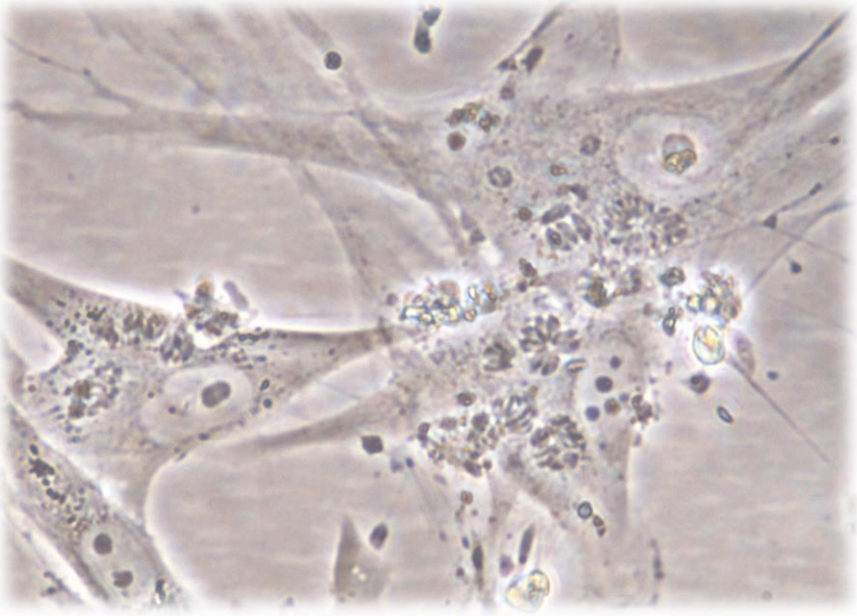

Determination of macrophage activation profile and T cell populations in the context of congenital toxoplasmosis

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Resumo

O *Toxoplasma gondii* é um parasita humano bem-sucedido que infecta milhões de pessoas em diversos países. Em indivíduos imunodeprimidos, o parasita causa uma doença severa com diversas manifestações clinicas. No entanto, os indivíduos imunocompetentes são assintomáticos, podendo este parasita persistir no hospedeiro por um longo período de tempo. Nas mulheres grávidas, uma primo-infeção com *T. gondii* pode levar a severas manifestações clinicas, como por exemplo, ao aborto ou a malformações do feto, como coriorretinite, hidrocefalia e encefalites. Assim, é importante entender os mecanismos e as células imunológicas envolvidas na resposta à infeção com este parasita tanto a nível sistémico como a nível da interface feto-materna. Neste trabalho, tivemos como principais objetivos: determinar o efeito da infeção nos parâmetros básicos da função reprodutiva durante a gravidez; quantificação do *T. gondii* em vários tecidos e células; e a análise de neutrófilos, macrófagos e populações de células T nas células esplénicas e do exsudado peritoneal. Para a infeção, usou-se uma estirpe do tipo II de *T. gondii* e murganhos Balb/c gestantes e não gestantes. Análises macroscópicas foram realizadas para avaliar os locais de implantação. Para a quantificação do parasita foi realizada a técnica de Real-Time PCR quantitativo. As populações de neutrófilos, macrófagos e células T foram avaliadas por citometria de fluxo. Os resultados obtidos neste trabalho demonstraram, que após 5 dias de infeção com *T. gondii* Me49 não ocorreu a disseminação do parasita desde do local da infeção (cavidade peritoneal) até ao pulmão, coração e rim. Foi no entanto, possível confirmar a disseminação sistémica do parasita após 7 dias de infeção. Neste trabalho foi possível efetuar a deteção e quantificação de células do exsudado peritoneal - *T. gondii*$^{+}$ pela técnica de citometria de fluxo para 5 e 7 dias após infeção. A análise da expressão de moléculas MHCII por citometria de fluxo, indicou um aumento significativo da expressão de MHCII nos *small peritoneal macrophages* comparativamente aos *large peritoneal macrophages*, nos murganhos não gestantes ou gestantes, não infetados. Foi demonstrado que após 7 dias de infeção a população dos *large peritoneal macrophages* é inexistente em murganhos não gestantes ou gestantes. Ao nível do baço, foi possível verificar uma diminuição significativa da percentagem da população CD11b^{+}F4/80^{+}MHCII^{+} e uma diminuição na expressão de MHCII nestas células, no dia 12 de gravidez durante a infeção com *T. gondii.* Estes resultados sugerem que *T. gondii* poderá influenciar vias dependentes da gravidez, interferindo com a capacidade da apresentação antigénica.

Palavras-chave

Toxoplasmose congénita, *Toxoplasma gondii*, Macrófago, Neutrófilo, Célula T, MHCII, Arginase-1, NOS2, Balb/c, Gravidez

Abstract

Toxoplasma gondii is a successful human parasite, which infects millions of people in several countries in the world. In immunosuppressed individuals, the parasite causes a severe disease with many clinical manifestations. However, immunocompetent individuals are asymptomatic, but this parasite persists for long periods of time. In pregnant women, *T. gondii* primo-infection can lead to a variety of severe clinical manifestations, like abortion or fetal malformations, as chorioretinitis, hydrocephalus and encephalitis. Therefore, is important to understand the immunological mechanisms and cell players involved in *T. gondii* immune response at the systemic level and maternal-fetal interface. In this work, the principal aims were: to determine the effect of infection in basic parameters of reproductive function in pregnancy; *T. gondii* quantification in tissues and cells; and neutrophil, macrophage and T cell populations analysis in spleen and peritoneal exudate cells. Infection using a type II strain of *T.gondii* of pregnant and non-pregnant Balb/c mice, allowed the follow-up of pregnancy. Macroscopic analyses were performed to evaluate implantation sites. The quantification of the parasite was performed by quantitative Real-Time PCR. Neutrophil, macrophage and T cell populations were evaluated by flow cytometry analysis. The results obtained, indicated that 5 days of infection were not sufficient for the parasite dissemination from the local of infection (intraperitoneal cavity) to the lung, heart and kidney. However, it was possible to confirm systemic parasite dissemination after 7 days of infection. The data obtained indicated that flow cytometry technique is sensible to detect peritoneal exudate cells-*T. gondii*$^{+}$ at 5 and 7 days post-infection. MHCII expression was significantly increased in small peritoneal macrophages compared to large peritoneal macrophages from uninfected non-pregnant or pregnant mice. It was demonstrated that large peritoneal macrophages are inexistent after 7 days of *T. gondii* infection in non-pregnant or pregnant mice. At spleen level, it was found a significant decrease in the percentage of CD11b^{+}F4/80^{+}MHCII^{+} and a MHCII^{+} expression down-regulation in CD11b^{+}F4/80^{+}, at day 12 of pregnancy during *T.gondii* infection. These results, suggest that *T. gondii* may be influencing pregnancy-dependent pathways, interfering with the capacity of antigen presentation.

Keywords

Congenital toxoplasmosis, *Toxoplasma gondii*, Macrophage, Neutrophil, T cell, MHCII, Arginase-1, NOS2, Balb/c, Pregnancy

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List of Abbreviations

Abs – Antibodies

AIDS – Acquired immune deficiency syndrome

Arg-1 – Arginase- 1

Balb/c - Balb\cByJ

CTLs- Cytotoxic T cells

DCs - Dendritic cells

dDCs - Decidual dendritic cells

DMEM - Dulbecco's modified eagle medium

DNA - Deoxyribonucleic acid

dNK - Decidual Natural Killer

E- Day of gestation

ELISA- Enzyme-linked immunosorbent assay

EVT – Extravillous trophoblast cells

FBS - Fetal bovine serum

FMI- Maternal-fetal interface

FP- Fetoplacental units

HFF- Human foreskin fibroblasts

Ig - Immunoglobulin

IL - Interleukine

IFN-ϒ – Interferon gamma

Inf – Infected

ip –Intraperitoneal

LAMP- Loop-mediated isothermal amplification

LPM - Large peritoneal macrophages

MFI – Mean fluorescence intensity

MHC- Major Histocompatibility complex

MIP-Macrophage inflammatory protein

NK – Natural Killer

dNK- Decidual natural killer cells

NO – Nitric oxide

NOS2 or iNOS – Inducible nitric oxide synthase

PBS – Phosphate-buffered saline

PCR - Polymerase chain reaction

PEC - Peritoneal exudate cells

q-PCR - Quantitative real-time polymerase chain reaction

ROI - Reactive oxygen intermediates

SAG-1 – surface-antigen 1

SPM - Small peritoneal macrophages

T. gondii – *Toxoplasma gondii*

Th - T helper cells

TLR - Toll-like receptor

TNF- Tumor necrosis factor

Treg – Regulatory T cells

TGF- β - Transforming growth factor beta

TOXO – *Toxoplasma gondii*

YFP - Yellow Fluorescence protein

Introduction

1. *Toxoplasma gondii*

T.gondii is an important group of obligate intracellular protozoan of the *Phylum Apicomplexa,* subclass coccidian, order Eimeriorina and family toxoplasmatidae (1,2). The cat constitutes the definitive host and other warm-blooded animals, such as humans, are the intermediate hosts (3). The first report of *T.gondii* occurred in 1908 by Charles Nicolle and Manceuax using tissues of hamster-like rodent, *Ctenodactylus gundi*, which was used in leishmaniasis research in the North Africa. Initially, they believed that the parasite found was Leishmania. However, they realized that it was a new organism and named it as *T. gondii* (4). This name derived from its crescent-shaped morphology of tachyzoite the Toxo means “arc form” in Greek and gondii derived from the first found host (5). At the same time another scientist, Alfonso Splendore, discovered a similar parasite in rabbits in Brazil (6).

In the next years, *T. gondii* was found in several other hosts, mainly in avian species (7). However, only in 1937 Sabin and Olitsky isolated, for the first time, viable *T. gondii* from the animal host and in 1941, they proved that human *T. gondii* was identical to the parasite present in other hosts (8). Later in 1940’s antibodies were detected and found to kill extracellular but not intracellular tachyzoites (8,9). In 1970 the life cycle of this parasite was discovered, allowing a better understanding of *T.gondii* transmission (10). In the course of 1980’s and 1990’s various methods, were developed to recognize genetic differences between *T. gondii* isolates from animals and humans (11–14). Many advances have been achieved due to *T. gondii* genes mapping helping in the search of better antigens for the diagnosis, prevention and the knowledge of disease mechanisms (15).

T.gondii infects millions of people in the world and is the most successful human parasite (16). This parasite can cause severe clinical manifestations specifically in two distinct cases: immunodeficient individuals and primo-infected pregnant women. The primo-infection in pregnant women can lead to a congenital toxoplasmosis, with diverse clinical manifestations such as hydrocephalus, macrocephaly, encephalitis, seizures, calcifications, chorioretinitis, blindness in the fetus and may also result in intrauterine death and spontaneous abortion (17).

2. Parasite morphology and Life-cycle

T. gondii present three infectious stages, such as sporozoites which disseminate in the environment within oocysts, tachyzoites which facilitate expansion during acute infection and bradyzoites which maintain the chronic infection being all the three stages haploid forms (Fig.1 ; 17,18).

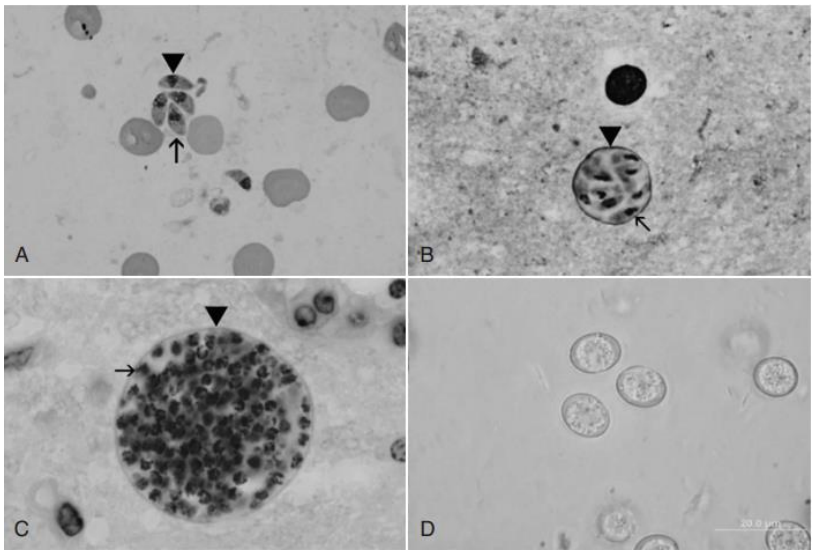

Fig. 1- Life cycle stages of *T. gondii.* A- Tachyzoites; B-Small tissue cysts; C- Tissue cysts; D- Unsporulated oocysts (2).

The life cycle of this parasite has both asexual (schizogony) and sexual (gametogony) reproduction, which occur in the small intestine of the definitive host (Fig.2; 19). Tachyzoites and bradyzoites divide asexually, while sporozoites are the parasite forms obtained after sexual reproduction (21).

The sexual phase or gametogony occurs throughout the small intestine and in the ileum. The microgamete (the male gamete) is biflagellate and fertilizes the macrogamete (the female gamete) within the enterocyte. The fertilization initiates oocysts wall formation, and the product of this sexual reproduction is the zygote. The oocysts are released into the intestinal lumen by rupture of the epithelial cells and thereafter excreted in cat feces (18). The oocysts release, from the epithelial cells, takes 3 to 10 days after ingesting of bradyzoites, >18 days after ingesting of sporulated oocysts and >13 days after ingesting of tachyzoites (22). Sporogony occurs outside of the host within 1-5 days after excretion, depending on atmospheric conditions, such as aeration, humidity, and temperature. Each oocyst contains two sporocysts, which contain four sporozoites (18).

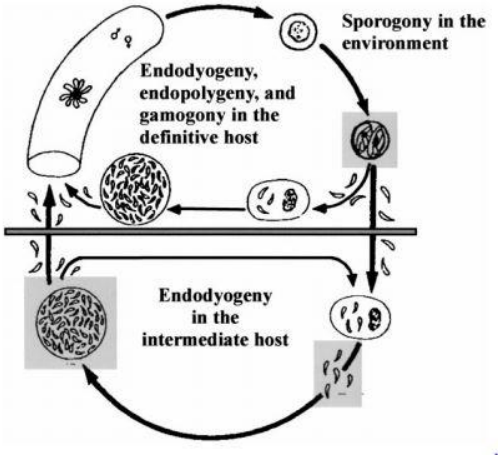

Fig. 2- The different infectious stages of *T.gondii* life cycle, i.e. tachyzoites, bradyzoites contained in tissues cysts and sporozoites contained in sporulated oocysts (23).

The asexual phase can occur after ingesting the tissue cysts or infectious oocysts (23). After this ingestion by an intermediate host (such as humans), the sporozoites are released from the oocysts, penetrate the intestine, invade a range of different cell types and multiply asexually, as tachyzoites (24). This parasite form is crescent or oval and is present during acute infection and multiply rapidly in a wide variety of nucleated cell hosts, within the parasitophorous vacuole (21,25). The parasite invasion is due to an actin-based motility, being the formation of this vacuole derived from the host cell plasma membrane and secretion of parasite proteins (26). The tachyzoites divide every 6-9h by a process denominated endodyogeny, in which daughter cells form multiply within the mother cell (27). After replication, the host cells are disrupted, and the tachyzoites disseminated through the bloodstream, infecting many tissues, including the CNS, eye, skeletal, and heart muscles, placenta leading that way to diverse clinical manifestations (25). Finally in response to environmental conditions, the tachyzoites converts into bradyzoites forming the tissue cysts, present in different organs and tissues (Fig. 3; 27). Bradyzoites replicated slowly by endodyogeny, they are morphologically identical to tachyzoites and are considered as the latent form able to remain in the tissues for the entire host life (18,21,25).

Depending on several environment factors, the tissue cysts can rupture and bradyzoites forms can convert to tachyzoites resulting in the reactivation of infection. This reactivation may result in various clinical manifestations, as toxoplasmic encephalitis, an opportunistic disease which can occur in immunodeficient patients (18).

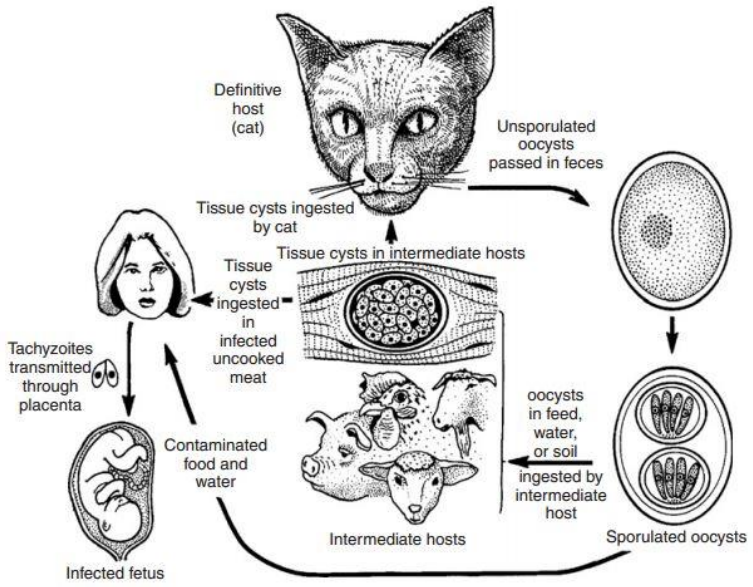

Fig. 3- The life cycle of *T. gondii* (2).

3. Transmission

The transmission of *Toxoplasma gondii* may occur in three different ways: Tissue cyst transmission, congenital transmission, and oocyst transmission. However, it can also occur by organ transplantation or laboratory infection (Fig. 4). In the next subtitles it each of the routes of transmission will be exposed.

3.1. Tissue cyst transmission

In 1954 Weinman and Chandler suggested that transmission could occur through the ingestion of undercooked meat. Later on, in 1960, Jacobs *et al*. provided evidence to support this idea, demonstrating that *T. gondii* was resistant to proteolytic enzymes due to *T. gondii* forms derived from cysts (29). Nowadays, we know that the primo-infection of cats can occur by eating infected prey, such as rodents, birds, initiating the sexual reproduction and production of oocysts in small intestine (30). Although, intermediate hosts can also acquire toxoplasmosis via ingestion of tissue cysts (20)

Intermediate hosts, such as pigs, sheep, and goats, can present tissue cysts in muscle and brain. Therefore, Human can acquire the infection with *T. gondii* through the ingestion or handling of undercooked or raw meat from infected animals (25). After ingestion, the cyst wall can usually suffer a rupture by proteolytic enzymes in the stomach, occurring the release of bradyzoites, which are resistant to proteolytic digestion, and therefore they survive and initiate the infection in the small intestine (20).

3.2. Congenital transmission

In 1939, three pathologists, Wolf, Cowen and Paige, identified the first case of Human congenital toxoplasmosis. They have then reported that the *T.gondii* infection was associated with encephalomyelitis, hydrocephalus, chorioretinitis and encephalitis in the developing fetus (31). Later on, these clinical manifestations were found in other animals species, such as sheep, goats, and rodents (32).

3.3. Oocysts transmission

For many years, the infection with *T. gondii* has been closely connected to carnivorism or congenital transmission. However, doesn't explaining the widespread infection in vegetarians and herbivores. Therefore, in 1965, Hutchison discovered, the oocysts, the parasite form present in the cat feces (33). This new form was able to transmit the infection to mice and other intermediate hosts, being stable and able to survive for up to 12-18 months in the environment, depending on the climatic conditions (34).

The cat is able to excrete up 10 million of oocysts per day, which sporulates in 1 to 5 days, becoming infective (19). The oocyst form can contaminate soil, water, and food, like vegetables and fruits. Therefore, transmission can occur by the ingestion of contamined water, vegetable and fruits (35).

3.4. Organ transplantation and laboratory infection

Another route of transmission, which is less frequent is by the organ transplantation. Heart, heart-lung, kidney, liver, or liver-pancreas transplantation, can lead to the transmission of *T. gondii* by a seropositive donor to a seronegative receptor (25). In the laboratory, the technician can be infected by contacting with contaminated needles, glassware or infected tissues or blood derived products (25).

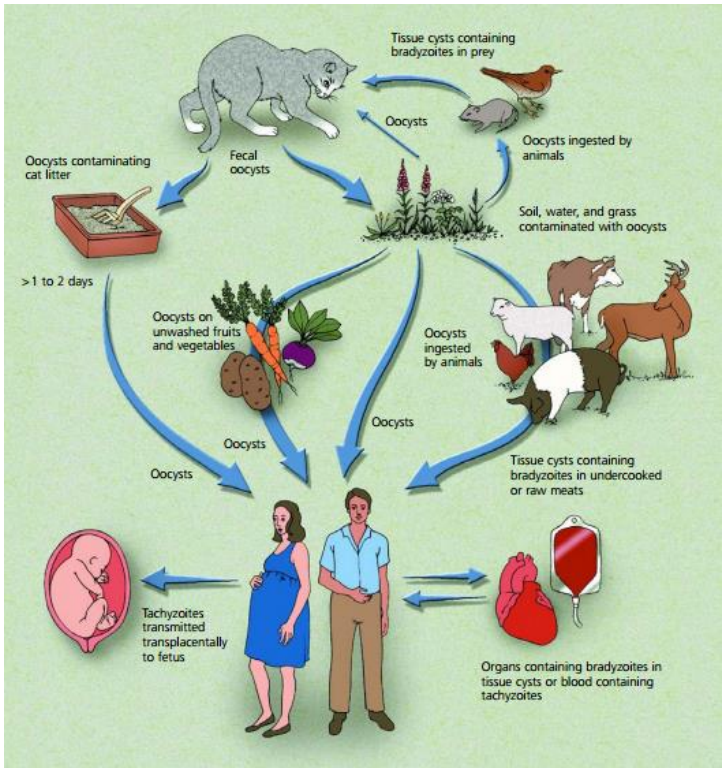

Fig. 4- Different pathways of *T. gondii* infection and transmission (36).

4. Animal hosts

T. gondii can cause infection, a severe disease and even mortality in diverse species of domestic and wild animals (37). In livestock animals, such as pigs, sheep and goats were, frequently, observed tissue cysts of *T.gondii* (23). In sheep and goats, the parasite may cause embryonic death and resorption, fetal death, abortion, stillbirth and neonatal death (38). However, in the case of cattle and horses, both presented resistance to the parasite (39). *T. gondii* infection is also very frequently in sea mammals, such as seals, dolphins, sea lions and others (37). Clinical toxoplasmosis has also been reported in several animals: carnivores (chinchillas, mink, ferrets, foxes, raccoons, skunk and black bears), wild pigs, wild felids, small mammals (rats, mice, rabbits, guinea pigs, squirrels), monkeys, marsupials and birds (38).

5. Humans infection

In immunocompetent individuals the primo-infection with *T.gondii* is asymptomatic, but about 10 % of infected individuals became symptomatic, with the development of lymphadenopathy (40), accompanied by fever, malaise, night sweats, myalgias, sore throat, maculopapular rash, abdominal pain, hepatosplenomegaly and small numbers of atypical lymphocytes (41).

In immunosuppressed patients, the clinical manifestation is more severe with splenomegaly, polymyositis, dermatomyositis, chorioretinitis, myocarditis, pneumonitis, hepatitis, encephalitis and multi-system organ failure (42). Ocular toxoplasmosis is the most common cause of chorioretinitis (43) and can be acquired congenitally, but it can also be developed at the post-natal period or can be a result of infection reactivation in immune-compromised and/or pregnant individuals (44). The toxoplasmic chorioretinitis cause decreased vision, blindness or glaucoma. (43). In the case of acute chorioretinitis symptoms like blurred vision, scotoma, pain, photophobia, epiphora or loss of central vision may occur (41). Encephalitis is the most frequent manifestation of *T.gondii* disease characterized by a unifocal or multifocal lesion at the central nervous system in immunosuppressed individuals (45,46). The symptomatology consists of headache, fever, psychomotor or behavioral changes, confusion, lethargy, hemiparesis, seizures, ataxia and cranial nerve palsies (46). AIDS positive individuals represent the majority of immunosuppressed patients exhibiting this clinical form (47).

5.1. Congenital toxoplasmosis

Congenital toxoplasmosis can occur when a woman acquires *T.gondii* infection for the first time during pregnancy. The infection occurs through the transplacental passage of the tachyzoites stage, thereby infecting the fetus (vertical transmission) (48). In the case of immunocompetent women that had been infected previously to their pregnancy, they are rarely reinfected (48). Although, it is described that reactivation of *T. gondii* latent infection occurs in immunocompromised women during pregnancy (49).

The severity and incidence of congenital infection depends the amount of weeks of pregnancy in which the woman acquires the infection. In the first weeks the risk of transmission is low (5-25%) but the offspring presents severe clinical signs, which can result in spontaneous abortion, hydrocephaly, and mental retardation. Otherwise, in later weeks of pregnancy the risk of transmission increase (to 90%), but with less

clinical signs, such as chorioretinitis and potential blindness (Table 1; 19,49). Although some other clinical manifestations can occur, including miscarriage, fetal developmental retardation, encephalitis, microcephaly, intracranial calcifications, strabismus and epilepsy (51).

The global maternal-fetal transmission rate in immunocompetent women who acquires primary *T. gondii* infection in pregnancy is approximately 29% (52) and in Europe, congenital toxoplasmosis affects normally 1 to 10 in 10 000 newborn babies (53). These numbers indicate that *T. gondii* infection is a worldwide problem and more research work is needed to understand parasite transmission and how the mother immune response can be determinant in controlling congenital toxoplasmosis.

Table 1 – Risk of *T. gondii* congenital infection in different gestational age and development of clinical signs in the infected offspring. Adapted from (49).

Gestational age at maternal seroconversion (weeks)	Risk of congenital infection (%)	Development of clinical signs in the infected offspring (%)
13	6	61
26	40	25
36	72	9

NOTE: this analysis was performed with 603 women, whose *T. gondii* infection was documented to have occurred during gestation.

6. Epidemiology

T. gondii infects approximately 25 to 30% of the human population in different areas of the world (Figure 6; 24). The seroprevalence depends on various factors, such as the environmental temperature. So in temperate countries, the range varies from 10 to 50% and in tropics countries the range reaches over 80% (54). The lower seroprevalence ranges 10 to 30% have been reported in South East Asia, in Sahelian countries of Africa, as well as in cold climate areas such as Northern Europe and North America. However, moderate seroprevalences between 30 to 50% have been observed in countries of Southern and Central Europe, but high incidences have been observed in Latin America and tropical African countries (55).

In the United States, a study of National Health and Examination Nutrition Survey (NHANES) demonstrate that *T. gondii* incidence decreased in U.S.-born persons aged between 12 to 49 years, from 14.1 % between 1988 to 1994 and to 9% in 1999 to 2004 (56). Additionally, recent studies have shown that prior infection to pregnancy is

common, however when congenital toxoplasmosis occurs as prior infection is relatively uncommon and estimated from 400 to 4000 cases per year of this disease (57).

In Europe, it is observed a declining in the *T. gondii* seroprevalence in pregnant women. In France, the seroprevalence in pregnant women were about 80% in the early 1960's, around 66% in the 1980's, 54% in 1995 and 44% in 2003. At the same time, the average age rate of pregnant women enhanced (58). In the Netherlands, the seroprevalence declined from 35,2% in 1995-1996 to 18,5% in 2006- 2007 in women of reproductive age (59).

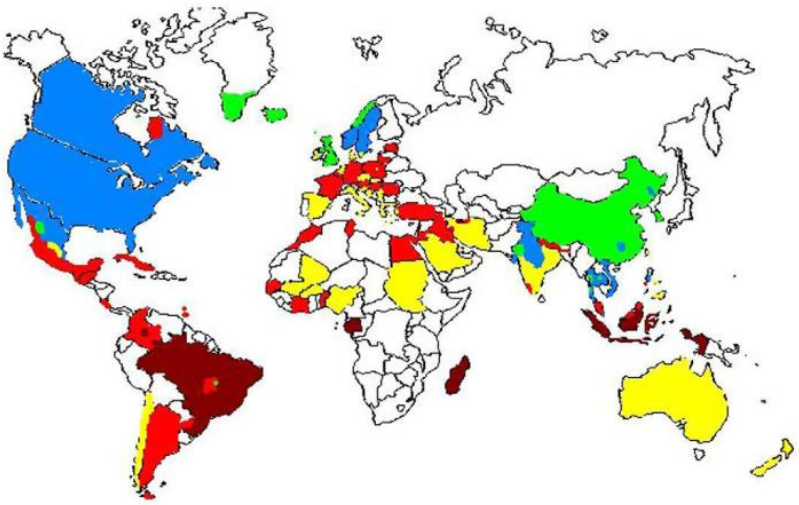

Fig. 5- Global status of *T. gondii* seroprevalence .Dark red corresponding to prevalence about 60%, light red to 40-60%, yellow 20-40%, blue 10-20 % and green < 10% of prevalence. The white zones equal absence data (60).

Toxoplasmosis can also occurs among worldwide laboratory workers. A study based on a total of 47 laboratory-acquired cases have reported 81% of them as symptomatic cases (61). Many factors can affect seroprevalence in humans in different countries as climatic factors, influencing the survival of oocysts in the environment. Several anthropogenic factors including economic, social or cultural habits, dietary habits (hand washing, method of cooking meat, vegetable cleaning and kinds of meat or vegetables consumed, etc.), quality of water and sanitation coverage (55). However, in the case of infection in Europe, for instance, the high prevalence was justified by the choice of people for eating raw or undercooked meat. Nevertheless, high prevalence in Central America and other developing countries has been attributed to the low socio-economics status and frequency of stray cats in a climate favouring survival of oocysts (62).

Another reason for the difference in prevalence in the several countries is the existence of three different genotypes, designated type I, II and III. Type I strain is

related to high-level virulence in mice and humans, and has been reported in patients with ocular toxoplasmosis, congenital disease, and AIDS patients. Type II is considered nonvirulent for mice but most commonly associated with human's infections in Europe and North America reported in patients with congenital diseases and AIDS patients. Type III is nonvirulent for mice, but it is the most common strain in animals and detected in lower degree in humans (63).

In conclusion, the parasite infects billions of people worldwide and the disease constitutes a public health problem. Therefore, it is necessary more and regular diagnosis in susceptible patients (pregnant and immunosuppressed people) and the search of more efficient treatment without secondary effects.

7. Diagnosis

The diagnosis of *T.gondii* can accomplish the three main assays:

- **Serological assays**: is the primary method of diagnosis consisting on the detection of specific antibodies to *T. gondii.* For the IgG antibodies detection the test used is the Dye test (DT), the enzyme-linked immunosorbent assay (ELISA), the Modified agglutination test (MAT), the indirect fluorescent antibody test (IFAT). The test used to measure the IgM antibody are double-sandwich or capture IgM-ELISA kits, the IFAT and the IgM-ISAGA. This test is still used by most laboratories to determine if a patient has been infected recently or in the distant past. Thus, IgM antibodies can appear earlier in patients with primo-infection, but decline more rapidly than IgG antibodies do. IgA antibodies can be detected in sera of infected adults or congenitally infected infants by the ELISA method. IgA antibodies can persist for many months or more than a year in the patient. Lastly, the IgE antibodies may be detectable by ELISA in congenitally infected infants, children with congenital toxoplasmic chorioretinitis and acutely infected adults. The period of IgE seropositivity is shorter compared to IgM or IgA antibodies being useful for identifying recently acquired infections (64). Although the diversity of serological tests, it's difficult to estimate when infection occurred in the case of maternal infection being dificult to manage the risk to the fetus properly. Recent assays have been proposed as the association of a sensitive test detecting *T.* gondii-specific IgM antibodies and allowing the measurement of the avidity of *T.gondii* IgG antibodies constituing a predictive assay to determine the time of infection. This assay has been denominated VIDAS avidity assay (65). Other recent methods, such as flow

cytometry analyses allow the detection of *T. gondii* antibodies, and proved to be quantitative and highly sensitive as compared with commonly used assays (66).

- **Molecular assays**: The polymerase chain reaction (PCR) can detect infection by amplification of the *T. gondii* DNA in various body fluids (as amniotic fluids or cerebrospinal fluid) and tissue samples. This technique is useful in diagnosing congenital, cerebral, ocular and disseminated toxoplasmosis (67–69). Other recent and promising method is the loop-mediated isothermal amplification (LAMP). This approach is highly specific, sensitive and very useful in the diagnosis of parasite infections. Using a diverse type of samples like blood samples, water and urine (70,71). The LAMP assay may represent a useful and practical tool for the current diagnosis and therapeutic evaluation of human toxoplasmosis (71).

- **Image assay**: it is frequently recommended ultrasound for women with suspected or confirmed acute infection acquired during or shortly before gestation. This imaging assay shows the presence of fetal abnormalities including splenomegaly, hydrocephalus, brain or hepatic calcification, and other lesions (49). To detect cerebral toxoplasmosis is actually used the magnetic resonance imaging (MRI), computed tomography (CT) or the positron emission tomography (PET) scans. These assays allow the monitorization of cerebral toxoplasmosis treatment (72,73).

8. Treatment

The current therapy of toxoplasmosis is based on drugs proved to be active against tachyzoites (the parasite form presents in the acute stage of toxoplasmosis), but not active to eliminate the tissue cysts stage (parasite form of chronic toxoplasmosis). Nowadays, the treatment recommended for acute toxoplasmosis is a combination of drugs: pyrimethamine and sulfadiazine plus folic acid (73; Table 2). The treatment of congenital toxoplasmosis may be applied at the prenatal period to prevent the risk of mother-to-child transmission and at the postnatal period. Thus, if the maternal infection is detected before 18 weeks of gestation, the treatment recommended is spiramycin, to prevent vertical transmission of the parasite. After 18 weeks of pregnancy, the treatment recommended is pyrimethamine, sulfadiazine plus folic acid, avoiding a reduction of platelet count (74; Table 2).

Table 2 – Toxoplasmosis manifestations and their treatment indications. Adapted from (76).

Syndrome	Treatment[a]
1. Asymptomatic infection: latent infection detected by a serological test being positive	Not required
2. Adenopathy, fever or malaise in the immunocompetent host	Not required[b]
3. Disseminated disease (i.e. CNS, heart or hepatitis) in the normal host	PYR/SULFA
4. Infection during pregnancy	SPR - PYR/SULFA[c]
5. Congenital toxoplasmosis	PYR/SULFA[d]
6. Ocular toxoplasmosis	PYR/SULFA and Steroids
7. Infection in immunocompromised hosts	
(A) AIDS	PYR/SULFA
(B) Transplantation	PYR/SULFA
(C) Acute disease	PYR/SULFA

Source: Petersen and Liesenfeld.[115]
[a] Recommended primary treatments: PYR, pyrimethamine; SULFA, sulphonamides; SPR, spiramycin; Steroids, corticosteroids.
[b] Painful adenopathy may respond to indomethacin, prolonged adenopathy may respond to PYR/SULFA.
[c] Infection during pregnancy as determined by seroconversion of the mother is treated with spiramycin. If the fetus is confirmed to have toxoplasmosis by ultrasound, amniocentesis or cordocentesis then PYR/SULFA is given, alternating with SPR.
[d] Congenital toxoplasmosis is treated until the infant is 6—12 months old. Most experts recommend a year of therapy. In Denmark postnatal treatment is given for 3 months.

Other studies using different drugs as sulphonamides, pyrimethamine, trimethoprim, and clindamycin, alone or in combination, as well as ponazuril proved to be active for domestic cat treatment (77).

Despite, the number of existent therapies, these are limited and not specific for toxoplasmosis. It is necessary the discovery of the new drugs able to eradicate the parasite form responsible for chronic toxoplasmosis and safer for the human organism. Recent studies have tested new drugs with a selective action on isoprenoid pathway, inhibitors of *T.gondii* enzymes as dihydrofolate reductase/thymidylate synthase and *T. gondii* adenosine kinase and *T. gondii* histone deacetylase (74,78). These new drugs may be a new promise and future treatment for *T. gondii* infection.

9. Prevention and control

Prevention of human toxoplasmosis can be done in two different ways:

- ✓ Primary prevention: Seronegative women should be informed about how to prevent toxoplasmosis during pregnancy (79). The implementation of simple strategies in daily life to avoid infection such as: soap washing of kitchen surfaces, the utensils and the hands after having contacted with unwashed fruits or vegetables or/and raw meat; wearing gloves when gardening or changing cat litter; fruits and vegetables should be peeled or washed before eating (57). Meat should be cooked at a high temperature (67ºC) to kill the parasite (80). The parasite can also be killed by exposure to extreme cold temperature (-13ºC) (81).
- ✓ Secondary prevention: The prenatal and postnatal screenings allow the detection and treatments of recent *T. gondii* infection in pregnant women

able to reduce congenital toxoplasmosis prevalence (82). In European countries such as France, Austria, Switzerland, Germany, Italy and Belgium, applied programs have also shown the decrease of the prevalence of this disease (82,83). Serological test is regularly realized during pregnancy to detect *T. gondii* infection (82).

In conclusion, the application of educational tools are necessary and extremely useful on the sensibilization to this disease, minimizing the risk of infection. Additionally, it is necessary the implementation of more effective screening programs in childbearing age or pregnant women.

10. Maternal-fetal interface (FMI) and immune cell populations

The maternal immune system, during a healthy pregnancy, changes in order to tolerate semi-allogeneic fetus without compromising the ability to protect the mother and the fetus against infection.

Pregnancy implies an intimate association between the mother and developing fetus. In a typical situation, maternal tissues are not contacting directly with the developing fetus. This intimate connection is formed by the maternal-fetal interface (FMI), which included the maternal and fetal components, such as the decidua and the placenta, respectively (84).

The maternal tissue, the decidua, is formed by a process called decidualisation. This process is vital for human pregnancy and responsible for providing a maternal immune tolerance, protection of the fetus and the modulation of the placentation (85). Placenta is a multifaceted organ that plays an essential role in maintaining and protecting the developing fetus. Placenta allows the nutrients apport from the mother to the fetus, substance secretion from the fetus, constituting a barrier to the fetus against pathogens and the maternal immune system, being an active endocrine organ too. It also has the capacity of synthesizing and secreting growth factors, hormones, cytokine and other bioactive products (86).

In the placenta, there are specialized cells denominated trophoblasts, which play a major role in the implantation and development of the maternal-fetal interface (87). These fetal trophoblast cells are in close contact with maternal immune cells and the reason for this process is intimately associated with the Extravillous Trophoblast cells

(EVT), which invade the maternal uterine mucosa, namely the decidua during the pregnancy. Trophoblast invasion assures placenta anchoring within the uterine wall and the access of the fetus to the maternal vascular system ensuring the supply of oxygen and nutrients. In the first trimester of pregnancy the invasive capacity of EVT is stronger, but declines afterwards (88). Trophoblasts cells also play an essential role in materno-fetal infection and have importance concerning parasite transmission, because of their location between maternal and fetal blood circulation (89).

During normal pregnancy, the maternal immune system can tolerate the paternal antigens present in placental tissue without immune rejection (84). To ensure materno-fetal tolerance a specific immune cell population plays a crucial role denominated regulatory T cells (Treg) (90). However, this tolerance to fetal antigens can occur in the presence of a variety of other cell population.

The immune populations present at the maternal-fetal interface are of maternal origin. Decidua is an active immunological tissue constituted by a broad population of maternal immune cells dynamically modifying in composition from ovulation throughout the course of pregnancy (91). More specifically the major population of decidual leukocytes present in the first trimester of pregnancy are: NK cells (~70%), Macrophages (~20%), T cell (~10-20%) including the regulatory T cells (Treg) and a small population of dendritic cells (DCs) (Figure 7; 88).

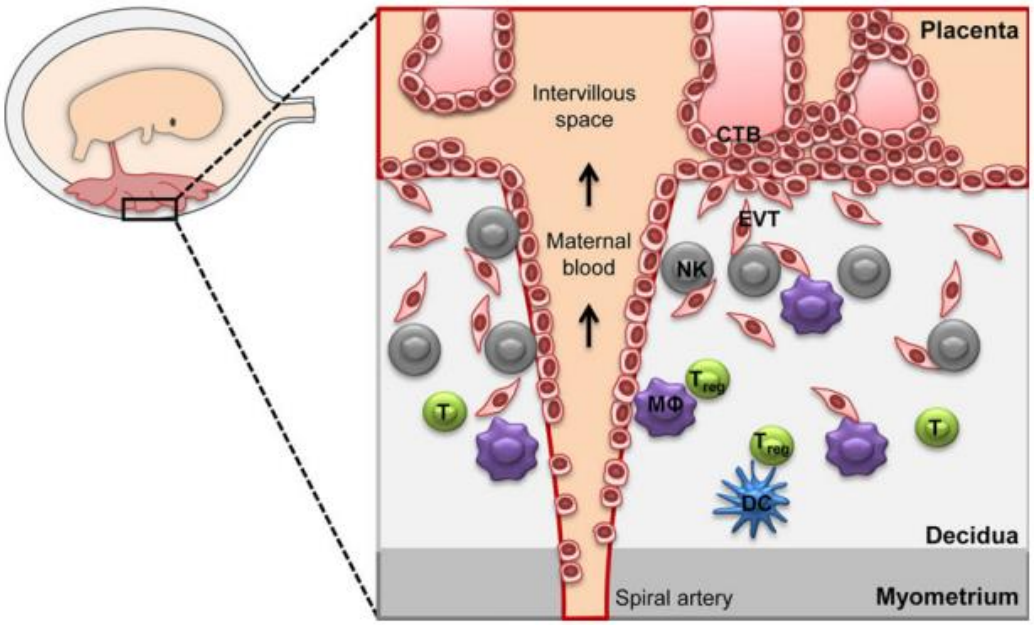

Fig. 6 – Schematic representation of the maternal-fetal interface (FMI). MΦ: macrophages; NK: Natural killer cells; T: T cells; Treg: regulatory T cells; DC: dendritic cells; EVT: extravillous trophoblast cells (93).

10.1. NK and Decidual NK cells

NK cells play an important role in host defense, killing tumor and/or fetus virally infected cells (94) by secreting a variety of cytokines (95). In the *T. gondii,* context NK cell contributes to the initial host resistance parasite through the production of the effector cytokine IFN-ϒ (96). The development of NK cells occurs in the bone marrow, migrating after to the spleen, peripheral blood, and liver. Nevertheless, this cells can migrate into tissues in response to infectious and inflammatory stimulus (97).

NK cells constitute the dominant maternal immune cell population in the first trimester of decidua. After the first trimester, decidual NK cell (dNK) numbers decrease becoming nearly absent at the term of pregnancy (95,98). The origin of dNK is not established, but several explanations have been proposed, such as: the maturation of endometrial NK cells in response to pregnancy-associated factors; the differentiation from hematopoietic precursors present in the decidua in response to decidual stromal factors; and the recruitment of peripheral blood NK cells that differentiate locally into dNK cells (99). dNK cells are in close contact with invasive placental trophoblasts, allowing the frequent interaction with other receptors (95). Specifically, dNK produce chemokines that attracting invasive trophoblasts, while trophoblasts express the receptors for that dNK produce chemokines (94). Thus, these cells have two important functions: helping the migration of trophoblast and promoting blood flow at the maternal-fetal interface (98). dNK cells are responsible for the secretion of high levels of the angiogenic factors: endothelial growth factor (VEGF), angiopoietin-2 and placental growth factor (PIGF), as promoting angiogenesis the maternal-fetal interface and vasculogenesis on the fetal side (95,99). Additionally, dNK cells have the capacity to produce cytokines and growth factors such as tumor necrosis factor-α (TNF-α), interleukin (IL)-10 and (IL)-1 β, transforming growth factor (TGF-β1), leukemia inhibitory factor (LIF), interferon gamma (IFN-ϒ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (98).

10.2. T cells

T cells have an important role in immunostimulation and immunoregulation (90). A variety of subpopulations expressing the CD4 marker ($CD4^+$ T cells) exist such as helper T cells (Th), type 1 (Th1) and type 2 (Th2), and regulatory T cells. Other subpopulations express CD8 marker ($CD8^+$ T cells) denominated by cytotoxic T cells (CTLs) (101). $CD4^+$ and $CD8^+$ T cells have an important role against intracellular

pathogens and have the capacity to produce IFN-Ɣ. The presence and profile of these cells subpopulations in the maternal-fetal interface can increase decidual inflammation leading to the detriment of pregnancy success (102).

The primarily function of Th1 cells is the destruction of intracellular pathogens and virus-infected cells, and are responsible for the production IL-2 and IFN-ϒ (101). These cells can be considered as the major players protecting fetal survival but may also lead to pregnancy pathologies. Otherwise, Th2 cells have functions in the eradication of helminths and allergic reactions, producing IL-4, IL-13 and IL-5 (102). However, these cells during pregnancy have the capacity to overrules Th1 response, constituting a major protection to the fetus from maternal Th1 cell response (103). It has been observed that Th1 cells are associated with spontaneous abortions or pregnancy pathologies (104,105). It have also been reported the presence of Th2 cells in abortion cases (106,107). Thus Th1/Th2 paradigm explanation is insufficient and another explanation emerged to understand the rejection of fetus by maternal immune cells (90). Recently Th1/Th2/Th17 and regulatory T (Treg) cells paradigm has been proposed (108). The Th17 cells also express CD4 marker playing an important role in the acute inflammatory response being involved in host defence against extracellular pathogens, such as bacteria, fungi or viruses. They are responsible for the production of pro-inflammatory cytokine IL-17 (102). An increase of Th17 subpopulation at systemic level has been suggested to be harmfull for the pregnant maintenance (109).

Although, regulatory T (Treg) cells play an essential role in prevention of autoimmune disease, they are also able to modulate immune responses during infection and exhibit a role in maintaining immune homeostasis (110). Treg cells have an important role in pregnancy, leading to the maintenance of the fetal immune tolerance (111). A decline or dysfunctional capacity of Treg cells during pregnancy are closely associated with the occurrence of spontaneous abortion or other complications (112). In human pregnancy during the first trimester, the Treg cells numbers are increased in decidua peaking during the second trimester and lastly declining postpartum (113).

10.3. Decidual Macrophages

Decidual macrophages are the second most abundant leukocyte population in the decidua, approximately 20% of total leukocytes (92). However, unlike NK cells, the number of macrophages remains high throughout pregnancy (114).

Macrophages are involved in a diversity of important processes for a successful pregnancy such as placental cell invasion, tissue remodeling, immune-cell activities and angiogenesis (115). Decidual macrophages in association with dNK cells, contribute to the migration of trophoblast and help the remodeling process of spiral arteries (93). Otherwise, these cells have a relevant function in apoptotic cells and debris clearance originated during placental and vascular remodeling (116).

Although the diverse roles of these cells, they have an essential role at the maternal-fetal interface, in the defence against pathogens protecting the fetus from infection (117).

- **Macrophages Activation**

Macrophages were regarded as phagocytic cells responsible for pathogens elimination and tissue homeostasis in a broad range of organisms. They have functions in both adaptive and innate immunity, being the major-antigen specific cells exhibiting different activation profiles (118).

Macrophages and dendritic cells (DCs) express a diversity of pattern-recognition receptors, such as Toll-like receptors (TLRs), allowing the recognition of pathogens (119). These receptors have an essential role in innate immunity and are responsible for the induction of pro-inflammatory cytokines production and upregulation of co-stimulatory molecules (120).

Activation states of macrophage can be divided in M1 (classically activation) or M2 (alternative activation). Classically activated macrophages can be induced by IFN-ϒ alone or in combination with lipopolysaccharide (LPS) and TNF-α. M1 macrophages have a microbicidal and tumoricidal capacity producing high levels of pro-inflammatory cytokines and mediators (121). Alternatively activated macrophages can be induced by IL-4, IL-13, IL-10 and TGF-β (122), having an important role in parasite clearance, tissue remodeling, inflammatory dampening, angiogenesis, allergy response, tumor promotion and immunoregulation (123). M2 macrophages can be divided into three subclasses: M2a induced by IL-4 and IL-13, M2b induced by exposed to TLR-receptors agonists or immune complexes and M2c induced by IL-10 and glucocorticoids (124). A study has proposed a fourth type of M2 macrophages profile, the M2d, which has similar features to tumor-associated macrophages (TAMs) and is characterized by an IL-10^{high} IL-12^{low} profile (Fig.7; 127).

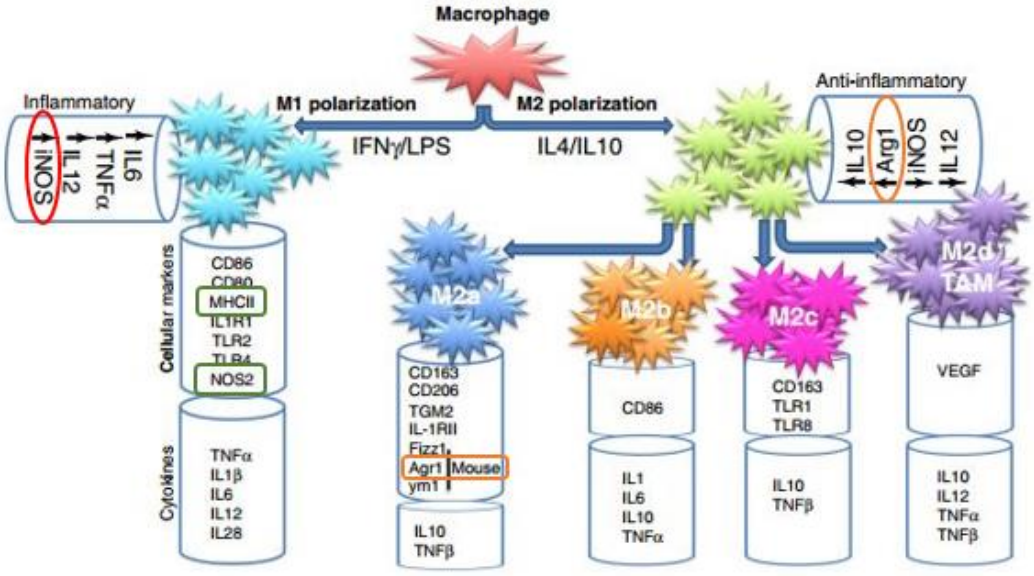

Fig.7 – Different types of macrophages polarization profile and the different subclasses of M2 profile. Adapted from (126)

M1 and M2 macrophages are in accordance with Th1/Th2 paradigm, because the Th1 cells can produce IFN-Y, which causes M1 activation. Otherwise, Th2 cells can produce IL-4 and IL-10 associated with M2 activation (127). However, it is notable that M1/M2 macrophages occur in all animals, whether they have T cells or not (128). More specifically in case of the presence of pathogens or altered self-antigens, M1/M2 macrophages response has an important role in induced Th1/Th2 responses (127).

It is known that macrophages have a flexibility in their programming, which means that, they have a capacity of switching from activation state to another in response to a variety of signals and factors depending on the micro-environmental conditions (129,130). Besides, recent *in vivo* studies have related that macrophages can also express, simultaneously, cell M1 and M2 markers in the context of *T. gondii* infection. (131). In accordance, M1/M2 macrophages can be associated with two opposing pathways for arginine metabolism. Arginine can be metabolized via inducible nitric oxide synthase (NOS2) to NO and citrulline, which is associated with M1 profile. Otherwise, arginine can also be metabolized via arginase 1 (Arg-1) to ornithine and urea, which is associated with M2 profile (132).

Actually, it is known that pregnancy is an active, functional and highly controlled immunologic process (133). A successful implantation is characterized by a transient inflammatory response initiated by cytokines and prostaglandins. In the peri-implantation period, decidual macrophages present mostly a M1 profile. However,

when trophoblast establishes a link to the endometrium and invades the uterine stromal, decidual macrophages initiate a transition to mixed M1/M2 profile (134). This mixed M1/M2 profile is maintained through the first trimester and in an initial phase of the second trimester (133). After placental development, the activation profile of decidual macrophages changes toward to M2 profile avoiding rejection of the fetus and allowing the fetal growth until parturition (115).

A recent *in vivo* study in rats demonstrated that *T. gondii* acute infection was associated with M1 profile, interfering with physiological balance of pregnancy and possibly leading to maternal pregnant failure, such as abortion (135).

10.4. Dendritic cells

Dendritic cells (DCs) are a type of antigen presenting cells (APC) with the capacity to activate maternal T cells with fetal/placental specificity. These cells have a capacity of recognizing pathogens, through the pattern-recognition receptors, as Toll-like receptors (136). dDCs are present in the first trimester of pregnancy, specifically of the decidua, (92) declining after implantation and remaining a few cell numbers near the developing placenta. The reduction of decidual DCs is critical for fetomaternal tolerance, being also responsible, in specifically conditions for inducing a T cell response to placental antigens and consequently leading to the failure of pregnancy (136). One possible explanation is the expression of indoleamine 2,3 dioxygenase (IDO) by DCs, at the maternal-fetal interface suppressing T-cell responses (137,138). Studies using decidua from early human pregnancy, have shown an increase of IL-12 production by myeloid dDCs, inducing a Th2 response and, consequently, leading to pregnancy maintenance (98).

11. The immunobiology response to *T. gondii*

T. gondii is frequently acquired by oral ingestion and actively crosses the intestinal epithelium, disseminating to other organs, tissues, and cells (139). The intestinal epithelium is the first line of defense against *T.gondii* (24). The infected cells have the capacity to secrete cytotoxic molecules which attract diverse immune cells, such as DCs, macrophages, neutrophils and T cells (24,140).

Neutrophils are important phagocytic cells that in presence of pathogens are rapidly recruited from bloodstream to the site of infection. These cells kill pathogens by releasing an anti-microbial compounds from their granules and production of ROI and NO (141). Furthermore, activated neutrophils can also produce a diversity of

chemokines, such as IL-8, macrophage inflammatory protein-1α (MIP-1α) and MIP-1β. The secretion of these MIP chemokines attract T cells, macrophages, monocytes and DCs (141). During the *T. gondii* infection early neutrophil induction is dependent on IL-17 signaling, which permits to eliminate a large number of parasites in the initial stage (142).

The maturation and activation of DCs in response to infection have a fundamental role in the initiation of innate immunity and consequently in adaptive immunity development. This maturation is characterized by an up-regulation of the expression of MHC molecules and diverse co-stimulatory molecules increasing the proliferation T cells (143). In response to microbial stimuli, DCs are also able to carry microbial antigens from the local of infection to the spleen accumulating in the T cells areas. Additionally, DCs are responsible for antigen-presenting activity leading to the polarisation of Th responses toward Th1 by the production of IL-12 (144,145). Indeed, DCs are the main source of IL-12 in response to *T. gondii* infection (146).

Macrophages, such as DCs, provide a first line of defense against initial dissemination and/or growth of infectious parasite (147). In the presence of *T. gondii* macrophages, DCs and neutrophils are important source of IL-12 in the initial stage (148). IL-12 is essential in the stimulation of NK cells and T cells to produce IFN-ϒ, as well as, promoting the proliferation of $CD4^+$ and $CD8^+$ T cells (144).

NK cells are the major source of IFN- ϒ and are able to recognize and kill infected cells (149). They can migrate to lymphoid tissue at the site of infection, where they release IFN- ϒ inducing classically activate macrophages with enhanced MHC class II expression (97). The classical activation is essential to the control of intracellular *T. gondii* infection, leading to the production of inflammatory cytokines, such as TNF, and NO.

There are two essentials signals for classical activation of macrophages. In the first place IFN-Ϋ is release by NK or/and T cells and prevented the macrophages. Then, in response to the activation of pattern recognition receptors (PRRs) by pathogens-associated molecules patterns (PAMPs), the endogenous TNF is produced by macrophages and completes the activation (24). After macrophages activation, they can migrate to the local of infections, phagocytose and destroy pathogens by the release of ROI, NO and lysosomal enzymes, as well as by inducing nutrient deprivation mechanisms (147,150). However, the overproduction of inflammatory cytokines and NO can promote severe pathology, thus the activity of these cells need be tightly regulated. So, signals provided by macrophages or surrounding cells, may down-

regulated macrophages activation resulting in the production of anti-inflammatory cytokines, including IL-10 and TGF- β (150).

$CD4^+$ and $CD8^+$ cells are important in the host survive during a chronic infections. $CD8^+$ cells have a main role as effector lymphocytes against the parasite (151). *T. gondii* induces CTLs that have an ability to lyse infected cells (152). Otherwise, $CD4^+$ are essential to regulate immune response to *T. gondii* and have the capacity to release several cytokines (153).

12. Animal study model

Murine infection with *T. gondii* has demonstrated to be one of the most powerful *in vivo* models for the study of fundamental aspects of immune mechanisms controlling intracellular parasite infections (154). Although, important parameters for the development of infection must be considered as, the strain of parasite, the mouse strain and the infection route (Table 3).

Mice and rats models are frequently used in the study of fetal-placental development. Obviously, there are several differences between rodents models and humans: the decidualization process in the case of humans can happen spontaneously during the late secretory phase of menstrual cycle, but in rat models this process occurs only in response to implantation or an artificial stimulus (155); the time of gestation in rodents models is approximately between 21-22 days and in humans are 9 months (92,155); and another critical difference is that placental trophoblast does not invade deeply in decidual arterioles in the murine model (92).

However, the murine and human pregnancy are similar in some events, such as placentation and accumulation of dNK cells (92). Although, NK cells are abundant in human and rat decidua, in the case of macrophages, they are more abundant in human decidua than in murine decidua (92,156).

Balb/c mice as human don't have the capacity to transmit *T. gondii* to the offspring during the chronic phase of infection. Vertical transmission occurs when exposition to the parasite occurs in specific conditions during pregnancy (157). Thus, this strain is commonly used in the study of vertical transmission of *T. gondii.* Normally, the *T. gondii* infection occurs in the first period of pregnancy (until 7 days of pregnancy) considering the first day of pregnancy as the day on which the presence of vaginal plug occur (155,157). Like in humans, the infection in the early stage can result in embryo loss

due to abortion or resorption, while infection in the second period (7- 14 days) of pregnancy can lead to congenital toxoplasmosis (157).

As the pregnancy period time is very short (only 21 days), infection in later period (14-21 days) typically, are not ethical approved by the competent European Entities (DGV;160).

Table 3 –Strain mice and susceptibility of *T. gondii* acute infection. Adapted from (154).

	Acute Infection Routes	
Strain	Oral	Intraperitoneal (ip)
Balb/c	Resistant	Susceptible

Aims

During the last years, several scientific advances, have been done to understand the immune response to *T. gondii*. However, there are fewer studies describing how maternal immune response during pregnancy may have a role in the pathology associated with congenital toxoplasmosis. A diversity of immune cell populations are described, as having an important role at the maternal-fetal interface. It is also known that under environmental conditions modification, like with infection, the activation profile of some of these cell populations, as macrophages and associated T cell responses might be implicated in pathological consequences for the fetus.

In this study, it was used Balb/c mice, a murine strain commonly used as a animal model of vertical transmission of *T. gondii*, allowing the follow-up of pregnancy. We aimed to understand the diversity of cell population present during *T. gondii* infection and pregnancy.

Specifically, we proposed to determine:

- the effect of infection on basic parameters of reproductive function in pregnancy, particularly in the implantation sites number.
- *T.gondii* quantification in liver, kidney, heart, lung, and spleen, decidua/placenta (E12), decidua (E14) and placenta (E14), embryo (E12, E14), PEC and spleen cells.
- *Ex-vivo* analysis of neutrophils, macrophages and lymphocytes at PEC and spleen cells by flow cytometry.

Materials and Methods

1. Parasite

The *T. gondii* strain used in this study was the Yellow Fluorescence protein (YFP), expressing type II strain, corresponding to the ME49 clone, kindly provided from Doctor Marcus Meissner laboratory (Parasitology department of the Faculty of Medicine, Heidelberg University, Germany).

2. Human Foreskin Fibroblast cells (HFF) *in vitro* infection

Viable tachyzoites were obtained by *in vitro* infection of HFF cells provided from American Type Culture Collection (ATCC). Cells culture were grown in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Paisley, UK) supplemented with L-glutamine (2mM), fetal bovine serum (10%), 100 U/ml penicillin and 100 µg/ml streptomycin. Cell culture were maintained in 75 cm^2 and 25 cm^2 flasks at 37 ◦C, in humidified atmosphere of 5% CO_2. Once confluent, HFF were either kept for maintaining *T. gondii* or split for maintenance of the HFF stocks.

For the maintenance of *in vitro* parasite cultures, the inoculum concentration obtained, was estimated depending on the microscopically observation of the density of intracellular tachyzoites, the size of the cell culture flask used, and on the parasite numbers needed for the maintenance of the parasite and/or *in vivo* infection. Specifically, when the majority of fibroblasts exhibited multiple intracellular tachyzoites, namely in rosettes, fibroblast cells were detached using a cell scraper and disrupted using a 25G needle and syringe. Cell suspension was filtered using a 0.45 µm filter and centrifuged at 500g for 6 minutes at room temperature, in order to eliminate HFF cell debris and resuspended in 1 ml of saline solution (0.9% NaCl). Tachyzoites were counted using a Neubauer chamber. When using a confluent 75 cm^2 flask of HFF, infection was done using $5x10^6$ to $6x10^6$ viable tachyzoites for an incubation period of 48h to 72h. When using a confluent 25cm^2 flask of HFF, infection was done using $2x10^5$ to $5x10^5$ viable tachyzoites for an incubation period of 48h to 72h. Parasites were passaged two or three times a week.

3. Pregnant mice and *in vivo* infection

Balb\cByJ (Balb/c) mice (7-8 weeks old) were purchased from Charles River laboratory (France). The maintenance and mating of the animals was done at the Institute of Biomedical Sciences Abel Salazar animal facilities (Porto, Portugal) supported by the medical veterinary and specialized technicians. Female and male animals were mated in cages (ratio 1:1). The appearance of the vaginal plug was considered day 1 of gestation (E1). For *in vivo* infection, the inoculum was prepared in saline solution (0.9%NaCl) to a final concentration of the $2{,}5x10^4$/ mL of viable tachyzoites. At the day 7 of gestation (E7), each mice were intraperitoneally (ip) infected with 5000 viable tachyzoites obtained, as previously described. After 5 or 7 days of *in vivo* infection [corresponding to day 12 and day 14 of gestation (E12, E14)] mice were sacrificed. In this study seven groups of mice were used: pregnant *T. gondii* infected (E12-Inf and E14-Inf), pregnant uninfected (E12-Ninf and E14-Ninf), non-pregnant (E0) 5 or 7 days *T. gondii* infected (E0-5 days Inf and E0-7 days Inf), non-pregnant uninfected (E0-Ninf). 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 competent national board authority, Direcção-Geral de Alimentação e Veterinária (0421/000/000/2014).

4. Collection of biological samples

At 5 and 7 days after infection, non-pregnant or pregnant mice (E12 and E14) were sacrificed with isofluorane. For each pregnant mouse 3 fetoplacental units (FP) were collected and preserved in 4% paraformaldehyde and included in paraffin for histological analyses. Other 3 FPs were dissected in decidua/placenta (E12) or decidua and placenta (E14) and embryo. For each pregnant or non-pregnant infected animal, portions of liver, kidney, lung, spleen and heart were collected and preserved in 4% paraformaldehyde and included in paraffin for histological analyses and others were stored at -80ºC for DNA extraction. For each animal (from each group) peritoneal exudate cells (PEC) were obtained after washing the peritoneal cavity with 7 ml of cold

Hanks solution. Spleen cells were obtained after homogenization of a half of the spleen, followed by treatment with ACK lysis buffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$) and ressuspended in DMEM supplemented with 10 % FBS. Spleen cells and PEC were counted using a Neubauer chamber and processed for flow cytometry and later parasite quantification by quantitative real-time PCR (q-PCR). The decidua/placenta (E12), decidua or placenta (E14) and embryo (E12 and E14) tissues, PEC, spleen cells and organs were stored at -80ºC in Trizol (1 ml per [50-100] mg of tissue or [5-10] x 10^6 cells), for later genomic DNA (gDNA), ribonucleic acid (RNA) and protein extraction according to manufacturer´s instructions (Sigma Aldrich, USA).

5. Quantitative real-time PCR (q-PCR)

Genomic DNA from the heart, liver, lungs, kidney, spleen, spleen cells, PEC, decidua/placenta (E12), decidua or placenta (E14) and embryo (E12, E14) was extracted using Trizol Reagent according to manufacturer's instructions (Sigma Aldrich,USA). The DNA concentration was determined using a Thermo Scientific Nanodrop 1000 spectophtometer.

T.gondii genomic DNA was detected using primers and a probe designed for the SAG-1 gene of *T.gondii*, 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: GGGTCCTTCCGCAGAC AAC), 0,2 µM of probe (6FAM-CTGTyTGCACCGTAGGAGCACCT-BBQ; all designed by Till Molbiol) and KAPA PROBE FAST qPCR Master Mix (Kapabiosystems, USA). The PCR programme run was as follows: 1) denaturation at 95ºC, 3 min; 2) amplification in 40 cycles (denaturation at 95ºC, 3 sec; combined annealing/extension 60ºC, 30 sec). The standard curve was obtained using DNA samples corresponding to 10^2 - 10^{-2} ng/µl of parasite DNA diluted in solution of 20 ng/µl of the host DNA. Quantitative evaluation of fluorescence signals from PCR products was performed with Step-One Plus (Applied Biosystems by Life Technologies) and analysed with Step One Software V2.3.

6. Flow cytometry analysis

Spleen cells and PEC were isolated from uninfected or infected animals. Briefly a single cell suspension was prepared from the spleen and PEC for each mouse, as

previously described. Cells were washed and the number of viable cells was counted by trypan blue exclusion. For the immunofluorescence staining, cells were labelled with distinct combinations of the following antibodies (Abs): 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); Purified anti-mouse CD16/32 Ab, Isotype rat IgG2a, λ (clone 93); 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 purchased from Biolegend); PE-Polyclonal Sheep IgG anti-Human/Mouse Arginase 1 (Arg-1); PE-Polyclonal Sheep IgG Isotype control; (both purchased from R&D Systems), PE anti-mouse NOS2 Ab, Isotype rat IgG2a, κ (clone CXNFT); PE rat IgG2a,κ Isotype control (clone eBR2a); (both purchased from eBioscience).

Briefly, for the surface staining, $1x10^6$ spleen cells and PEC 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 4ºC. Cells were then incubated for 20 minutes 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; CD11b-Alexa Fluor647, Ly6G-PercP/Cy5.5 and MHCII-PE or isotype control; CD3-APC and CD4-PE; CD3-APC and CD8-PE. Before acquisition, all samples were fixed with PBS-Paraformaldehyde 2% (2% PFA) and washed twice in PBS containing 2% FBS.

For intracellular staining, $2x10^6$ spleen cells were seeded in 96-well flat-bottom plates. After the surface staining for CD11b-Alexa Fluor647, F4/80-PercP/Cy5.5 or CD11b-Alexa Fluor 647, Ly6G-PercP/Cy5.5 cells as previously described, the cells were fixed with 2% PFA, washed in FACS buffer (PBS, 0.5% BSA, 0.1% NaN_3) , and washed with permeabilization buffer (PBS, 0.5% BSA, 0.1% NaN_3, 0.5% Saponin). Cells were then incubated with anti-Arg1-PE or anti-NOS2-PE or isotype-matched controls for 30 min at room temperature in the dark. The cells were then washed twice with FACS buffer (PBS, 0.5% BSA, 0.1% NaN_3) and acquired using a BD accuri C6 Flow cytometer and software (BD Biosciences).

Cells were selected on the basis of FSC-A/SSC-A and the singlets were gated according to FSC-H versus FSC-A. Lymphocytes TCD4$^+$ cells were defined as CD3$^+$CD4$^+$ and lymphocytes TCD8$^+$ were defined as CD3$^+$CD8$^+$. For each sample, acquisition of 10000 events and 1200 events of total CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$, was done, to spleen cells and PEC cells, respectively.

Neutrophils and macrophages were defined as CD11b$^+$Ly6G$^+$ and CD11b$^+$Ly6G$^-$ F4/80$^+$ respectively. For PEC, the data analysis was based on previous studies (158). Briefly, small peritoneal macrophages (SPM) were considered as CD11b^{int}F4/80i^{int} and large peritoneal macrophages (LPM) were considered as CD11b^{high}F4/80i^{high}. For spleen cells, M1 macrophages were defined as CD11b$^+$F4/80$^+$NOS2$^+$ and M2 macrophages defined as CD11b$^+$F4/80$^+$Arg-1$^+$. The population of neutrophils expressing NOS2 or Arg-1 was also defined as CD11b$^+$Ly6G$^+$NOS2$^+$ or CD11b$^+$Ly6G$^+$Arg-1$^+$. The number of the events acquired for neutrophil and macrophage analysis was 10 000 events of total CD11b$^+$ cells. The percentage of infected spleen cells and PEC were determined by flow cytometry analysis by the evaluation of YFP$^+$ cells in singlets. The number the events acquired for YFP$^+$ cells analysis was 10 000 events of total CD11b$^+$ cells. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

To determine the spleen cell number, the number of gated events for each cell population was multiplied by the total cell number (counted using Neubauer chamber) and divided by total number of singlets. To determine the percentage of CD11b$^+$Ly6G$^-$ F4/80$^+$ in spleen and PEC cells the correction was done taking account the number of events acquired inside the gate CD11b$^+$Ly6G$^-$F4/80$^+$ divided by the total number of singlets.

7. Statistical analyses

Results were expressed as mean + SD. Statistical significance was calculated by using the one-way ANOVA test (Fig. 12 C, 13 B, 15, 16, 24 A), two-way ANOVA test (Fig. 11 B, 18, 22 A) with a Tukey's post-test and paired T test (Fig. 22 B and C, 24 B). The *P* values <0.05 were considered statistically significant.

Results

1. Maintenance of parasite culture

As previously described in material and methods, viable tachyzoites were obtained after the *in vitro* infection of HFF cells (Fig. 8 A). After 48h or 72h of infection, parasites were obtained either to infect Balb/c mice for *in vivo* experiments and/or used to infect new HFF cells for the maintenance of the parasite culture. In Fig. 8 (B-G) images of *in vitro* parasite cultures are shown in different stages of infection.

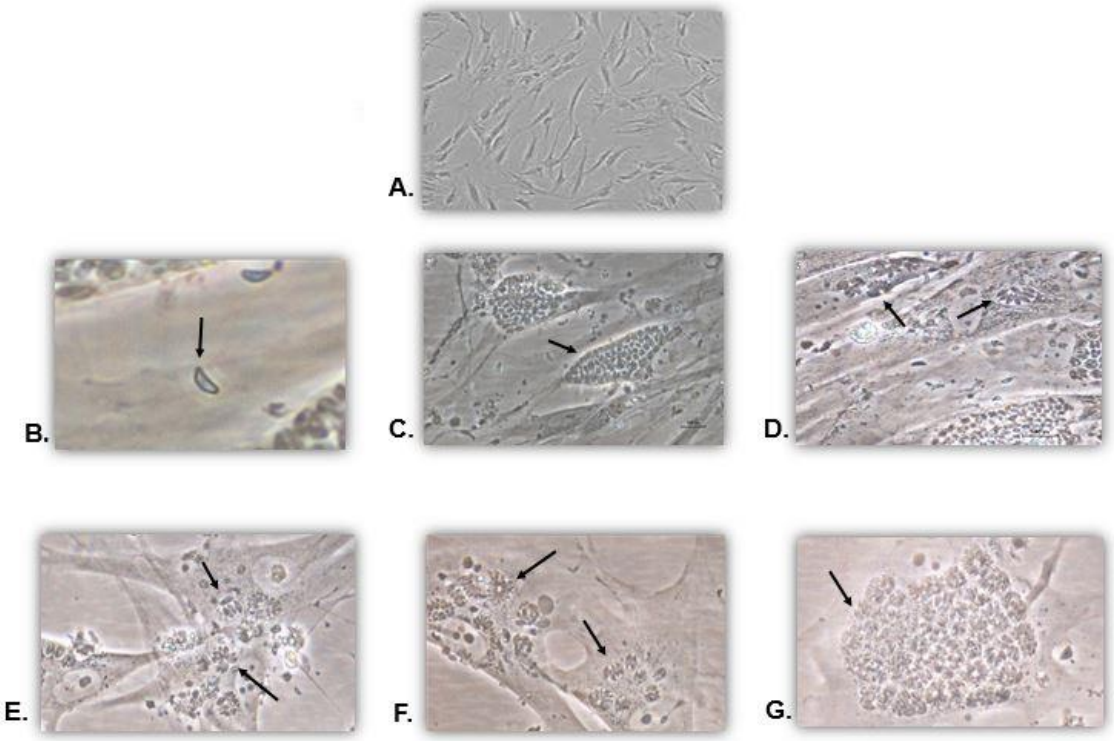

Fig. 8- *In vitro* culture of *T. gondii*-infected HFF cells. [A] Uninfected HFF; [B] Extracellular tachyzoite; [C-G] several stages of *T. gondii* multiplication within HFF; [E,F]-parasite pairs; [D, E,F]- rosettes, in the initial stage of formation; [C, G]- rosettes in the final stage of formation. Photos were taken with an inverted light microscope TS100 with a digital Sight DS-Fi1 camera using the NIS Elements F Imaging Software (original magnification x200 for [A] and x400 for [B-G]; Nikon)

It was also observed that *in vitro* cultures of *T. gondii,* they exhibited a non-synchronized multiplication. As observed in Fig. 9, the parasite inside one cell was at the initial stage of multiplication (parasite pair) and in other adjacent cell was already in active final stage of multiplication (rosettes). Thus, the HFF cell rupture by the parasite occurred at different times.

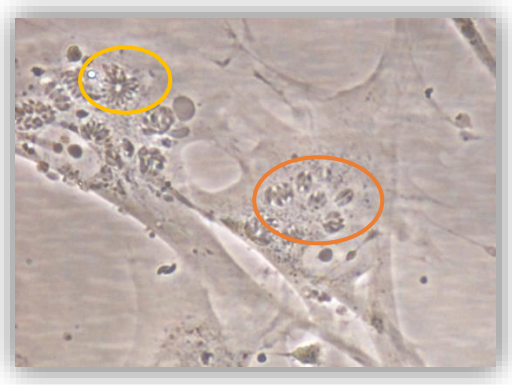

Fig. 9 - *T. gondii*- infected HFF cells. Yellow circle: Rosette form; Orange circle- Parasite pairs. Photo was taken with an inverted light microscope TS100 with a digital Sight DS-Fi1 camera using the NIS Elements F Imaging Software (original magnification x400 Nikon)

2. *In vivo* infection and pregnant Balb/c

In this work, it was used a total of 48 Balb/c animals for *in vivo* experiments. A total of 34 animals were inoculated *ip* with 5000 viable tachyzoites of *T. gondii*-YFP$^+$. Parasite infection was confirmed after 7 days (E14-Inf and E0-7 days Inf) and 5 days (E12-Inf and E0-5 days Inf) post-infection, by flow cytometry analysis of PEC. Unfortunately, PEC TOXO–YFP$^+$ were detected in only 18 out of the 34 inoculated animals. In the Fig. 10, is shown the evaluation of PEC TOXO YFP$^+$ through of the representative histogram of one animal from each group. As it can be observed the E14-Inf (B) and E0-7 days Inf (A) mice presented positive PEC-TOXO$^+$ YFP$^+$. Therefore, an infection ratio of ≅1:2 (infected animal: inoculated animals) was achieved. This could be explained due to several technical problems, concerning HFF cell culture, the influence of the metabolic conditions of host cells can affect the non-synchronized multiplication of intracellular *T. gondii* tachyzoites and it may have an influence in the number of the viable tachyzoites obtained. However, technical errors in the inocula preparation may also affect the obtaining of the viable tachyzoites.

In this work, were only shown the results of the confirmed infected animals by the detection of PEC-TOXO YFP$^+$ cells (18 animals) and non-inoculated animals (14 non-infected animals). A total number of 18 pregnant (9 animals for E14 and 9 animals for E12) and a total number of 14 non-pregnant animals were considered in this study (Table 4 and 5).

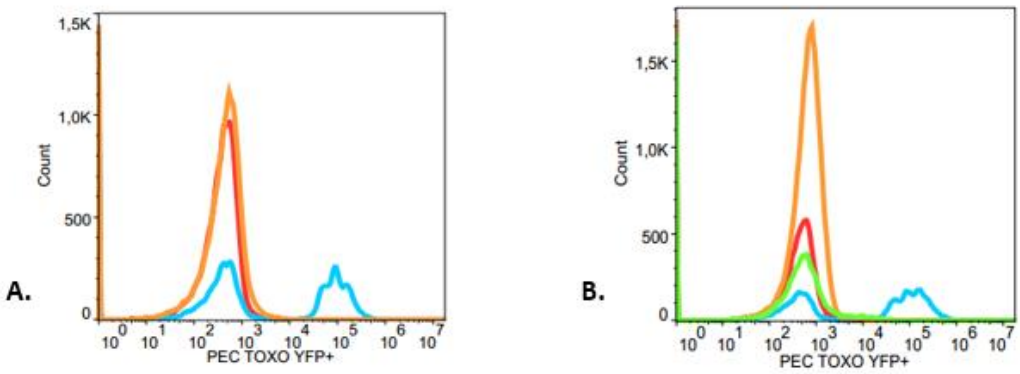

Fig. 10- Representative histogram overlay of PEC TOXO YFP$^+$ [A] E0 7 days Inf (blue), E0-5 days Inf (red) and E0-Ninf (orange). [B] E14-Inf (blue), E12-Inf (red), E14-Ninf (green) and E12-Ninf (orange).

Table 4 – Number of pregnant Balb/c mice considered in this study.

Pregnant	E14	E12
Infected	3	5
Non-infected	6	4

Table 5- Number of non-pregnant Balb/c mice considered in this study.

Non-pregnant	E0-7 days	E0-5 days
Infected	5	5
Non-infected	4	

3. Implantation units

Several studies using the mice model, describe that *T. gondii* infection induces an increase in the number of resorpted implantation sites, therefore affecting negatively pregnancy outcome (159,160). Resorpted units are characterized by a reduction in the size and local haemorrhaging as described (159). In a first attempt, total implantation units (FP and resorpted units) were macroscopic evaluated and quantified for each mouse (Fig.11). The results obtained, show a statistically significant increase of the FP unit numbers compared to the resorpted unit numbers for the groups of the analysis pregnant animals (Fig. 11B). However, it was not found any difference between E12-Inf and E12-Ninf or E14-Inf and E14 Ninf (Fig.11B).

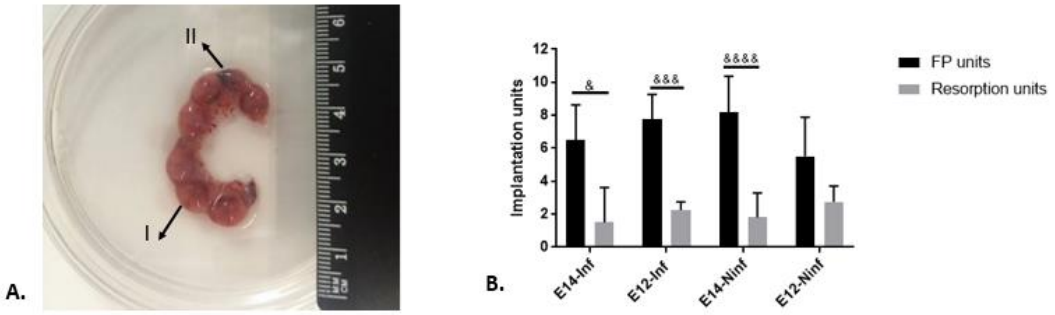

Fig. 11- **Macroscopic evaluation of Implantation units. [**A] Representative image from an E12-Ninf mouse uterus, I- FP unit; II- Resorpted unit. [B] Quantification of implantation units. Data represent the mean + SD. Each group comprised three to six animals analysed individually. $^{\&}P<0.05$, $^{\&\&\&}P<0.001$, $^{\&\&\&\&}P<0.0001$ (differences between FP units and resorpted units).

4. Pregnancy induces splenomegaly independently of *T. gondii* infection

Using the mice models, infection with different parasites induces splenomegaly, indicating an expansion of the immune cells at this secondary lymphoid organ in response to infection (161–164). Here is shown that E14-Ninf mice exhibit splenomegaly compared to E0-Ninf mice (Fig. 12 A). The same is true, when comparing E14-Inf with E0-7 days inf (Fig. 12 B). These observations indicate that pregnancy *per si* induces an expansion of spleen independently of infection.

However, by analysing the total spleen cell numbers of the different groups of animals it is observed a significant increase in both E14-Inf and E0-7 days Inf compared to E14-Ninf and E0-Ninf mice, respectively. No statically differences were observed between E12-Inf and E0-5 days Inf compared to E12-Ninf and E0-NInf mice, respectively. These results are not in accordance with spleen macroscopic observation, showing that *T. gondii* induces an expansion of spleen cells independently of the pregnancy at day 7 post-infection (Fig. 12 C).

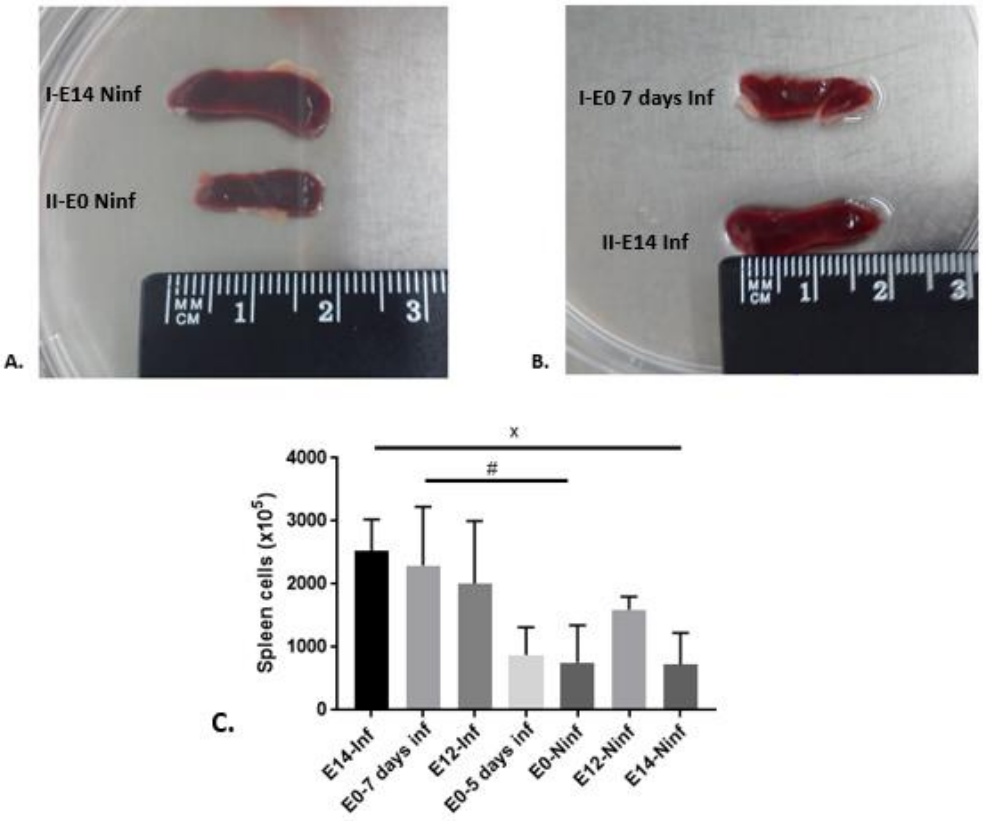

Fig. 12- **Pregnancy and *T. gondii* infection influence on spleen size and spleen cell numbers.** [A] Representative images of spleens from E14-Ninf (I) and E0-Ninf (II). [B] Representative images of spleens from E0- 7 days Inf (I) and E14-Inf (II). [C] Total spleen cells numbers obtained using a Neubauer chamber. Data represent the mean + SD. Each group comprised three to six animals analysed individually. ^{x} $P<0.05$ (differences between E14-Inf and E14-Ninf, respectively) $^{\#}$ $P<0.05$ (differences between E0-7 days Inf and E0-Ninf).

5. Parasite load quantification

As previously mentioned, the Balb/c strain is considered susceptible to *T. gondii* acute infection when *ip* inoculated (154). In this work, the quantification of parasite load was performed using samples of several organs (liver, lung, heart, kidney and spleen), PEC and spleen cells from infected pregnant or non-pregnant mice by amplification of SAG-1 gene by q-PCR. In the case of pregnant mice, parasite quantification was also performed using one FP unit from each animal by amplification of the SAG-1 gene by q-PCR. For this, a FP unit was dissected and in E14-Inf mice it was possible to analyse the decidua, the placenta and the embryo, and in E12-Inf mice it was analysed decidua/placenta tissue (not possible to separate them due to the small size of placenta) and the embryo.

In table 6 and 7, is presented DNA parasite quantification in the different organs, PEC and spleen cells from E14-Inf, E0-7 days Inf, E12-Inf and E0-5 days Inf mice. In two animals E12-Inf, it was possible to detect the parasite in the liver and in the spleen cells, confirming the systemic infection in these animals (Table 6). Additionally, in two animals E0-5 days Inf, it was also possible to detect the parasite in spleen cells, in one animal the liver and in other one in spleen cells, PEC and spleen (Table 6). Otherwise, in E14-Inf and E0-7 days Inf it was possible to detect parasite in the majority of the organs, in spleen cells and PEC (Table 7).

Table 6- Parasite load quantification in E12-Inf and E0-5 days Inf mice

		Parasite DNA (ng/µl)						
	Animal	**Spleen cells**	**PEC**	**Liver**	**Lung**	**Heart**	**Kidney**	**Spleen**
E12- Inf	1	ND	ND	0,0024	ND	ND	ND	ND
	2	0,0020	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND
	4	ND	ND	ND	ND	ND	ND	ND
	5	ND	ND	ND	ND	ND	ND	ND
E0-5 days Inf	1	0,0012	ND	ND	ND	ND	ND	ND
	2	0,0068	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND	ND
	4	ND	ND	21,81	ND	ND	ND	ND
	5	0,0351	4,6776	ND	ND	ND	ND	0,3110

*ND- not-detected

Table 7 - Parasite load quantification in E14-Inf and E0-7 days Inf mice

		Parasite DNA (ng/µl)						
	Animal	**Spleen cells**	**PEC**	**Liver**	**Lung**	**Heart**	**Kidney**	**Spleen**
E14- Inf	1	0,0374	9,0965	0,0485	0,1549	ND	ND	0,0528
	2	0,0277	4,2048	0,0020	ND	ND	0,0036	0,0039
	3	ND	ND	ND	ND	ND	ND	ND
E0-7 days Inf	1	0,1171	5,6795	0,0169	0,0062	0,0058	0,0017	0,0739
	2	0,0252	2,0300	0,0148	ND	ND	ND	0,0533
	3	0,0835	7,5774	0,0039	ND	ND	ND	0,0128
	4	0,0007	ND	ND	ND	ND	ND	ND
	5	0,0007	ND	ND	ND	ND	ND	ND

*ND-not-detected

In E12-Inf group analysed it was not detected the parasite in the spleen cells, PEC, liver, lung, heart, kidney and spleen, but paradoxically it was detected in decidua/placenta tissues in animals 4 and 5 (Table 6 and 8).

For the E14-Inf and E0-7 days Inf was possible to detected parasite in the liver and spleen, indicating a systemic infection. These organs are very important in response to *T. gondii* infection, being that the spleen is the secondary lymphoid organ essential in the immunological system and the liver play an essential role in removing of pathogens, such as parasites, of the blood (165,166). The results for parasite quantification by q-PCR for E14-Inf in the spleen cells (32,6 ± 6,8), PEC (6650,7 ± 3458,9) and liver (25,2 ± 32,9), and for E0-7 days Inf in the spleen cells (321,6 ± 46,5), PEC (5095,6 ± 2819,4), liver (11,8 ± 7,0), they did not allow the achievement of the differences between the groups of the analysis animals (Table 7).

Concerning parasite quantification at the maternal-fetal interface by q-PCR it was possible to detect the parasite in three out of the five animals E12-Inf in the decidual/placental tissue, but not in the embryo (Table 8). Otherwise, it was possible to detect the parasite in two animals E14-Inf in decidua and placenta, and one out of three in the embryo, conforming congenital infection in the FP units analysed for this animal (Table 9).

Table 8 - Parasite load quantification at FP interface and embryo from E12-Inf mice.

		Parasite DNA (ng/µl)	
	Animal	**Decidua/placenta**	**Embryo**
E12- Inf	1	0,0017	ND
	2	ND	ND
	3	ND	ND
	4	0,0016	ND
	5	0,0071	ND

*ND-Not- detected

Table 9- Parasite load quantification at FP interface and embryo from E14-Inf mice.

		Parasite DNA (ng/µl)		
	Animal	**Decidua**	**Placenta**	**Embryo**
E14- Inf	1	0,0084	0,0059	0,0025
	2	0,0014	0,0007	ND
	3	ND	ND	ND

*ND-Not- detected

Parasite detection was determined in spleen cells and PEC by flow cytometry analysis. In Fig. 13 A is presented the gating strategy accomplished, to evaluate the percentage of PEC TOXO YFP$^+$ cells. The results indicate that the percentage of PEC TOXO YFP$^+$ was increased in E14- Inf and E0-7 days Inf compared to E12-Inf and E0-5 days Inf mice respectively (Fig.13-B). The sensitivity of flow cytometry method for the detection of cell TOXO YFP$^+$ was determined by analysing animals uninfected (1,5 %). Therefore, flow cytometry analysis of spleen cells was not able to detect spleen cells TOXO YFP$^+$ (Fig.13 C). Since the sensitivity of flow cytometry analysis for the quantification of cells TOXO YFP$^+$ was of 1,5% and no parasite was detected by q-PCR, the animal 5 from E12-Inf (0,53%) and the animal 3 from E14-Inf (1,09%) were excluded from the data analysis presented in Fig 13 B and T cells, macrophages and neutrophils flow cytometry analysis. The data obtained by flow cytometry analysis for E12-Inf and E0-5 days Inf mice, indicate detection of the parasite in PEC (up to 1,5%).

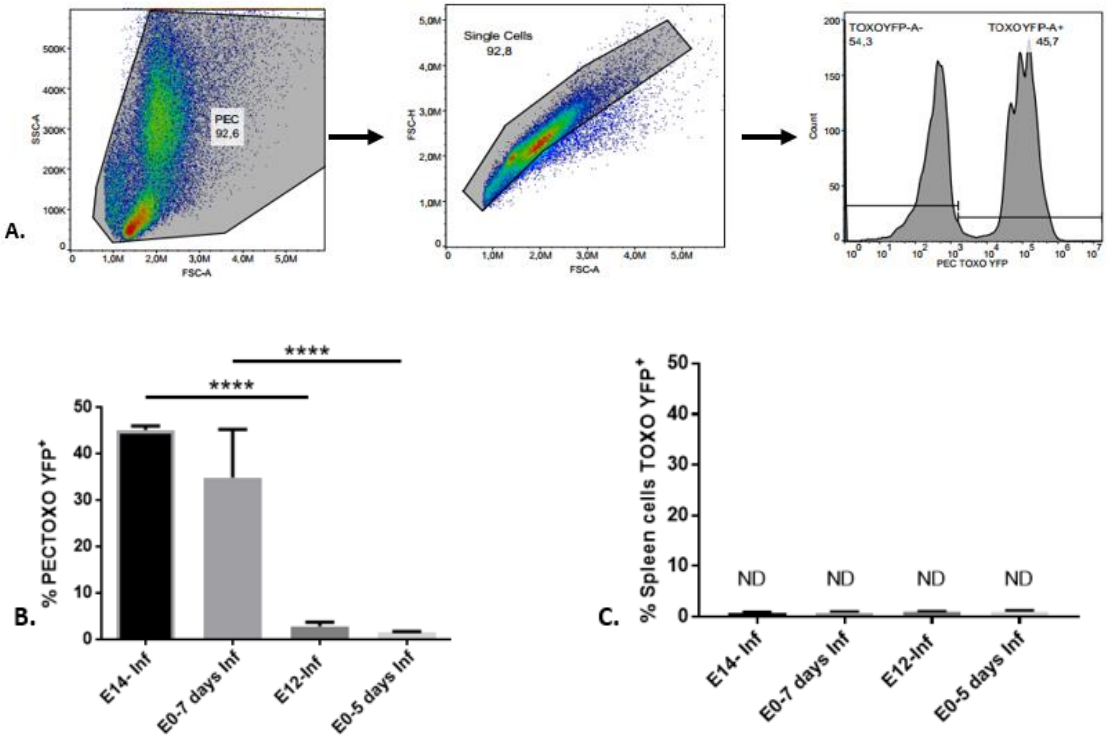

Fig. 13 –Quantification of PEC and spleen cells TOXO-YFP⁺ by flow cytometry. [A] Flow cytometry gating strategy to PEC TOXO YFP⁺ analysis, using the data from E14-Inf mice. [B] Percentage of PEC-TOXO YFP⁺. [C]Percentage of spleen cells TOXO YFP⁺. Data represent the mean + SD. Each group comprised two to four animals analysed individually (E14-Inf, n=2; E0-7 days Inf, n=3; E12-Inf, n=4; E0-5 days Inf, n=5) ****P<0.0001 (difference between E14 Inf vs E12-Inf and E0-7 days Inf vs E0-5 days Inf).

6. T cell populations

In order to understand how *T. gondii* infection alters immune cells populations during pregnancy it was evaluated by flow cytometry analysis CD4⁺ T cell and CD8⁺ T cell populations, neutrophils and macrophages in the spleen cells and PEC.

T cell populations are important players in response to *T. gondii* infection (152,153). To evaluate the CD4⁺ T cell ($CD3^+CD4^+$) and CD8⁺ T cell ($CD3^+CD8^+$) populations at systemic level, the flow cytometry was performed using the spleen cells and PEC from different groups of mice: E12-Inf, E14-Inf, E0-7days Inf, E0-5 days Inf, E0-Ninf, E12-Ninf and E14-Ninf. In Fig. 14, it is presented the gating strategy performed for analysis of PEC and spleen cells.

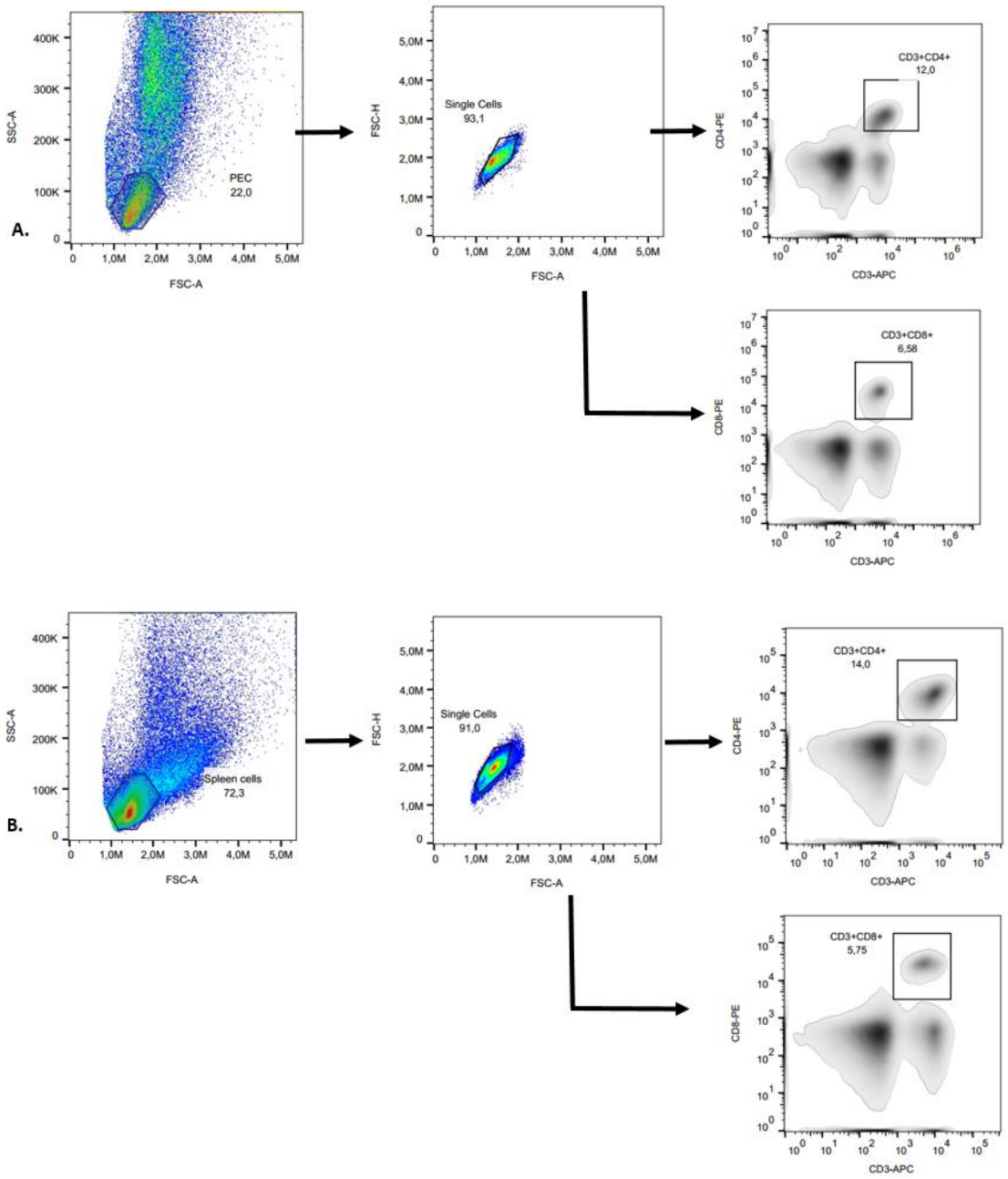

Fig. 14 – Representative flow cytometry gating strategy used to define $CD4^+$ and $CD8^+$ T cells in PEC [A] and in spleen cells [B].

Fig. 15, presents the percentage of the $CD3^+CD4^+$ and $CD3^+CD8^+$ for the different groups analysed. The results obtained for $CD3^+CD4^+$ indicate a significant increase in E0-5 days Inf compared to E12-inf and E0-Ninf. It is also observed an increase in $CD3^+CD4^+$ in E0-7 days comparatively to E0-Ninf (Fig. 15 A). However, E12-Inf animals present a significant decrease of percentage of $CD3^+CD4^+$, which indicates that infection, in the context of pregnancy, doesn't induce the recruitment of $CD3^+CD4^+$ for the peritoneal cavity.

In the case of $CD3^+CD8^+$, the results indicate a significant increase in E14-Inf compared to E14-Ninf and a significant increase in E0-7 days Inf comparatively with E0-Ninf.

The evaluation in terms of $CD3^+CD4^+$ and $CD3^+CD8^+$ numbers was not considered due to technical problems during the collection of PEC. The numbers achieved were dependent on the volume collected of exudates obtained from each mice and were highly variable [between 2-8 $x10^6$ cells].

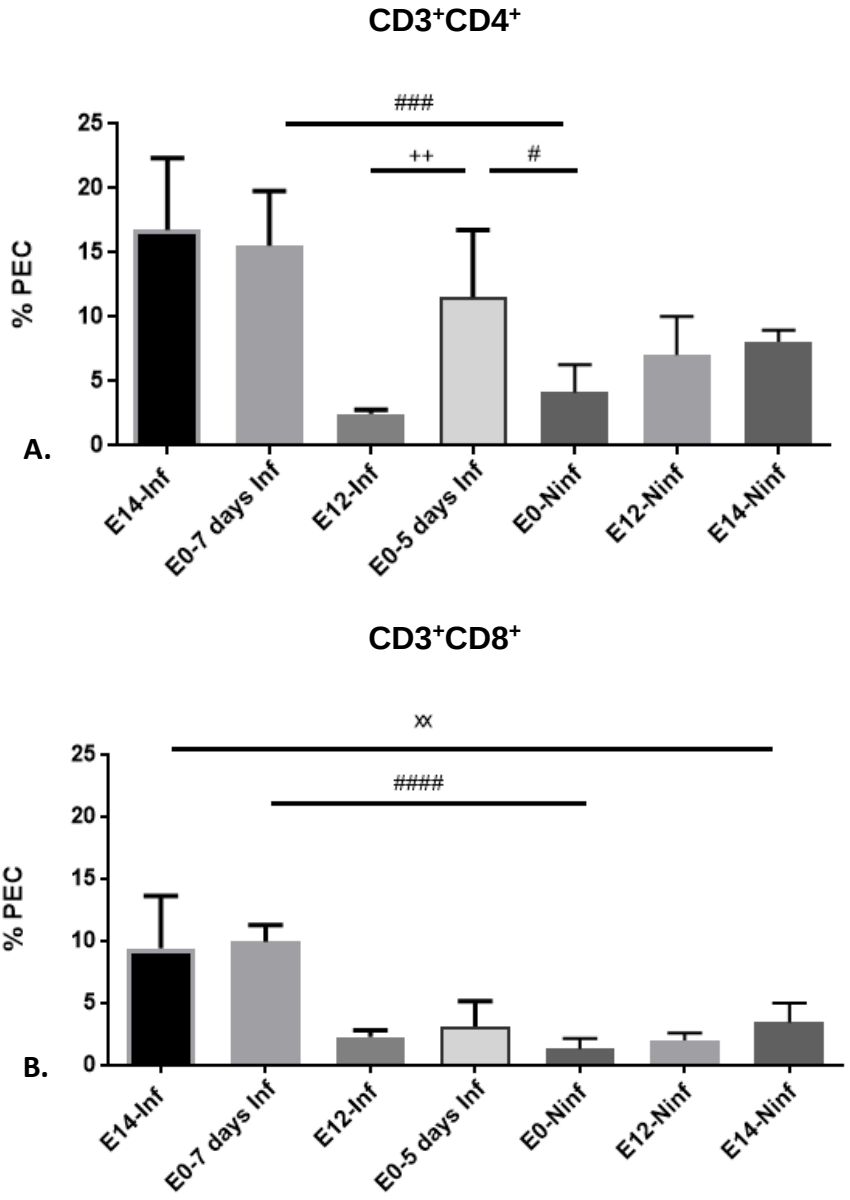

Fig. 15- Evaluation of T cell populations in PEC. [A] Percentage $CD4^+$ T cell population. [B] Percentage of $CD8^+$ T cell population. Data represent the mean + SD. Each group comprised two to four animals analysed individually. ^{xx} $P<0.01$ (differences between E14-Inf and E14-Ninf). $^{++}$ $P<0.01$ (differences between E12-Inf and E0-5 days Inf). $^{\#}$ $P<0.05$, $^{\#\#\#}$ $P<0.001$, $^{\#\#\#\#}$ $P<0.0001$ (differences between E0-7 days Inf/E0-5 days Inf and E0-Ninf).

The results concerning to spleen cell analysis indicate a significant decrease in percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ in E14-Ninf and E12-Ninf comparatively to E0-Ninf (Fig. 16 A, C). These results indicate that pregnancy induces a decrease in the percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ independently of the infection. No differences, in terms of percentages were obtained, analysing the other animals groups. When analysing spleen cells numbers a similar decrease trend of $CD3^+CD4^+$ and $CD3^+CD8^+$ was observed in E12-Ninf and E14-Ninf compared to E0-Ninf but not statically significant. However, when analysing the number of the $CD3^+CD4^+$ a significant increase was found in E14-Inf compared to E14-Ninf and a significant decrease in E14-Inf comparatively with E0- 7 days Inf (Fig. 16 B). No differences were observed in the $CD3^+CD8^+$ spleen cell numbers for the other animal groups analysed.

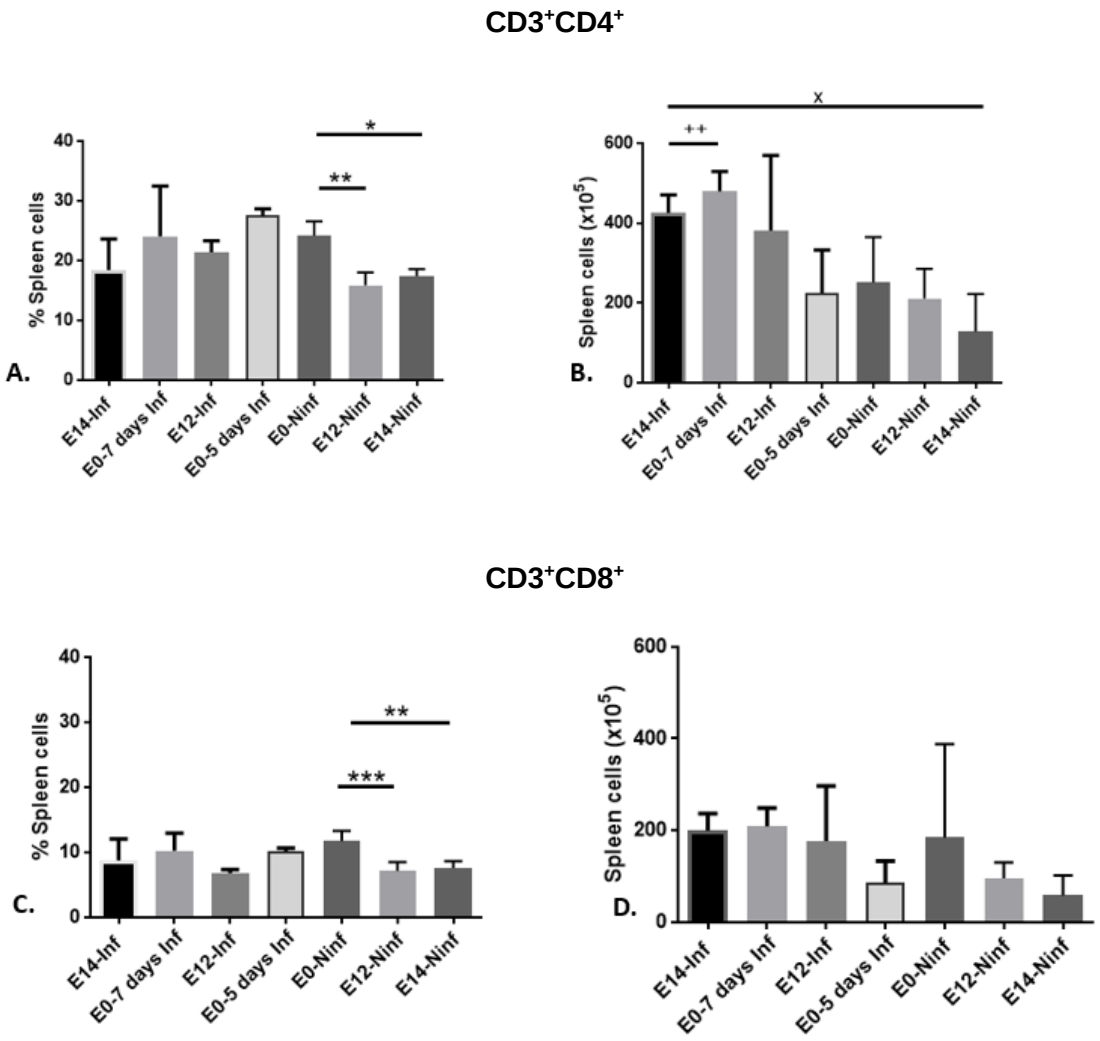

Fig. 16 – Evaluation of T cell populations in spleen cells. [A] Percentage of $CD4^+$ T cells population. [B] Number of $CD4^+$ T cell population. [C] Percentage of $CD8^+$ T cells population. [D] Number of $CD8^+$ T cell population. Data represent the mean + SD. Each group comprised two to four animals analysed individually. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, (differences between E14-Ninf, E12-Ninf and E0-Ninf). ^{x} $P<0.05$ (differences between E14-Inf and E14-Ninf). $^{++}$ $P<0.01$, (differences between E14-Inf and E0-7days Inf).

7. Macrophage and neutrophil populations

Neutrophils and macrophages constitutes the first line of defense in the initial stage of *T. gondii* acute infection (142,147). To evaluate these two immune cell populations at a systemic level and in the local of infection, during *T. gondii* infection and in the pregnancy, flow cytometry analysis were performed using spleen cells and PEC. The cells were labelled with a combination of CD11b, Ly6G and F4/80 antibodies, as described in material and methods section. The gating strategy used for this analysis is presented in Fig. 17. PEC analysis indicated a significant increase of granulocytes ($CD11b^+$) and neutrophils ($CD11b^+Ly6G^+$) in E14-Inf relatively to E14-Ninf and E0-7 days Inf comparatively to E0-Ninf mice (Fig.18 A). These results indicate that at 7 days post-infection a recruitment of the neutrophils occurs independently of the pregnancy.

For spleen analysis, the results obtained for percentage and cell number were concordant, concerning the comparison between E14-Inf and E14-Ninf, E0-7 days Inf and E0-Ninf, E12-Inf and E0-5 days Inf. Thus, an increase of $CD11b^+$ (percentage and cell number) was found in E14-Inf compared to E14- Ninf, and in E0-7 days Inf compared to E0-Ninf animals. These results indicate that granulocyte recruitment occurs in response to infection, independently of the pregnancy. It was found a significant decrease of $CD11b^+$ (percentage and cell number) in E12-Inf compared to E0-5 days Inf. In terms of percentage it was found a decrease of total $CD11b^+$ in E12-Inf compared to E0 Ninf. However, no differences were obtained for $CD11b^+Ly6G^+$ or $CD11b^+Ly6G^-F480^+$ in terms of percentage in all animals groups analysed. It was found an increase in spleen cells number of $CD11b^+Ly6G^+$ in E14-Inf vs E14-Ninf, E0-7 days Inf vs E0-Ninf and E12-Inf vs E12-Ninf. These results may indicate an increase of the neutrophils recruitment in response to infection independently of pregnancy. It also may be noted that in terms of percentage there is an increase of $CD11b^+$ in PEC (59,9%) compared to spleen cells (5,0%) of E0-7 days Inf animals.

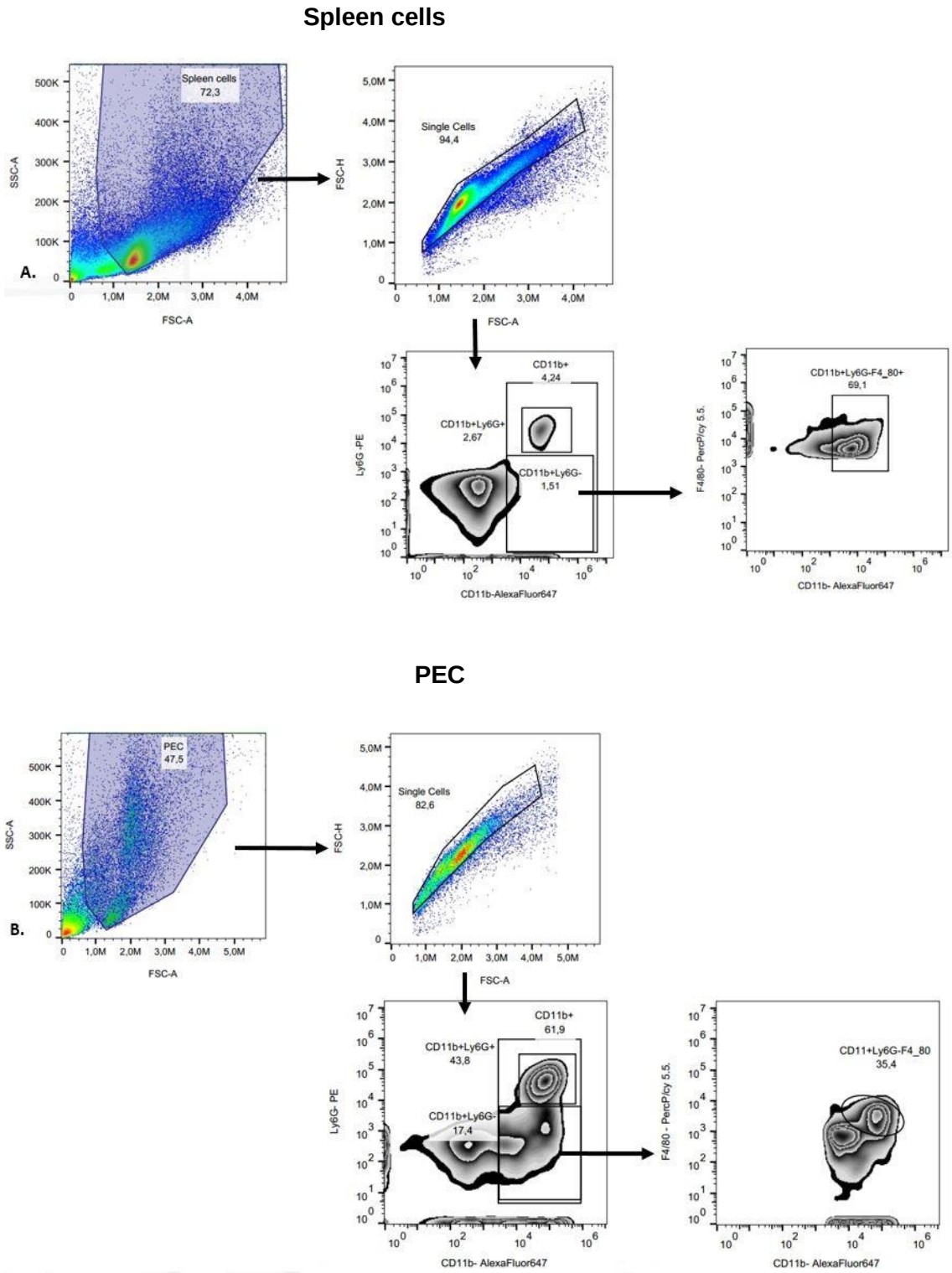

Fig. 17 –Representative flow cytometry gating strategy used to define granulocytes ($CD11b^+$), neutrophils ($CD11b^+Ly6G^+$) and macrophages ($CD11b^+$ $Ly6G^-F4/80^+$) in PEC [A] and spleen cells [B].

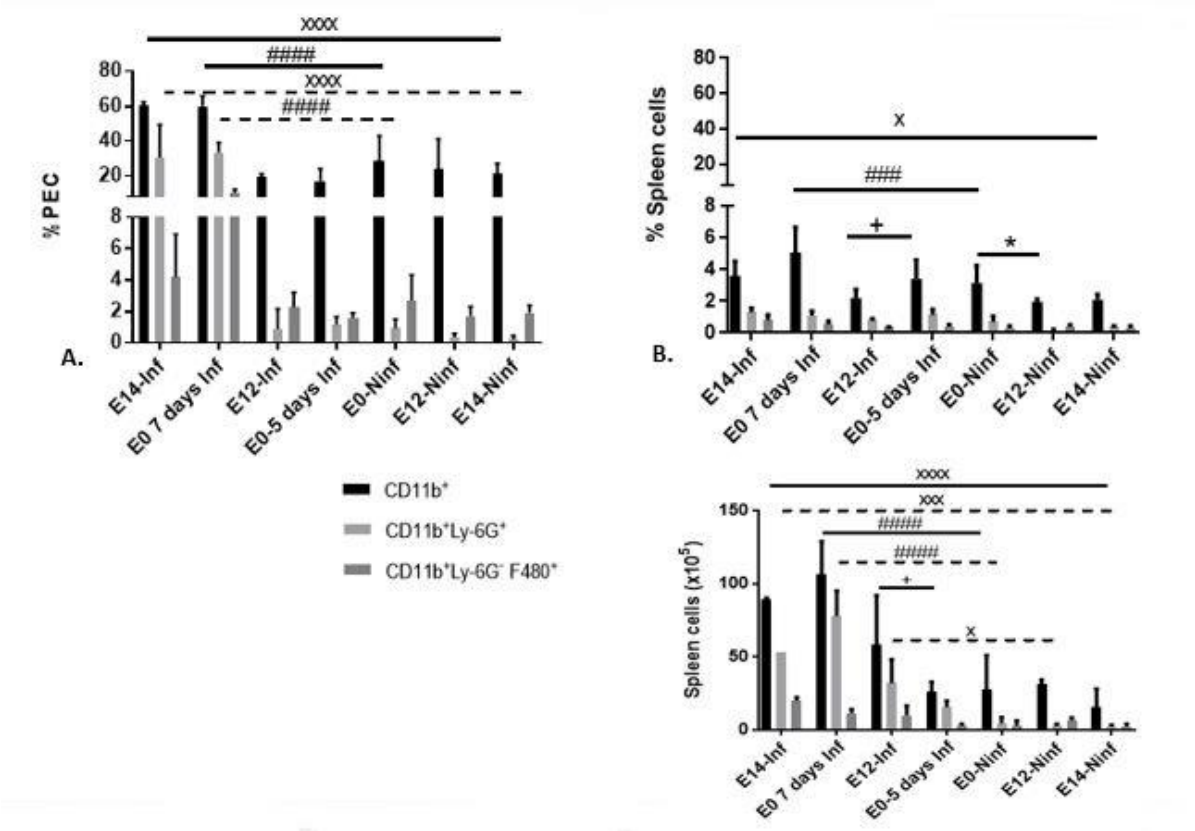

Fig. 18– Evaluation of granulocytes (CD11b$^+$), neutrophils (CD11b$^+$Ly6G$^+$) and macrophages (CD11b$^+$Ly6G$^-$ F4/80$^+$) in PEC [A] spleen cells percentage [B] and spleen cells number [C.]. Each group comprised two to four animals analysed individually. Data represent the mean + SD. * P<0.05, (differences between E12-Ninf and E0-Ninf). ^{x} P<0.05,^{xxx} P<0.001, ^{xxxx} P<0.0001 (differences between E14-Inf/E12-Inf and E14-Ninf/E12-Ninf, respectively). $^+$ P<0.05, (differences between E12-Inf and E0-5 days Inf,). $^{###}$ P<0.001, $^{####}$ P<0.0001 (differences between E0-7 days Inf and E0-Ninf).

8. Arg-1 and NOS2 expression macrophages and neutrophils

As previously mentioned, macrophages have two activation profiles: M1 and M2 stages (150). To evaluate the macrophages activation at systemic level during *T. gondii* infection, the flow cytometry analysis was performed using spleen cells from the different groups, previously described. Flow cytometry was used for the analysis of the neutrophils (CD11b$^+$Ly6G$^+$) and macrophages (CD11b$^+$F4/80$^+$) expressing Arg-1 and NOS2. The gating strategy used for this analysis is shown in Fig.19.

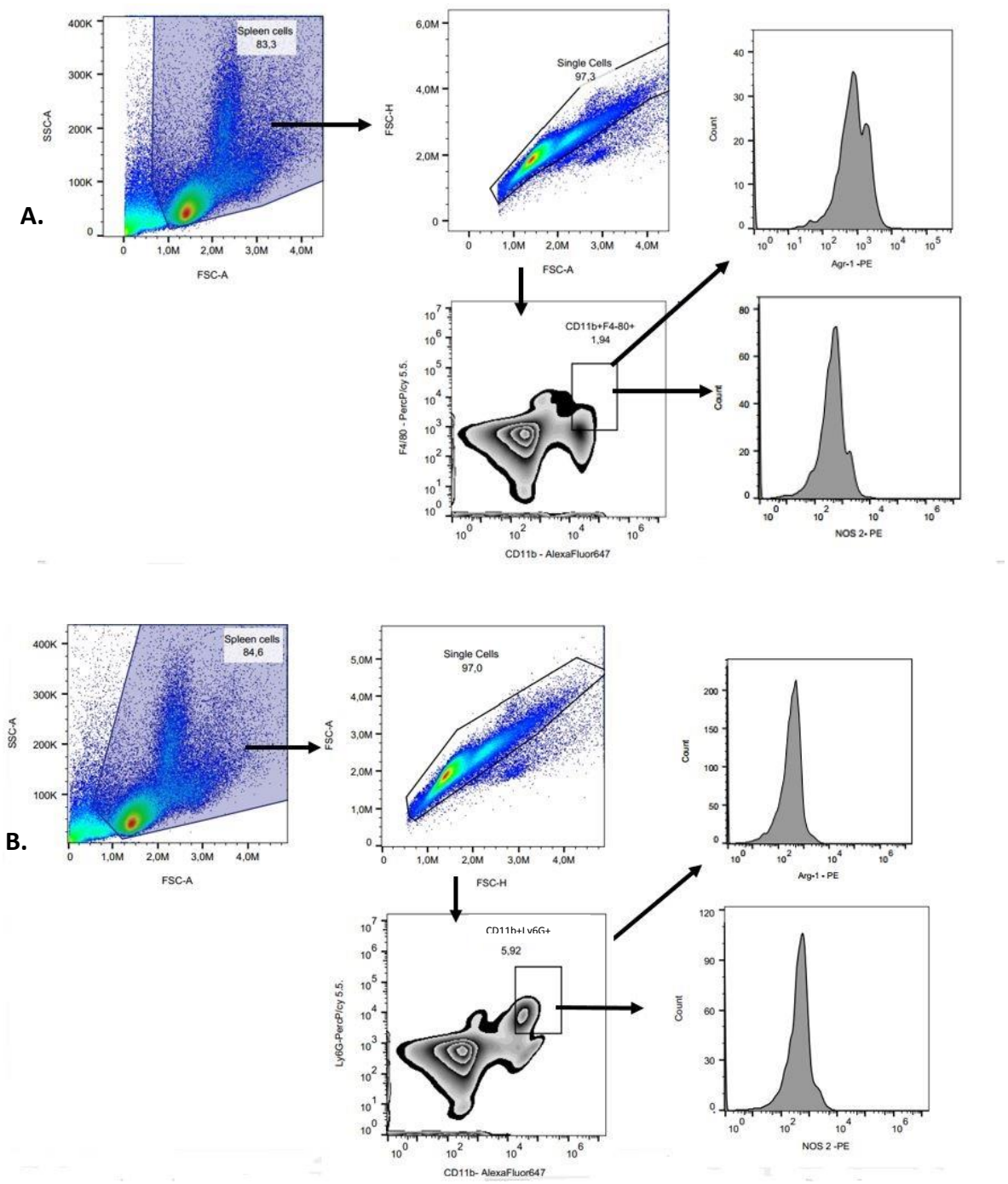

Fig. 19 – Representative flow cytometry gating strategy used to define spleen M1 ($CD11b^+F4/80^+NOS2^+$) and M2 ($CD11b^+F4/80^+Arg\text{-}1^+$) macrophages [A] and spleen neutrophils expressing NOS2 ($CD11b^+Ly6G^+NOS2^+$) and Arg-1 ($CD11b^+Ly6G^+Arg\text{-}1^+$) [B].

Arg-1 and NOS2 were not detected in our experimental conditions. The results indicate negative staining for Arg-1 and NOS2 in the spleen cell *ex-vivo* analysis for all the groups of mice analysed (Fig.20).

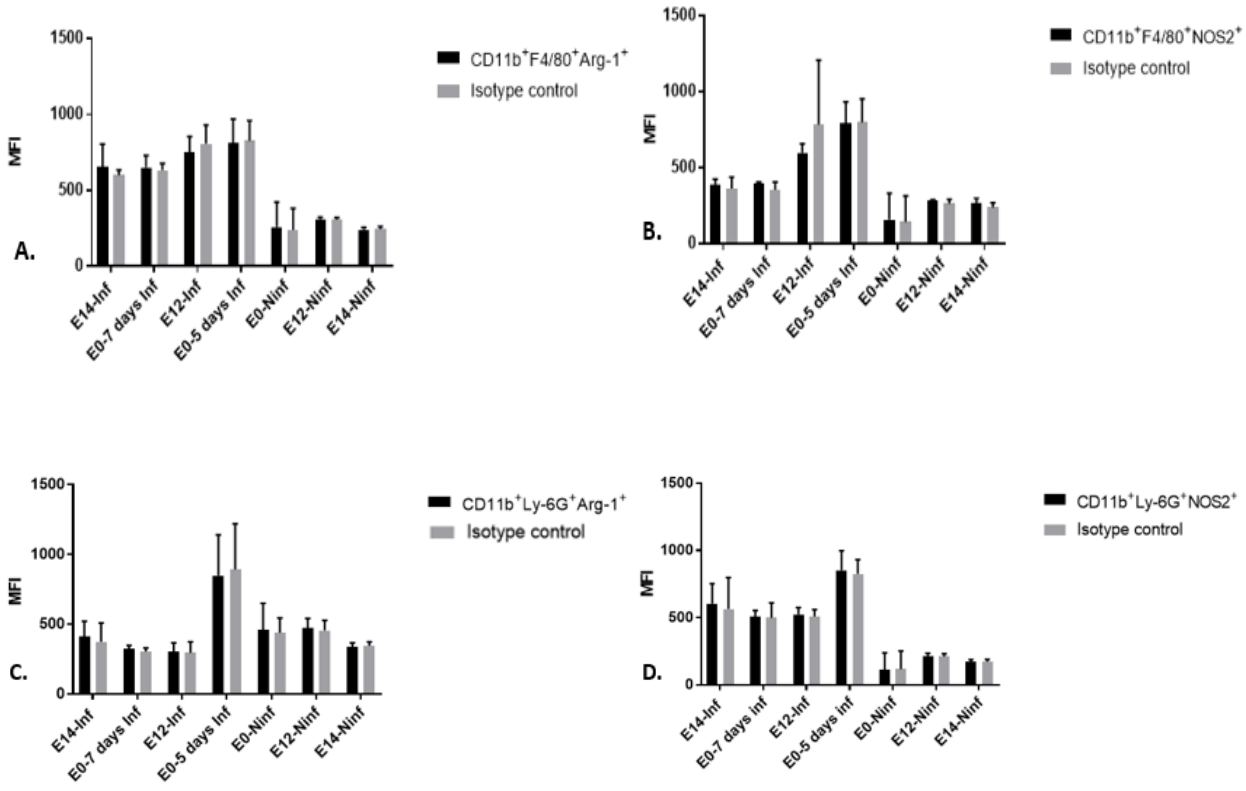

Fig. 20 – Evaluation of macrophages [A, B] and neutrophils expressing Arg-1 and NOS2 [C, D]. The cells were labelled with two combinations (CD11bF4/80 or CD11bLy6G) and subsequently labelled with intracellular antibodies (Arg-1, NOS2 and isotype antibody control). Each group comprised two to four animals analysed individually. Data represent the mean + SD.

9. MHC II expression in macrophages

Macrophages and other phagocytic cells (like dendritic cells) have the capacity of *T. gondii* phagocytosis and are able to present antigens to $CD4^+$ T cells. Normally, these cells are denominated antigen-presenting cells (APCs), which expressed MHCII molecules. Macrophages are also characterized by a MHCII increase expression after pro-inflammatory stimulation (167). To evaluate the percentage of $MHCII^+$ cells during *T. gondii* acute infection, flow cytometry analysis were performed using spleen cells and PEC. The values are expressed as Mean Fluorescence Intensity (MFI) for MHCII inside $CD11b^+F4/80^+$ cell population. The gating strategy used for PEC analysis is shown in Fig. 21.

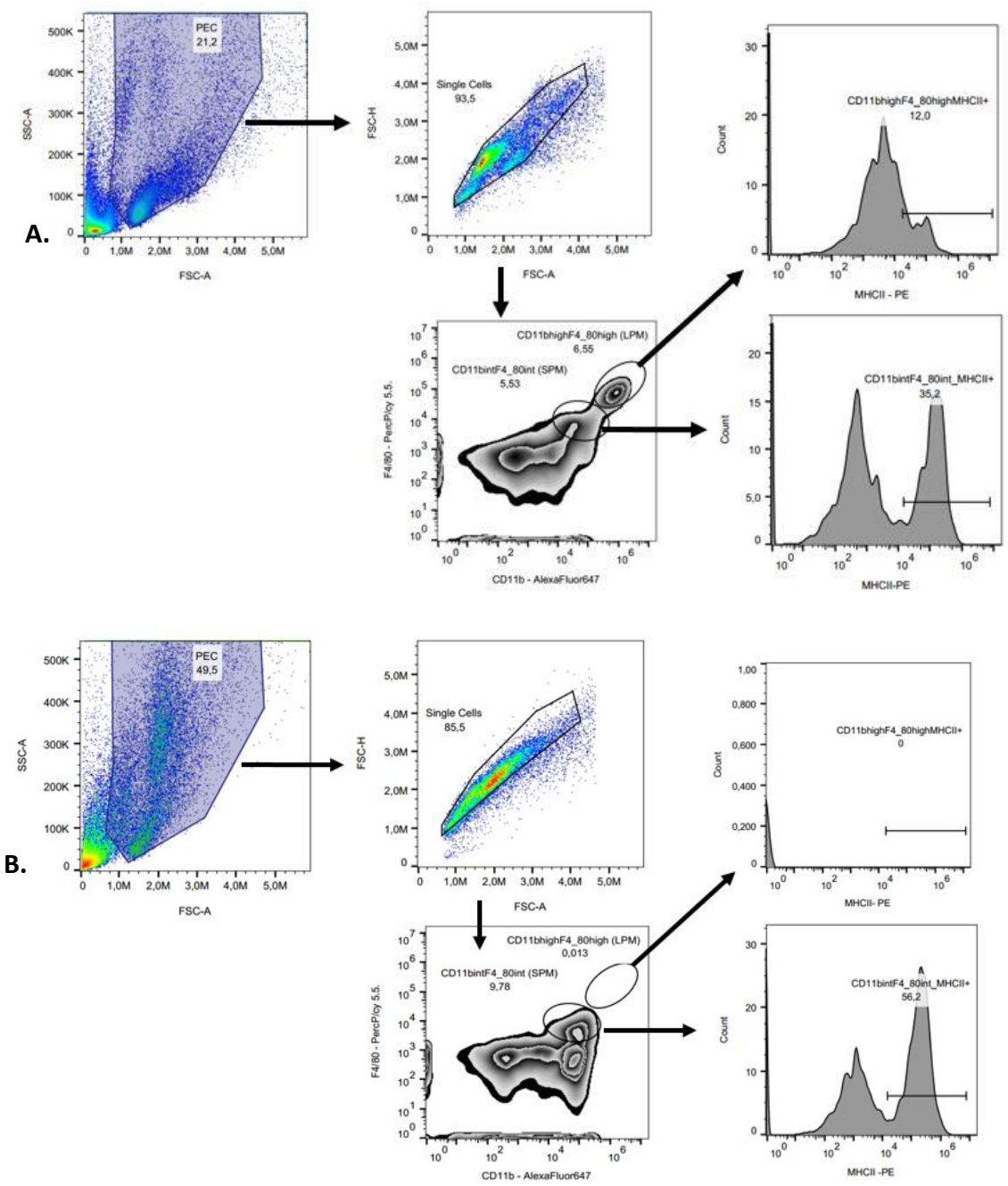

Fig. 21- Representative flow cytometry gating strategy used to define CD11b^{high}F4/80^{high}MHCII^{+} (LPM) and CD11b^{int}F4/80^{int}MHCII^{+} (SPM) in PEC. [A] Data from E0-Ninf animal. [B] Data from E14-Inf animal.

First, we performed the evaluation of the MHCII MFI versus an isotype control to verify the antibody binding specificity (Fig.22 B, C and 24 B).

The analysis of PEC flow cytometry data was performed according to other studies (158). In this study, LPM are defined as CD11b^{high}F4/80^{high} and SPM are defined as CD11b^{int}F4/80^{int}. To verify the specificity and the expression of MHCII in LPM and SPM, the isotype control was used at the same time in the surface stanines.

The data indicate that LPM disappear in response to *T. gondii* infection (E14-Inf and E0-7 days Inf compared to E14-Ninf and E0-Ninf, respectively; Fig. 22 A). A significant decrease of LPM was observed in E0-5 days Inf compared to E0-Ninf (Fig. 22 A). SPM were also detected in all animal groups (Fig. 22 A). However, there seems to be an increase between E0-7 days Inf vs E14-Inf and E0- 7 days Inf vs E0-Ninf.

As expected to MHCII expression was higher in SPM population (154300 ± 765,8), when compared to LPM population (4291 ± 872,3) in E0-Ninf animal group, as previously described (158). For MHCII analysis in spleen cells, we used the gating strategy shown in Fig. 23.

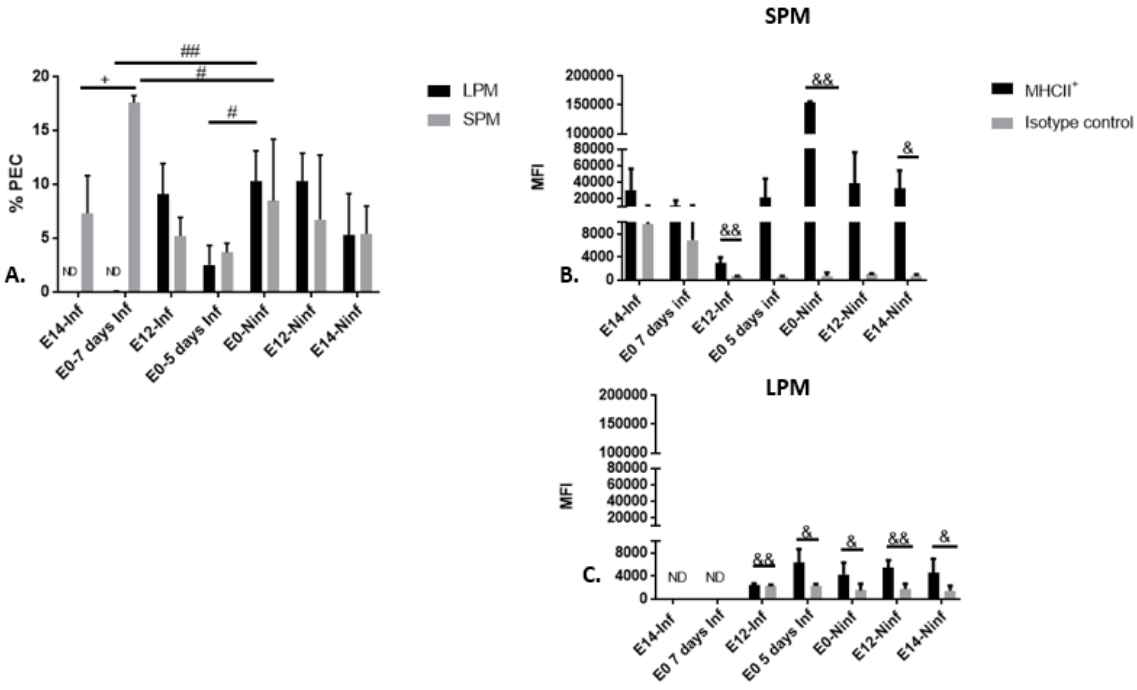

Fig.22- Evaluation of MHCII in LPM and SPM. [A] Percentage of LPM and SPM. [B] Evaluation of the MHC II MFI versus isotype control for SPM. [C] MHCII MFI versus isotype control for LPM. Each group comprised two to four animals analysed individually. Data represent the mean + SD. $^{\&}P<0.05$, $^{\&\&}P<0.01$ (differences between MHCII MFI versus isotype control). $^{+}P<0.05$ (differences between E14-Inf and E0-7days Inf). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, (differences between E0-7 days Inf, E0-5 days Inf and E0-Ninf).

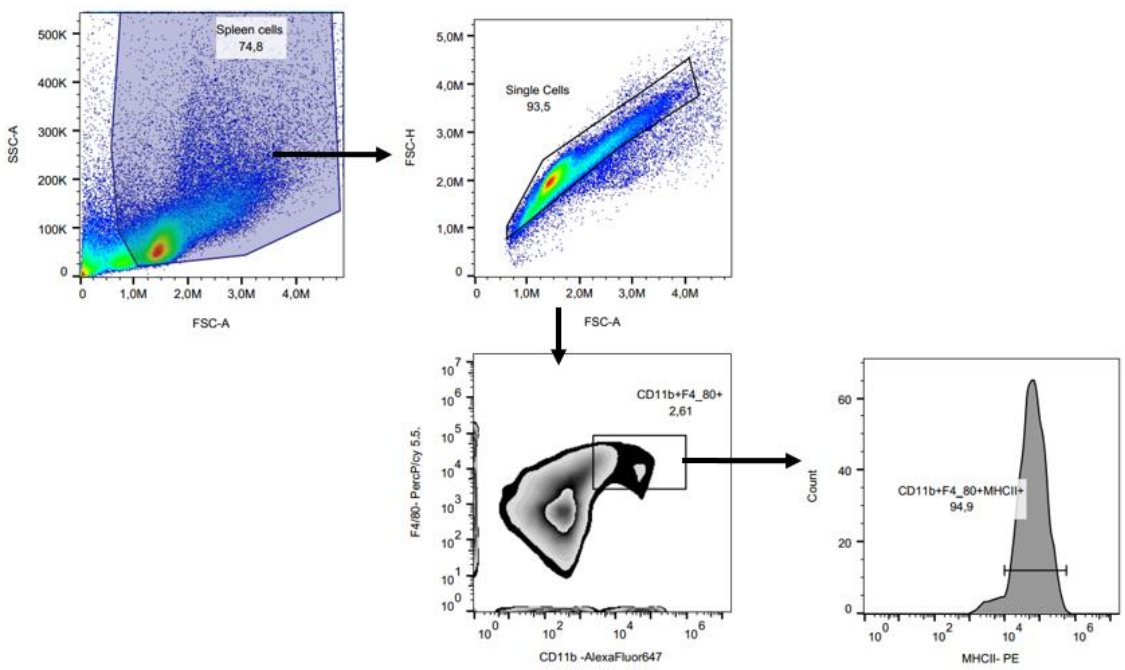

Fig.23- Representative flow cytometry gating strategy used to define MHCII CD11b^{+}F4/80^{+} spleen cells.

The results indicate a specific MHCII expression in spleen CD11b^{+}F4/80^{+} cells except for E0-Ninf and E14-Ninf (Fig.24 B). A decrease of CD11b^{+}F4/80^{+}MHCII^{+} percentage was observed in E12-Inf vs E12-Ninf and E12-Inf vs E0-5 days Inf indicating that *T. gondii* infection interferes during pregnancy with macrophages MHCII expression.

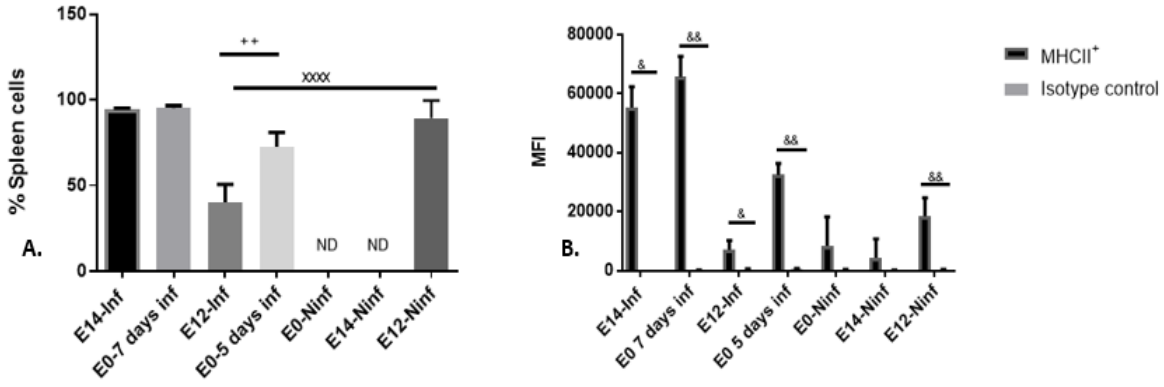

Fig.24- Evaluation of MHCII in spleen cells. [A] Evaluation spleen CD11b^{+}F4/80^{+}MHCII^{+} cells [B] Evaluation of MHCII expression**.** Each group comprised two to four animals analysed individually. Data represent the mean + SD. $^{\&}P$<0.05, $^{\&\&}P$<0.01, (differences between MHCII MFI versus isotype control), ^{xxxx}P<0.0001 (differences between E12-Inf and E12-Ninf). ^{++}P<0.01 (differences between E12-Inf and E0-7days Inf).

Discussion

T. gondii is one protozoan parasite that infects billions of people in diverse areas of the world. The parasite infection is normally asymptomatic, but in immunocompromised hosts it has a diversity of the clinical manifestation (55). Also, a primo-infection during pregnancy can lead to fetus clinical manifestation or even abortion depending on the gestation period (168). *T. gondii* has the capacity of crossing biological barriers, such as the intestine or the placenta, thereby, disseminating through the blood flow and reaching several organs of the intermediate host (169). Murine models are used in the study of *T. gondii* infection and in the understanding of the fundamental immune mechanisms, which control intracellular parasite infection (154). Also, murine models are frequently used in the study of fetal-placental development. In this work, it was used the Balb/c strain infected with viable tachyzoites of *T. gondii* type II strain. However, the obtainment of the infected mice was very difficult.

As previously described to obtain viable tachyzoites *in vitro* is needed a host cell. In this study we used the HFF cells. The use of HFF cells have several advantages, such as the large and flat morphology permitting multiple cycles of parasite replication within each cell, the presence of contact inhibition, thus allowing the confluent monolayer to remain many weeks until being needed and HFF cells also supply a host comparable in some aspects to a clinical infection (170). However, HFF has two important disadvantages: the slow growth, particularly of the cells with elevated passage number, and a limitation of the replication lifespan (170).

Differences in the culture, such as passage number, age and density, can lead to significant differences in their capacity of keeping parasite growth (170). Therefore, in this work, some problems in the maintenance of the HFF culture occurred, because the stock vial cells weren't in the best conditions, due to the cells age. During the first couple of months, HFF didn't grow easily, and subsequently, affected the multiplication of the parasite. Moreover, as shown in this work is very difficult to get synchronous infection with *T. gondii*, being the time between infection and cell lysis very variable. In this study, 16 of the inoculated mice didn't present infection and the number of infected mice was much reduced. The data from flow cytometry analysis of spleen cells and PEC populations were related to only two E14 - Inf mice presenting similar infection, three E0-7 days Inf mice, four E12-Inf mice and four E0-5 days Inf mice.

A recent work describes that a non-virulent *T. gondii* infection may cause pathological consequences without vertical transmission (160). After maternal *T. gondii* infection, parasite can cause different types of damage including increased of resorpted units, extensive haemorrhages or local haemorrhaging and offspring absence (160). Macroscopically evaluation of implantation sites and spleen of infected and non-infected mice was evaluated. It was observed between 2-3 resorpted units and between 8 to 9 FP units in all groups of animals, indicating that in our study model, *T. gondii* infection was not inducing increase resorption rate. Additionally, one of the E12-Inf mice presented offspring absence (data not show). At present, volumetric analysis of decidua and placenta collected during this work are being performed in order to verify the effect of *T. gondii* infection at the maternal-fetal interface. Another macroscopic observation was splenomegaly in E14-Inf and E14-Ninf mice. These observations suggest that pregnancy *per si* induces an expansion of spleen in both infected and uninfected mice. The literature also suggests that pregnancy and infection with *T. gondii* in murine model induces a severe splenomegaly (163,164).

One of the aims of this study, was the quantification of the parasite in several organs, such as liver, lung, heart, kidney and spleen by q-PCR. These results indicate that after 5 days of infection, the parasite was not detected in the majority of the organs. Otherwise, in animals with 7 days of infection, it was possible to detect parasite in the majority of the organs. In the context of pregnancy, the quantification of the parasite was also performed in decidua/placenta and embryo tissues. After 5 days of infection, was detected the presence of the parasite in decidua/placental tissues of two animals, but not in the embryo indicating no vertical transmission. After 7 days of the infection is detected parasite in the decidua and in the placenta of two animals analysed and in the embryo of one of three animals, indicating vertical transmission. Flow cytometry analysis of PEC-TOXO YFP^+ allowed the parasite detection in all considered infected mice in our experimental conditions, compared with q-PCR technique. Also, it allowed parasite detection, indicating an increase in cells–TOXO YFP^+ in E14-Inf mice compared to E12-Inf mice.

In the literature it is described that pregnant mice are more susceptible to *T.gondii* infection compared with non-pregnant mice (171). Previous studies by us suggested that pregnancy has a protective effect against *T. gondii* infection in Balb/c mice (data not show). However, since the data obtained by us are contradictory between the two experimental sets, concerning parasite load in pregnant infected compared to non-pregnant infected mice, more experiments will be needed.

T cell populations have an important role in the host defence against *T. gondii* infection. During early acute infection an increase of the $CD4^+$ and $CD8^+$ T cells proliferations occurs (152,172). $CD4^+$ T cell has an essential role to regulate the *T. gondii* immune response and $CD8^+$ T cell plays an important role in intracellular pathogen clearance (102,153). In this study, it was evaluated T cell populations at systemic level (spleen cells) and in the local of infection (PEC). The results obtained indicated that during pregnancy, a decreased percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ were observed in the spleen. It is known, that maternal immune system tolerate the presence of the fetus by a suppression of immune responses (173). This way, the decrease percentage of T cell populations in spleen cells from pregnant non-infected mice in our experiments may be associated to a decrease in the maternal immune system response that contributes to fetal tolerance. It was observed an increase of PEC $CD4^+$ T cells in E0- 7 days Inf and E0-5 days Inf mice compared to E0-Ninf, and an increase of the PEC $CD8^+$ T cells in E14-Inf and E0- 7 days Inf mice, compared to E14-Ninf and E0-Ninf, respectively. These results indicating that infection induces the T cell recruitment and/or proliferation at the local of infection in early stages of *T. gondii* infection, as already reported in other studies (172,174,175).

Neutrophils are phagocytic cells that during *T. gondii* infection are rapidly recruited to the local of infection (24). Here, is observed a significant increase of the neutrophils percentage in E14-Inf and E0-7 days Inf compared to E14-Ninf and E0-Ninf. The results obtained indicate a significant increase of spleen neutrophils ($CD11b^+Ly6G^+$) in number from E14-Inf, E0-7 days Inf and E12-Inf mice, but not in E0-5 days Inf mice when compared to the corresponding uninfected animal groups. Parasite quantification allowed the detection of the parasite in the spleen of E14-Inf mice, indicating that *T. gondii* induces the recruitment of neutrophils to the spleen.

Macrophages are phagocytic cells with an essential role in the recognition and elimination of pathogens, such *as T. gondii* (24). Polarity macrophages can be divided in M1 or M2 activation profile. M1 macrophages are characterized by the expression of NOS2, responsible for the metabolization of arginine to nitric oxide and citruline. M2 macrophages express the enzyme arginase-1, which is responsible for the hydrolysing arginine into ornithine (132). Macrophages analysis was evaluated in PEC and spleen. The results obtained for PEC and spleen cells (percentage and number) did not presented significant differences in macrophages ($CD11b^+Ly6G^-F4/80^+$) between the different groups analysed. However, when analysing results obtained seems to be an increased (but not statically significant) of $CD11b^+Ly6G^-F4/80^+$ in PEC of the E14-Inf and E0- 7 days Inf mice, when compared with the other groups. Macrophages

activation was evaluated by flow cytometry analysis of NOS2 and Arg-1 MFI. Nevertheless, the results obtained were not conclusive, since specific binding was not found. Mores studies will be performed in order to evaluate NOS2 and Arg-1 expression by flow cytometry analysis of PEC. Other markers are used to define the activation profile of macrophages, like MHCII which it is a marker for the M1 profile or classical activation (126). MHCII molecules have an important function in peptide presentation by APC (macrophages, dendritic cells) to T cells. These molecules can be divided into class I and the class II, which interact with $CD8^+$ and $CD4^+$ T cells, respectively (176).

In literature, is described the peritoneal macrophages characterization in two subtypes: LPM and SPM (158). Differences in MHCII expression using control Balb/c mice are described allowing the description of SPM with increased MHCII MFI (60000) compared to LPM (Not detected; 159). Our results are in agreement with this observation, being the MHCII MFI for SPM increased in E0-Ninf mice (154300) compared to LPM for E0-Ninf mice (4291). In literature, it was suggested that the peritoneal macrophages after an *in vivo* stimulation with lipopolysaccharide (LPS) or thioglycolate lead to a decrease of LPM and an increase of SPM by *ex-vivo* analysis (158). Our results indicate that *T. gondii* infection induces the disappearance of the LPM.

Spleen cells analysis indicated that specific detection of MHCII were observed in E14-Inf, E0-7 days Inf, E12-Inf, E0-5 days Inf and E12 Ninf, but not in E0-Ninf and E14-Ninf. The data concerning the evaluation of $CD11b^+F4/80^+MHCII^+$ percentage indicates that pregnancy at day 12 decreases the MHCII expression independently of infection (E12-Inf vs E0-5 days inf), but infection in the context of pregnancy can also decrease the percentage of $CD11b^+F4/80^+MHCII^+$ (E12-Inf vs E12-Ninf). These may suggest that *T. gondii* may be influencing pregnancy-dependent pathways (as pregnancy hormones-dependent) interfering with the capacity of MHCII expression (177–179).

Conclusion

Toxoplasmosis is a prevalent disease in a large number of countries worldwide, and it became a health public problem. Therefore, it is important to understand the immunological mechanisms and cell players involved in *T.gondii* immune response at the systemic level during pregnancy and specifically at the maternal-fetal interface during *T.gondii* infection. In this work, the results obtained can be divided into two levels: the maternal-fetal interface and the systemic level.

Thus, using our experimental study model, the results obtained by macroscopic analysis indicated that *T.gondii* infection did not induce an increase of resorption rate. *T.gondii* parasite load quantification at the maternal-fetal interface was indicated that the parasite did not reach the embryo after 5 days of infection. However, it was possible to detect the parasite in one embryo from 1 out of the 3 mice analyzed after 7 days of infection. At the systemic level, it was observed that 5 days after *T.gondii* infection, the parasite was not able to disseminate from the local of infection (intraperitoneal cavity) to the lung, heart, and kidney. Otherwise, it was possible detected *T.gondii* in the majority of the organs after 7 days of infection.

Concerning the characterization of immune cell populations, the results indicated that *T.gondii* infection induced an increase of $TCD4^+$, $TCD8^+$ and $CD11b^+Ly6G^+$ in the local of infection and an increase of $TCD4^+$ and $CD11b^+Ly6G^+$ in the spleen independently of the pregnancy. The results obtained did not show differences in the $CD11b^+Ly6G^-$ $F4/80^+$ between the animal groups analysed. However, it was possible to show a decrease of $CD11b^+F4/80^+MHCII^+$ spleen cells at day 12 of pregnancy, in response to infection.In our experimental conditions, Arg-1 and NOS2 expression was not detected in spleen cells by flow cytometry. Lastly, flow cytometry analysis indicated that was not possible to detect LPM after *T.gondii* infection independently of pregnancy. The results obtained, indicate that further functional characterization of the immune cell populations studied here, must be performed at the systemic level, but also at the maternal-fetal interface, specifically at day 7 of infection, in order to deep understand a putative role during congenital toxoplasmosis. Also, the results obtained suggests that *T.gondii* may be influencing pregnancy-dependent pathways (as pregnancy-hormone dependent) interfering with the capacity of MHCII expression.

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