Universidade de Aveiro Departamento de Biologia
2015

Carina Vanessa Fernandes Brito

Determinação da expressão da Arginase 1 e Sintase do Óxido Nítrico Induzível durante a gravidez e infeção por *Toxoplasma gondii*

Determination of Arginase 1 and Nitric Oxide Synthase expression during pregnancy and *Toxoplasma gondii* infection

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Margarida Maria Coutinho Nogueira Marta Borges, Professora Auxiliar do Departamento de Ciências Biológicas da Faculdade de Farmácia da Universidade do Porto e Doutor Mário Jorge Verde Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

"*Depois de termos conseguido subir a uma grande montanha, só descobrimos que existem ainda mais grandes montanhas para subir*"

Nelson Mandela

o júri

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Artigos em revista de circulação Internacional com arbitragem Científica:

T.M.Silva, C.Brito, N.Teixeira, and M.Borges.
The role of Arginine at the fetomaternal interface during *Toxoplasma gondii* infection.
Manuscript *in preparation*

C.Brito, T.M.Silva, A.Gonçalves, N.Teixeira and M.Borges.
Determination of Arginase 1 and Inducible Nitric Oxide Synthase expression during pregnancy and *Toxoplasma gondii* infection.
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Resumo em revista de circulação Internacional com arbitragem Científica:

C. Brito, A. Gonçalves, T. M. Silva, N. Teixeira, M. Borges.
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C. Brito, T. M. Silva, N. Teixeira, M. Borges.
Arginase 1 and iNOS expression at systemic level and fetomaternal interface during Toxoplasma gondii infection
YES Meeting, Faculdade de Medicina, Porto, Portugal, 15-18 de Setembro de 2015

C. Brito, A. Gonçalves, T. M. Silva, N. Teixeira, M. Borges.
Determination of macrophage activation profile during Toxoplasma gondii infection and implications on pregnancy outcome using the mice model
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Palavras-chave

Toxoplasmose congénita, Aborto, *Toxoplasma gondii,* Arginase 1, NOS2, Macrófagos

Resumo

Toxoplasma gondii é considerado um parasita zoonótico, causando toxoplasmose congênita, uma doença prevalente em todo o mundo com implicações graves para o feto. Os mecanismos que desempenham um papel, induzindo patologia durante a infeção por este parasita não são claros, mas são potencialmente associados com alterações dos processos homeostáticos imunológicos normais, incluindo a ativação de macrófagos. Os macrófagos apresentam uma variedade de estadios de ativação descritos como ativação inata (ligação TLR), clássica/M1 (ligação TLR com IFNγ) ou alternativa/M2 (ligação IL4Ralpha). Assim, enquanto os macrófagos M1 controlam a replicação dos parasitas pela indução de sintetase do óxido nítrico induzível (NOS2), os macrófagos M2 controlam a replicação dos parasitas induzindo a arginase-1 (Arg-1) e depletando a arginina.

O nosso estudo incidiu sobre os efeitos da infeção por *T. gondii,* na expressão de Arg-1 e de NOS2 na interface feto-materna (FMI) e a nível sistémico (células de exsudado peritoneal e células do baço) utilizando murganhos como modelo de estudo.

A infeção de animais gestantes Balb/c e C57BL/6 (B6) com uma estirpe de *T. gondii* tipo II permitiu o acompanhamento da gravidez. A análise morfométrica da decídua e da placenta foi realizada utilizando seções de unidades feto-placentárias coradas com hematoxilina-eosina. A determinação da carga parasitária foi realizada por PCR quantitativo em tempo real utilizando sondas *Taqman* (q-PCR). A expressão de Arg-1 e NOS2 foi avaliada por qRT-PCR, Imuno-histoquímica e Western-Blotting.

Observou-se uma diminuição significativa na área da placenta e da decídua em B6 infetados em comparação com os animais controlo, mas não nos animais Balb/c. A análise imunohistoquímica indicou um aumento da expressão de Arg-1 e uma diminuição da expressão de NOS2 na decídua de animais infetados em comparação com os animais controlo em ambas as estirpes. Os resultados obtidos sugerem que a infeção interfere com o processo de placentação atrasando a decidualização, podendo existir uma relação com as alterações da expressão de Arg-1 e NOS2 nos B6, mas não nos animais Balb/c. O trabalho desenvolvido visa não só a compreensão do papel da ativação de macrófagos na infeção congénita pelo *T. gondii*, mas poderá fornecer informações essenciais sobre o papel dos macrófagos durante uma gravidez saudável e uma gravidez com infeção.

Keywords Congenital toxoplasmosis, Abortion*, Toxoplasma gondii,* Arginase 1, NOS2, Macrophages.

Abstract

Toxoplasma gondii is considered to be the world's most successful zoonotic parasite, causing congenital toxoplasmosis, a prevalent disease worldwide with serious implications for the fetus. The mechanisms playing a role in the induction of pathology during infection are not clear, but are potentially associated with disruption of normal homeostatic immunological pathways including macrophage activation. Macrophages have a variety of activation states, being able to adapt their functions to environmental changes of cytokines, described as innate (TLR ligation), classical/M1 (TLR ligation with IFNγ) or alternative/M2 (IL4Ralpha ligation) activation states. Thus, while classical macrophage activation can control replication through induction of inducible nitric oxide synthase (NOS2), alternative activation can control parasite replication through induction of arginase 1 (Arg-1) and depletion of arginine.

Our study was focused on the effects of *T. gondii* infection on Arg-1 and NOS2 expression at the fetomaternal interface (FMI) and systemic level (peritoneal exudate cells and spleen cells) using the mice model.

Infection of pregnant BALB/c and C57Bl/6 (B6) mice with a type II strain of *T. gondii* allowed the follow-up of pregnancy. Morphometric analysis of decidua and placenta was performed using haematoxylin-eosin sections of the fetoplacental units. The parasite loads' evaluation was done by quantitative Real Time-PCR (q-PCR) using *Taqman* probes. Arg-1 and NOS2 expression were evaluated by qRT-PCR, immunohistochemistry and Western Blotting.

It was observed a significant decrease in placental and decidual areas in infected B6 compared to control but not in Balb/c mice. Immunohistochemical analysis indicated an increased expression of Arg-1 and a decreased expression of NOS2 in the decidua from infected compared to control animals from both strains of mice. This indicates that infection interferes with the process of placentation delaying decidualization and this might be correlated with altered expression of Arg-1 and NOS2 in B6 but not in Balb/c mice. This work aims not only understanding the role of macrophage activation in congenital infection by *T. gondii,* but also should provide valuable information regarding the role of macrophages during healthy pregnancy and infection complicated pregnancy.

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List of Abbreviations

AIDS	Acquired immune deficiency syndrome
Arg-1	Arginase 1
Arg-2	Arginase 2
B6	C57Bl/6 mice
Balb\c	Balb\c/cByJ
CCR	C-C chemokine receptor
CXCR	C-X-C chemokine receptor
DAPI	4’,6-diamidino-2-phenylindole
DCs	Dendritic cells
dDCs	Decidual dendritic cells
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
gDNA	Genomic DNA
E	Day of gestation
FMI	Fetomaternal interface
H&E	Hematoxylin and Eosin
HFF	Human foreskin fibroblasts
HPRT	Hypoxanthine guanine phosphoribosyl transferase
Ig	Immunoglobulin
IL	Interleukin
IFN-γ	Interferon gamma
ip	Intraperitoneal
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NK	Natural killer cells
dNK	Decidual natural killer cells
NO	Nitric oxide
NOS	Nitric oxide synthase

NOS2 or iNOS	Inducible nitric oxide synthase
ODC	Ornithine decarboxylase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEC	Peritoneal exudate cells
q-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
SAG1	Surface antigen 1
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
T. gondii	*Toxoplasma gondii*
Th	T helper cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T lymphocytes
UFs	Fetoplacental units
VEGF	Vascular endothelial growth factor

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Chapter I. **INTRODUCTION**

1. *Toxoplasma gondii*

Toxoplasma gondii (*T. gondii*) is a protozoan parasite of the Apicomplexa phylum (1) and Coccidiasina subclass (2), with cats as definitive host and other warm-blooded animals as intermediate hosts (3). It was first discovered by Nicolle and Manceaux during a leishmaniosis research project at the Pasteur Institute in Tunis (4). In the beginning, they have confused the parasite as a piroplasm, later designated as Leishmania, but soon they realized that had discovered a new organism (5). Based on the morphology (toxo=arc, plasma=life) and the host (*Ctenodactylus gundi*) it was named *Toxoplasma gondii* (Fig. 1) (6). Thereafter, *T. gondii*-like organisms were detected, but the first viable *T. gondii* was isolated by Sabin and Olitsky (7).

T. gondii has a worldwide distribution, but there is only one specie (8). Different methods were established to identify genetic differences between *T. gondii* isolates from humans and animals (9,10). In 2005, Khan *et al.,* performed the mapping of *T. gondii* genes, which definitely was determinant in understanding the disease pathology mechanisms, and for the search of better antigens for diagnosis and vaccination (11).

T. gondii infection is common, affecting about one third of the world population (12), while developing toxoplasmosis only occurs in rare situations such as in immunocompromised hosts and in pregnant women (13).

The primary infection in pregnant women can cause congenital toxoplasmosis, that might lead to abortion, fetal growth restriction, mild chorioretinitis, mental retardation microcephaly, hydrocephalus, seizures (14) or ocular disease, being this late sequel, undoubtedly the most common of congenital toxoplasmosis (15).

Figure 1. Tachyzoites analysed by scanning electron microscopy. Scale 2 µm. (16)

2. Structure and Life Cycle

There are three infection stages of *T. gondii*: tachyzoites (Figure 2A), bradyzoites (Figure 2B-C) and sporozoites (Figure 2G) (15).

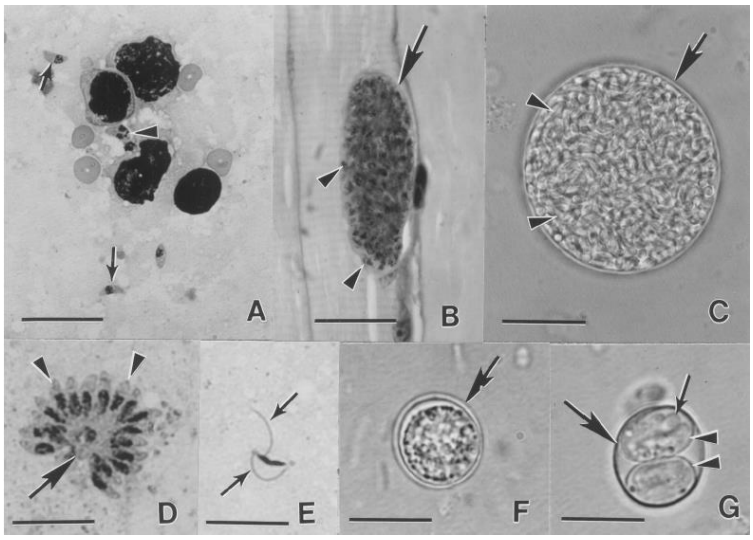

Figure 2. Stages of *T. gondii.* (A) Tachyzoites in impression smear of lung. Note: crescent-shaped individual tachyzoites (arrows) and dividing tachyzoites (arrowheads) compared with size of host red blood cells and leukocytes. (B) Tissue cists with bradyzoites in a section of muscle. Note: tissue cyst wall (arrow) and bradyzoites (arrowheads). (C) Brain tissue cists with sporozoites. Note: tissue cyst wall (arrow) and sporozoites (arrowheads). (D) Schizont (arrow) with several merozoites (arrowheads), impression smear of infected cat intestine. (E) A male gamete with two flagella (arrows). (F) Unsporulated oocyst in fecal float of cat feces. Note: double layered wall (arrow). (G) Sporulated oocyst. Note: oocyst wall (large arrow), two sporocysts (arrowheads). Each sporocyst has four sporozoites (small arrow). Scale bar in (A-D) = 20 mm, in (E-G) = 10 mm. (15)

After the ingestion of tissue cysts by cats, the cyst wall is dissolved in the small intestine and the bradyzoites are released and penetrate the epithelial cells, thereby initiating the development of asexual and sexual cycles of *T. gondii* (Fig. 3) (17). After penetrating into the epithelial cells, the bradyzoites can multiply as tachyzoites and, within a few hours, may disseminate to extraintestinal tissues forming cysts in the heart and brain persisting several months and possible for the lifespan of the cat (17).

As the asexual cycle progresses, the sexual cycle also occurs, as *T. gondii* multiplies as schizonts (Fig. 2D) in intestinal epithelial cells of cats. The merozoites released from schizonts form the male gamete, with two flagella (Fig. 3E), and the female gamete after fertilization form the oocyst. After maturation, the oocysts are released into the intestinal lumen (15). Upon ingesting any of the infection stages

of *T. gondii,* cats release oocysts that take 1-5 days to sporulate and become infectious (Fig. 2G) (15,18,19). Oocysts are the resistant stage and are only formed in felids (definitive hosts, where the sexual cycle occurs) (19).

Oocysts, containing sporozoites, spread in the environment and contaminate water, soil, fruits and vegetables, which can be then ingested by humans and other animals (intermediate hosts, where occurs asexual phase). Humans can also be contaminated by ingestion of cysts, containing bradyzoites, found in raw or undercooked meat from previously infected animals (Fig.3) (20). *T. gondii* transmission can also occur through blood transfusions and organ transplants (21).

Sporozoites and bradizoites invade the gastro-intestinal tube, undergoing multiplication in the form of tachyzoites that disrupt the infected cells and invade new cells (20). With the onset of immune response, there is a reduction in parasite load. The development of tissue cysts (with bradyzoites) remain latent throughout host lifespan in tissues such as lymph nodes, muscle, brain, retina, myocardium, lung and liver (14). However in immunosuppressed individuals, bradyzoites can restart their division and spread again as tachyzoites (14).

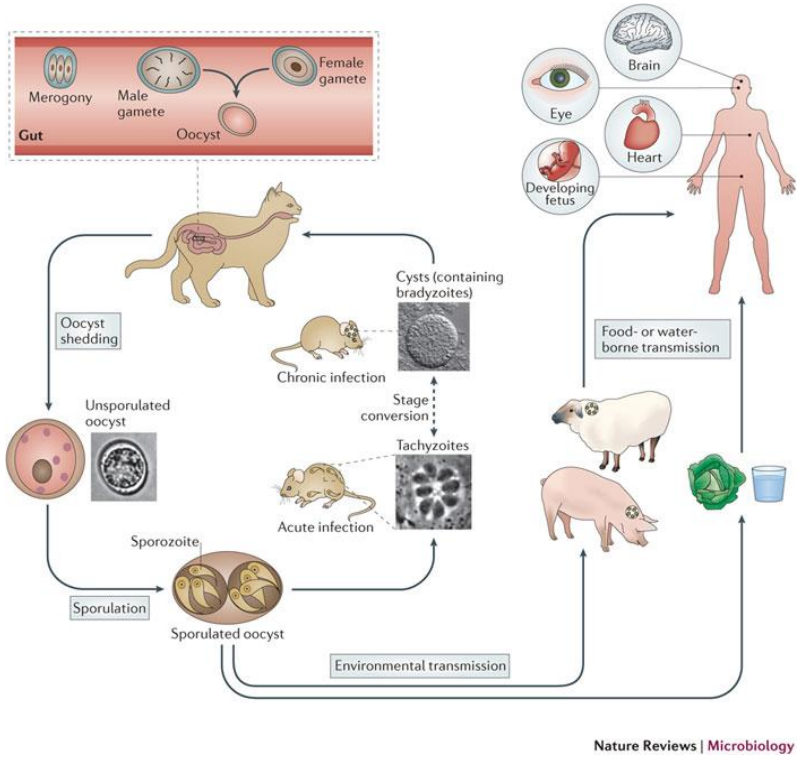

Figure 3. Life cycle of *Toxoplasma gondii* (22)**.**

3. Transmission

In 1939, Wolf *et al*, described congenital *T. gondii* infection in a human child and later, it was also found in several animal species (23). However, congenital transmission occurs very rarely, which does not explain the worldwide spread of the infection. In 1954, Weinman and Chandler proposed that transmission could occur through the ingestion of undercooked meat (24), which was later comproved through the demonstration of *T. gondii* cysts resistance to proteolytic enzymes (25).

While congenital transmission and ingestion of undercooked meat explained some of the *T. gondii* transmission, it did not explain infection of vegetarian and herbivore hosts. In 1965, Hutchison found *T. gondii* infectivity associated with cat feces (26–29). Finally, *T. gondii* life cycle was completely described, with the discovery of the sexual phase of the parasite in the cat gut. It was found that *T. gondii* oocysts derived from gametogony and schizogony (30,31). These oocysts released with cat feces could contaminate food and water being subsequently ingested by animals and humans (12), originating the fecal-oral route of infection, explaining vegetarians and herbivores infection.

4. Infection in humans

Most human infections are asymptomatic, but sometimes the parasite can produce devastating disease. Congenital infection occurs, only when a woman becomes infected during pregnancy.

Infection may occur in any organ, but the most common observed clinical form of toxoplasmosis in humans are enlarged lymph nodes, associated with fever, fatigue, headache, among other symptoms (32). In immunosuppressed patients, encephalitis is the most important clinical form of toxoplasmosis, causing serious damage, such as hemiparesis, convulsions and coma (33). In acquired immune deficiency syndrome (AIDS) patients, brain infection is the most frequently clinical form reported. The predominant lesion in the brain is necrosis, especially of the thalamus (34).

4.1. Congenital Toxoplasmosis

Congenital Toxoplasmosis is caused by vertical transmission of *T.gondii* tachyzoites from a primary acute infected pregnant woman to the fetus. In immunocompetent hosts, infection usually results in life-long immunity, so if a primary infection is acquired at least 4-6 months before conception, protective immunity usually prevents vertical transmission on subsequent exposures, except in immunocompromised woman whose infection can be reactivated (e.g AIDS) (35,36).

Besides causing complications to the fetus, as abortion and neonatal mortality, this disease can also reduce the quality of life in children, who survive prenatal infection, causing mild chorioretinitis, mental retardation microcephaly, hydrocephalus, seizures and ocular disease (35).

Worldwide, 3-8 infants per 1000 live births are infected in the uterus, and there are multiple factors associated with this infection, including transmission route, cultural behavior, eating habits, hygienic standards and climatic conditions (37,38).

The risk of congenital infection increases with gestational age (Table 1). However, when infection occurs at later gestation stages, less serious will be the consequences for the fetus (36).

Table 1. Risk of Human *T. gondii* congenital infection and development of clinical signs in offspring, according to maternal gestational age. Adapted from (39).

Gestational weeks when maternal seroconversion occurs	Risk of congenital Infection %	Development of clinical signs in the infected offspring %
13 (≈ 3 Mo)	6	61
26 (≈ 7 Mo)	40	25
36 (≈ 9 Mo)	72	9

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

5. Infection in animals

In addition to humans, *T. gondii*, is also able to cause infection and severe disease in domestic animals (38). In sheep and goats, it causes embryonic death, abortion, stillbirth and neonatal death, causing great losses for producers. Outbreaks of toxoplasmosis in pigs and also sporadically in rabbits, mink and other domestic animals have also been reported (40).

According to serologic surveys, *T. gondii* infections have also been demonstrated in carnivores (chinchillas, mink, ferrets, foxes, raccoons and raccoon dogs), wild pigs, wild felids (feral cats, lynx, bobcats, jaguars and ocelots), sea mammals (sea lions, seals, dolphins and whales), wild ungulates (gazelles, deers, bisons and reindeers), small mammals (rats, mice, rabbits, porcupines, guinea pigs and squirrels), monkeys, marsupials and birds, with more serious consequences for some than for others (15).

6. Epidemiology

T. gondii infections occur all around the world, although infection rates differ significantly depending on the geographic localization (Fig. 4) (41). A survey performed in the 1990s following childbearing age women, indicated that seroprevalences ranged between 37-58% in Central European countries, 51-72% in several Latin-American countries and 54-77% in West African countries. Lower seroprevalences have been reported in Southeast Asia, China, Korea (4-39%) and in areas with a cold climate, such as the Scandinavian countries (11-28%) (35).

Toxoplasmosis is also a risk for laboratory workers since, 47 laboratory-acquired cases have been reported, being 81% symptomatic cases (42). In the United States, data from the National Health and Nutrition Examination Survey (NHANES), between 1999 and 2004, found 9% of US-born persons, with ages between 12-49 years, compared to a previously survey performed, between 1988 and 1994 indicating 14,1% of seroprevalence (43). These surveys and others, indicated a trend of decreasing seroprevalence in the United States and many European countries (41).

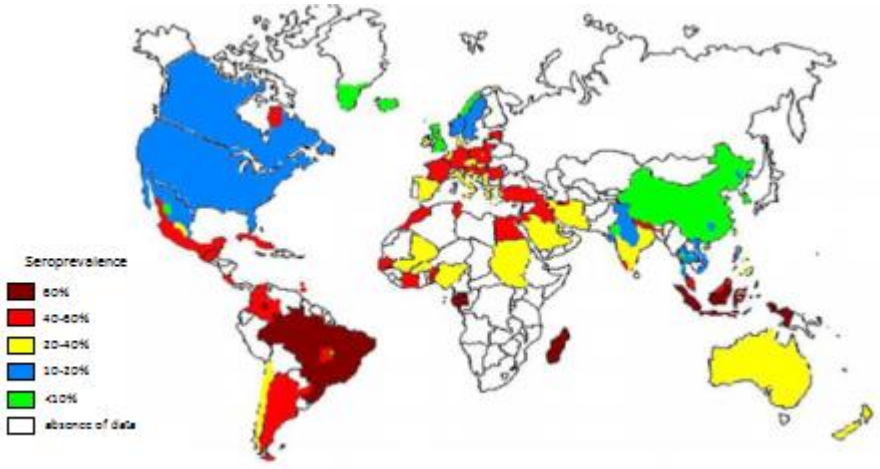

Figure 4. Global status of *T. gondii* seroprevalence. Adapted from (41)**.**

There are about 225 thousand cases of *T. gondii* infection per year worldwide, resulting in about 5000 hospitalizations and 750 deaths, making *T. gondii* the third most common cause of fatal diseases foodborne (14).

The reasons for the dissemblance in the prevalence of toxoplasmosis in different countries have been attributed to different causes. For instance, the high prevalence of infection in Europe, has been related to a preference for raw or undercooked meat, while the high prevalence in Central America and in other developing countries has been related to low socio-economic levels and the frequency of stray cats in a friendly climate for oocysts survival. Besides, the infection is most common in warm climates and at lower altitudes than in cooler, drier climates and mountainous regions (44).

There are three major genotypes of *T. gondii* responsible for the Toxoplasmosis throughout the world: type I, II, and III. These *T. gondii* strains have different effects depending on the host, due to their genotype variations (45).

- Type I: virulent in mice and humans, detected in AIDS patients.
- Type II: non-virulent in mice, virulent in humans (found in Europe and North America), detected in AIDS patients.
- Type III: non-virulent in mice, most frequent strain from animals, detected in a lower degree in humans.

Current serotyping techniques only allowed differential identification of strain I and III from strain II (46).

Because the parasite is a threat to the fetus when contracted during pregnancy, most of the global epidemiological information concerning *T. gondii* comes from seropositivity tests in woman of childbearing age. Seropositivity tests guarantee only the exposure to the parasite, but not chronical infection (47).

7. Diagnosis

The diagnosis of toxoplasmosis can be made by three different assays:

- Immunological assays: *T. gondii* infection induces the production of antibodies against specific *T. gondii* antigens (48). These antibodies can be detected in the blood using several immunoassays as VIDAS avidity assay (49) and Dot-ELISA (50). Acute toxoplasmosis can be differentiated from chronic toxoplasmosis using flow-cytometry or vitek immune diagnostic assay system (VIDAS) avidity assays to quantify the presence of different types of antibodies (49,51). Acute patients are positive for immunoglobulin (Ig) M antibodies, but have a low IgG avidity index, whereas, chronic patients can be positive or negative for IgM antibodies but will have a high IgG avidity index (Fig. 5). Differentiation between acute and chronic infections is important, since acute infection of pregnant women can be transmitted to the fetus and cause complications in fetal development (52). However, chronic infections may also be reactivated during pregnancy and infect the fetus in immunocompromised individuals (53).

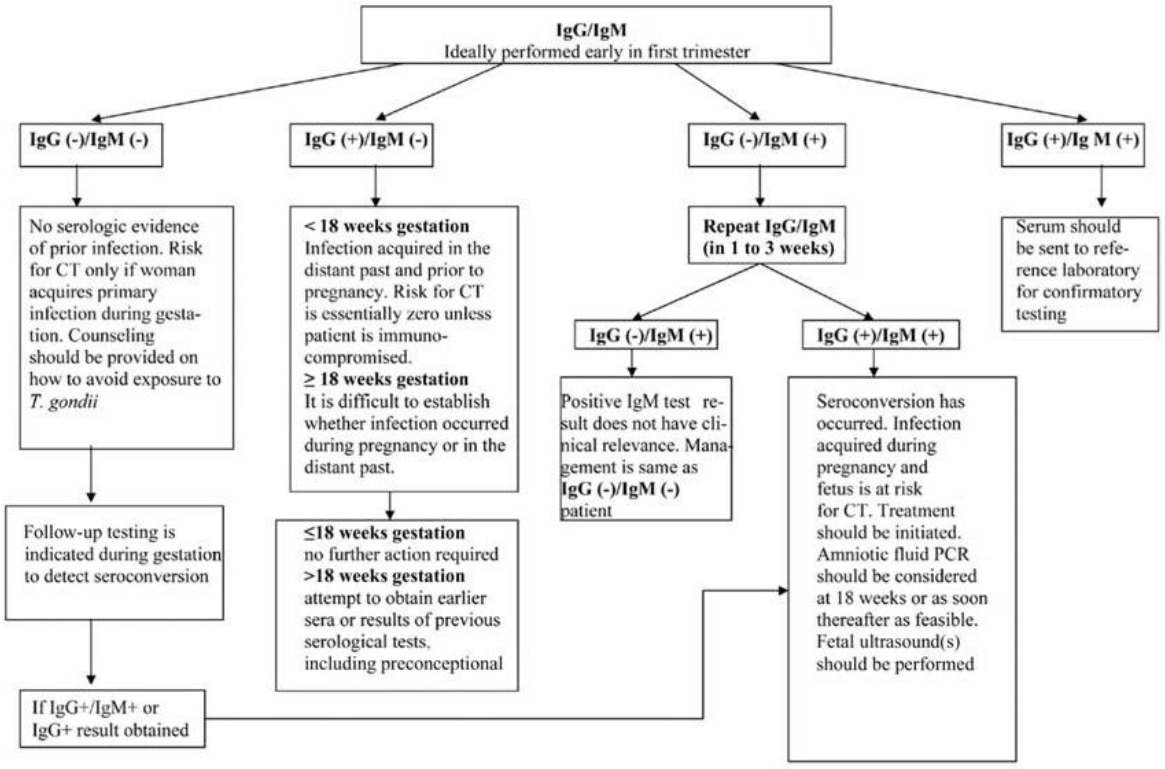

Figure 5. Guidelines for serological testing and management of toxoplasmosis during pregnancy on the basis of initial results obtained from *T. gondii* IgG and IgM antibody tests. CT= Congenital Toxoplasmosis (36)**.**

- Deoxyribonucleic acid (DNA) assays: toxoplasmosis can be detected in various tissues or body fluids by polymerase chain reaction (PCR) using probes for *T. gondii* specific DNA sequences (49,54). PCR is useful to diagnose fetal toxoplasmosis because it can detect *T. gondii* in amniotic fluid (52). Recently, a new and faster method called loop-mediated isothermal amplification (LAMP) has been developed. It can detect *T. gondii* DNA in mouse urine and, in the future, it will be useful for routine diagnosis and therapeutic evaluation in humans (55).

- Imaging: toxoplasmosis is a common treatable cause of brain lesions in immunocompromised patients. These lesions can be detected on an magnetic resonance imaging (MRI), computed tomography (CT), or positron emission tomography (PET) scans (54,56). When lesions are detected, treatment is immediately initiated and if symptoms improve, then toxoplasmosis is inferred as being the cause of the lesions.

8. Prevention and Control

Prevention of *T. gondii* infection might be done in two stages:

- Primary prevention: the efforts of prevention have primarily been directed towards health education, avoiding personal exposure to the parasite. So, hands, cutting boards, sink tops, knives and other materials should be washed with soap and water after handling meat (57). *T. gondii* parasite present in meat can be killed by exposure to extreme cold (-13ºC) (58) or heat (67ºC) temperatures (59). Thus, meat of any animal should be frozen or well-cooked before consumption. Pet cats should be fed only with dry, canned or cooked food and the litter box should be emptied and washed every day. The vegetables should be well-washed before consumption (60).

The development of a vaccine can prevent human disease, by immunization of humans as well as animals. Much work has been done focused on a surface *T. gondii* antigen (SAG1), attempting to induce host protective immune response, but, until now, there is no effective vaccine preventing toxoplasmosis in humans (61).

- Secondary prevention: in many countries routine toxoplasmosis screening programs for pregnant women have been established (62). Woman screening should begin before the conception with follow-up of monthly tests during pregnancy (63). A recent study in the United States screened neonates for *T. gondii* specific IgM and the confirmed cases of congenital toxoplasmosis where treated. Seven years after treatment, patients revealed no symptoms or at least no disease progression. Based on this, authors suggested that toxoplasmosis neonatal screening programs should be implemented in order to prevent disease (64).

In conclusion, it is crucial the population education in order to minimize risks of infection. Also screening programs implementation, of childbearing age or pregnant women, as well as, of newborn babies should be highly effective for early treatment and prevention of sequelae.

9. Treatment

In immunocompetent individuals, chronic toxoplasmosis is usually untreated because tissue cysts are asymptomatic and resistant to drugs (65).

There are available a number of drug therapies for acute toxoplasmosis treatment. In humans, the recommended treatment for acute toxoplasmosis is the administration of a combination of pyrimethamine and sulfadiazine (given with folinic acid to prevent a reduction in platelet count) (66). To prevent congenital transmission in pregnant women, spiramycin has been used, because is non-toxic and does not cross the placenta (67,68). However, a study in rhesus monkeys showed that spiramycin may prevent transmission of infection to the fetus, but, most probably, cannot interrupt brain infection, which is the most severe outcome of congenital toxoplasmosis in humans (69). Sulphonamides, trimethoprim, pyrimethamine and clindamycin, either alone or in combination, as well as ponazuril are being studied as possible treatments for cats (70).

Treatments in immunosuppressed individuals with chronic disease are being tested (71), consisting of drugs that target specifically the isoprenoid pathway, the dihydrofolate reductase, the *T. gondii* histone deacetylase, or type II fatty acid biosynthesis pathway showing some promise as possible future treatments (54,71).

10. Immune Cell Populations at the fetomaternal interface (FMI)

A successful pregnancy requires that the maternal immune system adapts properly to avoid rejection of semi-allogeneic fetus, without compromising the ability to protect the mother and the fetus from infections.

There is an intimate association between mother and the fetus during pregnancy, however the maternal tissue never comes into direct contact with the developing fetus. This indirect connection is formed at FMI constituted by decidual cells (maternal component) and trophoblast cells (fetal component) (72).

The human decidua is a dynamic immunological tissue within the uterus composed by a large population of maternal immune cells, able to actively change its constitution along the pregnancy process (73). After decidua differentiation, an

extensive uterine vasculature remodelling occurs with the enlargement of maternal blood vessels duo to trophoblast invasion (74,75). The human placenta is a multifunctional organ responsible for secretion and synthesis of several important hormones, cytokines and growth factors, crucial for pregnancy maintenance and fetal protection, being highly active during the first trimester of pregnancy and it slowly decreases by the end of gestation (76).

Placental tissue contains paternal antigens that under normal conditions would trigger a maternal immune response, however during normal pregnancy this immune response does not occur (72). The fetomaternal tolerance is ensured by a tight and complex regulation of T cell functions, particularly T-helper (Th) cells, ensuring fetal protection and survival. Among several mechanisms, it is described a limited immunosuppressive state of the maternal immune response, allowing tolerance to fetal alloantigens expressed by the placenta, thus, preventing fetal rejection (77,78). Interestingly, this tolerance to fetal alloantigens occurs in the presence of a large number of maternal leukocytes. Therefore, intense reproductive research has its focus on leukocyte populations present in the endometrium and decidua from infertile women and women with obstetric complications (79).

The major populations of leukocytes present in the decidua in the 1st trimester are: natural killer cells (NK) (70%), macrophages (20%), regulatory T lymphocytes (Treg) (10-20%) and dendritic cells (DCs) (rare) (80). Macrophages together with NK cells, contribute to tissue remodeling process, and together with Treg cells play a central role in keeping a homeostatic environment between maternal and fetal components (Fig. 6) (81).

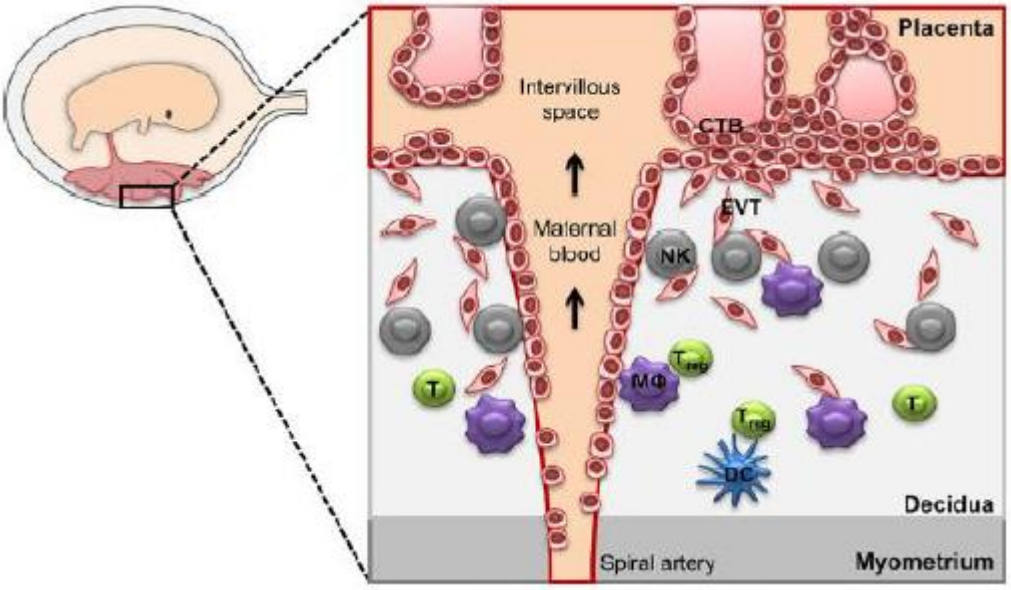

Figure 6. Schematic representation of FMI. MΦ: macrophages; NK: natural killer cells; DC: dendritic cells; T: lymphocytes; Treg: regulatory T lymphocytes; EVT: trophoblasts; CTB: cytotrophoblasts (81)**.**

10.1. Trophoblasts

Trophoblast cells protect the fetus against infection and are considered as a component of the innate immune system (82).

Initially trophoblast cells from the trophectoderm of blastocyst that proliferate, differentiate and invade the decidua giving rise to the placenta (83). These cells, express paternal antigens and exhibit unique features, not expressing classical major histocompatibility complex (MHC) class I receptors, avoiding recognition by maternal T cells and thus preventing maternal immune rejection (84). However, they do express non-classical receptors, escaping from NK cell-mediated toxicity that may occur during pregnancy (83,85).

The trophoblasts main functions include fetus protection, nevertheless, in some situations, placental trophoblast cells may also initiate signals that are able to promote fetal rejection (82). These cells are able to recognize pathogens that induce toll-like receptor (TLR)-mediated trophoblast inflammatory or apoptotic responses, impacting in the production of chemokines and cytokines. The production of these factors influence the differentiation, migration and activation of macrophages, NK, Treg, DCs at the decidua, which may shift from a protective to

an aggressive phenotype promoting fetal rejection (73,82). Trophoblasts are then required for parasite transmission since they are strategically located between the maternal and fetal blood circulation systems, therefore being efficiently infected for instance with *T. gondii* (83).

10.2. NK and Decidual NK cells

Decidual NK (dNK) cells are the major leukocyte cell population at the decidua and uterine mucosa, during implantation and early pregnancy (79,86). At the first trimester of pregnancy, NK cells constitute about 70% of total human decidual leukocytes (80), decreasing in number during the second trimester (87). Due to its close contact, dNK cells interact with the invading fetal-derived trophoblast cells, helping them during migration and contributing to the remodeling of decidual spiral arteries into high conductance blood vessels, thereby allowing a sufficient blood flow supply at the FMI, determinant in a healthy gestation (74,81,85). Rather than work as cytotoxic effector cells, the major function of NK cells during pregnancy comprises the secretion of a variety of angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietin-2, nutritional cytokines and growth factors, such as tumor necrosis factor-α (TNF-α), TNF-β, interleukin (IL)-10, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon gamma (IFN-γ) (79,87). The secretion of IFN-γ by dNK cells, an essential Th1-type cytokine controlling trophoblasts invasion at the FMI during pregnancy, induces trophoblasts apoptosis via a caspase mediated-pathway (88).

NK cells contribute to early resistance to *T. gondii* (89,90) by recognizing and killing infected cells. In fact, they have shown to be the major source of IFN-γ early in infection (89). After *T. gondii* infection, the traffic of NK cells is first increased into the lymph node and then into the site of infection, producing IFN-γ and therefore inducing classically activate macrophages with increased MHC class II molecules expression (91). These cells possess a number of chemokine receptors such as C-C chemokine receptor (CCR)-5 facilitating this process (91).

10.3. Decidual Macrophages

Decidual macrophages constitute the second most abundant immune cell population in decidua, being about 20% of the decidual leucocyte population (81,92).

Macrophages are responsible for decidual homeostasis, placental development and tolerance to the semi-allogeneic trophoblast. They also form a major line of defense against invading pathogens in the decidua, protecting, therefore, the fetus from infection (93). Decidual macrophages are able to decrease the cytolytic activity of dNK cells against the invading trophoblasts, contributing to the maintenance and support of uterine tissue homeostasis and remodelling (75,79,81). Decidual macrophages recognize, phagocyte, control and eliminate pathogens, promoting the normal fetal development and acceptance, preventing tissue damage and fetal rejection (75,79,81,84).

10.3.1. Macrophage Activation

Macrophages have long been recognized as phagocytic cells, responsible for eliminating pathogens that invade any living vertebrate or invertebrate (94). They play not only a critical role in innate immunity, but also help in the adaptive immunity, by recruiting other immune cells such as lymphocytes, that are the major antigen-specific cells responsible for macrophage activation (95). Beyond increasing inflammation and stimulating the immune system, macrophages also play an important anti-inflammatory role through the release of cytokines (including chemokines, interferons, ILs, lymphokines and TNF) (96).

TLRs are a proteins class expressed in sentinel cells (such as macrophages and DCs) that play a key role in the innate immune system. They recognize structurally conserved molecules derived from pathogens, such as lipopolysaccharide (LPS) and activate immune cell responses. TLRs and IL-1 receptors form a receptor superfamily having in common the toll-IL-1 receptor (TIR) domain (97).

A number of different activation states for macrophages are considered: innately activated through LPS stimulation (or other TLR ligands), classically activated (M1) through a combination of LPS and IFN-γ, and alternatively activated (M2) through IL4/IL13 signaling. M1-type macrophages are critical for host defense against intracellular pathogens and interfere in antitumor immunity and autoimmune inflammation. M2 macrophages are protective against helminth parasites and are important regulators of the wound healing response, tissue homeostasis and adiposity (98).

In the presence of pathogens, M1 and M2 macrophage responses induce a Th1/Th2 responses, although both M1 and M2 macrophages functions, occur in all animals whether they have or not T cells (99).

During the course of an inflammatory response, phenotypic switching of macrophages from an M1 to an M2 profile has been observed, underscoring the dual role of macrophages in initiating and subsequently resolving inflammation. Whether this switch occurs by local conversion of M1 macrophages to anti-inflammatory M2 cells (100) or by sequential recruitment from distinct precursor populations from the blood is currently being debated (101).

The macrophages can respond to divergent environmental stimulation and simultaneously express M1 and M2 phenotypes (102). This ability stems from the metabolism of arginine through two enzymatic pathways that negatively regulated each other. M1 profile is associated with inducible nitric oxide synthase (NOS2) pathway, which leads to arginine conversion in citrulline and nitric oxide (NO) (75). M2 profile is associated with arginase 1 (Arg-1) pathway, whereas arginine is hydrolyzed to ornithine and urea (Fig. 7) (75).

The success of the zygote implantation in the uterus during pregnancy, requires a transient inflammatory phase initiated by cytokines and prostaglandins (75). During the peri-implantation period, the pattern of decidual macrophages polarization is shifted to a M1 profile. However, while trophoblasts establish connection with the endometrium and invade the uterus stroma, decidual macrophages begin transition to a M1/M2 mixed profiles (103). This mixed pattern remains in the first trimester and in the beginning of the second trimester of pregnancy (104). After the development of the placenta, macrophage activation

profile is directed to an M2 profile, which prevents the fetus rejection and allows their growth until delivery (75).

A recent *in vivo* study in rats, reported that acute infection with *T. gondii* may direct the macrophage activation profile preferably to an M1 profile, producing NO and pro-inflammatory cytokines. This can interfere with the maternal-fetal immune balance and induce negative consequences in pregnancy and even cause abortion (105).

10.3.2. F4/80 Marker

The monoclonal antibody F4/80 has been widely used as a marker for mouse macrophages (106). This marker is highly expressed in the plasma membrane and reveals potential interactions with surrounding cells (107). The antigen recognized by the antibody was characterized as a member of the epidermal growth factor transmembrane 7 (EGF-TM7) family. F4/80 molecule is expressed in a variety of macrophage subsets, including liver Kupffer cells, bone marrow macrophages, thymic macrophages, red pulp splenic macrophages, bone marrow macrophages of the lymph nodes and microglial cells in the brain (106).

The F4/80 marker has become widely used for mouse macrophages detection. However, not all mouse macrophages express this marker. Thus, in the spleen of mice F4/80+ macrophages are present in the red pulp, but not in the marginal zone (107). In areas with large amounts of T lymphocytes, such as lymph nodes and Peyer's patches, the F4/80 marker is expressed in small amounts, or is even non-existent (107). Eosinophils are F4/80+, especially after parasitic infection and during tissue remodeling (107). There are also reduced levels of F4/80 found in monocytes (106). This protein is absent in neutrophils (107). F4/80+ macrophages also exist in mice placenta (108) and mesometrial decidua (109), and the numbers may change depending on the gestation day (109).

10.4. Dendritic cells

DCs are antigen-presenting cells priming T-cell responses, but decidual DCs (dDCs) fail to initiate immunogenic T-cell responses to placental antigens contributing, therefore, to the fetomaternal tolerance (79,95,110). One of the explanations, is that dDCs, by expressing indoleamine-2,3-dioxygenase (IDO), inhibit human T-cell responses (111). *In vitro* studies showed that *T. gondii*-infected human dDCs increased the cytotoxicity of dNK cells, suggesting a possible mechanism for abnormal pregnancy outcomes caused by this parasite (112). Previous studies in human early pregnancies have shown that IL-12-producing myeloid dDCs promote a Th2 environment leading to the maintenance of pregnancy (79).

10.5. Regulatory T cells

During pregnancy, Treg cells are recognized as highly important elements for maternal immunological tolerance to the allogeneic fetus (73). Dysfunctional or a decreased number of Treg have been found associated with human infertility and miscarriage (73,79,111,113). Previous studies have shown that the levels of T cell populations rise dramatically in early gestation, reaching a maximum in the second trimester, which is concomitant with the extreme trophoblast invasion into the maternal decidua, and then decrease to lower levels till post-delivery when the allogeneic stimulus has been already removed (79,111,113). During this period, fetus-specific Treg cells are being selectively recruited from maternal peripheral blood into the decidua, supporting local regulation of fetus-specific responses at the FMI (73,79,85,111).

11. L-Arginine metabolism

Arginine is an important amino acid for adult mammals and must be provided in the diet in some physiological (such as pregnancy) or pathological

conditions, when the need exceeds the capacity of production by the body itself (114).

The enzyme arginase hydrolyzes L-arginine to the products L-ornithine and urea. In the mammalian liver, arginase was first discovered by Kossel and Dakin in 1904 (115). When the existence of a second form of arginase was proven, in 1986 (116), the liver arginase was designated Arg-1, and the second isoform arginase 2 (Arg-2). They catalyze the same reaction but differ in cellular expression, regulation and localization (117). Thus, Arg-1 is a trimeric cytosolic protein that is constitutively expressed only in hepatic tissues or erythrocytes of humans and higher primates (118). Arg-2 is also a trimeric mitochondrial protein expressed in extrahepatic tissues predominantly in the kidney and prostate (118–120). In addition to this, some pathogens possess their own arginase and can use it to metabolize L-arginine from the host. Bacteria and parasites explore the effects of the depletion of L-arginine as a survival strategy, using its own or host arginase to exhaust L-arginine (114). *T. gondii* is one of the parasites that induces host Arg-1 protein expression within 1 hour of infection (118).

Arginase is important in several metabolic pathways because it leads to the synthesis of L-ornithine that can be further metabolized to polyamines (putrescine, spermidine, spermine) via ornithine decarboxylase (ODC) or to L-proline via ornithine aminotransferase (OAT), which is an important component of collagen (121). Polyamines are small cationic molecules, that participate in a several fundamental cellular functions, such as proliferation and cell membrane transport (121). One of the enzymes that competes with arginase for L-arginine is nitric oxide synthase (NOS). There are three types of nitric oxide synthases: inducible NOS (iNOS or NOS2), neuronal NOS, and endothelial NOS. NOS2 is not constitutively expressed but is highly induced by LPS and Type-1 cytokines like IFN-γ, tumor necrosis factor alpha (TNF-α), IL-1 and IL-2 (122), and metabolize L-ornithine to L-citrulline (121).

In the last years, literature concerning the arginase role in the immune system has increased, due to the fact that this enzyme is involved in a critical manner in inflammation. Arginase participates in inflammation pathways, triggering immune cell dysfunction, and play a role on fibrosis and infectious diseases (114).

L-Arginine is transported from the blood into the cells by the cationic amino acid transporter (CAT) (123). In myeloid cells (macrophages, granulocytes and DCs) the NOS2 and the Arg-1 are competitively regulated by Th1/Th2 cytokines and by complex intracellular biochemical pathways, that include the negative feedback and competition for the same substrate. Pro-inflammatory signals (such as IL-1, TNFα, IFN-γ and IFN-β) and the anti-inflammatory signals (such as IL-10, transforming growth factor (TGF)-β, cyclic adenosine monophosphate (AMP), and dexamethasone) may contribute to the balance regulation between NOS2 and Arg-1. The most important factor for the transcription of Arg-1 is the signal transducer and activator of transcription (STAT) 6, but there are other STAT6 independent pathways. In particular, STAT1 may be necessary for the expression of both NOS2 and Arg-1 genes, but the molecular pathway involved has not been fully elucidated. Furthermore, Arg-1 and NOS2 directly activate several metabolic pathways that are negatively regulated. Low concentration of extracellular L-arginine, increased Arg-1 expression or reduction of L-arginine absorption capacity can decrease intracellular levels of L-arginine and inhibit mRNA translation encoding NOS2, thus, reducing this enzyme activity (114). NG-hydroxy-L-arginine (NOHA) is an intermediate in the biosynthesis of NO and is a potent competitive inhibitor of Arg-1 (123).

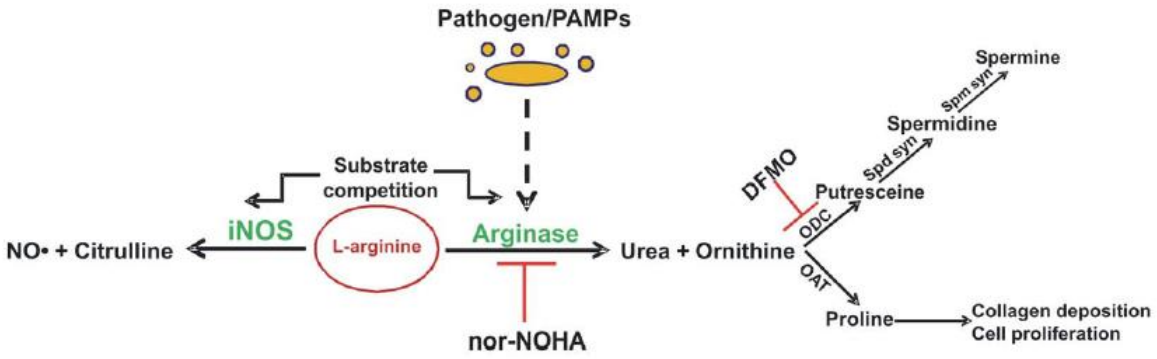

Figure 7. Overview of mammalian L-arginine metabolism. DFMO: difluoromethyl ornithine; iNOS: inducible nitric oxide synthase; NO: nitric oxide; nor-NOHA: nor-NG-hydroxy-l-arginine; OAT: ornithine aminotransferase; ODC: ornithine decarboxylase; PAMPs: pathogen-associated molecular patterns; Spd Syn: spermidine synthase; Spm Syn: spermine synthase (118).

The relation between NO and L-arginine are intrinsically connected. L-ornithine promotes parasite growth, since it is a precursor for a variety of

polyamines (putrescine, spermidine and spermine), through the ornithine ODC pathway as previously described (121). On the other hand, NO is an effective antimicrobial agent that controls pathogens by restricting their access to micronutrients (124). Thus, the balance between the expression of NOS2 and Arg-1 seems to be involved in resistance or susceptibility to infection with *T. gondii* (125).

Several protozoan parasites develop strategies to circumvent the antimicrobial activity mediated by NO. *T. gondii* inhibits transduction signal cascade of IFN-γ induced in murine macrophages, suggesting that NOS2 expression induced by IFN-γ can also be changed after the parasite infection and that *T. gondii* regulates the production of NO induced by IFN-γ (126).

12. The immunobiology response to *T. gondii*

Epithelial intestinal cells are the first line of defense against *T. gondii*, since this parasite is often transmitted via oral ingestion. However, the parasite can also spread and survive in other cells, tissues and organs, such as in DCs and macrophages, by taking advantage of their migratory capacity to disseminate throughout the body (127). The migration of the immune cells from blood into the infection site is highly controlled by diffusible chemotactic factors and cell surface adhesion molecules. These migrated acute inflammatory cellular infiltrates consist of cells such as neutrophils, DCs, macrophages and NK cells, that play an important role in the resistance to *T. gondii* (128).

Upon infection, neutrophils are the earliest phagocytic cells recruited to the site of infection, phagocyting and killing pathogens by the release of anti-microbial compounds and production of reactive oxygen intermediates (ROI) and NO (129). Neutrophils have also an immunoregulatory function mediated by the release of pro-inflammatory chemokines and cytokines that are able to attract and stimulate other immune cells (129).

After intraperitoneal tachyzoites' inoculation, there is a local recruitment of IL-12-positive neutrophils, whose influx is dependent on the C-X-C chemokine receptor (CXCR)2 (130) and the CCR1 (131). During early infection, depletion of

neutrophils results on a Th1 shift to a Th2 predominant response, thereby increasing susceptibility to the parasite. However, at later stages of infection, the removal of neutrophils has no effect, thus highlighting their major role in the initial response to *T. gondii* (129,132). Macrophage inflammatory proteins (MIP)-1α, MIP-1β, TNF and monokines acting on CCR5 and CXCR3 expressed by Th1 cells, enable neutrophils to support the immune response through Th1 cells (129). MIP-1α and MIP-1β also attract monocytes, macrophages and DCs to the infection site (129). The induction of neutrophils resulting in the elimination of a large number of parasites during the initial stages of *T. gondii* infection is dependent on IL-17 signaling, a cytokine responsible for neutrophil recruitment and development (133). The maturation and activation of DCs in response to infection plays an important role in the initiation of innate immunity and in the development of adaptive immunity. During infection, DCs migrate into inflammatory sites in response to chemokines, such as CCR5 ligands (CCL3, 4 and 5), secreted by neutrophils (134). DCs maturation is characterized by the up-regulation of the MHC and other stimulatory molecules, inducing the proliferation of naive T cells, while DCs activation is associated with production of inflammatory cytokines regulating T cell differentiation (135). DCs carrying microbial antigens migrate from the infection site to the spleen and accumulate in the T cell areas. In fact, these cells are responsible for the most of the antigen-presenting activity and drive the polarization of the Th response towards Th1 through the production of IL-12 (136,137). In addition, since DCs are the major producers of IL-12 in response to a *T. gondii* infection, they became very important for *in vivo* resistance against infection (138). Depletion of DCs has shown to suppress IL-12 production and increase mice susceptibility to acute infection (139). The chemokine receptor CCR5 has been proven to be involved in DCs mobilization and IL-12 production upon *T. gondii* infection.

Macrophages, together with DCs, provide the first line of cell-mediated defense against pathogens since they are essential to prevent the spread and growth of these infectious organisms through the modulation of the resulting immunological reactions (140). Macrophage functions include the production of cytokines, such as IL-12, presentation of antigens to T cells through the MHC and

co-stimulatory molecules leading to microbicidal mechanisms activation, as phagocytosis and phagolysosomal degradation, and to adaptive immune responses (140,141).

Macrophages, DCs and neutrophils infected with *T. gondii* are an important source of IL-12 early in infection (142). IL-12 acts first on NK cells and later on T cells, and promotes the production of IFN-γ, which, in turn, induces the classical activation of macrophages, thus creating a positive feedback. There are two signals required for classical activation of macrophages: IFN-γ produced by NK and T cells and also, the endogenous TNF-α production by macrophages, in response to the activation of pattern recognition receptors (PPRs) by pathogen-associated molecular patterns (PAMPs) completes activation. These activated macrophages in the site of infection phagocyte and destroy the microorganisms by releasing lysosomal enzymes, ROI and NO, as well as by inducing nutrient deprivation mechanisms, as iron deprivation and tryptophan starvation (140,143).

Since macrophage overproduction of inflammatory cytokines and NO may induce the development of pathology, the activity of these cells needs to be tightly regulated. Therefore, signals arising from macrophages or surrounding cells down-regulate macrophage activation and result in the production of anti-inflammatory cytokines, as IL-10 and TGF-β (143).

13. Animal Model

Murine models of infection by *T. gondii* have proved to be useful for the study of immune mechanisms that control intracellular parasitic infections (144). Nevertheless, the parasite strain used in infection, the mouse strain and infection route are critical factors for the development of infection, as can be seen in Table 2 (144).

The mice and the rat animal models are often used to study the fetal-placental development. However, in contrast to humans, the mouse uterine natural killer cells (uNK) appear to be very important for arterial remodeling (74). NK cells are the predominant cell type in human decidua, rat and mice, whereas macrophages are abundant in human decidua but not in mice decidua (80,109).

The main difference between the decidualization process in mice, rats and humans, is that decidualization starts spontaneously in humans, while in mice or rats occurs only in response to a stimulus induced by blastocyst or artificially (74). The concern is that pregnancy in mice, rats and humans, although similar in some aspects (e.g., in placentation mode and the accumulation of dNK cells), are also quite different for other potentially important aspects. Besides the obvious differences in the anatomy of the placenta and gestational time (20-21 days versus 9 months in humans), a critical difference is that the placental trophoblasts do not invade deeply arterioles and decidua in the mouse model (80). As in chronically infected humans, Balb/c mice do not transmit the disease to their fetuses even if re-exposed to infection. The infection of Balb/c mice on the early pregnancy stage (until day 7 of gestation), which corresponds to the 1st trimester of human pregnancy, results in the loss of embryos due to abortion or resorption. The infection in intermediate pregnancy stages results in congenital transmission. However, as pregnancy in the mouse is only 21 days, the infection on the later pregnancy stages does not seem to be sufficient to occur congenital transmission (145).

Table 2. Strain mice susceptibility to *T.gondii* acute infection. Adapted from (144)**.**

	INFECTION ROUTE	
STRAIN	**Oral**	**Intraperitoneal (ip)**
BALB\C	Resistant	Susceptible
B6	Susceptible	Resistant

14. Aims

In the last years, scientific breakthroughs have been made in order to understand the immune response to toxoplasmosis, but there is still a lack of knowledge about which immune players are responsible for the pathology associated with this infection during pregnancy. This immune players are potentially associated with the disruption of normal homeostatic immunological mechanisms including macrophage activation.

As it is well known, classical macrophage activation can control parasite replication through induction of NOS2, while alternative activation can control parasite replication through Arg-1 induction. Therefore, in this work, in a first attempt, we hypothesized that Arg-1 and NOS2 expression could be altered during pregnancy and *T. gondii* infection, in order to determine if hypothetical alterations could be related to a different macrophage activation profile.

In this study Balb\c and B6 mice were used. Balb\c and B6 have different haplotypes (H-2d and H-2b, respectively), and therefore, antigenic presentation and the trigger of the T cell dependent immune responses and consequently susceptibility to infection is different, as described (144). Thus, it would be expected to find differences in the expression of gene and protein Arg-1 and NOS2, and in the polarity of macrophage activation between these two strains of mice.

For this, it was analyzed: I) the effect of infection at the basic parameters of reproductive function in pregnancy, by morphometric analysis of decidua and placental areas; II) the quantification of parasite burden by Q-PCR; III) the evaluation of Arg-1 and NOS2 gene expression by qRT-PCR, immunohistochemistry and Western Blotting; and IV) the co-localization of Arg-1 or NOS2 and F4/80 by immunofluorescence assay.

Chapter II. **MATERIAL AND METHODS**

1. Parasite and *in vitro* infection

The *T. gondii* strain used in this study, was the Prugniaud – *Yellow Fluorescence Protein* (YFP), a type II strain (ME49 clone), from Doctor Marcus Meissner laboratory (Parasitology department of the Faculty of Medicine, Heidelberg University, Germany). Viable tachyzoites were maintained *in vitro,* using Human Foreskin Fibroblasts (HFF) from American Type Culture Collection (ATCC), cultivated in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Paisley, UK) with L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.

For the maintenance of *in vitro* parasite cultures, a ratio of 1:10 (HFF cells: parasite) was used. After 48h of *in vitro* infection, parasite suspension was obtained, after the disruption of HFF using a needle (26G) and a syringe, centrifugation and filtration (0,45 µm), in order to eliminate HFF cell debris. Parasites were counted using a Neubauer chamber.

For *in vivo* infections, viable tachyzoites were resuspended in saline solution to a final concentration of 2,5 x 10^4/mL.

2. Mice and *in vivo* infection

Balb\c/cByJ (Balb\c) and C57Bl/6 (B6) mice were bred at IBMC facilities from breeding pairs purchased from Charles River (Barcelona, Spain). Female and male mice with 8-10 weeks were kept in cages in the ratio 2:1, respectively. The appearance of the vaginal plug marked the first day of gestation (E1).

Mice were intraperitoneally infected with 5000 viable tachyzoites obtained from *T. gondii in vitro* infection of HFF. Pregnant Balb\c mice, were infected at day 7 of gestation (E7) and 5 or 7 days post infection corresponding to day 12 and day 14 of gestation (E12, E14), mice were sacrificed. B6 pregnant mice were infected at E7 of gestation and 5 days pos-infection (E12) mice were sacrificed.

Four groups of mice were used in this study: pregnant infected, pregnant non-infected, non-pregnant infected and non-pregnant non-infected.

After mice euthanasia, the fetoplacental units (UFs) were collected from pregnant animals. The peritoneal exudate cells (PEC) were obtained after washing

the peritoneal cavity with 5 ml of cold Hanks solution. Spleen cell suspensions were prepared individually using Potter-Elvehjem tissue homogenizer and resuspended in DMEM supplemented with 10% fetal calf serum (FCS, Life Technologies). For each mice, three UFs were fixed in 4% paraformaldehyde and included in paraffin for histological analysis; and three other UFs were dissected, being decidua/placental tissues isolated from the embryo. Decidua/placental tissues, PEC and spleen cells were stored at -70ºC in Trizol, ([50-100] mg of tissue or [5-10] x 10^6 cells/ mL of trizol), for later genomic DNA (gDNA), ribonucleic acid (RNA) and protein extraction according to manufacturer´s instructions (Invitrogen, Carlsbad, CA).

All procedures involving animals were performed in accordance with the recommendations of the European Convention for 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).

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

Genomic DNA from the decidua/placenta, PEC and spleen cells from infected mice was extracted using Trizol Reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). *T. gondii* genomic DNA was detected using primers and a probe designed for the Sag1 gene of *T. gondii*, giving rise to an amplification product of approximately 100 bp. Product amplification was performed with 1 µl of template DNA, corresponding to 50 ng (Spleen cells) and 40 ng (PEC) of DNA in a final volume of 10 µl containing 0.2 µM of each primer (Sag1 forward: CCAGAGCCTCATCGGTCGTC; Sag1 reverse: GGGTCCTTCCGCAGAC AAC), 0.2 µM of probe (6FAM-CTGTyTGCACCGTAGGAGCACCT-BBQ) (all designed by Till Molbiol) and KAPA PROBE FAST qPCR Master Mix (Bio-Rad). The PCR programme run was as follows: 1) denaturation at 95°C, 3 min; 2)

amplification in 40 cycles (denaturation at 95°C, 3 sec; combined anneling/extension 60°C, 30 sec). DNA samples corresponding to 10^2 to 10^{-2} ng/µL of parasite DNA, diluted in a solution of 20 µg/µL of host DNA were included for creating a standard curve. Quantitative evaluation of fluorescence signals from PCR products was performed with Step-One Plus (Applied Biosystems by Life Technologies) and analyzed with Step One Software V2.3.

4. Histology and Morphometric analysis

After fixation in 4% paraformaldehyde, the UFs were dehydrated, embedded in paraffin and sections (4 µm) were obtained using a microtome.

For morphometric analysis of decidua and placental areas, were used three adjacent sections from the center of the UFs, from three UFs, from individual animals from each group (n=3). The slides were stained with hematoxylin and eosin (H&E) dyes (Sigma-Aldrich), using the standard staining histological technique. After staining, slides were mounted in 1,3-diethyl-8-phenylxanthine (DPX) mounting medium (VWR Prolabo).

Decidua and placental areas were determined using the ImageJ image analysis software.

5. Quantitative Reverse Transcriptase PCR (qRT-PCR)

Total RNA extracted from samples was quantified using Nanodrop ND-1000 apparatus (Thermo Scientific). Then, 10 µg of total RNA was purified using DNA-free™ kit (Ambion). Reverse transcription to complementary (cDNA) was performed using 1 µg of total RNA in a final volume of 10 µL, using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, USA), according to manufacturer's instructions. qRT-PCR was performed for Arg-1 and NOS2 mRNA expression levels quantification, using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), in a Step-One Plus™ thermocycler (Applied Biosystems by Life Technologies). Hypoxanthine guanine phosphoribosyl transferase (HPRT), was used as a house keeping gene. The amplification reaction was performed in a final

volume of 10 µL, containing 0.4 µM of each specific primer: Arg1 forward: CTCCAAGCCAAAGTCCTTAGAG, Arg1 reverse: AGGAGCTGTCATTAGGGACA TC (Tib Molbiol, Berlin, Germany); NOS2 forward: CCAAGCCCTCACCTACTTCC, NOS2 reverse: CTCTGAGGGCTGACACAAGG (Tib Molbiol, Berlin, Germany); Hprt forward: GCTGGTGAAAAGGACCTCT, Hprt reverse: CACAGGACTAGAACA CC (GeneCust, Dudelange, Luxembourg) and 1x Master Mix plus 1 µL of newly-synthesized cDNA. The PCR program run was as follows: 1) denaturation at 95°C, 3 min; 2) amplification in 40 cycles (95°C, 15 seg; 60°C, 1 min). The specificity of the amplification conditions and primer was assessed by the analysis of the melting curve. The fold change in gene expression was calculated using the comparative cycle threshold: $2^{-\Delta\Delta C_T}$ (146).

6. Western blotting

Protein concentrations were determined by the Bradford assay (147). Samples were then subjected to SDS-PAGE (10% running gel; 5% stacking gel) and transferred onto a nitrocellulose membrane. Incubation with the primary antibodies: goat polyclonal IgG anti-Arg-1 antibody (sc-18351, Santa Cruz Biotechnology) diluted to 1:400 in phosphate saline solution (PBS) or rabbit polyclonal IgG anti-β-Tubulin antibody (sc-9104, Santa Cruz Biotechnology) diluted to 1:500 in PBS, was done at 4ºC overnight. After washing and incubation with secondary antibody mouse anti-goat IgG (sc-2354, Santa Cruz Biotechnology) diluted to 1:1000 in PBS, or mouse anti-rabbit IgG (sc-2357, Santa Cruz Biotechnology) diluted to 1:1000 in PBS, a chemiluminescent substrate (ECL Western blotting detection reagent, Super Signal West Pico; Pierce, Rockford, USA) was added and film exposed. Bands were quantified by densitometry (BIO-PROFIL Bio-1D, Vilber Lourmat, France). Data are expressed as volume of the western blotting bands, normalized to the volume of β-tubulin, used as a loading control.

7. Immunohistochemistry

The UFs sections obtained as previously described were used for the detection of Arg-1 and NOS2 protein expression. The sections were incubated at 100ºC in 10 mM citrate buffer, pH 6.0, for 30 minutes. The primary antibodies used were: goat polyclonal IgG anti-Arg-1 (sc-18351, Santa Cruz Biotechnology) diluted to 1:100 in PBS; and goat polyclonal IgG anti-NOS2 (sc-650, Santa Cruz Biotechnology) diluted to 1:50 in PBS. The immunoreactivity was analyzed using an avidin-biotin alkaline phosphatase complex immunohistochemical technique, according to manufacturer's instructions (Vectastain ABC kit, Vector Laboratories, CA, USA). Tissue sections were counterstained with Hematoxylin, and mounted using Aquatex Mounting Medium (Merck).

8. Immunofluorescence

Following dewaxing and hydration, tissues sections were washed with PBS and then incubated at 100°C in 10 mM citrate buffer, pH 6.0 for 30 minutes in order to proceed to the antigenic recovery. Then, sections were incubated with horse normal serum blocking solution diluted to 1:50 in PBS containing 5% bovine serum albumin (BSA) for 1 hour. Thereafter the sections were washed with PBS and the primary antibody solution diluted to 1:50 in PBS was added and left to incubate overnight at 4°C in a humid chamber. The primary antibodies used were the rat monoclonal IgG anti-mouse F4/80 (Abcam) and the goat polyclonal IgG anti-Arg-1 (sc-18351, Santa Cruz Biotechnology) or the goat polyclonal IgG anti-NOS2 (sc-650, Santa Cruz Biotechnology). Next day, the sections were washed with PBS and incubated with the secondary antibody solution diluted to 1:200 in PBS and allowed to incubate 1 hour at room temperature using a dark humid chamber. The secondary antibodies used were monkey anti-rat IgG antibody labeled with AlexaFluor488 (Molecular Probes) and the bovine anti-goat IgG antibody labeled with Texas Red (sc-2786, Santa Cruz Biotechnology). Then, the tissue sections were mounted on Fluoroshield with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich). The images were observed and analyzed in the Nikon fluorescence microscope using NIS-Elements imaging system software.

9. Statistical analysis

The results are presented as mean ± SD for morphometric analyses and ± SE for q-PCR, qRT-PCR and Western Blotting analysis. Statistical differences between two groups were determined by the unpaired Student t-test. For multiple group comparisons, the one-way ANOVA test with a Tukey's post-test was performed using GraphPad Prism 5.0 software (San Diego, CA, USA). Statistically significant differences between infected and non-infected animals were labelled as * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$, and between the two strains of mice were labelled as $^{\#}$ for $P<0.05$, $^{\#\#}$ for $P<0.01$ and $^{\#\#\#}$ for $P<0.001$.

Chapter III. **RESULTS**

1. Morphometric analysis of decidua and placenta

During the collection of the UFs, it was macroscopically observed that the UFs from infected mice exhibited a reduced size compared to the UFs from non-infected mice from both mice strains. To determine if these qualitative differences were correlated to alterations at the FMI, it was performed morphometric analysis of mesometrial decidua and placental areas. H&E stained sections were used from infected and non-infected mice from both strains of mice at E12 (Figure 8).

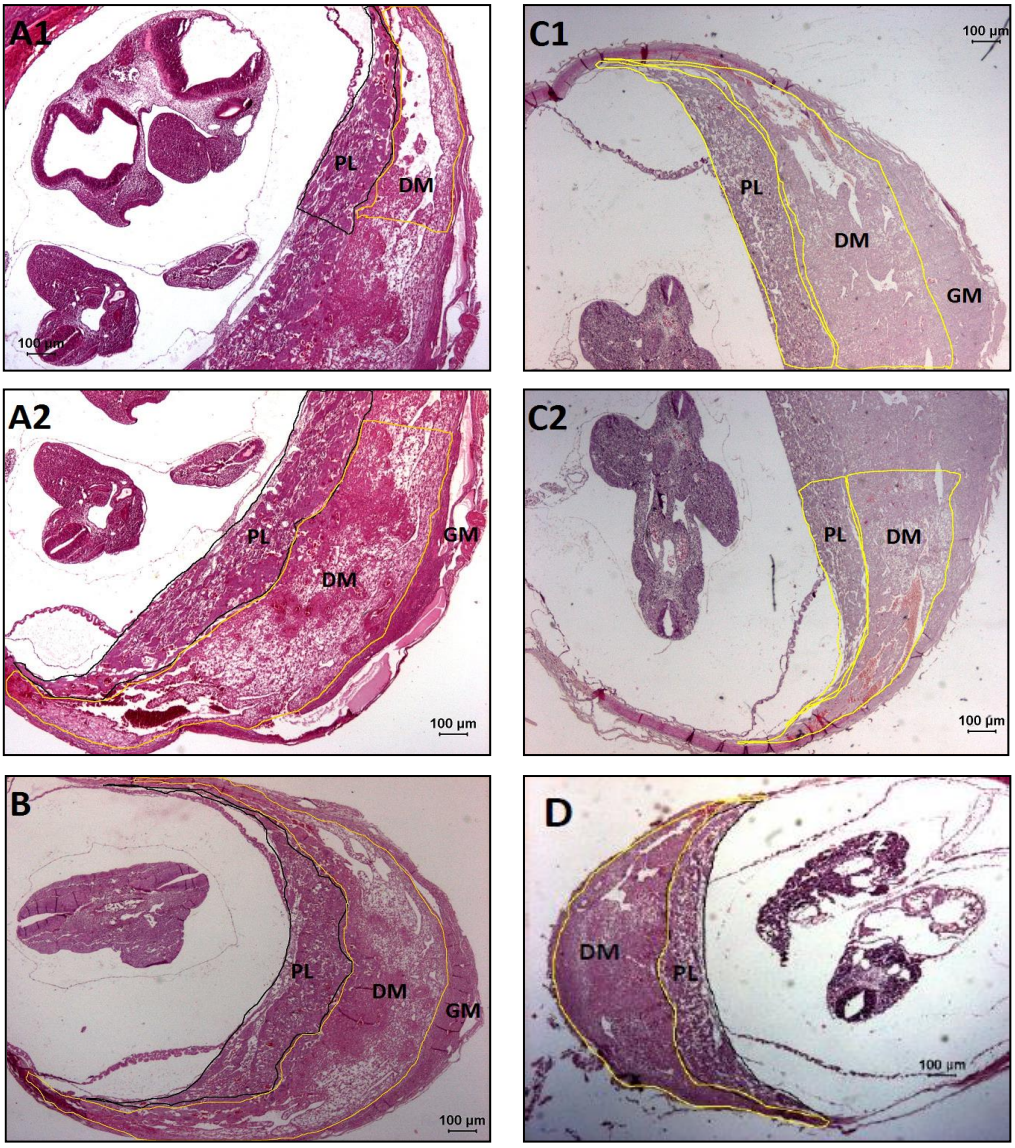

Figure 8. Representative histological images of UFs sections stained with H&E. (A1, A2) E12 non-infected B6; (B) E12 infected B6; (C1, C2) E12 non-infected Balb\c; (D) E12 infected Balb\c. PL: placenta; DM: mesometrial decidua; GM: mesometrial gland.

To overlap the complete section from non-infected and infected mice, at the same magnification, two images were captured from UFs from non-infected mice (A1, A2 and C1, C2 for B6 and Balb\c respectively) compared to only one image captured for infected mice (B, D for B6 and Balb\c respectively). This histological analysis confirmed the previously macroscopical observation.

Morphometric analysis was performed using three sections from three UF of three individual mice from each mice group. Decidua and placental areas were delimitated as it is marked in the images (Figure 8).

In Balb\c mice, no significant differences were observed between infected and non-infected mice, probably due to the variability found between the different animals analyzed (Figure 9).

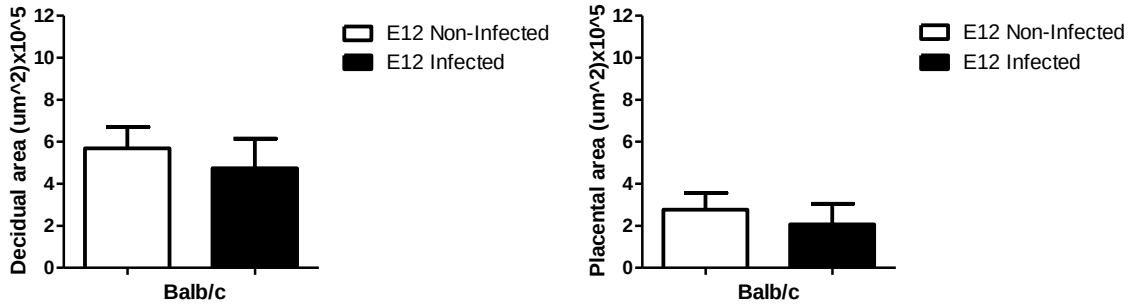

Figure 9. Morphometric analysis of decidua and placental areas from E12 pregnant Balb\c mice. Each group was comprised of 3 animals. All data are expressed as means ± SD from 3 sections of 3 individual animals.

However, in B6 mice it was possible to find a significant decrease in the decidua and in the placental areas from infected compared to non-infected mice, thus confirming earlier macroscopical and microscopical observations (Figure 10).

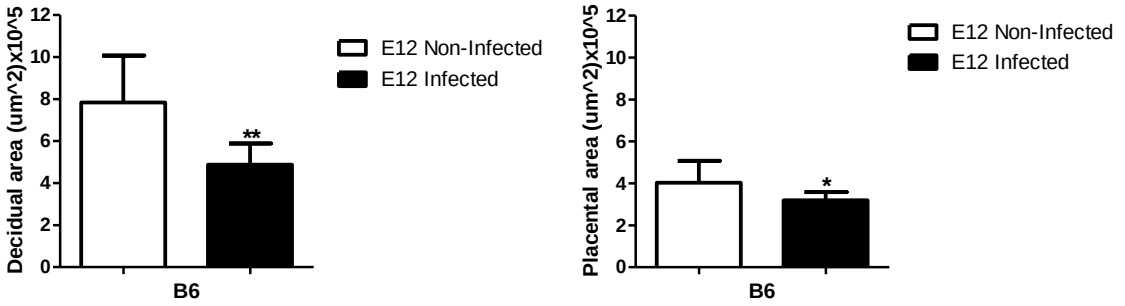

Figure 10. Morphometric analysis of decidua and placental areas from E12 pregnant B6 mice. Each group of mice was comprised of 3 animals. All data are expressed as means ± SD from 3 sections of 3 individual animals. **P<0.01, *P<0.05 for comparisons of E12 infected versus E12 non-infected animals.

These results suggest a delay in the process of decidualisation and placentation in B6 mice infected with *T.gondii.*

2. Parasite load quantification

As previously described B6 and Balb\c strains have different susceptibilities to *T. gondii* infection, depending on the route of infection (144). In this work, the quantification of parasite load was done using decidua/placental tissues, spleen cells and PEC from infected B6 and Balb\c at E12 by amplification of SAG-1 gene by q-PCR.

The quantification of the parasite load in decidua/placental tissues was inconclusive for both B6 and Balb\c mice, as in some animals there was no amplification of the SAG1 gene, leaving insufficient results for statistical analysis (Table 3). This indicates that five days of infection, via ip route, have not been sufficient in some animals to allow parasite dissemination from the site of infection to the decidua/placental tissues.

Table 3. Parasite load quantification in the Decidua/Placental tissues from E12 infected mice.

Strain		ct	Parasite Quantity (ng/µL)
B6	**Animal 1**	Undetermined	0
	Animal 2	Undetermined	0
	Animal 3	38,49	0,003366
Balb\c	**Animal 1**	38,96	0,002472
	Animal 2	Undetermined	0
	Animal 3	40,42	0,000943

NOTE: Parasite load was quantified using a standard curve of known concentrations of parasite genomic DNA diluted in host genomic DNA ranging from 10^2 (100 ng/µL) to 10^{-2} (0,01 ng/µL).

The parasite burden found in spleen cells and PEC from pregnant Balb\c was significantly decreased when compared to non-pregnant Balb\c mice. These results indicate that pregnant Balb\c mice are more resistant to infection compared to non-pregnant Balb\c mice. Further, the parasite load found in spleen cells and PEC from non-pregnant Balb\c mice were significantly increased compared to non-pregnant B6 mice confirming, as previously described, that Balb\c strain is more susceptible to *T. gondii* infection than B6 strain, when infected by ip route (144).

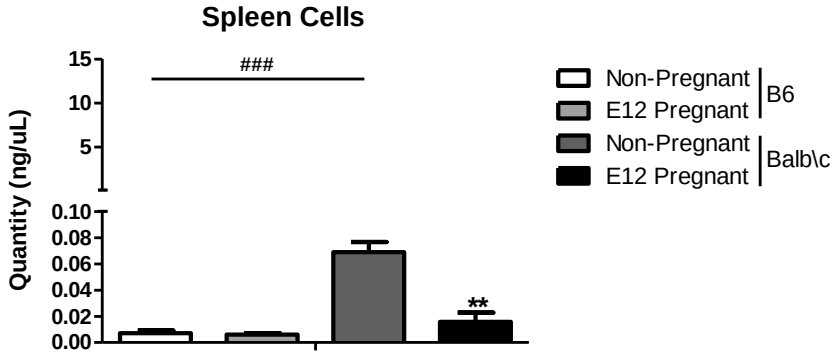

Figure 11. Parasite load quantification in spleen cells from E12 pregnant or non-pregnant B6 or Balb\c mice, either infected with *T. gondii*. Values are presented as means ± SE from three individual mice experiments. **P<0.01 (Differences between pregnant and non-pregnant), ###P<0.0001 (Differences between strains).

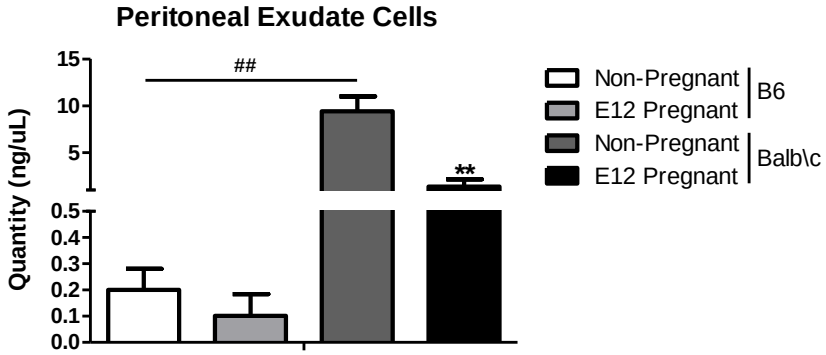

Figure 12. Parasite load quantification in PEC from E12 pregnant or non-pregnant B6 or Balb\c mice, either infected with *T. gondii*. Values are presented as means ± SE from three individual mice experiments. **P<0.01 (Differences between pregnant and non-pregnant), ##P<0.01 (Differences between strains).

No significant differences were found comparing B6 pregnant with non-pregnant mice, probably due to the lower parasite burden found in this strain and the infection time-point chosen to perform this study. However, in Balb\c mice, pregnancy causes a decrease in the parasite load of both cell types (Figure 11 and 12). This could be explained by the immunological status that is changed during pregnancy, allowing host survival and the maintenance of pregnancy. By contrast, since B6 mice are more resistant to infection compared to Balb\c, it was not possible to determine a pregnancy effect on the parasite load. It was also observed an increased parasite load in PEC compared to spleen cells from both mice strains, due to the infection route chosen in this study.

3. Arg-1 and NOS2 gene expression

To evaluate Arg-1 and NOS2 gene expression at the FMI and at systemic level, during *T. gondii* infection, qRT-PCR was performed using decidua/placental tissue and spleen cells from different groups of mice: non-infected, infected, E12 pregnant non-infected and E12 pregnant infected mice.

The results obtained for decidua/placental tissues indicated a decreased Arg-1 and NOS2 gene expression in infected animals, compared to non-infected ones (Figure 13A, B). However, a significant decrease was only found for NOS2 gene expression in infected B6 mice compared to non-infected B6 mice (Figure

13B). It was also noticed that Balb\c and B6 strains, have different Arg-1 and NOS2 gene expression patterns. Therefore, a increased expression of Arg-1 and a decreased expression of NOS2 was found in both non-infected and infected B6, compared to Balb\c. Indeed, it was found a significant decrease in Arg-1 gene expression comparing non-infected or infected Balb\c with non-infected or infected B6 mice, respectively (Figure 13A). It was found a significant increase of NOS2 gene expression in infected Balb\c compared to infected B6 mice (Figure 13B).

Moreover, Arg-1 and NOS2 pathways are antagonics, since when we observe high levels of Arg-1, NOS2 levels are low and vice versa. These results are consistent with the literature, which shows that Arg-1 and NOS2 compete for the same substrate (L-arginine), thereby regulating one another (121).

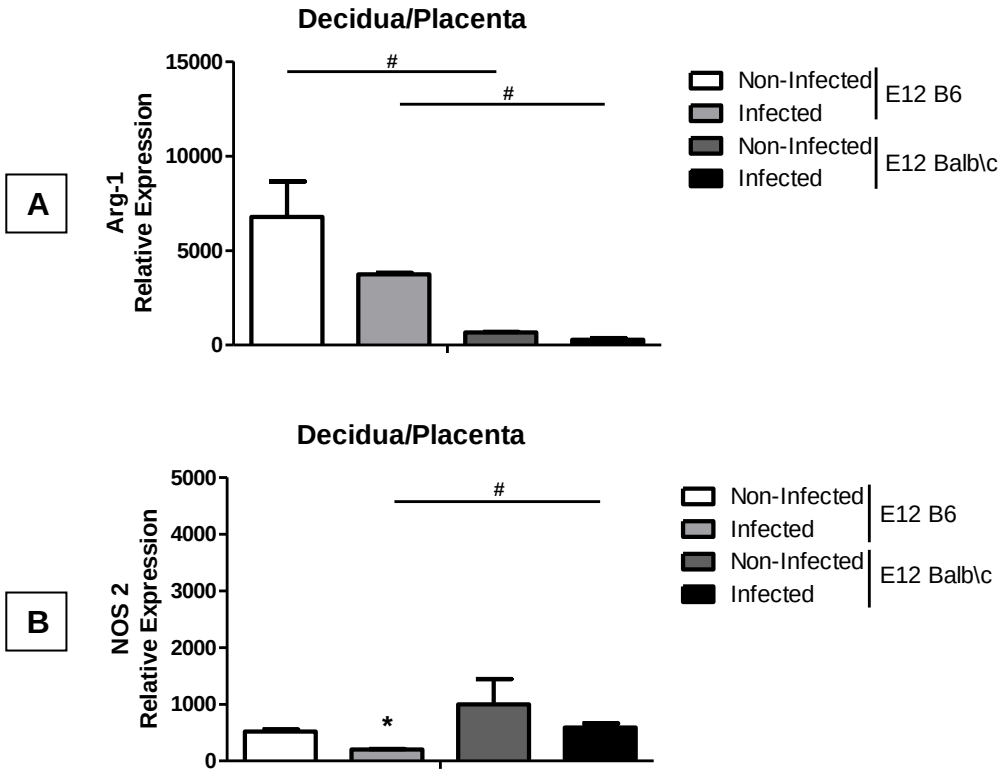

Figure 13. Relative expression levels of Arg-1 (A) and NOS2 (B) normalized to HPRT in the Decidua/Placenta of E12 pregnant B6 or E12 pregnant Balb\c mice. Values are presented as means ± SE from three individual mice. (*) Represent differences in infected animals compared with not-infected animals; (#) represent difference between strains. $^{*,\#}$ P<0.05.

In the context of pregnancy, the results obtained for spleen cells indicated that there were no significant differences for Arg-1 gene expression between the

different groups of mice. However, according to the results obtained for decidua/placental tissues was found, a significant decrease of NOS2 gene expression in E12 pregnant infected B6 mice compared to E12 pregnant non-infected B6 mice. This may indicate that the same pattern of NOS2 gene expression is observed at the FMI and at systemic level.

However, in spleen cells it was observed that NOS2 gene expression levels were increased compared to Arg-1 expression levels in B6 pregnant mice (Figure 14). This data is not in accordance to what is occurring in the decidua/placenta in the same group of animals, that might be explained by the fact of Arg-1 role in different organs (UFs versus spleen). Paradoxically, it was observed a significant decrease of NOS2 gene expression between E12 pregnant non-infected B6 and E12 pregnant non-infected Balb\c (P<0.05).

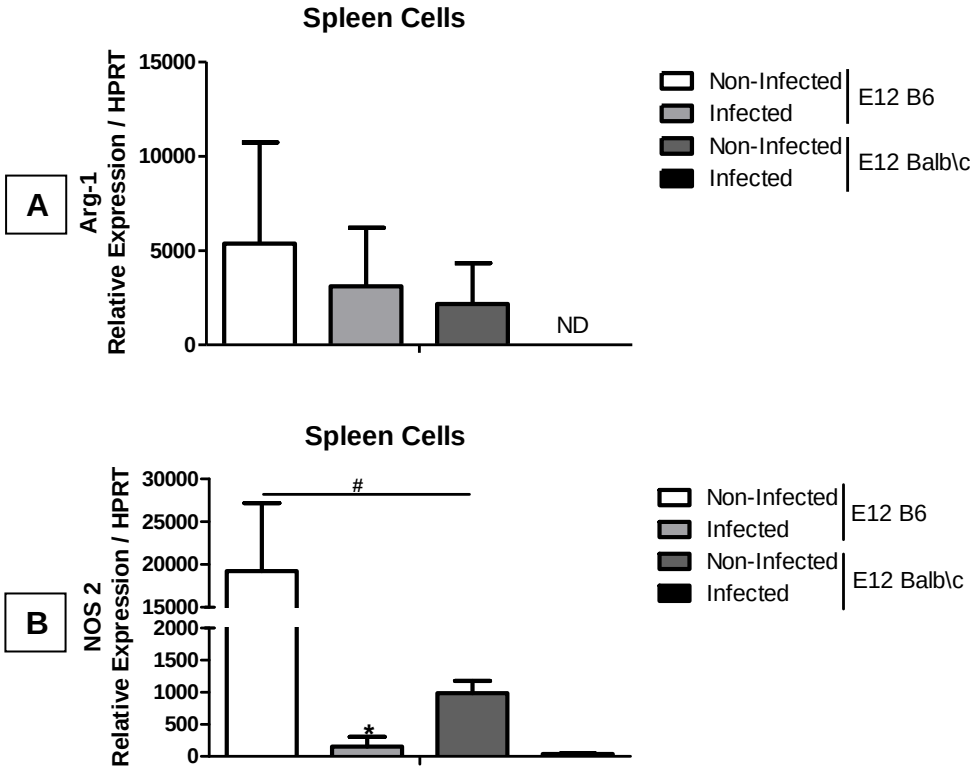

Figure 14. Relative expression levels of Arg-1 (A) and NOS2 (B) normalized to HPRT in the Spleen cells of E12 pregnant B6 or E12 pregnant Balb\c mice. (*) Represent differences in infected animals compared with not-infected animals; (#) represent difference between strains. Values are present as means ±SE from three individual mice. (ND = Not detected). $^{*,\#}$ P<0.05.

At systemic level, no significant differences were obtained from Arg-1 and NOS2 gene expression between the different groups of non-pregnant animals (Figure 15).

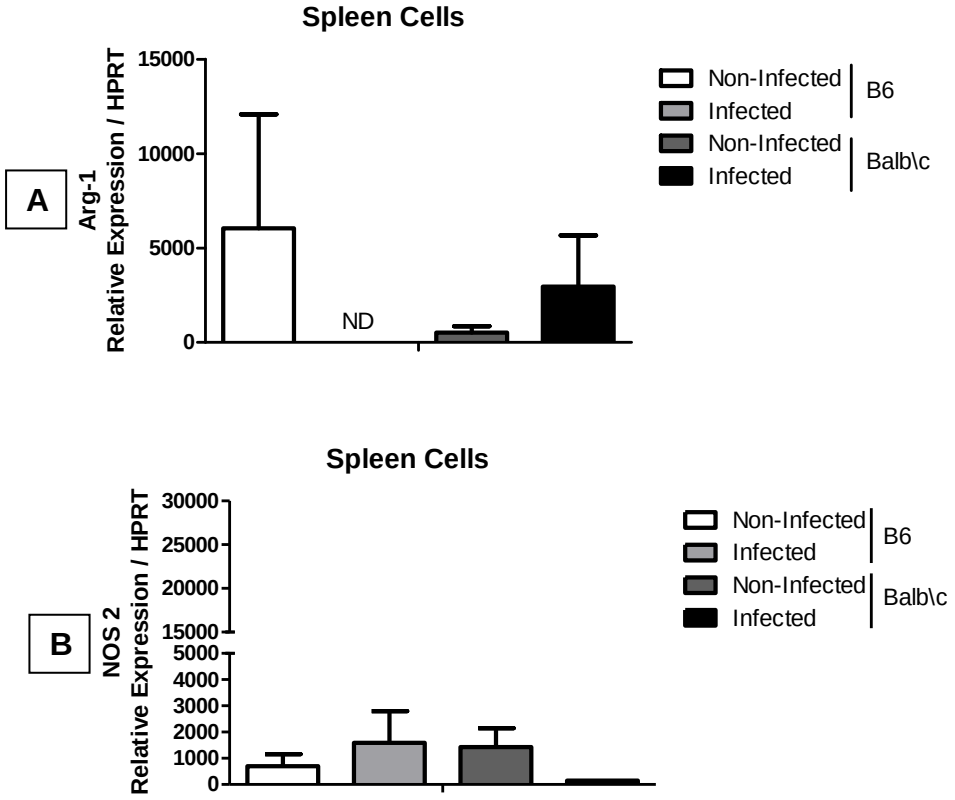

Figure 15. Relative expression levels of Arg-1 (A) and NOS2 (B) normalized to HPRT in the Spleen cells of B6 and Balb\c mice. Values are present as means ±SE from three individual mice. (ND = Not detected).

It was also observed a significative increase of NOS2 expression in pregnant non-infected B6 compared to non-pregnant non-infected B6 mice. This result suggest that during pregnancy, NOS2 gene expression is increased in B6 mice, which is somehow controversial, since NOS2 induces the production of NO, known to be harmful for pregnancy. This may also explain the fact that pregnant B6 strain is more resistant to *T. gondii* infection compared to Balb\c strain.

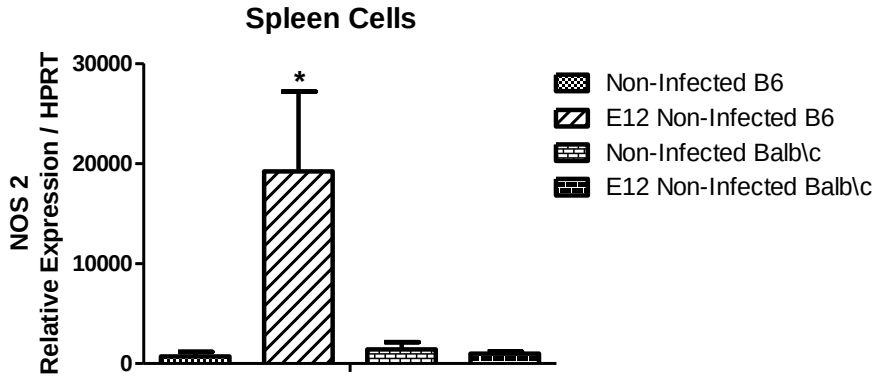

Figure16. Comparing the levels of NOS2 relative expression between E12 pregnant non-infected and non-infected, B6 or Balb\c mice. Values are present as means ± SE from three individual mice. (*) Represent differences between pregnant and non-pregnant animals. *P<0.05.

4. Arg-1 protein expression

To evaluate Arg-1 protein expression at the FMI and at systemic level during *T. gondii* infection, western blotting was performed using protein samples extracted from decidua/placental tissues, spleen cells and PEC from the four groups previously described.

The results obtained for the decidua/placental tissues indicated a significant increase in Arg-1 protein expression of infected B6 compared with non-infected B6 mice (Figure 17). These results were not in accordance to the results observed for the Arg-1 gene expression. This results may suggest, an alternative activation of macrophages upon infection with *T. gondii* at FMI.

Unfortunately, we have not achieved conclusive results for Balb\c pregnant mice due to the decidua/placental tissue samples degradation, thus further experiments must be performed.

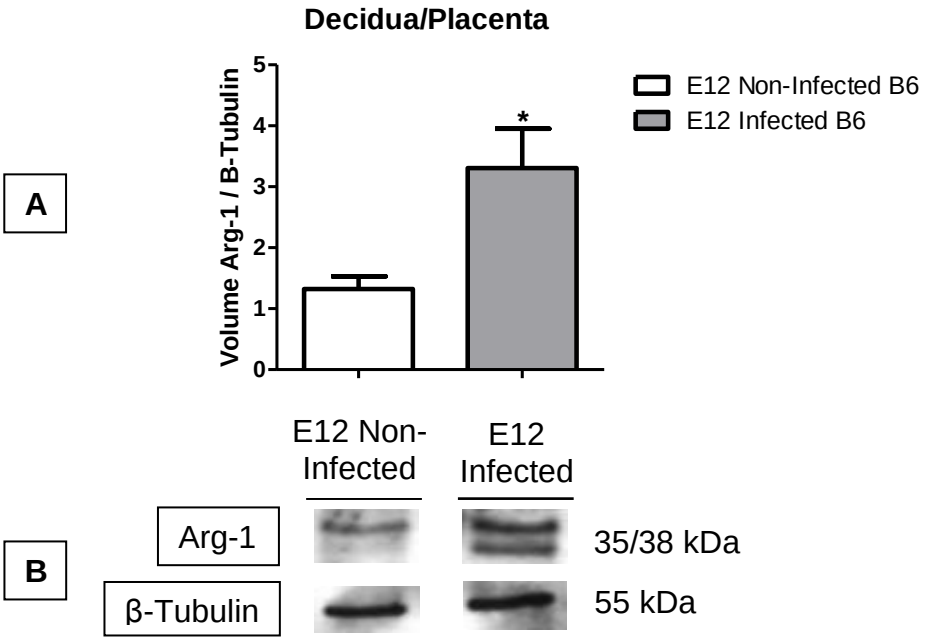

Figure 17. Densitometry analysis (A) and representative western blotting (B) of Arg-1 in decidua/placental tissues obtained from E12 pregnant B6 mice. Data are shown as Arg-1/β-tubulin ratio and expressed as means ±SE from three individual mice. (*) Represent differences in infected animals compared with non-infected animals *P<0.05.

In the pregnancy context, in spleen cells, a tendency for decreasing Arg-1 protein expression occurs upon *T. gondii* infection in B6 mice (Figure 18). This data constitutes the opposite of what happens in non-pregnant B6 mice, suggesting, therefore, that gestation alters the pattern of Arg-1 protein expression upon infection with *T. gondii* in B6 mice (Figure 19).

It is also possible the observation of differences in the basal levels of this protein expression among the two strains of pregnant mice.

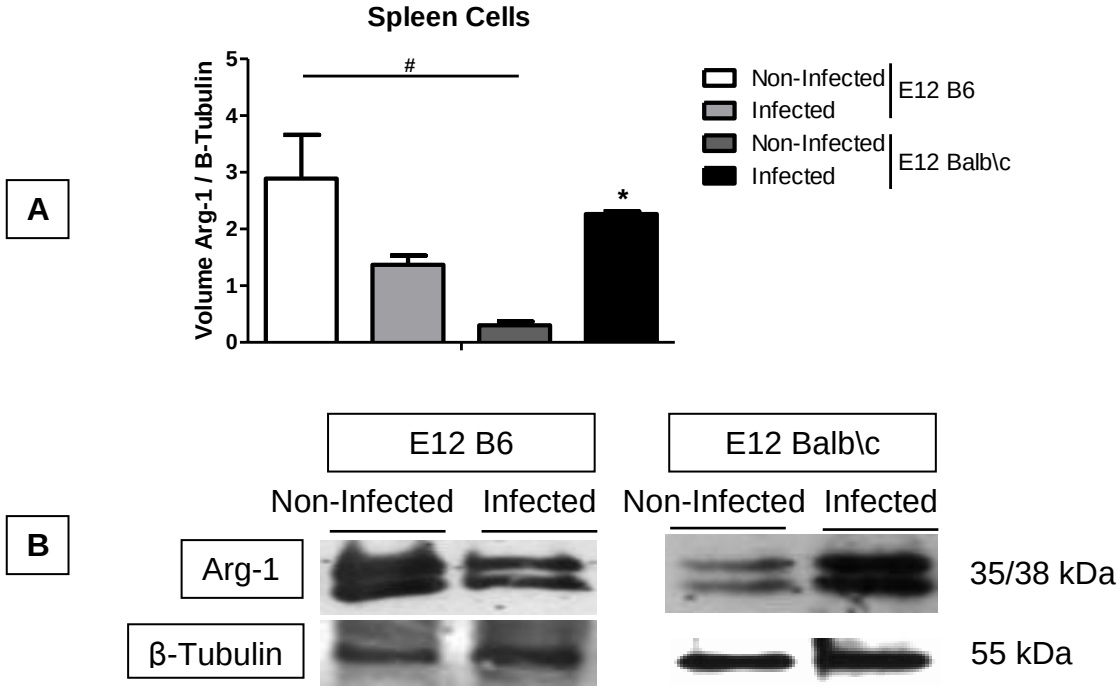

Figure 18. Densitometry analysis (A) and representative western blotting (B) of Arg-1 in spleen cells obtained from E12 pregnant B6 or Balb\c mice. Data are shown as Arg-1/β-tubulin ratio and expressed as means ±SE from three individual mice. (*) Represent differences in infected animals compared with non-infected animals; (#) represent difference between strains. $^{*,\#}$ P<0.05.

Regarding Balb\c mice, in both E12 pregnant infected and non-pregnant infected animals, an increased Arg-1 protein expression is observed, however, no significant differences were observed (Figure 18 and 19). Significant differences were achieved, between E12 pregnant infected compared to E12 pregnant non-infected Balb\c mice (Figure 18). These data indicates that pregnancy synergizes with infection in the induction of Arg-1 protein expression in Balb\c mice.

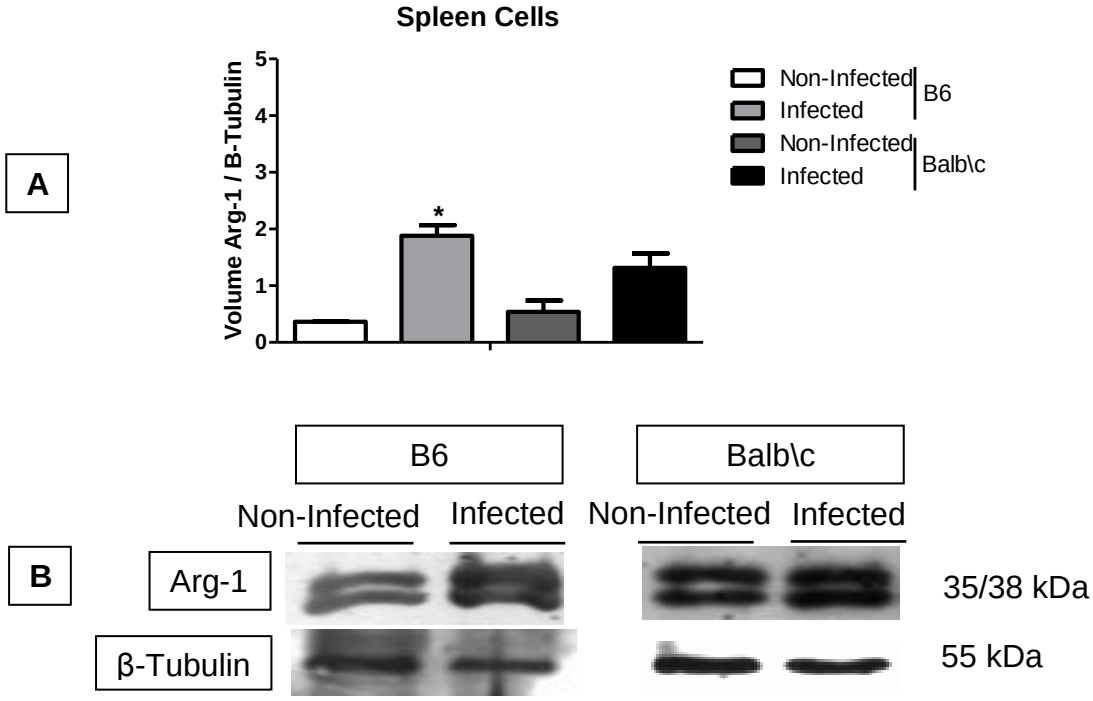

Figure 19. Densitometry analysis (A) and representative western blotting (B) of Arg-1 in spleen cells obtained from B6 and Balb\c mice. Data are shown as Arg-1/β-tubulin ratio and expressed as means ±SE from three individual mice. (*) Represent differences in infected animals compared with non-infected animals. * P<0.05.

Pregnancy increased the Arg-1 protein expression in spleen cells of E12 non-infected B6 mice (Figure 20). These results suggest that pregnancy and infection separately increase Arg-1 expression, but the two conditions combined seemed to decrease Arg-1 expression in B6 mice (Figure 18, 19 and 20).

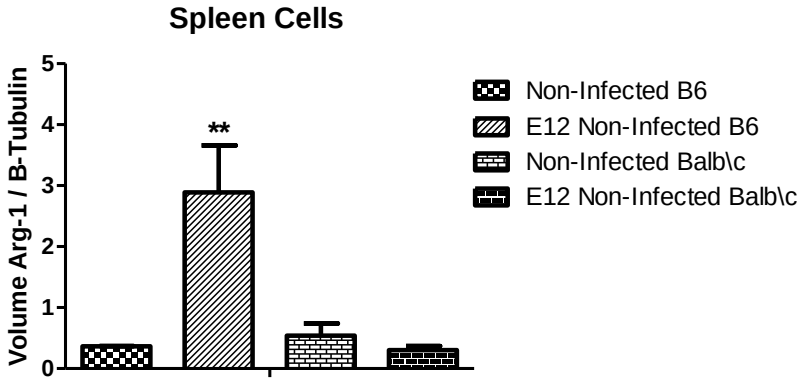

Figure 20. Comparing the levels of Arg-1 protein expression between E12 pregnant non-infected B6 and non-infected B6 mice. Data are shown as Arg-1/β-tubulin ratio and expressed as means ±SE from three individual mice. (*) Represent differences in pregnant animals compared with non-pregnant animals ** P<0.01.

In the context of pregnancy, in PEC, there is an increase and a decrease of Arg-1 protein in infected B6 and Balb\c mice, respectively. In this case, it is also possible to observe a significant difference between the Arg-1 protein levels of E12 pregnant non-infected B6 compared to E12 pregnant non-infected Balb\c mice (P<0.001) (Figure 21). These results already suggest a different pattern of Arg-1 protein expression in pregnant B6 mice compared to pregnant B6 mice.

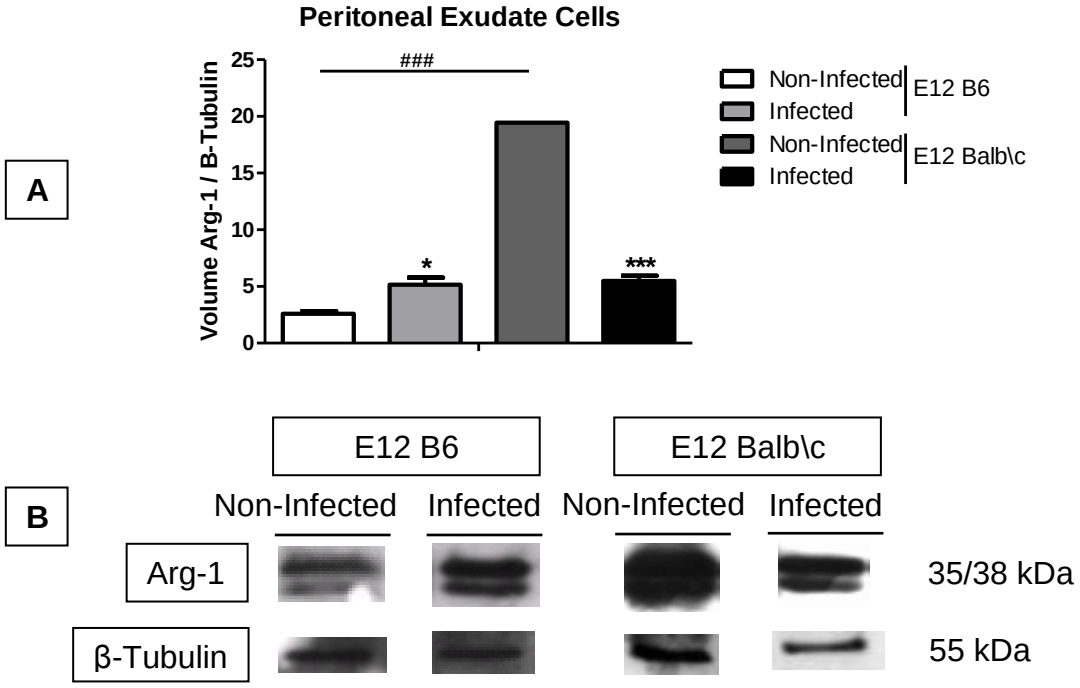

Figure 21. Densitometry analysis (A) and representative western blotting (B) of Arg-1 in PEC obtained from E12 pregnant B6 or Balb\c mice. Data are shown as Arg-1/β-tubulin ratio and expressed as means ±SE from three individual mice. (*) Represent differences in infected animals compared with non-infected animals; (#) represent difference between strains. *P<0.05, ***, ### P<0.001.

In the PEC it's possible to see an increase in Arg-1 protein expression in infected Balb\c mice compared to non-infected Balb\c mice; however, for B6 mice there are no differences. A difference between the two strains in the context of infection, with higher levels of Arg-1 protein in Balb\c was observed (Figure 22).

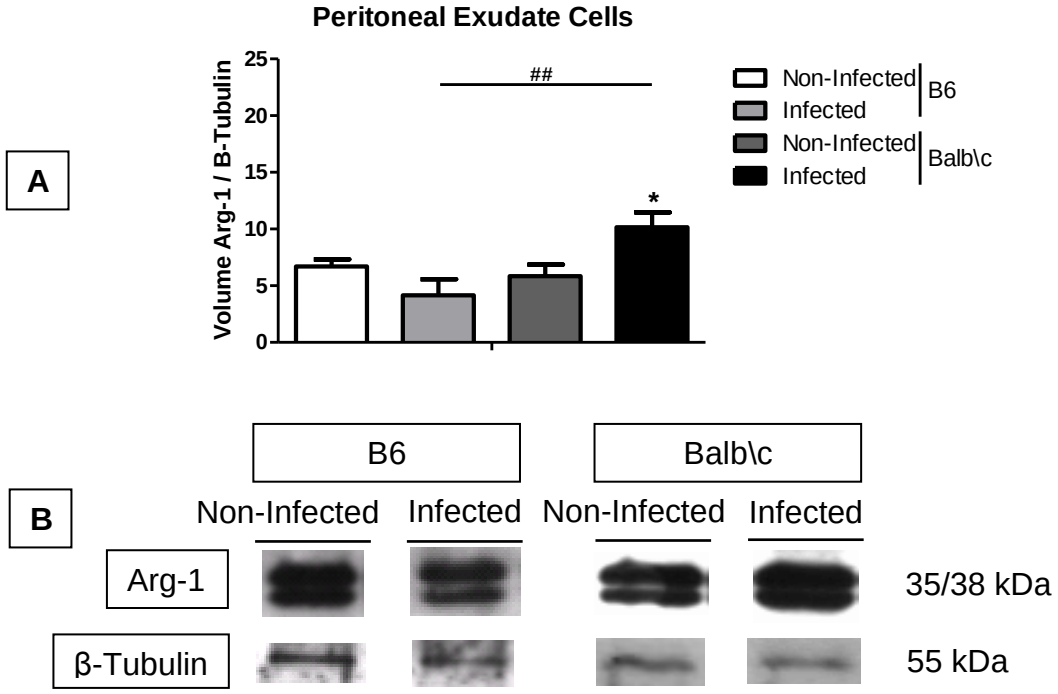

Figure 22. Densitometry analysis (A) and representative western blotting (B) of Arg-1 in PEC obtained from B6 and Balb\c mice. Data are shown as Arg-1/β-tubulin ratio and expressed as means ±SE from three individual mice. (*) Represent differences in infected animals compared with non-infected animals; (#) represent difference between strains. * P<0.05, ## P<0.01.

5. Immunohistochemistry for Arg1 and NOS2

To confirm the results obtained by western blotting, immunohistochemical analysis for Arg-1 was performed in order to study protein expression and distribution, using UFs sections from the Balb\c E12 or E14, non-infected and infected mice, and from B6 E12, non-infected and infected mice.

In parallel, liver tissue sections, where the Arg-1 is constitutively expressed, from non-infected Balb\c mice, were used as internal negative control (no primary antibody) and as internal positive control (with primary antibody) (Figure 23A-B) (120).

The figures indicate an increase in specific labeling for Arg-1 in infected animals compared to the non-infected animals, for Balb\c E12 and E14 (Figure 23C-F) and for the B6 E12 (Figure 23G-H).

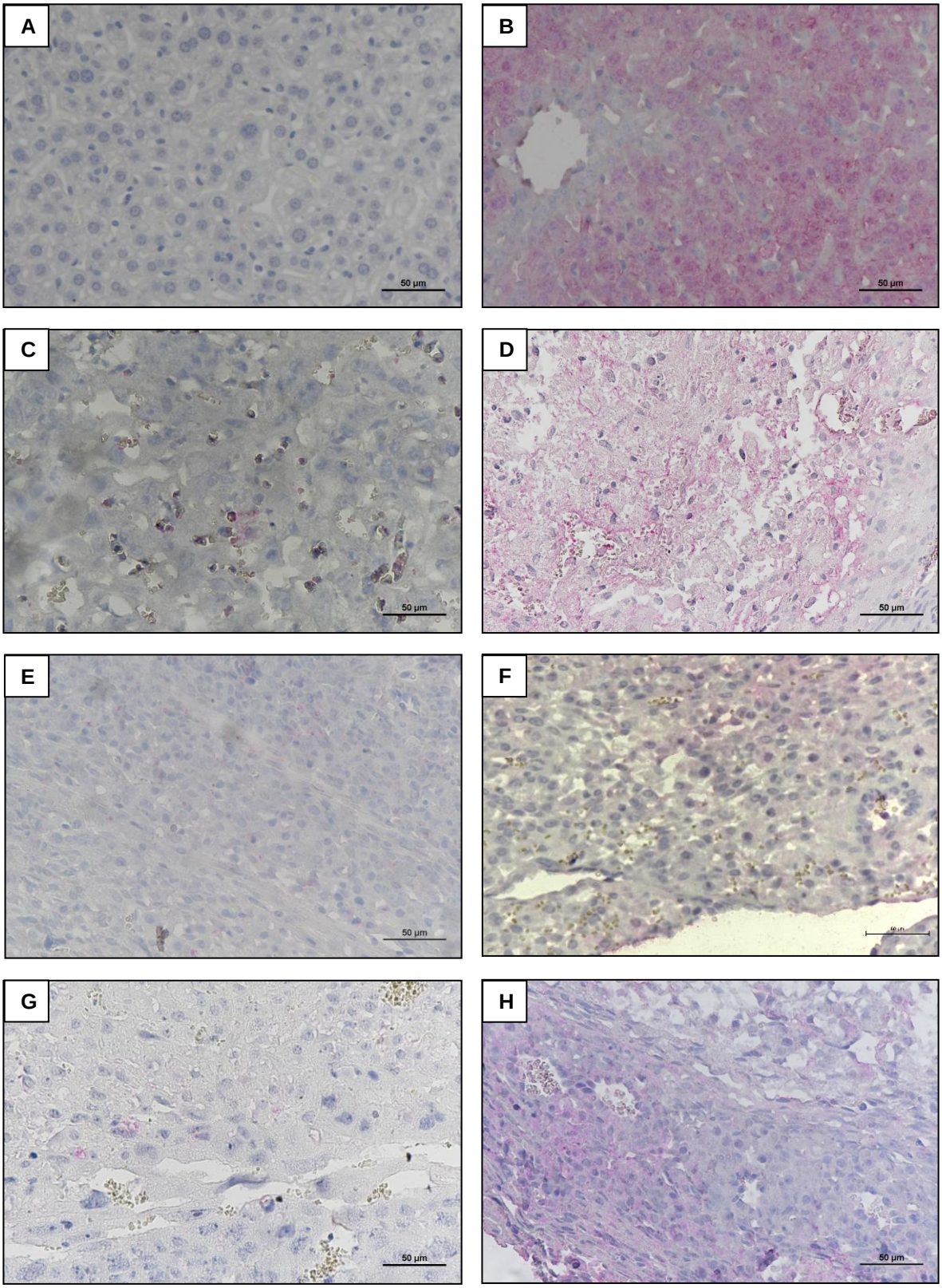

Figure 23. Representative images of immunohistochemical staining of Arg-1 positive cells using UFs sections from E12, E14 Balb\c and E12 B6 mice at the mesometrial decidua. [A] Liver of infected Balb\c, negative control; [B] Liver of infected Balb\c, positive control; [C] E14 not-infected Balb\c; [D] E14 infected Balb\c; [E] E12 not-infected Balb\c; [F] E12 infected Balb\c; [G] E12 not-infected B6; [H] E12 infected B6.

Immunohistochemical analysis for NOS2 was performed in order to study protein expression and distribution, using UFs sections from the Balb\c E12 or E14, non-infected and infected mice, and from B6 E12, non-infected and infected mice.

In parallel, liver tissue sections from *T. gondii* infected Balb\c mice were used as internal negative control (no primary antibody) and as internal positive control (with primary antibody), since NOS2 is induced in response to infection (Figure 24A-B) (121).

The results for the immunohistochemical analysis of NOS2 showed a decrease in specific staining in infected animals compared to the non-infected animals for the E12 and E14 Balb\c and for the E12 B6 (Figure 24C-H).

This decrease of NOS2 expression might be consistent with the literature, having in mind that NOS2 and Arg-1 pathways are antagonics. This increased Arg-1 expression in infected animals can be related to a decreased NOS2 expression.

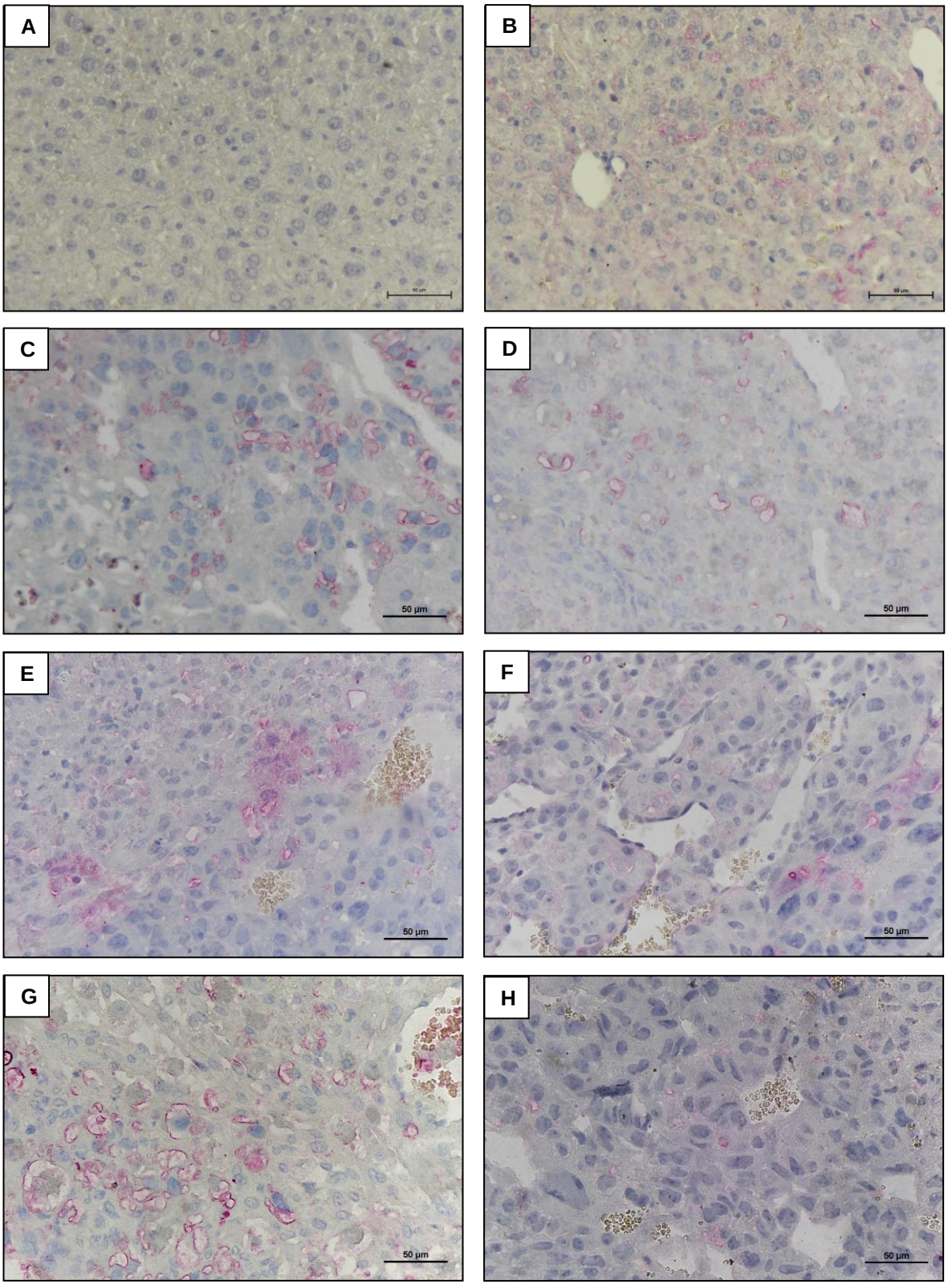

Figure 24. Representative images of immunohistochemical staining of NOS2 positive cells using UFs sections from E12, E14 Balb\c and E12 B6 mice at the mesometrial decidua. [A] Liver of infected Balb\c, negative control; [B] Liver of infected Balb\c, positive control; [C] E14 not-infected Balb\c; [D] E14 infected Balb\c; [E] E12 not-infected Balb\c; [F] E12 infected Balb\c; [G] E12 not-infected B6; [H] E12 infected B6.

6. Immunofluorescence for Arg1 and F4/80

To verify if Arg-1 specific labeling detected by immunohistochemistry is linked to alternative macrophage activation, it was performed immunofluorescence allowing co-localization of the macrophage marker F4/80 and Arg-1 proteins using UF sections from both mice strains.

It was possible to detect F4/80$^-$ cells expressing Arg-1 (F4/80$^-$ Arg-1$^+$) in infected Balb/c mice, indicating that other cells than macrophages were expressing Arg-1, compared with non-infected (Figure 25A-B). In non-infected Balb\c it was possible to detect both green and red fluorescence corresponding to background, indicating no specific staining for F4/80 or Arg-1 (F4/80$^-$ Arg1$^-$) cells (Figure 25A).

In non-infected B6 mice, it was possible to detect F4/80$^+$ cells expressing Arg-1 (F4/80$^+$ Arg-1$^+$) indicating that macrophages were expressing Arg-1, compared with infected mice (Figure 26A-B). In infected B6 it was possible to detect both green and red fluorescence corresponding to background indicating no specific staining for F4/80 or Arg-1 (F4/80$^-$ Arg1$^-$) cells (Figure 26B).

This data supports immunohistochemical analysis confirming an increasing Arg-1 protein expression in the mesometrial decidua upon infection in Balb/c but not in B6 mice.

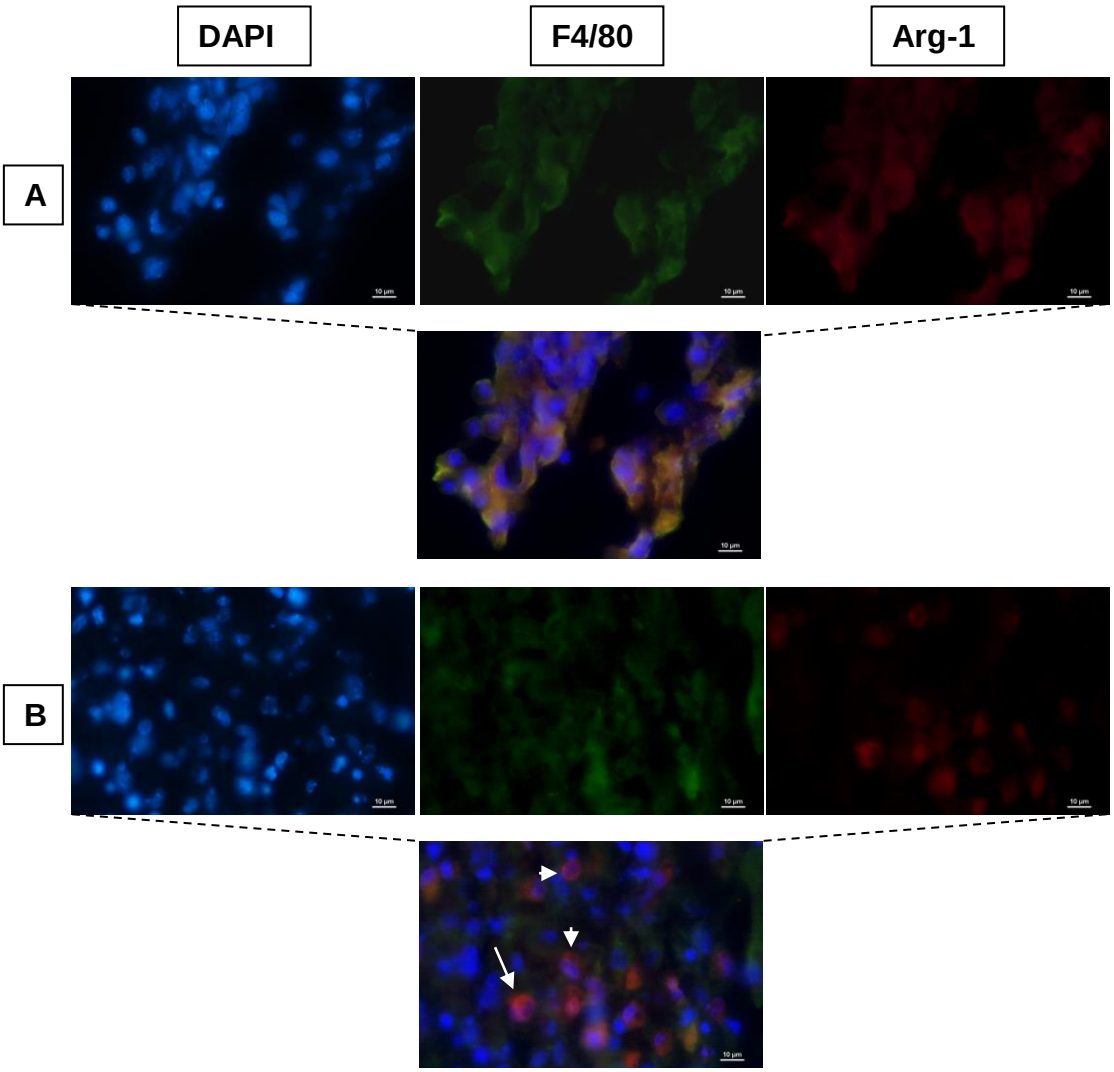

Figure 25. Identification of Arg-1 positive cells in the mesometrial decidua from E12 Balb\c mice. (A) E12 non-infected Balb\c; (B) E12 infected Balb\c. Representative UFs sections stained with antibodies specific for F4/80 and Arg-1. Arg-1 (red), F4/80^{+} cells (green). Nuclei were stained blue by DAPI.

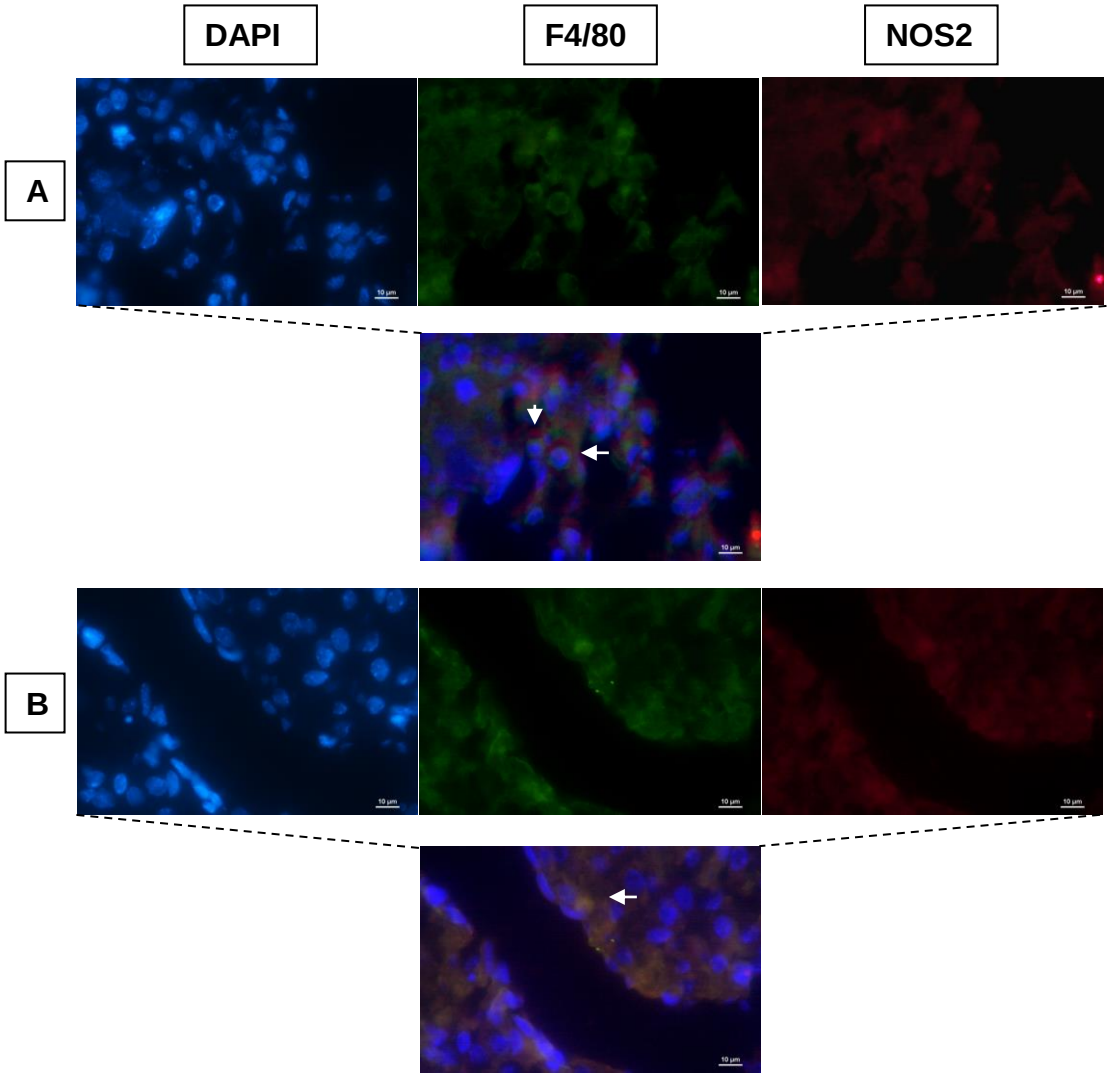

Figure 26. Identification of Arg-1 positive cells in the mesometrial decidua from E12 B6 mice. (A) E12 non-infected B6; (B) E12 infected B6. Representative UFs sections stained with antibodies specific for F4/80 and Arg-1. Arg-1 (red), F4/80^{+} cells (green). Nuclei were stained blue by DAPI.

7. Immunofluorescence for NOS2 and F4/80

To verify if NOS2 specific labeling detected by immunohistochemistry is linked to classical macrophage activation, it was performed immunofluorescence allowing co-localization of the macrophage marker F4/80 and NOS2 proteins using UF sections from both mice strains.

In relation to NOS2 it was possible to detect $F4/80^-$ cells expressing NOS2 ($F4/80^-$ $NOS2^+$) in both non-infected and infected Balb/c mice, indicating that other cells than macrophages were expressing NOS2 independent of infection (Figure 27A-B). It was possible to detect green fluorescence corresponding to background indicating no specific staining for F4/80 (Figure 27A-B). In non-infected B6 mice, it was possible to detect $F4/80^-$ cells expressing NOS2 ($F4/80^-NOS2^+$) indicating that other cells than macrophages were expressing NOS2 (Figure 28A). In infected B6 it was possible to detect $F4/80^+$ cells expressing NOS2 ($F4/80^+NOS2^+$) indicating that macrophages were expressing NOS2 (Figure 28B). This data do not support immunohistochemical analysis, therefore further experiments must be performed to confirm these results.

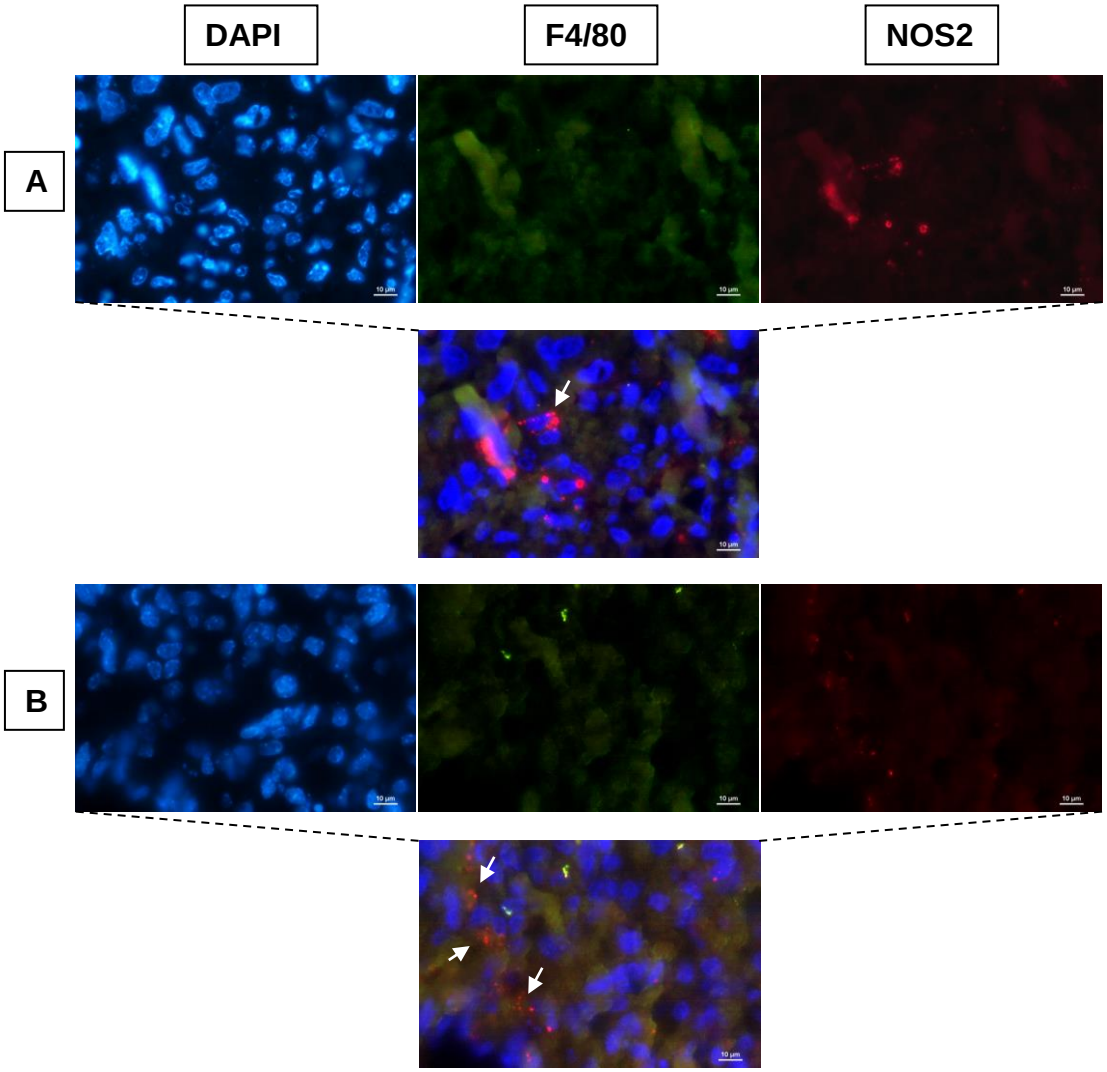

Figure 27. Identification of NOS2 positive cells in the mesometrial decidua from E12 Balb\c mice. (A) E12 non-infected Balb\c; (B) E12 infected Balb\c. Representative UFs sections stained with antibodies specific for F4/80 and NOS2. NOS2 (red), F4/80^{+} cells (green). Nuclei were stained blue by DAPI.

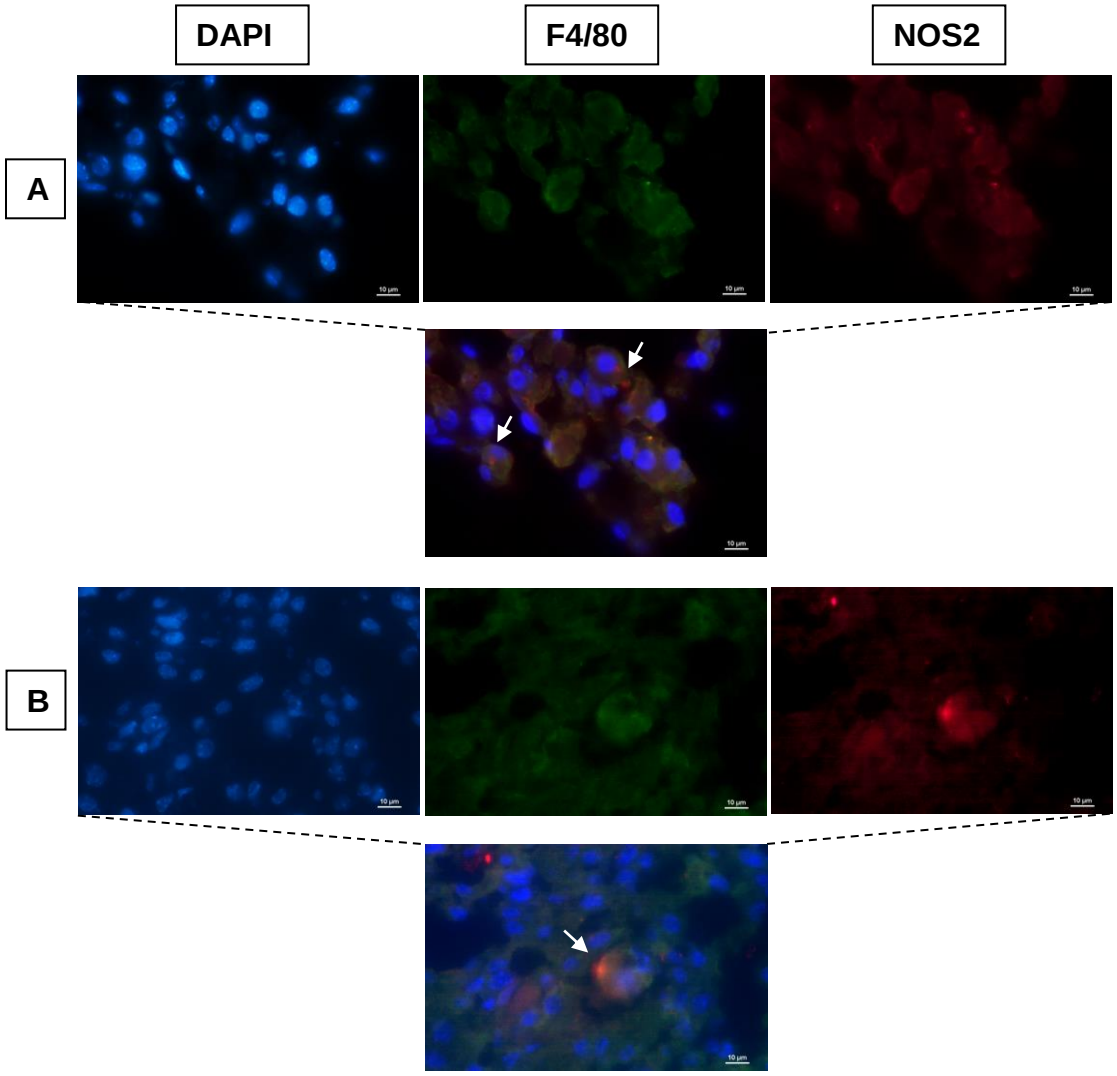

Figure 28. Identification of NOS2 positive cells in the mesometrial decidua from E12 B6 mice. (A) E12 non-infected B6; (B) E12 infected B6. Representative UFs sections stained with antibodies specific for F4/80 and NOS2. NOS2 (red), F4/80^{+} cells (green). Nuclei were stained blue by DAPI.

Chapter IV. **DISCUSSION**

T. gondii is one of the most frequent worldwide parasitic infections, usually asymptomatic, when occurs in an immunocompetent host. However, the pathophysiology of toxoplasmosis is far more complex when primary infection is acquired during pregnancy (52). During primary infection, parasites cross the intestinal barrier and invade monocyte cells in contact with the lamina propria, which allow them to disseminate through the blood flow, towards virtually all organs, including placenta (148).

In this study, we can observe a delay in the process of decidualization and placentation upon infection, in B6 mice, which may have consequences in the normal progression of pregnancy, since the decidua/placenta prevents the passage of infectious agents towards the foetal compartment. Decidua and placenta are key tissues in the mother-to-foetus relationship, not only because of their trophic role, but also because they provide a tolerant immune microenvironment necessary for gestation progression (149).

The results indicate, that five days after infection, it was not possible to detect in decidual/placental tissues in B6 and Balb\c mice, the *T. gondii* parasite by q-PCR, indicating that probably this period of time was not sufficient to allow parasite dissemination from the site of infection (ip route) to the FMI. However, analysing the parasite load at systemic level (spleen cells and PEC) we conclude that Balb\c mice are more susceptible to *T. gondii* infection compared to B6 mice, which is in agreement with the literature (144). The literature also suggests that pregnant mice are more susceptible to infection with *T. gondii* (150). However, our results for Balb\c mice suggest that pregnancy has a protective effect against infection, reducing the parasite load of pregnant animals compared to non-pregnant mice. Since Balb\c mice are more susceptible to infection compared to B6 mice during pregnancy, this susceptibility might be camouflaged by some mechanisms allowing host survival and pregnancy maintenance. This might not occur in B6 strain since they are already more resistant to infection, explaining probably the fact that no differences were detected in the parasite load between pregnant and non-pregnant B6 mice.

Macrophages support a variety of essential processes for successful pregnancy such as remodelling the uterine connective tissues and blood vessels,

regulation of trophoblast implantation, immune-tolerance toward fetal antigens, immunomodulation of neighboring leukocytes, and initiation of parturition (151–155). All of these processes are manifestations of macrophage polarity or state of activation, which have been referred to as M1 and M2 (156). M1/M2 activities arise from arginine metabolism via two enzymatic pathways, NOS2 and Arg-1 (157). Successful pregnancy requires a regulation of macrophage activation state throughout pregnancy (158).

In this study, Arg-1 gene expression was increased in decidua/placental tissues from B6 mice, that might indicate a shift toward M2 profile, which is in agreement with recent studies indicating that in the second pregnancy stage (7-12 days), decidua begins to shift towards a pro-M2 environment, which prevents rejection of the fetus and allows fetal growth until parturition (153). It is observed an increasing NOS2 gene expression in Balb\c mice that might indicate a shift towards M1 profile. These results suggest a different gene expression profile between these two strains, which can be explained by the need of more NOS2 production in Balb\c mice, a more susceptible strain of mice, compared to B6, since NOS2 is responsible for the production of NO, that can directly kill the parasite and prevent congenital infection. There are no significant differences in the expression of these genes at systemic level (spleen cells) of non-pregnant mice. However, in the context of pregnancy, there is a decrease in NOS2 gene expression in spleen cells of infected B6 compared to non-infected B6 mice, suggesting that pregnancy in the context of infection leads to an M2 profile in B6 mice. However, pregnancy alone induces NOS2 expression in B6 mice, which may explain the more resistant profile of pregnant B6 mice compared to pregnant Balb\c.

The results for the Arg-1 protein expression suggest an increase of this protein upon infection at FMI and spleen cells but not in PEC in non-pregnant B6 mice. It is also observed that pregnancy itself increases Arg-1 expression. Suggesting therefore that pregnancy and infection separately increase Arg-1 expression, which is consistent with the literature (153). However, the two conditions combined (pregnancy and infection) decrease Arg-1 expression. For Balb\c mice it is also observed an increase in Arg-1 expression, only in spleen

cells of infected pregnant mice and PEC of infected non-pregnant mice.

The results for Arg-1/NOS2 gene and protein expression showed a tendency for M2 profile, however this results were not consistent, especially in Balb\c mice. In conclusion, more studies using Balb\c strain will be needed to obtain valid results.

In an attempt to confirm the previous results, immunohistochemical analyses were performed at the decidua. The immunohistochemical results are in agreement with the previously described hypothesis, which indicates that *T. gondii* infection induces the expression of Arg-1 and this, in turn, may induce an increasing production of polyamines that promote cell division and proliferation of the parasites (125,159,160). Furthermore, it is also settled that M2 macrophages control the replication of the parasite inducing arginase and depleting arginine which is decisive for the microorganisms multiplication (160). The imunohistochemical results also suggested a decreasing NOS2 expression that leads to a decreasing production of NO, a potent microbicide (125,126,159). The increase of the Arg-1 and decrease of NOS2 expression can be indicative of the presence of alternative activated macrophages (102), allowing parasite multiplication on one hand, but, on the other hand, prevent the production of NO which is hurtful to pregnancy.

This increase of Arg-1 and decrease of NOS2 upon infection might indicate a decreased macrophage activation by the classical pathway (as these are characterized by the expression of NOS2) and an increased macrophage activation by the alternative pathway (as these are characterized by the expression of Arg-1) (75).

Alternative activation pathway is described, as allowing the maintenance of pregnancy preventing abortion, since successful gestation has been thought to be accompanied by a bias away from T helper (Th) 1-type cytokines (M1 profile) and towards Th2- type cytokines (M2 profile) (161,162).

Immunofluorescence analysis indicates that other cells, other than macrophages express NOS2 and Arg-1 at the FMI. Further experiments are being performed in order to clarify the connection between alterations in Arg-1/NOS2 pathways and macrophage activation profiles at the FMI.

Chapter V. **CONCLUSION**

Toxoplasmosis is a disease with high incidence in men and some animals. In immunosuppressed individuals, the development and dissemination of the parasite, occurs leading to clinical manifestations. In healthy individuals, the parasite can be in the organism for long periods of time and do not offer any kind of danger to the host, unless a breakdown in the normal functioning of the immune system occurs. *T. gondii* may also cause danger if it is acquired during pregnancy, passing through the decidua to the fetus resulting in congenital toxoplasmosis and can cause abortion or fetal malformations.

Despite the immune response to toxoplasmosis being well understood nowadays, few is known about the immune response to this parasite during pregnancy, at FMI and systemic levels.

As expected, in this study infection with *T. gondii* affects negatively pregnancy, causing a delay in the development of the UF in the B6 strain, but not in Balb\c strain. Further experiments using Balb\c mice will be needed to confirm these results. Interestingly, the results for the parasite load quantification suggest that pregnancy has a protective effect against *T. gondii* infection, since there is a decrease in parasite load of infected Balb\c mice.

Arg-1 and NOS2 gene showed to be reduced upon *T. gondii* infection at the FMI. However, concerning the evaluation of the Arg-1 protein expression, was observed an increase during infection, indicating that although the gene is being less expressed, the transcript of the gene is being translated multiple times, and this is probably because the protein is being required in greater quantities during pregnancy and infection.

Immunohistochemical analyses confirmed that infection induces an increase in Arg-1 protein expression and a decrease in NOS2 protein expression at the decidua in B6 and Balb\c mice. These results may indicate the existence of M1 and M2 polarized macrophages, proving that both activations occur in macrophages in response to infection with *T. gondii* at the FMI. They also, suggest an increase of M2 activated macrophages, indicating an alteration of macrophage polarization, in order to maintain pregnancy, but also promoting parasite replication and therefore congenital toxoplasmosis.

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