argentina strain plus immune-stimulating complexes (ISCOMs; Abisco-300, ISCONOVA, Uppsala, Sweden), 3 weeks apart (Hecker et al., 2013); Group C) heifers (n = 4) sc-immunized with 2 doses of a vaccine formulated with a mixture of rNcSAG1, rNcHSP20 and rNcGRA7 recombinant proteins of *N. caninum* strain NC-1 (Dubey et al., 1988) and ISCOMs, 3 weeks apart (Hecker et al., 2014); Group D) (n = 4) and Group E) (n = 4) heifers sc-sham-inoculated twice, 3 weeks apart, with sterile PBS and ISCOM-MATRIX, respectively (Hecker et al. 2013, 2014).

Four weeks after the first immunization, all heifers were estrus synchronized and then allocated with 4 healthy Angus bulls for natural breeding over 7 days. 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 tachyzoites at day 70 of gestation. Fetal viability was confirmed by ultrasonography every week following challenge and until euthanasia at day 104 of pregnancy. Post-mortem examination was carried out on all dams and fetuses.

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, 2015). 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) 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

The live NC-6 Argentine strain (6.25×10^7 live tachyzoites in 2 ml PBS) used to immunize heifers from group A. Similarly, the production of the native antigen extract and recombinant proteins used for the formulation of the experimental vaccines of groups B and C were as previously described (Hecker et al., 2013, 2014). Either the native antigen extract (containing approximately 500 µg/ml) or a mixture (containing 30 µg of each protein) were formulated with 200 µl of ISCOMs (with approximately 750 µg/ml), kindly provided by Dr. Bror Morein, Uppsala University, Sweden.

2.4. Tissue collection

Following euthanasia, fetuses were immediately removed and examined following standard gross pathology procedures (Campero et al., 2003). Fetal tissues, including brain and lung were collected and fixed in 10% buffered formalin, processed by standard histologic methods and embedded in paraffin. Five µm-thick sections of tissue were cut, mounted on glass microscope slides and stained with hematoxylin and eosin (H&E) for histopathologic examination by light microscopy.

2.5. Fetal N. caninum infection status

Fetal infection was established by PCR in fetal tissues and/or the presence of specific antibodies in fetal fluids (Hecker et al., 2013, 2014).

2.6. Immunohistochemical analysis

Immunohistochemistry (IHC) for CD3, CD20, and MHC II was performed on 4um sections from fetal lung and brain mounted on charged slides and using an automated staining instrument (Ventana Benchmark XT, Ventana Medical Systems Inc., Tuscon, AZ USA). All methods used online heat-induced epitope retrieval (HIER) in tris-based buffer (pH 8), and an alkaline phosphatase-linked goat anti-rabbit / anti-mouse polymer detection kit with Fast Red chromogen (ultraView Universal alkaline phosphatase red detection kit, Ventana). Sections of bovine fetal spleen and of normal canine lymph node and spleen were used to optimize the protocol for each antibody, and sections from the same canine tissues were used as positive tissue controls for each subsequent run. For negative reagent controls, duplicate sections of each control and test tissue were subjected to the same immunohistochemical procedure with substitution of antibody diluent alone for mouse monoclonal primary antibody, and substitution of non-immune rabbit serum at similar protein concentration for the rabbit polyclonal antisera Table 1.

All sections were analyzed by 1 pathologist blinded as to treatment group of the dam and *N. caninum* infection status of the fetus. Lesions were subjectively scored based on the percentage contribution to inflammatory lesions or anatomic sites by each labelled cell type, as follows: 0 = no immunolabeled cells, 1 = few immunolabeled cells, 2 = small groups of immunolabeled cells, 3 = large groups of immunolabeled cells.

2.7. Statistical analysis

In order to establish differences in the immunolabeling of cells, an ANOVA of two factors, tissue and group, with a significance level of 0.05 was performed. To establish the association between fetal infection and cell immunomarking, an accurate Fisher test was performed. All statistical analyses were performed using Graph-Pad Prism 5 v.5.01

Table 1
Antibodies used for IHC evaluation of fetal lung and brain.

Antibody	Clone / Source	Dilution
CD3	rabbit polyclonal/Dako	1:400
CD20	rabbit polyclonal/Fisher-Thermo (Neomarkers/LabVision)	1:4000
MHC II	mouse monoclonal TAL.1B5 / Dako	1:200

Table 2
Animals groups, fetuses status and immunolabeling for different types of cells in the brain and lung (0 = No marked cells, 1 = few marked cells, 2 = small groups of marked cells, 3 = Large groups of marked cells).

Group	Fetal #	Status for *N. caninum*	Immunolabeling Brain-score			Immunolabeling Lung-score		
			MHC II$^+$	CD3$^+$	CD20$^+$	MHC II$^+$	CD3$^+$	CD20$^+$
A	5	+	0	0	1	0	0	1
	8	–	0	0	0	1	1	1
	12	–	0	0	0	0	1	1
	15	–	0	0	0	0	0	0
B	9	+	3	1	0	3	2	0
	10	+	3	1	0	3	1	1
	17	–	0	0	0	2	0	1
	20	+	2	1	0	2	1	1
C	4	+	1	0	0	3	1	1
	6a*	–	1	0	0	1	1	1
	6b*	+	2	1	0	1	1	1
	13	+	1	1	1	3	2	1
	14	+	2	1	0	3	2	1
D	1	+	1	1	1	3	2	1
	7	–	2	1	0	3	2	0
	18	+	0	0	0	2	1	1
	16	–	2	1	1	3	2	1
E	2	+	2	1	1	3	2	1
	3	+	2	2	2	2	1	0
	11	–	1	1	1	2	1	1
	19	–	3	0	0	3	2	0

* 6a and 6b were twins.

(GraphPad Soft-ware, San Diego, CA, USA).

3. Results and discussion

3.1. MHC II $^+$ cells

Inflammatory infiltrates in brain and lung were mostly MHC II$^+$ cells (Table 2) (Fig. 1A and B). Such immunolabeling varying from 70 to 90% of the total cellular infiltrate was associated with the status of *Neospora*-infection but was higher in lung compared with brain tissue ($p = 0.003$). Although non suppurative necrotic encephalitis is a typical finding in *Neospora*-aborted bovine fetuses (Dubey et al., 2017), the severe interstitial pneumonia, probably associated to the intravenous route of challenge (Hecker et al., 2013, 2014), may explain the higher detection of MHC II$^+$ cells in that tissue. A massive load of parasites at early gestation has been suggested in systemic fetal neosporosis where many tissues are severely affected including lungs (Dubey et al., 2017). It is noteworthy that extensive peribronchial and interstitial infiltrate clusters, sometimes like hyperplasia of the bronchial-associated lymphoid tissue (BALT) were observed (Fig. 1B). Since BALT are not frequently observed in bovine fetuses, these structures would correspond to an important reaction of the fetus to parasite infection (Liebler-Tenorio and Pabst, 2006)

The blood -brain barrier may also explain the lower amount of MHC II immunolabeling among inflammatory cells in the brain of infected fetuses (Fig. 1A). On the other hand, MHC II molecules can be expressed not only by microgial cells but also by astrocytes in the CNS (Amills et al., 1998). Interestingly, different subpopulations of MHC II cells, mainly glial cells and astrocytes, were described in inflammatory infiltrates of brain tissue in adult goats and aborted goat fetuses infected by *N. caninum* (Costa et al., 2014). MHC II$^+$ cells were either not present or scarce in brain and lung of fetuses from group A ($p = 0.0001$) (Fig. 2A) mainly because exposure to live parasite before mating protects against both congenital infection and abortion (Innes et al., 2001, Hecker et al., 2013). We can speculate that the non-suppurative inflammatory infiltrates frequently observed in histopathological lesions in *N. caninum*-infected bovine fetuses (Benavides et al., 2012; Gibney et al., 2008) are mostly macrophages presenting *Neospora*-antigens. Overall, the association of immunolabelling of these cells with fetal infection demonstrates that *N. caninum* is capable of stimulating a proimmflamatory immune response in bovine fetuses even at early gestation in dams receiving inactivated vaccines or without previous exposure.

3.2. CD3$^+$ cells

CD3$^+$ cells were the second most frequent cell population in inflammatory infiltrates, both in brain and lung tissue (Table 2). Since this immunolabeling was associated with fetal infection ($p = 0.01$) and considering that MHC II$^+$ cells, presumptively macrophages, were also observed in those tissues, it is obvious that T lymphocytes and probably γδ lymphocytes were involved in the immune reaction (Tizard, 2009). A mixed mononuclear inflammatory infiltrate, including lymphocytes, has been observed in several works (Gibney et al., 2008; Benavides et al., 2012; Hecker et al., 2013).

Of the tissues examined, lung had more immunostaining for CD3+ cells ($p = 0.002$) (Fig. 1C and D), with CD3$^+$ cells contributing to approximately 30% of the observed cells. That immune marking was independent from that observed in CNS ($p = 0.7$); however differences were also observed between in the groups ($p = 0.01$). Fetuses from group A did not have (or was very scarce) positive CD3 neither in the brain nor in the lung. On the contrary such immunolabeling was higher in the other experimental groups. Similar to the finding for MHC II$^+$ cells, the differences observed between the groups (Fig. 2B) and tissues for the immunolabelling of CD3$^+$ cells would be explained not only by the anatomical differences in the tissues but also by the difference in the immune protection induced by different treatments. Based on these results, fetal *N. caninum* infection triggered a cell-based immune response involving CD3$^+$ cells, which was a common finding in fetal tissues from experimentally challenged dams that were not previously exposed to live parasites.

3.3. CD20$^+$ cells

CD20$^+$ cells were presented as a scarce marginal group (between 10 and 25%), in both brain and lung tissues (Fig. 1E and F). In spite of the immunolabeling was higher in lung tissues ($p = 0.04$), the marking was similar for all treatments ($p = 0.6$) including those fetuses from dams in

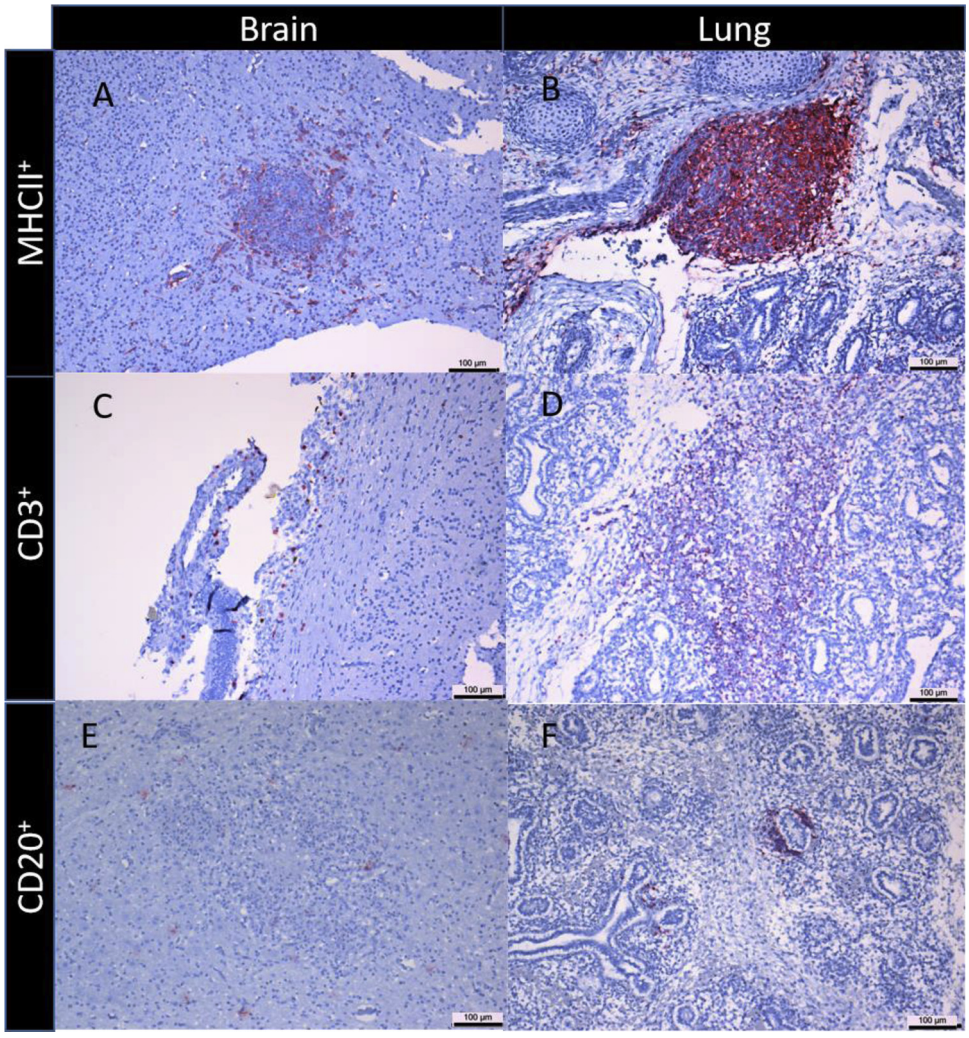

Fig. 1. Microphotographs of IHC for immunolabelling different cells in bovine fetal brain and lung. A) Gliosis focus in cortical neuropil with abundant MHC II$^+$ cells in the center and periphery (fetus #9); B) Abundant inflammatory infiltrate with MHC II$^+$ cells forming a peribronchial cluster (fetus #6a); C) Scarce CD3$^+$ cells scattered in meninges and underlying neuropil (fetus #6b); D) Abundant interstitial and peribronchial infiltrate of CD3$^+$ cells (fetus #7); E) Scarce CD20$^+$ cells on the periphery of a gliosis focus (fetus #10); F) Scarce CD20$^+$ cells at peribronchial level (fetus #1).

group A. There was no association between immunolabelling of these cells and *N. caninum* infection ($p = 0.5$) (Fig. 2C). CD20 marker is mainly expressed in mature and immature B lymphocytes, but is not expressed by plasma cells (Tizard, 2009). The main role of B lymphocytes is the production of antibodies and the presentation of antigens. In agreement with our findings Costa et al (2014) described that this type of cell as rare or scarce in the cerebral inflammatory infiltrate of aborted goat fetuses even when a different marker (CD79α) was used. On the other hand, production of specific antibodies could be detected in fetuses from groups B, C, D and E but only 1 fetus from group A (Hecker et al., 2013, 2014).

Over all our results clearly show that fetuses from all experimental groups (except those from dams previously exposed to live parasites) developed a cellular immune response. This is in agreement not only with the higher protection achieved against congenital transmission in heifers immunized with live tachyzoites before mating (Hecker et al., 2013) but also with the light lesions and low levels of pro-inflammatory cytokines observed in their placentas (Hecker et al., 2015). We may speculate that cows having a protective immune response may kill the parasites in the blood and the spleen quickly before they rich the materno-fetal interfase (Hecker et al., 2013, 2015). Although efficient immunization using live parasites have been reported by others (Williams et al., 2003; Mazuz et al., 2015; Weber et al., 2013), safety and efficacy at field conditions need further research.

4. Conclusion

Infection with *N. caninum* at 70 days of gestation was capable of triggering inflammatory responses involving at least two populations of immune cells in bovine fetal brain and lung. Experimental inactivated vaccines do not limit inflammatory processes in bovine fetuses during early gestation.

Authors' contributions

DPM conceived and supervised the study; YPH designed this study; JD conducted laboratory testing; and MGM analyzed data; JEMR, JD and YPH wrote the manuscript; GJC, CMC and ACO contributed to the study design and provided funding acquisition, access to animals, and laboratory oversight. All authors read and approved the manuscript.

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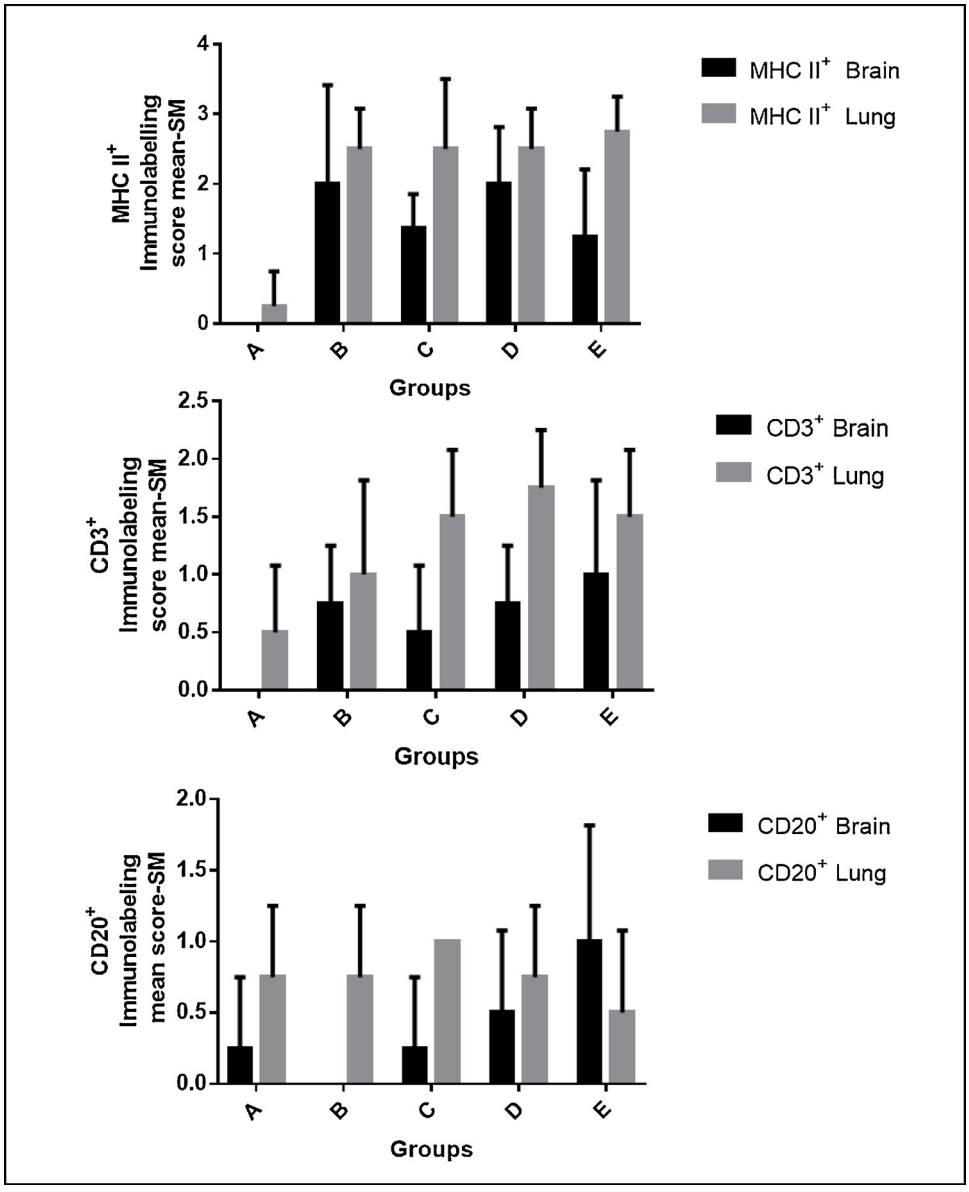

Fig. 2. Average score values for the immunolabelling of MHC II^+ (A); $CD3^+$ (B) and $CD20^+$ (C) cells in bovine fetal brain and lung in the different experimental groups.

Declaration of Competing Interest

The authors declared no potential conflicts of interest with respect this research and publication of this article.

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References

Amills, M., Ramiya, V., Norimine, J., Lewin, H.A., 1998. The major histocompatibility complex of ruminants. Rev. Sci. Tech. (Int. Office Epizoot.) 17, 108–120.

Andrianarivo, A.G., Barr, B.C., Anderson, M.L., Rowe, J.D., Packham, A.E., Sverlow, K.W., Conrad, P.A., 2001. Immune responses in pregnant cattle and bovine fetuses following experi- mental infection with *Neospora caninum*. Parasitol. Res. 87, 817–825.

Barr, B.C., Anderson, M.L., Dubey, J.P., Conrad, P.A., 1991. *Neospora*-like protozoal infections associated with bovine abortions. Vet. Pathol. 28, 110–116.

Benavides, J., Katzer, F., Maley, S.W., Bartley, P.M., Cantón, G., Palarea-Albaladejo, J., Purslow, C.A., Pang, Y., Rocchi, M.S., Chianini, F., Buxton, D., Innes, E., 2012. High rate of transplacental infection and transmission of *Neospora caninum* following experimental challenge of cattle at day 210 of gestation. Vet. Res. 43, 83.

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

Costa, R.C., Orlando, D.R., Abreu, C.C., Nakagaki, K.Y.R., Mesquita, L.P., Nascimento, L.C., Silva, A.C., Maiorka, P.C., Peconick, A.P., Raymundo, D.L., Varaschin, M.S., 2014. Histological and immunohistochemical characterization of the inflammatory and glial cells in the central nervous system of goat fetuses and adult male goats naturally infected with *Neospora caninum*. Vet. Res. 10, 291.

Cantón, G.J., 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., 2014. Inflammatory infiltration into placentas of *Neospora caninum* challenged cattle correlates with clinical outcome of pregnancy. Vet. Res. 45, 247–254.

Dubey, J.P., Carpenter, J.L., Speer, C.A., Topper, M.J., Uggla, A., 1988. Newly recognized fatal protozoan disease of dogs. J. Am. Vet. Med. Assoc. 192, 1269–1285.

Dubey, J.P., Hemphill, A., Calero-Bernal, R., Schares, G., 2017. Neosporosis in Animals. Published by CRC Press, pp. 1–529.

Gibney, E.H., Kipar, A., Rosbottom, A., Guy, C.S., Smith, R.F., Hetzel, U., Trees, A.J., Williams, D.J.L., 2008. The extent of parasite-associated necrosis in the placenta and foetal tissues of cattle following *Neospora caninum* infection in early and late gestation correlates with foetal death. Int. J. Parasitol. 38, 579–588.

Hecker, Y.P., Cantón, G., Regidor-Cerrillo, J., Chianini, F., Morrell, E., Lischinsky, L., Ortega-Mora, L.M., Innes, E.A., Odeón, A., Campero, C.M., Moore, D.P., 2015. Cell mediated immune responses in the placenta following challenge of vaccinated pregnant heifers with *Neospora caninum*. Vet. Parasitol. 214, 247–254.

Hecker, Y.P., Cóceres, V., Wilkowsky, S.E., Jaramillo Ortiz, J.M., Morrell, E.L., Verna,

A.E., Ganuza, A., Cano, D.B., Lischinsky, L., Ángel, S.O., Zamorano, P., Odeon, A.C., Leunda, M.R., Campero, C.M., Morein, B., Moore, D.P., 2014. A *Neospora caninum* vaccine using recombinant proteins fails to prevent foetal infection in pregnant cattle after experimental intravenous challenge. Vet. Immunol. Immunopathol. 162, 142–153.

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.

Innes, E.A., Andrianarivo, A.G., Bjo¨rkman, C., Williams, D.J.L., Conrad, P.A., 2002. Immune responses to *Neospora caninum* and prospects for vaccination. Trends Parasitol. 18, 497–504.

Liebler-Tenorio, E.M., Pabst, R., 2006. MALT structure and function in farm animals. Vet. Res. 37, 257–280.

Mazuz, M.L., Fish, L., Wolkomirsky, R., Leibovich, B., Reznikov, D., Savitsky, I., Golenser, J., Shkap, V., 2015. The effect of a live *Neospora caninum* tachyzoite vaccine in naturally infected pregnant dairy cows. Prev. Vet. Med. 120, 232–235.

McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 28, 1473–1479.

Pescador, C.A., Corbellini, L.G., Oliveira, E.C., Raymundo, D.L., Driemeier, D., 2007. Histopathological and immunohistochemical aspects of *Neospora caninum* diagnosis in bovine aborted fetuses. Vet. Parasitol. 150, 159–163.

Tizard, I.R., 2009. Introducción a la Inmunología Veterinaria. Elsevier, España ISBN: 978-84-8086-431-2.

Weber, F.H., Jackson, J.A., Sobecki, B., Choromanski, 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 *Neospora caninum* for prevention of *Neospora*-associated fetal loss in cattle. Clin. Vaccine Immunol. 20, 99–105.

Williams, D.J.L., Guy, C.S., Smith, R.F., Guy, F., McGarry, J.W., Macay, J.S., Trees, A.J., 2003. First demonstration of protective immunity against foetopathy in cattle with latent *Neospora caninum* infection. Int. J. Parasitol. 33, 1059–1065.