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TOXOPLASMA GONDII INFECTION REDUCES SERUM PROGESTERONE LEVELS AND ADVERSE EFFECTS AT THE MATERNAL-FOETAL INTERFACE

Short running title: **Impact of *Toxoplasma gondii* on pregnancy**

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ABSTRACT

Aims: Pregnant BALB/c mice infected with a *Toxoplasma gondii* type II strain were used to determine how pregnancy interferes with the development of maternal immunity to *T. gondii* and how infection disrupts pregnancy and foetal development.

Methods: Maternal and foetal parasite loads were assessed by amplification of *T.gondii SAG1* using qPCR. Adverse effects of infection were evaluated on foetal placental development by quantification of implantation units undergoing resorption and by histopathological analyses. Serum progesterone levels were quantified by immunoassay. The effect of *T. gondii* infection on maternal immunity was determined by assessing the cellular composition of spleens by flow cytometry.

Results: Infected pregnant mice exhibited clinical signs of infection, inflammation and necrosis at the maternal-foetal interface and decreased serum progesterone levels. In infected mice, there was a clear effect of pregnancy and infection on macrophage cell numbers. However, no differences in the parasite load were detected between non-pregnant and pregnant mice.

Conclusions: Maternal *T. gondii* infection induced adverse effects at the maternal-foetal interface. Alterations were found in immune spleen cells, dependent on the day of pregnancy, relative to non-pregnant animals. The results obtained suggest a pregnancy-dependent mechanism during *T. gondii* infection able to interfere with macrophage numbers.

Keywords: Zoonosis; Pregnancy; Pathology; Congenital infection; *Toxoplasma gondii*; Immunology; Progesterone

INTRODUCTION

Infection with *Toxoplasma gondii* during pregnancy can lead to congenital toxoplasmosis, which, depending on the gestational period of infection, may induce disruption of pregnancy (abortion, stillbirth or pre-term deliveries), serious neurological and ophthalmological or other sequelae in the newborn [1-3]. The pathophysiology of congenital toxoplasmosis is complex, since it is not only a consequence of foetal infection during pregnancy, but it is also due to the inflammatory immune response to infection during pregnancy [4-6]. Furthermore, elements of the immune response that are normally associated with a protective immune response to *T. gondii* infection in non-pregnant mice contribute to the pathology associated with congenital infection [7]. In addition, normal pregnancy is dependent on a series of sequential alterations in the maternal immune system, which facilitate implantation, decidualization and prevent rejection of the semiallogeneic foetus [8]. *T. gondii* infection during pregnancy modifies systemic maternal cellular immune responses and immune mechanism at the maternal-foetal interface and, thus, influence pregnancy outcome [9]. It is known that progesterone, a pregnancy-specific factor, is able to modulate the maternal immune response by affecting macrophages and lymphocytes activation [10-13]. In addition, *T. gondii*-infected pregnant women exhibit decreased serum progesterone levels, although the mechanisms by which this modulation occurs remain unclear [14]. Accordingly, there is still a lack of knowledge about the immune players and pregnancy-dependent factors, such as progesterone, that are implicated in the pathology associated with congenital toxoplasmosis [14].

In the present study, BALB/c mice were used as an animal model of congenital toxoplasmosis [15,16]. A type II strain of *T. gondii* (Me49), commonly found in Europe and the United States, and associated with human disease, was used as infection agent, because it allows following up the pathology without inducing death of pregnant mice [17-19]. Mice were infected with *T. gondii* early in pregnancy to determine the effect on decidualization, a process that involves proliferation and differentiation of endometrial stromal cells, and on placenta development, consisting of trophoblast cells of foetal origin. Another aspect addressed in this study was the characterization of maternal spleen lymphoid, macrophage and neutrophil cell populations, which are known to be modulated by progesterone and critical to control parasites and successful pregnancy. Finally, progesterone serum levels were quantified during pregnancy, in order to evaluate the impact of *T. gondii* on the endocrine function on mice during pregnancy.

MATERIAL AND METHODS

Parasite and *in vitro* infection

ME49 strain of *T. gondii* constitutively expressing Yellow Fluorescence Protein (YFP) was kindly provided by Dr Marcus Meissner (Parasitology department of the Faculty of Medicine, Heidelberg University, Germany). Viable tachyzoites were obtained by *in vitro* infection of HFF cells and cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), fetal bovine serum (10%), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Life Technologies Europe, Porto, Portugal). Cell cultures were maintained in flasks at 37°C, in humidified atmosphere of 5% CO_2. For the maintenance of *in vitro* parasite cultures, a ratio of 1:10 (HFF cells: parasite) was used. When the majority of fibroblasts exhibited multiple intracellular tachyzoites, namely in rosettes, they were detached using a cell scraper and disrupted using a 25G needle and syringe for 10 times. Cell suspension was filtered using a 5 µm filter (Sartorius, Germany) and centrifuged at 500g for 6 minutes at room temperature, in order to eliminate HFF cell debris. Tachyzoites were counted using a Neubauer chamber.

Mice and *in vivo* infection

Balb\c/cByJ (Balb\c) mice were obtained from Charles River (L'Arbresle, France) and maintained in the Animal facility of the Institute of Biomedical Sciences Abel Salazar (Porto, Portugal). BALB/c virgin females, aged 8-12 weeks, were mated with fertile males (1 male/ 2 females/ cage). For each female, day 1 of pregnancy was set as the day when vaginal plug became apparent, designated day 1 of pregnancy. For *in vivo* infection, an inoculum of 2.5 x 10^4/ mL viable tachyzoites was prepared in saline solution (0.9 % NaCl) . At day 7 of gestation, each mouse was infected intraperitoneally (i.p.) with 5 x 10^3 viable tachyzoites, obtained as described above. Mice were sacrificed 5 or 7 days post-infection (corresponding to day 12 and day 14 of gestation). In this study, seven groups of mice were used: non-infected and non-pregnant mice; non-infected mice at day 12 of pregnancy; non-infected mice at day 14 of pregnancy; non-pregnant mice at day 5 of infection; non-pregnant mice at day 7 of infection; mice at day 5 of infection and day 12 of pregnancy; mice at day 7 of infection and day 14 of pregnancy. For histological and immunohistochemistry analysis, two additional groups of animals were used: non-infected mice at day 18 of pregnancy and mice at day 7 of infection and day 18 of pregnancy. These mice were

infected i.p. with 5 x 10^3 viable tachyzoites at day 11 of pregnancy and were sacrificed 7 days post-infection. All pregnant mice obtained were primiparous. The sacrifice of animals consisted of anesthetic induction with 5% isofluorane, followed by cervical dislocation. All procedures involving animals were performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123) and directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of the animals used for scientific purposes, and Portuguese rules (DL 113/2013). The authorization to perform the experiments was issued by IBMC.INEB Animal Ethics Committee (MacroGondii reference) and by the responsible national board authority, Direcção-Geral de Alimentação e Veterinária (0421/000/000/2014).

Collection of biological samples and resorption rate determination

For each pregnant mouse, all implantation units were collected, and the number of normal or resorpted units quantified. Implantation units undergoing resorption were identified by their small sizes and confirmed by histology, as previously described [5,20]. For each mouse, the resorption rate was calculated as the ratio between the number of resorpted units and the total number of implantation units. The average resorption rate was calculated for each group. Essentially, for each mouse, three implantation units, as well as resorpted implantation units, were preserved in 4% paraformaldehyde (PFA) and included in paraffin for histological analysis. At day 12 of pregnancy, mesometrial decidua plus placenta (decidua/placenta) and foetus were dissected from three implantation units from each animal. At day 14 of pregnancy the mesometrial decidua, the placenta and the foetus were collected from three implantation units from each animal. All the tissues were stored at -80^{0}C in Trizol (50-100 mg of tissue/ ml; Sigma Aldrich, USA). The weights of placenta, mesometrial decidua and foetus were determined for day 14 of pregnancy. For each infected pregnant or non-pregnant animal, portions of spleen, liver, kidney, lung and heart were collected and stored at -80^{0}C in Trizol, for posterior parasite load quantification (Sigma Aldrich, USA). Spleens from all the animals were collected for flow cytometry experiments. Blood from non-infected or infected pregnant mice was collected for serum progesterone evaluation.

Quantitative real-time PCR (qPCR)

Genomic DNA (gDNA) from the heart, liver, lung, kidney, spleen, decidua/placenta, decidua or placenta and foetus, was extracted using Trizol Reagent according to the manufacturer's instructions (Sigma Aldrich, USA). The DNA concentration was determined using a Thermo Scientific Nanodrop 1000 spectrophotometer. *T.gondii* gDNA was detected using primers and a probe designed for the *T.gondii surface antigen 1* (*SAG-1;*GenBank: X14080), giving rise to an amplification product of approximately 100 bp. Product amplification was performed with 1 µl of sample DNA, corresponding to 50 ng of DNA, in a final volume of 10 µl containing 0,2 µM of each primer (*SAG-1* forward: CCAGAGCCTCATCGGTCGTC; *SAG-1* reverse: GGGTCCTTCCGCAGACAAC), 0,2 µM of probe (6FAM-CTGTyTGCACCGTAGGAGCACCT-BBQ; all designed by Tib Molbiol, Germany) and Kapa probe Fast qPCR Master Mix (Kapabiosystems, USA). The PCR programme run was as follows: 1) denaturation at 95^0C, 3 min; 2) amplification in 40 cycles (denaturation at 95^0C, 3 sec; combined annealing/extension 60^0C, 30 sec). The standard curve was obtained using samples ranging from 10^2 to 10^{-2} ng/µl of parasite gDNA diluted in a solution of 20 ng/µl of host gDNA. Quantitative evaluation of fluorescence signals from PCR products was performed with Step-One Plus and analyzed with Step One Software V2.3 (Applied Biosystems by Life Technologies, USA). The detection limit was determined by performing *SAG-1* qPCR using gDNA from non-infected animals. Limit detection of 27 parasites per 2000 ng DNA. The infection rate was calculated as the percentage of tissues testing positive for *T. gondii SAG1*.

Histology and morphometry

After collection, the implantation units were fixed in 4% PFA, dehydrated and embedded in paraffin. The sections (4 µm) were stained with hematoxylin and eosin (H&E; Sigma-Aldrich, USA) and mounted in 1,3-diethyl-8-phenylxanthine (DPX) mounting medium (VWR, England). Slides were observed under a bright-field microscope (Eclipse Ci-L, Nikon, Japan) and NIS-Elements image software was used for image processing. Per animal, three fetoplacental sections from the cross-sectional area of three independent implantation units (9 slides from each animal) were analyzed (27 slides for each group of mice). The determination of the maternal component (blood vessels, mesometrial gland and metrial gland) and placenta was performed as previously described [21].

Immunohistochemistry (IHC)

Paraffin sections were dewaxed, rehydrated, and permeabilized in PBS containing 0.1% Triton X-100 and 0.1% Tween. After antigen retrieval with 10 mM sodium citrate buffer for 30 min at 96°C, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase in methanol for 35 min at RT, followed by blocking the nonspecific Ab binding with normal horse serum (Vector Laboratories, Burlingame, CA, USA). Sections were incubated overnight at 4°C with rat IgG2a monoclonal anti-mouse CD68 (dilution 1:200; clone FA-11; AbD serotec) or rat IgG monoclonal anti-mouse F4/80 (final concentration 20 μg/ml; clone Cl:A3-1; Abcam). After washing with PBS, sections were incubated with goat anti-rat IgG and horseradish peroxidase-conjugated Ab (GE Healthcare). Development was performed with a 3,3-diaminobenzidine (DAB) labelling system (Vector Laboratories, USA). Sections were then counterstained with Gill's hematoxylin, dehydrated, and mounted in DPX (VWR, England) and analyzed under a bright field microscope (Eclipse E400, Nikon, Japan) equipped with image analysis software LeicaQWin.

Fluorescence microscopy

For fluorescence microscopy, paraffin sections were dewaxed, rehydrated and mounted in 4=,6-diamidino-2-phenylindole (DAPI)-Vectashield (Vector Laboratories, USA). *T. gondii*-YFP and nuclei were detected using a fluorescence microscope (Eclipse Ci-L, Nikon, Japan) equipped with excitation filters with maximum transmission at 515 nm (Epi-FL filter block YFP-LP) or 400 nm, respectively. Images were processed using a camera (Nikon Instruments Camera Heads DS-Ri2) and NIS-Elements image software.

Determination of serum progesterone level

After animal anesthetic induction with 5% isofluorane, blood samples were collected directly from the heart into microtubes. After complete clot formation, each sample was centrifuged at 965*g* for 10 min. Serum was removed, collected in aliquots and stored at −80°C until further analysis. Progesterone serum concentrations were measured by enzyme-linked fluorescent assay using VIDAS Progesterone Kit and mini VIDAS analyser (bioMerieux S.A., Marcy L'Etoile, France). According to the manufacturer, the assay has a measurement range of 0.25–80 ng/ml for progesterone.

Flow cytometry

Spleen cells were isolated from uninfected and infected animals. Briefly, a single cell suspension was prepared from the spleen for each mouse, as previously described [22]. Cells were washed, and viable cells were counted by trypan blue exclusion. For the immunofluorescence staining, cells were labelled with distinct combinations of the following antibodies: Alexa Fluor 647 anti-mouse/human CD11b antibody (Ab), Isotype rat IgG2b, κ (clone M1/70); PercP/Cy5.5 anti-mouse Ly6G Ab, Isotype rat IgG2a, κ (clone 1A8); Phycoerythrin (PE) anti-mouse Ly6G Ab, Isotype rat IgG2a, κ (clone 1A8); PercP/Cy5.5 anti-mouse F4/80 Ab, Isotype rat IgG2a, κ (clone BM8); PE anti-mouse I-A/I-E (MHCII) Ab, Isotype rat IgG2b, κ (clone M5/114.15.2); PE rat IgG2b, κ Isotype control (clone RTK4530); allophycocyanin (APC) anti-mouse CD3ε Ab, Isotype Armenian hamster IgG (clone 145-2C11); PE anti-mouse CD4, Isotype rat IgG2a, κ (clone RM4-5); PE anti-mouse CD8a Ab, Isotype rat IgG2a, κ (clone 53-6.7); All of the Abs were obtained from BioLegend (San Diego, CA, USA). Briefly, for the surface staining, 1 x 10^6 spleen cells were seeded in 96-well round-bottom plates. For macrophage and neutrophil analysis, Fc blocking was performed, incubating cells with anti-mouse CD16/32 Ab during 20 min at 4^0C. Cells were then incubated for 20 min with saturating concentrations of the different combinations of the following antibodies: CD11b-Alexa Fluor647, F4/80 PercP/Cy5.5 and Ly6G-PE; CD11b-Alexa Fluor647, F4/80 PercP/Cy5.5 and MHCII-PE or isotype control; CD3-APC and CD4-PE or CD3-APC and CD8-PE. After cell surface staining, all samples were fixed with PBS-2% PFA and washed twice in PBS containing 2% FBS. The acquisition of cells was performed using a BD Accuri C6 using BD AccuriC6 Software (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Cells were selected on the basis of forward scatter/side scatter (FSC/SSC), and the singlets were gated according to size *versus* width. For lymphocyte analysis we used CD3, CD4 and CD8 to label T cells. CD4+ T cells were defined as CD3+CD4+ and CD8+ T cells were defined as CD3+CD8+. The number of events acquired for CD4+ or CD8+ T cells was 10000 events of total CD3+CD4+ or CD3+CD8+ cells. $CD11b^{high}F4/80^{high}$ $Ly6G^{-}$and $CD11b^{high}F4/80^{-}$ $Ly6G^{high}$ were defined as macrophages and neutrophils, respectively. MHCII expression was defined by median of fluorescence intensity (MFI) in mononuclear phagocytes defined as $CD11b^{high}F4/80^{high}$. The specificity of anti- MHCII antibody was evaluated by the use of isotype control antibody. The number of the events acquired for neutrophil and macrophage analysis and MHCII expression was 10 000 events of total CD11b+ cells. To determine the cell number, the number of gated events for each cell population was multiplied by the total cell number (counted

using Neubauer chambers) and divided by the total number of events selected by FSC/SSC parameters.

Statistical analysis

Comparisons were carried out using analysis of variance and *t*-test. When normality or homogeneity of variances was not observed, the comparisons were carried out using the Mann-Whitney-Wilcoxon test and Friedman Test (Figure 1A, day 12 and day 14 of pregnancy, respectively) or Kruskal-Walkis test (Figure 7). When normality or homogeneity of variances was observed the two-way analysis of variance (ANOVA) test was performed with multiple comparisons (Figure 1B, 2C, 6, 8 and 9). The limited number of animals analyzed rendered inadequate the use of the *t*-test for statistical comparison between the groups pf mice for each tissue (Figure 3). Graphs were plotted using using GraphPad Prims 8.1.2. Statistical significance was assessed for $P < 0.05$. Statistical analysis was carried out using IBM SPSS Statistics 25.

RESULTS

Maternal *T. gondii* infection has adverse effects on foetal placental development irrespective of their infection status

BALB/c mice were infected by i.p. route with 5000 viable tachyzoites of *T. gondii* Me49 at day 7 of pregnancy. *T. gondii* were quantified by qPCR for *SAG-1* in mesometrial decidua, placenta and foetus at day 12 and day 14 of pregnancy to confirm maternal-foetal interface and congenital infections [23]. At day 12 of pregnancy, it was technically challenging to separate mesometrial decidua from placenta, hence parasite load was quantified in the total tissue sample (mesometrial decidua and placenta). A significant difference in the parasite load was observed between decidua/placenta and the foetus at day 12 of pregnancy. No significant differences were recorded between tissues at day 14 of pregnancy (Figure 1A). An infection rate of 47% was observed on day 12 at the foetal placental interface (Decidua/Placenta) while on day 14 it was found 30% and 20% (Decidua and Placenta, respectively). Congenital infection rates, as determined by PCR positive foetuses, were 46% and 34% for day 12 and day 14 of pregnancy, respectively (Table 1). Furthermore, the positive detection of *T. gondii* in the foetus coincided with to its presence in the placenta.

The adverse effects on pregnancy outcome were evaluated by comparative morphometric analyses of normal implantation units from non-infected with implantation units from infected pregnant animals and quantification of implantation units undergoing resorption. Morphometric analyses of the implantation units revealed no differences at days 12 and 14 of pregnancy between control and *T. gondii* infected pregnant mice. However, a discreet reduction of the cross-sectional area (maternal component and placenta) was observed in infected mice at day 14 of pregnancy relative to non-infected mice at this time point (Figure 1B).

The numbers of normal and resorpted implantation units per mouse revealed a scattered distribution of values within each experimental group (Figure 2A and B). Specifically, the resorption rates were found to be similar between days of pregnancy and between infected and non-infected mice (Figure 2C). A small decrease in the placenta and foetus weights, from infected mice at day 14 of pregnancy was found, compared with non-infected pregnant mice (Figure 3).

In addition, histopathological analyses were performed at the maternal-foetal interface, in order to study the possible histological alterations and co-localization of *T. gondii* identified by YFP expression. *T. gondii* was generally detected at the myometrium layer. However, in the resorbed foetal-placental units from infected mice at day 14 of pregnancy, small numbers of tachyzoites were also observed in the mesometrial decidua (Figure 4A-E). Additional experiments using infected day-18 pregnant animals indicated the occurrence of necrosis at the maternal-foetal interface (Figure 4F-H), and *T. gondii* parasites were present in the placenta (Figure 4I-J). No parasites were detected by fluorescence microscopy in the foetus (data not shown), despite the previous positive results obtained by qPCR for *SAG-1*.

T. gondii-infection induces inflammatory changes in implantation units

To assess the presence of macrophages at the maternal-foetal interface during *T. gondii* infection, IHC was performed for CD68 and F4/80 markers that are highly and specifically expressed by murine macrophages. Negative controls were performed in order to verify the specificity of primary antibodies (Figure 5A, 5D). CD68+ cells were detected at the mesometrial decidua from infected mice at day 14 of pregnancy (Figure 5B-C). CD68+ cells were not detectedin implantation units from non-infected pregnant mice (data not shown). F4/80+ macrophages were found in the anti-mesometrial decidua from infected mice at day 18 of pregnancy (Figure 5E-F). These cells were also detected in non-infected day-18 pregnant mice, albeit in fewer numbers (data not shown). These results demonstrate the presence of macrophages in the implantation units of mice irrespective of infection. However, CD68+ activated monocytic cells were unique to the implantation units from *T. gondii*-infected pregnant mice.

T. gondii-infected pregnant mice exhibit decreased serum levels of progesterone

Levels of progesterone were quantified in sera from non-infected or infected pregnant mice by immunoassay. Progesterone levels were similar between infected and non-infected mice at day 12 of pregnancy. However, a significant decrease of serum progesterone levels was observed in infected mice at day 14 of pregnancy, compared with non-infected pregnant mice ($P<0.05$, Figure 6).

Pregnancy does not affect maternal systemic parasite load

Maternal parasite loads were assessed in the spleen, lung, liver, heart and kidney by amplification of *SAG-1* using qPCR. No differences were obtained in any of the organs analyzed between non-pregnant and pregnant mice for each day of pregnancy examined (Figure 7, Table 2). These results suggest that parasite proliferation is not influenced by pregnancy.

Spleen immune cells during pregnancy and *T. gondii* Me49 infection

The effect of *T. gondii* infection in spleen cellular composition during pregnancy was assessed by flow cytometry analysis. The total number of CD4+ and CD8+ T cells, macrophages and neutrophils, in the spleens from non-infected or infected non-pregnant or pregnant mice were quantified (Figure 8). At day of pregnancy, the numbers of CD4+ and CD8+ T cells were similar, to the numbers found in non-pregnant mice, indicating that pregnancy has no effect on the number of spleen T lymphocytes. However, at day 14 of pregnancy, without infection, it was observed a significant decrease in the numbers of CD4+ and CD8+ T cells in pregnant mice compared with non-pregnant mice, indicating an effect of pregnancy in the number of these T cell subpopulations. At days 5 and 7 of infection, the CD4+ T population significantly increased in both non-pregnant and pregnant mice ($P<0.05$; $P<0.001$). The number of CD8+ T cells remained low at day 5 of infection in both non-pregnant or pregnant mice. Despite this, at day 7 of infection, the number of CD8+ T cells significantly increased in both infected non-pregnant or pregnant mice, compared with non-infected non-pregnant or pregnant mice ($P<0.001$). This indicated that at day 7 of infection, CD4+ and CD8+ T cells increased in response to *T. gondii* infection independently of pregnancy. No significant differences were noticed between non-infected non-pregnant and infected pregnant animals, indicating no interaction effect between pregnancy and infection in CD4+ and CD8+ T cell numbers. Analysis of macrophage cell numbers revealed that both non-infected and infected mice at day 12 of pregnancy exhibited significantly higher number of macrophages compared with non-pregnant mice ($P<0.01$), suggesting a pregnancy-dependent, but an infection-independent effect in this cell population. However, 7 days post-infection, a significant increase in macrophages numbers was observed in both non-pregnant and pregnant mice. Furthermore, it was found an interaction effect between infection and pregnancy, suggesting a pregnancy effect on macrophage numbers during infection or vice-versa ($P<0.05$). Neutrophils remained low during pregnancy in non-infected animals. Notably, A significant increase in spleen

neutrophils in both infected non-pregnant or pregnant mice was observed at days 5 and 7 of infection ($P<0.001$). No interaction effect was observed between infection and pregnancy on neutrophil cell numbers ($P>0.05$)

MHCII expression in spleen CD11b^{high}F4/80^{high} in response to *T. gondii* infection during pregnancy

MHCII expression was evaluated in spleen mononuclear phagocytes (CD11b^{high}F4/80^{high}) from non-infected or infected, non-pregnant and pregnant mice. The results showed that MHCII expression remained low in non-infected animals in both non-pregnant and day-12 pregnant mice. However, a significant increase in MHCII expression was found in non-infected day-14 pregnant mice compared with non-infected, non-pregnant mice ($P<0.05$), indicating a pregnancy-dependent effect. Upon infection, an increase in MHCII expression was observed in both non-pregnant and pregnant mice at day 5 of infection ($P<0.05$). At day 7 of infection, a significant increase in MHCII expression was observed between non-infected and infected non-pregnant mice ($P<0.001$). Surprisingly, a significant decrease in MHCII expression was found between non-infected and infected pregnant mice ($P<0.05$). No interaction effect was found between infection and pregnancy ($P>0.05$, Figure 9).

DISCUSSION

BALB/c mice have been previously validated as model of human congenital toxoplasmosis. This model mimics some aspects of human congenital toxoplasmosis, specifically the fact that mothers only transmit infection to foetus if their first contact with parasites takes place during pregnancy. After exposure to *T. gondii*, females develop a protective immune response that impedes congenital transmission of parasites to the offspring, even if mothers are challenged with during pregnancy. [15]. This model has been shown to be useful for testing the ability of putative vaccines to prevent vertical disease transmission. Herein, we used a type II strain of *T. gondii* stably transfected with YFP to allow easy visualization of the parasite in tissues. We focused on the impact of *T. gondii* infection in early pregnancy, as this, is known to cause pathology during pregnancy in humans and mice. Thus, mice were infected on day 7 of pregnancy. We examined the effects on day 12 and 14 of pregnancy as these time points are two important periods during pregnancy. The former corresponds to the maximum development of decidua, while the latter corresponds to the fully establishment of placenta. Later time-points were generally avoided as we did not want to risk maternal mortality. In this study, a significant difference in parasite load was observed between decidua/placenta and fetus at day 5 of infection and no significant differences in parasite load were detected between mesometrial decidua, placenta or fetus at day 7 of infection, suggesting no parasite tissue tropism in our experimental conditions. These observations are in contrast with other studies where placenta was put forward as a privileged site for *T. gondii* replication and, constituting a real barrier to *T. gondii* transmission to the fetus [9,24]. In this work , infection rates of 47% (Decidua/Placenta) at day 5 of infection and of 30% (Decidua) and 20% (Placenta) at day 7 of infection were found. These results suggest that *T. gondii* has been controlled by an effective innate immune response, similar to that described elsewhere [25]. To determine the occurrence of vertical transmission, parasite quantification in the fetus was assessed. A fetus infection rate of 46% and 34% was obtained at day 5 and day 7 post-infection, respectively. Once again these results demonstrate parasite containment, and are in agreement with a previous study indicating that *T. gondii* infection led to approximately 50% of the pups being infected [26].

Pregnancy outcome was evaluated by the quantification of the resorpted units, as well as by morphometric analysis of implantation units. No statistical differences were observed in the rate of resorption or in the area of the maternal foetal interface from *T. gondii*-infected mice at days 12

and 14 of pregnancy. However, a slight reduction in placenta and fetus weightswas also found in infected mice at day 14 of pregnancy.

Several studies suggest that the necrosis and apoptosis that occur at the maternal-foetal interface during *T. gondii* infection are associated with inflammatory immune response [5,6,27]. Other research indicates that increased resorption rate, abortion and foetus damage are not necessarily associated with parasite presence [4]. In accordance with these studies, our histopathological analysis revealed the occurrence of necrosis at maternal-foetal interface late in pregnancy [5]. Moreover, fluorescence microscopy indicated no co-localization of tissue necrosis and *T. gondii*. This suggests other mechanism, such as the induced inflammatory response, rather than parasite-mediated tissue destruction, as responsible for the observed pathology [4,9]. Immunohistochemistrty identified CD68+ cells at the mesometrial decidua and higher immunoreactivity for F4/80+ cells at the antimesometrial decidua in *T. gondii* infected animals. These observations confirmed an inflammatory response taking place at maternal-foetal interface, as a consequence of *T. gondii* infection, as already described previously [5]. *T. gondii* infection is known to alter the cross-talk, by inducing toll-like receptor (TLR)-mediated trophoblast inflammatory or apoptotic responses, hence altering the recruited and resident maternal immune cells. Decidual macrophages are recruited to the maternal-foetal interface by stromal and trophoblast cells. Here, additionally to their specialized functions, such as decidual homeostasis, placental development and tolerance to the semi-allogeneic trophoblast, they also form a major line of defence against the invading pathogens in the decidua, protecting the fetus from infection [28,29]. In murine models of adult-acquired *T. gondii* infection, these classically-activated macrophages induced by a Th1 cells contribute to parasite killing [30]. However, the data reported here, in agreement with some previous studies, suggest that these cells are also associated with immunopathology and adverse pregnancy outcomes [31]. Our studies demonstrate that maternal *T. gondii* infection has adverse effects on the maternal foetal interface concomittant with inflammation.

We observed a decrease in serum progesterone levels in *T. gondii*-infected pregnant mice. This is in accordance with a previous study that described low levels of progesterone in *T. gondii*-infected pregnant women, proposing that the parasite may also affect progesterone production [14] . Progesterone is synthesized in breast, endometrium, brain, ovaries and placenta. In the latter, it has a key role during pregnancy, since it regulates immune cells, essential for maintenance of pregnancy [10,11,32,33] . In fact, progesterone has an essential role in maternal-foetal immunological interactions, which are fundamental for pregnancy [10,13,32,33]. This hormone has been shown to

contribute to a local Th2-associated cytokine production by murine fetoplacental tissues and, recently, it has been demonstrated to negatively regulate the differentiation of Th cells into Th1 and Th17 [12,34]. Consequently, it is not possible to understand if the reduced levels of progesterone reflect reduced prodution in the placenta secondary to the pathology or if the pathology in the placenta reflects immunological changes at the placenta due to reduced progesterone production.

All infected mice had clear clinical signs of infection and exhibited alterations in their immunological cell composition, including increased splenic neutrophil numbers, relative to non-infected controls. However, not all tissues in all infected mice tested positive for *T. gondii* by PCR. No differences were found between non-pregnant and pregnant mice, when comparing themean parasite load or the percentage of *T. gondii*-positive spleen, lung, liver, heart or kidney. Indeed, there is a significant amount of controversy in whether pregnancy affects parasite number, with some authors suggesting a higher infection susceptibility during pregnancy with other pathogens, such as influenza, hepatitis E, herpes simplex, human immunodeficiency víruses, *Plasmodium falciparum,* and *Listeria monocytogenes*, among others [35,36]. Previous studies in murine models of maternal *T. gondii* infection have demonstrated that mice infected during pregnancy are more susceptible in terms of mortality and this was associated with reduced IFNγ production. Furthermore, this can be ameliorated, by administration of IFNγ or IL-2 [37,38]. These studies did not report the effect of *T. gondii* on maternal parasite loads. Our results demonstrate no differences in the number of mice having detectable levels of parasites in their spleen by PCR.

In agreement with others, a significant reduction in the numbers of CD4+ and CD8+ T cells was observed, in day 14-pregnant animals compared with non-pregnant animals, showing that pregnancy itself leads to an alteration of the immune status namely in the number of T cells[39,40]. As previously reported, *T. gondii* infection induced increased CD4+ and CD8+ T cell numbers in the spleens of mice at day 7 post-infection, , and this was not affected by pregnancy [19,41]. Differential quantification of macrophages and neutrophils was performed by using common macrophage and neutrophil markers ($CD11b^{high}F4/80^{high}Ly6G^{-}$ and $CD11b^{high}F4/80^{-}Ly6G^{high}$ respectively). The analysis of spleen macrophages clearly showed that pregnancy *per se* was able to exert a significant increase on this cell population by day 12 of pregnancy independent of infection. By day 14 of pregnancy, *T. gondii*-infected mice had a further increase in this cell population compared to non-infected non-pregnant mice ($P<0.05$). Neutrophils numbers were increased in mice following infection, but no differences were observed between infected non-pregnant andinfected mice at day 12 or day 14 of pregnancy.

MHCII expression was determined by flow cytometry analysis on mononuclear phagocytes ($CD11b^{high}F4/80^{high}$). MHC II expression on spleen mononuclear phagocytes was upregulated following infection in both non-pregnant mice and day-12 pregnant mice. Further, MHCII expression was upregulated in non-infected day-14 pregnant animals, suggesting that pregnancy itself is able to interfere with the expression of this molecule in mononuclear phagocytes. However, infected day-14 pregnant mice showed a down-regulation of MHCII expression compared with non-infected day-14 pregnant mice. Our observations are in agreement with others. Indeed, it has been shown the ability of *T. gondii* infection to down regulate MHCII gene expression and antigen presentation on macrophages and DCs, thought this has only previously been reported *in vitro* [42-44]. Our results suggest a pregnancy-related mechanism interfering with MHCII expression, and therefore, with antigen presentation during *T. gondii* infection.

Taken together, our data suggest that maternal *T. gondii* infection induces recruitment of inflammatory cells to the foetal maternal interface, which causes delay or damage in the development of placenta. The pathology at the placenta likely contributes to the ability of placenta to produce progesterone, which may have both systemic and local immunological consequences. Furthermore, the ability of pregnancy to interfere with MHCII expression by mononuclear phagocytes may affect T cell responses and, therefore, also the susceptibility of pregnant mice to *T. gondii* infection.

Conflicts of interest

The authors state no conflict of interest.

Acknowledgements

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Fig. 1. Effect of *T. gondii* Me49 infection in the maternal-foetal interface.

(A) Parasite load assessed by quantitative PCR for *SAG-1* in implantation units from mice at day 5 post-infection and day 12 of pregnancy (5 dpi Day-12 pregnancy; decidua/placenta or foetus; n=10) or mice at day 7 post-infection and day 14 of pregnancy (7 dpi Day-14 pregnancy; decidua, Placenta or foetusn=11). Data represent the mean of the parasite load of each tissue, from 3 normal implantation units, from 10 and 11 mice analyzed individually for day 12 and 14 respectively. Horizontal lines correspond to the median value in each groupDetection limit (DL) is indicated by a horizontal dashed line. * $P<0.05$ (statistically significant differences between tissues for day 12 of pregnancy). (B) Morphometric analysis of maternal-foetal interface. Cross-sectional area corresponded to the sum of the maternal component (blood vessels, metrial gland and mesometrial decidua) and foetal component (placenta). Data represent mean areas ± SEM of the mean from the analysis of three slides from each normal implantation units, from three mice analysed individually in each group. + $P<0.05$, (statistically significant differences between days of pregnancy).

Fig. 2: Effect of *T. gondii* Me49 infection in the implantation units development.(A) Number of normal and resorpted implantation units from non-infected mice at day 12 of pregnancy (Ninf Day-12 of pregnancy; n=7) and mice at day 5 post-infection and day 12 of pregnancy (5 dpi Day-12 pregnancy; n=15). (B) Number of normal and resorpted implantation units from non-infected mice at day 14 of pregnancy (Ninf Day-14 pregnancy; n=16) and mice at day 7 post-infection and day 14 of pregnancy (7 dpi Day-14 pregnancy; n=19). (C) Resorption rates for each day of pregnancy. The resorption rate for each mouse consists of the ratio between the number of resorpted units and the total number of implantation units. Data represent the mean.

Fig. 3: Effect of *T. gondii* Me49 infection in the weights of mesometrial decidua, placenta and foetus.

(A) Data represent the weight of each tissue from normal implantation units from non-infected day-14 pregnant mice (Ninf, n=3) and infected day-14 pregnant mice (7 dpi, n=3). (B) Data represent the mean of the means of the weights of each tissue, from all the normal implantation units, from three mice analyzed individually (involving a total of 25 deciduas, 25 placentas and 25 foetus from non-infected day-14 pregnant mice and 21 deciduas, 21 placentas and 21 foetus from mice at day 7 post-infection and day 14 of pregnancy).

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Fig. 4. Histopathological analysis of implantation units from pregnant BALB/c mice infected with *T. gondii* Me49.

(A) Representative image of H&E-stained sections of a resorpted implantation unit from mice at day 7 post-infection and day 14 of pregnancy. (B) Magnification of the inset shown in A. (C-E) Fluorescence images showing the localization of *T. gondii* Me49 expressing yellow fluorescence protein (YFP; green fluorescence). Nuclei were stained blue by DAPI. (C-D) Magnifications of the insets shown in A and B, respectively. (E) Representative image of *T. gondii*-YFP localization at the myometrium from mice at day 7 post-infection and day 14 of pregnancy (magnification of 630). (F, H) Representative image of H&E-stained sections of implantation units from mice at day 7 post-infection and day 18 of pregnancy showing the evidence of necrosis. (G) Magnification of the inset shown in F. (I-J) Fluorescence images showing the localization of *T. gondii* Me49-YFP. Nuclei were stained blue by DAPI. (I) Magnification of the inset shown in H. (J) Magnification of the inset shown in I. White arrows – presence of the parasite. M: Myometrium; MG: metrial gland; MD: mesometrial decidua; PL: placenta; CH: chorion.

Fig. 5. Detection of inflammatory cells by immunohistochemical staining at decidua from BALB/c mice infected with *T. gondii* Me49 at day 14 of pregnancy (A-C) and at day 18 of pregnancy (D-F). (A) Negative control for CD68 staining. (B) Detection of $CD68^+$ cells at the mesometrial decidua from mice at day 7 of infection and day-14 of pregnancy (C) Magnification of the inset shown in B. (D) Negative control for F4/80 staining. (E) Detection of $F4/80^+$ cells at the anti-mesometrial decidua from mice at day 7 of infection and day-18 of pregnancy. (F) Magnification of the inset shown in E**.** BV- blood vessel; MD- mesometrial decidua; PL- placenta.

Fig. 6. Progesterone levels in *T. gondii* Me49 -infected mice at day 14 of pregnancy.

Serum levels were determined by immunoassay. Results correspond to pooled data from: non-infected mice at day 12 of pregnancy (Ninf Day-12 pregnancy, n=8); mice at day 5 of infection and day 12 of pregnancy (5 dpi Day-12 Pregnancy, n=17); non-infected mice at day 14 of pregnancy (Ninf Day-14 pregnancy, n=6) and mice at day 7 of infection and day 14 of pregnancy (7 dpi Day-14 Pregnancy, n=13). Each dot represents an individual mouse. Data are expressed as mean ± SEM. *P<0.05 (statistically significant differences between non-infected and infected mice for each day of pregnancy); Detection limit (DL)=0.25 ng/ml.

Fig. 7. Quantification of parasite load in different tissues from non-pregnant and pregnant BALB/c mice infected with *T. gondii* Me49.

Parasite load was assessed by qPCR for *SAG-1*. Results for spleen are pooled from mice at day 5 of infection and non-pregnant (5 dpi Non-pregnancy, n=19), mice at day 7 of infection and non-pregnant (7 dpi Non-pregnancy, n=10), mice at day 5 of infection and day 12 of pregnancy (5 dpi Day-12 Pregnancy, n=13) and mice at day 7 of infection and day 14 of pregnancy (7 dpi Day-14 Pregnancy, n=11). Results for liver, heart and kidney are pooled from 5 dpi Non-pregnancy (n=10), 7 dpi Non-pregnancy (n=11), 5 dpi Day-12 Pregnancy (n=10) and 7 dpi Day-14 Pregnancy (n=12) groups of mice. Each dot represents an individual mouse. Horizontal lines correspond to the median value in each group. A horizontal dashed line indicates detection limit (DL).

Fig. 8. Spleen cellular composition during pregnancy and *T. gondii* Me49 infection**.**

Results are pooled from 11 non-infected and non-pregnant mice (Ninf Non-Pregnancy); 6 non-infected mice at day 12 of pregnancy (Ninf Day-12 pregnancy); 7 mice non-infected at day 14 of pregnancy (Ninf Day-14 pregnancy); 9 mice at day 5 of infection and non-pregnant (5 dpi Non-pregnancy); 7 mice at day 5 of infection and day 12 of pregnancy (5 dpi Day-12 Pregnancy); 9 mice at day 7 post-infection and non-pregnant (7 dpi Non-pregnancy); and 8 mice at day 7 post-infection and day 14 of pregnancy (7 dpi Day-14 Pregnancy). Data represents the mean+SEM values from mice analyzed individually in each group. *P<0.05, ***P<0.001 (statistically significant differences between non-infected and infected mice for each day of pregnancy). ++P<0.01 (statistically significant differences between pregnant and non-pregnant mice for each day of pregnancy). • P<0.05 (interaction effect between infection and pregnancy; statistical

significant difference between non-infected non-pregnant mice and infected pregnant mice at day 14 of pregnancy).

Fig. 9. MHCII expression in spleen mononuclear phagocytes in non-pregnant and pregnant mice with or without *T. gondii* Me49 infection.

(A) MHCII expression by CD11b^{high}F4/80^{high} spleen cells. Results are pooled from: non-infected and non-pregnant mice (Ninf Non-Pregnancy, n=4); non-infected mice at day 12 of pregnancy (Ninf Day-12 pregnancy, n=4); non-infected mice at day 14 of pregnancy (Ninf Day-14 pregnancy, n=6); mice at day 5 of infection and non-pregnant (5 dpi Non-pregnancy, n=10); mice at day 5 of infection and day 12 of pregnancy (5 dpi Day-12 Pregnancy, n=11); mice at day 7 post-infection and non-pregnant (7 dpi Non-pregnancy, n=10); and mice at day 7 post-infection and day 14 of pregnancy (7 dpi Day-14 Pregnancy, n=10). Data represents the mean+SEM of the median of fluorescent intensity (MFI) values for MHC-II from mice analyzed individually in each group. *P<0.05, ***P<0.001 (statistically significant differences between non-infected and infected mice for each day of pregnancy). +P<0.05 (statistically significant differences between pregnant and non-pregnant mice for each day of pregnancy).

Table 1

T. gondii infection rate assessed by quantitative PCR for SAG-1 in implantation units at day 5 and day 7 post-infection, from pregnant BALB/c mice.

Tissue	Day 5			Day 7		
	+	-	%	+	-	%
Decidua/Placenta	14	16	47	ND	ND	ND
Decidua	ND	ND	ND	8	22	30
Placenta	ND	ND	ND	7	28	20
Fetus	13	15	46	12	23	34

+: number of decidua/placenta, decidua, placenta or embryo with parasite load above detection limit; -: number of decidua/placenta, decidua, placenta or embryo with parasite load below limit detection; %: Percentage of tissues where it was possible to quantify the parasite load. Detection limit: 27 parasites per 2000 ng DNA; nd: not done.

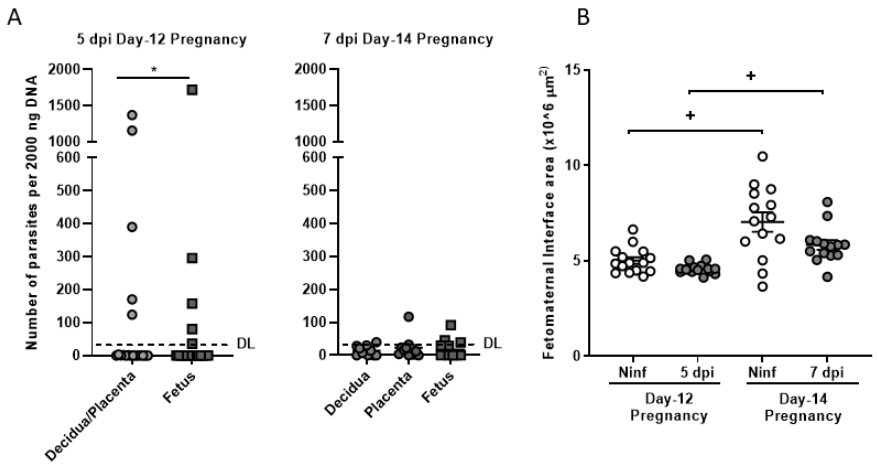
Table 2

T. gondii infection rate assessed by quantitative PCR for SAG-1 in tissues at day 5 and day 7 post-infection, from non-pregnant and pregnant BALB/c mice.

Tissue	Day 5						Day 7					
	Non-pregnant			Pregnant			Non-pregnant			Pregnant		
	+	-	%	+	-	%	+	-	%	+	-	%
Spleen	13	6	68	6	7	46	8	2	80	8	3	73
Lung	2	8	20	4	6	40	3	8	27	6	6	50
Liver	3	7	30	3	7	30	5	6	45	5	8	42
Heart	1	9	10	5	5	50	2	9	18	1	11	8
Kidney	2	8	20	2	8	20	5	6	46	4	8	33

+: number of mice with parasite load above detection limit; -: number of mice with parasite load below detection limit; %: Percentage of animals whereas it was possible to quantify the parasite load. Detection limit: 27 parasites per 2000 ng DNA.

Figure 1



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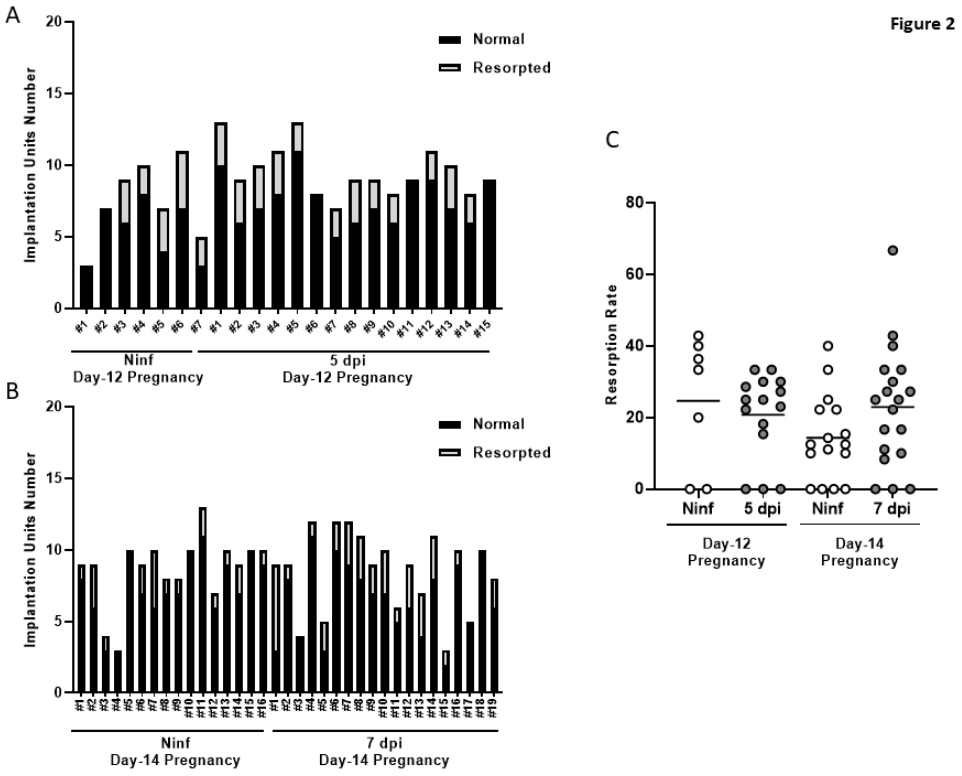

Figure 2

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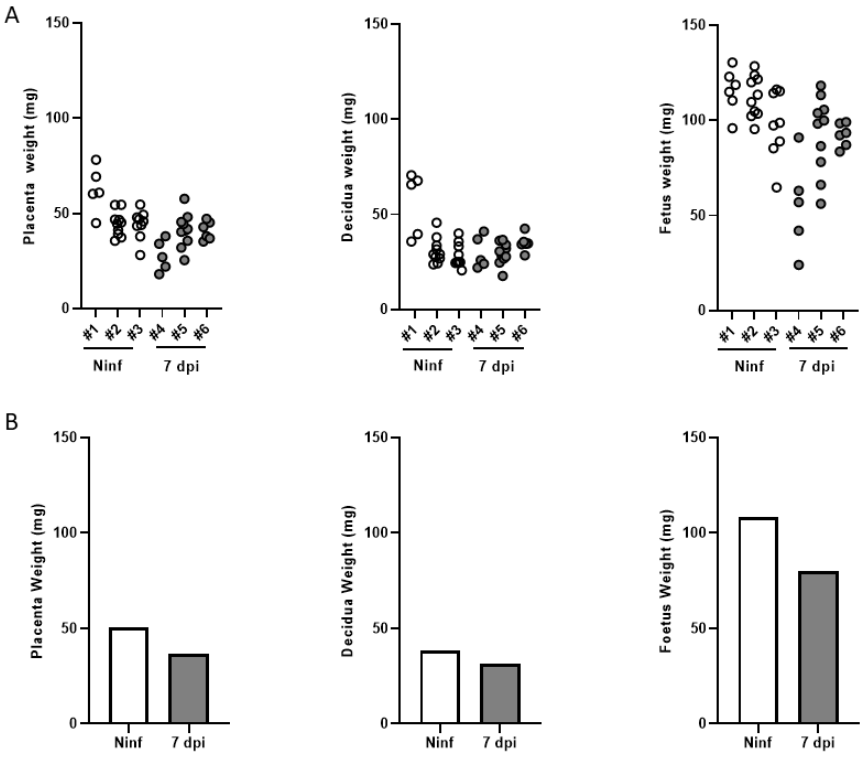

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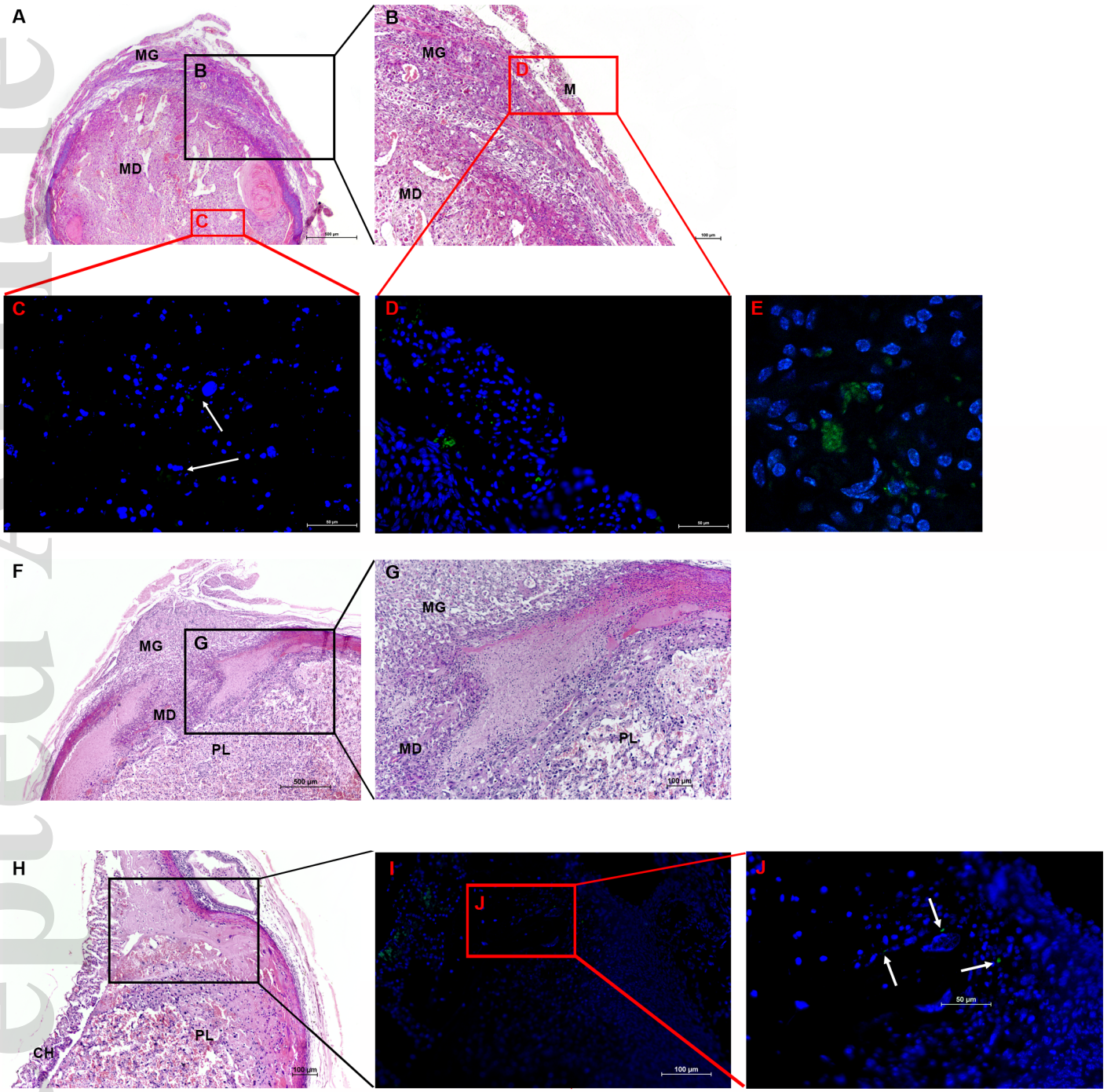

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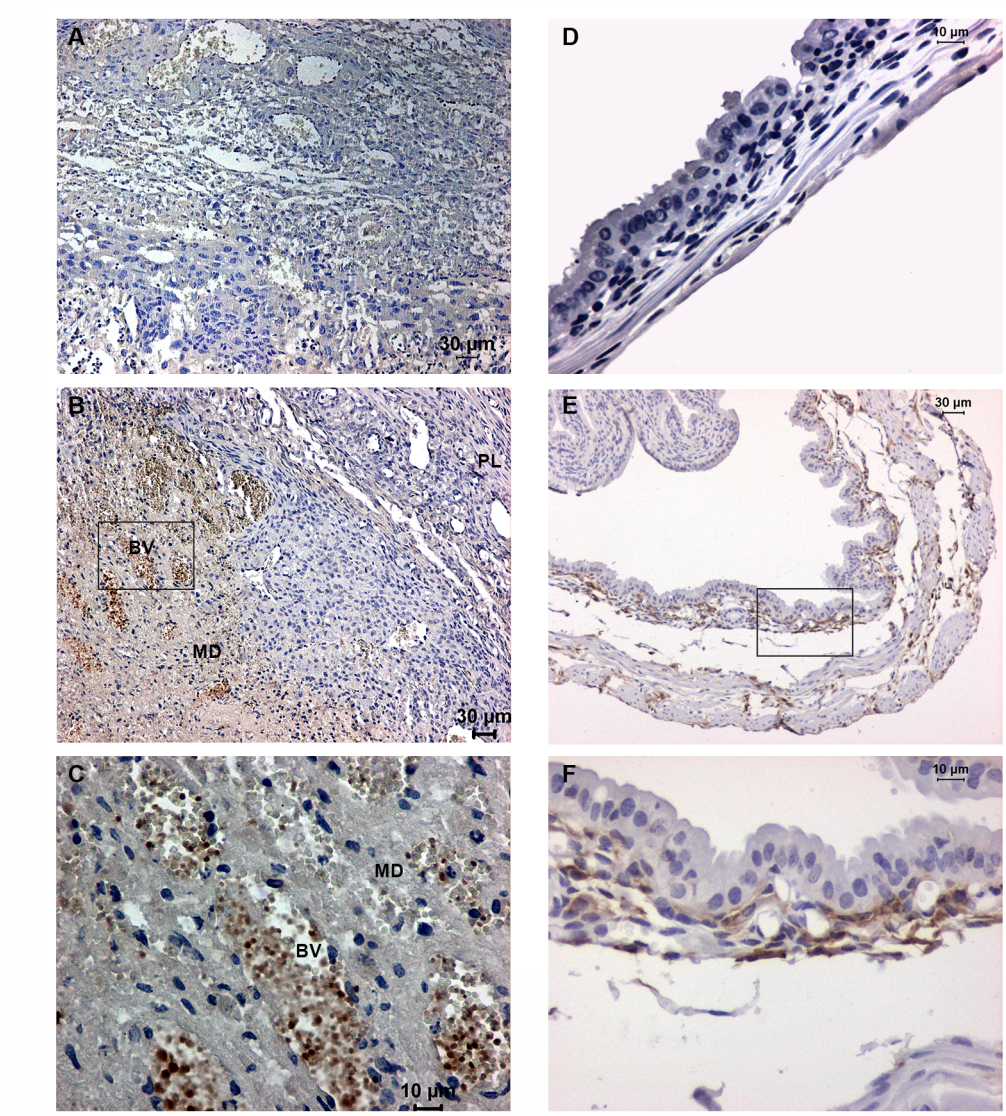

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Figure 6

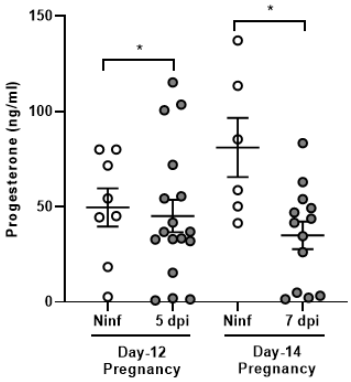

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Figure 7

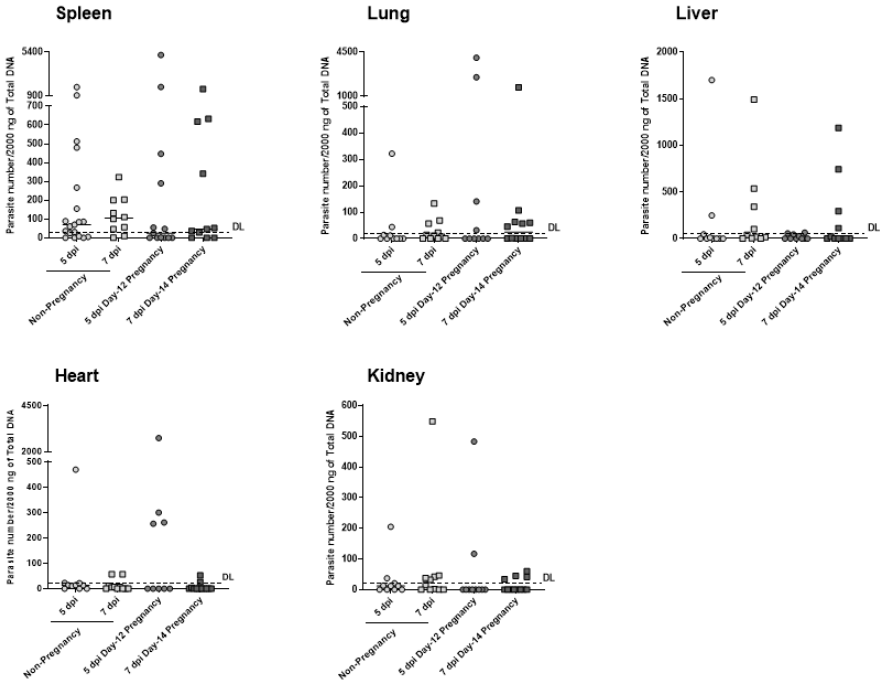

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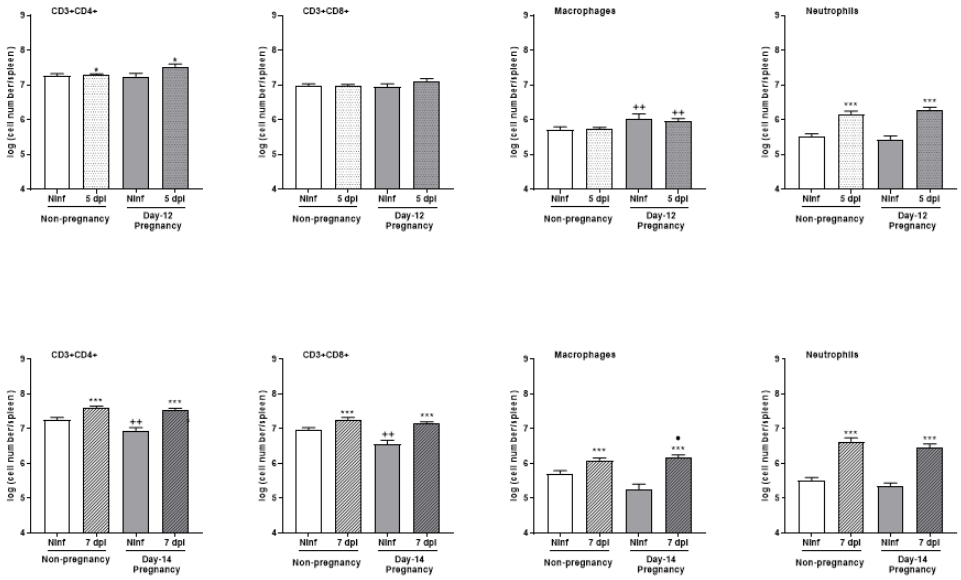
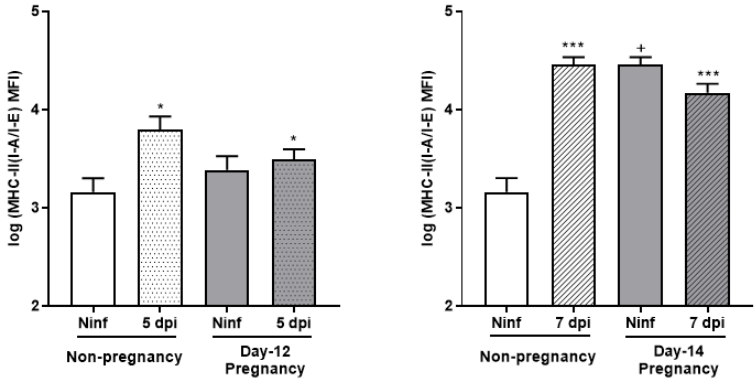
Figure 8



pim_12690_f8.tif

Figure 9



pim_12690_f9.tif