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FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

MESTRADO INTEGRADO EM BIOENGENHARIA
BIOTECNOLOGIA MOLECULAR
2015

Host response to *Neospora caninum* in naive and immunized mice

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Thesis developed in:

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Approved in public defence

Date
October 14, 2015

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Acknowledgments

In the course of this work, several persons helped realized this thesis, allowing me to learn not only new methods, but also how to develop an investigative work.

Firstly, I must thank Professor Manuel Vilanova for giving me the opportunity to work within the Laboratório de Imunologia Mário Arala Chaves, and giving very valued advice in how to develop the thesis.

I must also thank Dr. Luzia Teixeira for allowing me to work with her. The guidance provided by Luzia during this work provided me a great knowledge of the laboratory work, greatly improving my skills development and production of this type of work.

I also express gratitude to Pedro Ferreirinha, being also a crucial help in the course of this work, not only with the practical work, but also giving valuable advice. Also, I am grateful to the entire Laboratório de Imunologia Mário Arala Chaves for allowing me to develop the work there, always being so helpful. Thank you Alexandra for all the help provided as well, and thank you Encarnação for the friendliness and helpfulness demonstrated.

I must thank Prof. Artur Águas, for allowing me to develop this project in the Departamento de Anatomia. Thank you also to the whole department for being so welcoming and helpful. The help and sympathy provided daily by Madalena, Ana, Ângela, Sofia, Tiago, Raquel and Gil was very important during this thesis. I also want to thank Sr. Duarte, Sr. Costa and D. Manuela for all the help and sympathy while I was present in the department.

Finally I have to thank my family and friends for supporting me in this journey. Without them, this wouldn´t have been possible.

For all of this, thank you.

This work was supported by:

FCT/MCTES (PIDDAC) and co-funded by FEDER through COMPETE, PTDC/CVT/115126/2009 and FCOMP-01-0124-FEDER-014679.

FEDER through COMPETE and by national funds through FCT - FCOMP-01-0124-FEDER-020158 (PTDC/CTV/122777/2010).

Abstract

Neospora caninum is an apicomplexan protozoan responsible for the development of neosporosis. Abortions in cattle remain the most common clinical sign of this disease. Therefore, industries dependent on cattle such as the dairy and beef industries are negatively impacted by this infection, with losses exceeding one billion dollars per annum.

Immunization remains the most promising protective strategy against infection with *N. caninum*. Consequently, the development of an efficient and safe vaccine that could prevent this infection is crucial. Previously, a protective effect against neosporosis in a murine model was found, through intranasal (i.n.) immunization with *N. caninum* membrane proteins. Although parasite-specific antibodies raised by the immunization showed effector function *in vitro*, the specific mechanism through which the *in vivo* protection was achieved is still to be determined.

Therefore, in order to assess whether protection could still be achieved in animals lacking an intact cell-mediated response, interleukin-12 (IL-12)/IL-23 p40-deficient ($p40^{-/-}$) C57BL/6 mice were immunized i.n. with *N. caninum* membrane proteins, using CpG as adjuvant, and challenged intraperitoneally (i.p.) with *N. caninum* tachyzoites. In these animals a protective effect of the i.n. immunization was observed. As previous reported for immunosufficient animals, elevated parasite-specific IgA and IgG serum antibody titres were observed in the immunized mice. However, and interestingly, IFN-γ and IL-4 production was not found altered in the immunized animals, as compared to similarly challenged sham-immunized controls. These results further suggested that antibodies could mediate protection in the immunized mice.

The long term effect of the used immunization in the immune response to *N. caninum* was also assessed in wild type (WT) C57BL/6 mice, inoculated intragastrically (i.g.) with *N. caninum* tachyzoites 18 weeks upon boosting immunization. In the immunized animals, elevated levels of both IgA and IgG parasite-specific antibodies were found as compared to sham-immunized or PBS treated controls. However, IFN-γ and IL-4 production did not present differences among immunized or non-immunized mouse groups. These results indicated that the assessed i.n. immunization induced a sustained humoral immune response.

The adipose tissue has been increasingly recognized as an organ involved in immunological processes. However, only a few studies have addressed the impact of infection in the adipose tissue immune cell composition and function. As it was previously demonstrated that *N. caninum* is also able to infect the adipose tissue, these studies were furthered here in order to understand how the immune response is

developed in the adipose tissue of hosts infected with this parasite. WT C57BL/6 mice were inoculated i.p. with *N. caninum* tachyzoites and the immune response in the adipose tissue of the infected mice was assessed 24 hours, and 1 year after challenge, as well as the parasitic load. Parasitic DNA was found in both liver and lungs of all mice euthanized 24 hours after infection, and at low levels in the brain of some animals 1 year after infection. At 24 hours after infection, IFN-γ production by immune cells of both mesenteric and subcutaneous adipose tissue was significantly higher than that of immune cells of control, non-infected mice. At 1 year after infection, the same was observed when the cells were stimulated with killed *N. caninum* tachyzoites. These results showed that an immune response against *N. caninum* is mounted within the adipose tissue of infected mice that was able to induce local memory.

As an endocrine organ, the adipose tissue is able to produce several adipokines such as leptin. This adipokine presents various functions, ranging from osteogenesis to hematopoiesis, being also able to influence the immune response. In a previous study, leptin levels in the serum were shown to be sustainedly elevated in mice infected with *N. caninum*. Therefore, a more detailed analysis of leptin production in infected mice was performed in this work, in order to determine the cell source of this hormone. WT C57BL/6 mice were inoculated i.p. with *N. caninum* tachyzoites and two months after challenge leptin was detected in several organs and adipose tissue by using immunohistochemistry. Moreover, leptin mRNA levels in those tissues were assessed by quantitative real-time PCR (qPCR). No significant differences were found between non-infected and infected mice in leptin production/presence in different analyzed tissue samples, either by immunostaining or qPCR. Therefore, the cell source of the elevated leptin serum levels of infected mice was not yet identified.

Finally, in order to confirm whether serum leptin levels remained elevated in chronically infected mice, these were measured in mice infected for 1 year with *N. caninum*. The analysis of serum leptin levels demonstrated that the leptin levels were still higher in infected mice than in non-infected controls. These results further highlight the long-term impact of infection in the physiology of adipose tissue.

Keywords: *Neospora caninum*, mucosal immunity, immunization, adjuvant, CpG, IFN-γ, adipose tissue, leptin

Resumo

Neospora caninum é um protozoário da família apicomplexa responsável pelo desenvolvimento de neosporose. O sinal clínico mais comum desta doença continua a ser abortos em bovinos. Portanto, as indústrias dependentes de gado, tais como a indústria leiteira e de produção de carne são impactadas negativamente por esta infecção, com perdas superiores a um bilhão de dólares por ano.

A imunização continua a ser a estratégia mais promissora de protecção contra a infecção com *N. caninum*. Por conseguinte, o desenvolvimento de uma vacina eficaz e segura que poderia evitar esta infecção é crucial. Anteriormente, um efeito protector contra a neosporose num modelo murino foi encontrado, através da imunização intranasal (i.n.) com proteínas de membrana de *N. caninum*. Embora os anticorpos específicos para o parasita, aumentados pela imunização, terem mostrado uma função efectora *in vitro*, o mecanismo específico através do qual a protecção foi obtida *in vivo* ainda está para ser determinada.

Por conseguinte, a fim de avaliar se a protecção poderia ainda ser obtida em animais com falta de uma resposta mediada por células intacta, ratinhos da estirpe C57BL/6 deficientes na expressão da subunidade p40 da interleucinas 12 e 23 ($p40^{-/-}$) foram imunizados pela via i.n. com proteínas de membrana de *N. caninum*, utilizando CpG como adjuvante, e infetados por via intraperitoneal (i.p.) com taquizoítos de *N. caninum*. Nestes animais foi observado um efeito protetor da imunização i.n.. Como relatado anteriormente para animais imunocompetentes, foram observados títulos elevados de anticorpos IgA e IgG específicos para o parasita no soro dos ratinhos imunizados. No entanto, e curiosamente, não houve alteração na produção de IFN-γ e IL-4 nos animais imunizados, em comparação com os controlos falsamente imunizados e igualmente infetados. Estes resultados sugerem assim que os anticorpos podem mediar protecção nos ratinhos imunizados.

O efeito a longo prazo da imunização utilizada, na resposta imunológica a *N. caninum* foi também avaliado em ratinhos *wild type* (WT) C57BL/6 inoculados, por via intragástrica (i.g.), com taquizoítos de *N. caninum* dezoito semanas após a imunização. Nos animais imunizados, níveis elevados de anticorpos IgA e IgG específicos para o parasita foram encontrados, em comparação com os controlos administrados com PBS ou falsamente imunizados. No entanto, os valores de IFN-γ e IL-4 não apresentaram diferenças entre os ratinhos imunizados e não imunizados. Estes resultados indicaram que a imunização i.n. sob estudo induziu uma resposta imune humoral sustentada.

O tecido adiposo tem sido cada vez mais reconhecido como um órgão envolvido em processos imunológicos. Apesar disto, apenas alguns estudos têm abordado o impacto da infecção na composição celular imunológica e função do tecido adiposo. Como foi demonstrado anteriormente que *N. caninum* é também capaz de infectar o tecido adiposo, estes estudos foram aqui desenvolvidos, a fim de compreender como a resposta imunitária é desenvolvida no tecido adiposo de hospedeiros infectados com este parasita. Ratinhos WT C57BL/6 foram inoculados por via i.p. com taquizoítos de *N. caninum* e a resposta imunitária no tecido adiposo de ratinhos infectados foi avaliada 24 horas, e 1 ano após inoculação, bem como a carga parasitária. Detectou-se DNA do parasita no fígado e pulmões em todos os ratinhos sacrificados 24 horas após a infecção, e em baixos níveis no cérebro de alguns animais 1 ano após a infecção. Após uma infecção de 24 horas, a produção de IFN-γ por células do sistema imunológico tanto do tecido adiposo mesentérico como subcutâneo foi significativamente mais elevada do que a de células do sistema imunológico de ratinhos controlo, não infectados. 1 ano após a infecção, o mesmo foi observado quando as células foram estimuladas com taquizoítos mortos de *N. caninum*. Estes resultados mostraram que uma resposta imunitária contra *N. caninum* é desencadeada do tecido adiposo de ratinhos infectados sendo capaz de induzir memória local.

Como um órgão endócrino, o tecido adiposo é capaz de produzir várias adipocitocinas, tais como a leptina. Esta adipocitocina apresenta várias funções, que vão desde a osteogénese a hematopoiese, sendo também capaz de influenciar a resposta imune. Num estudo anterior, os níveis de leptina no soro mostraram-se elevados de forma sustentável em ratinhos infectados com *N. caninum*. Portanto, uma análise mais detalhada da produção de leptina em ratinhos infectados foi utilizada neste trabalho, a fim de determinar a fonte celular desta hormona. Ratinhos WT C57BL/6 foram inoculados por via i.p. com taquizoítos de *N. caninum* e dois meses após a infecção a leptina foi detectada em vários órgãos e tecido adiposo utilizando imunohistoquímica. Além disso, os níveis de mRNA de leptina nos tecidos foram avaliados em tempo real por PCR quantitativo (qPCR). Não foram encontradas diferenças significativas entre os ratinhos não infectados e infectados na produção/presença de leptina em diferentes amostras de tecidos analisados, quer por marcação imunohistoquímica ou qPCR. Portanto, a fonte celular dos níveis séricos elevados de leptina em ratinhos infectados não foi ainda identificada.

Finalmente, a fim de confirmar se os níveis de leptina no soro permaneceriam elevados em ratinhos cronicamente infectados, estes foram medidos em ratinhos infectados durante 1 ano com *N. caninum*. A análise dos níveis de leptina no soro

demonstrou que os níveis de leptina estavam ainda elevados nos animais infectados comparativamente com os controlos não infectados. Estes resultados realçam ainda mais o impacto a longo prazo da infecção na fisiologia do tecido adiposo.

Palavras-chave: *Neospora caninum*, imunidade de mucosa, imunização, adjuvante, CpG, IFN-γ, tecido adiposo, leptina

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List of abbreviations

AP - Alkaline phosphatase

APC - Antigen-presenting cell

AT - Adipose tissue

BAT - Brown adipose tissue

BSA - Bovine serum albumin

CMI - Cell-mediated immunity

CNS - Central nervous system

CTL - Cytotoxic T lymphocyte

DAB - 3,3'diaminobenzidine

DC - Dendritic cell

DL - Detection limit

EDTA - Ethylenediamine tetraacetic acid

ELISA - Enzyme-Linked Immunosorbent Assay

FACS - Fluorescent-activated cell sorter

FBS - Fetal bovine serum

FITC - Fluorescein isothiocyanate

GAT - Gonadal adipose tissue

GALT - Gut-associated lymphoid tissue

HBSS - Hanks Balanced Salt Solution

HEPES - hydroxyethyl piperazineethanesulfonic acid

HPRT - Hypoxanthine phosphoribosyl-transferase I

IFN-γ - Interferon gamma

Ig - Immunoglobulin IL - Interleukin

IMAT- Intramuscular adipose tissue

i.n. - Intra-nasal

i.g. - Intragastric

i.p. – Intraperitoneal

KO - Knock out

MAT - Mesenteric adipose tissue

MALT - Mucosa-associated lymphoid tissue

MEM - Minimal Essential Medium

MHC - Major histocompatibility complex

MLN - Mesenteric lymph node

N. caninum - *Neospora caninum*

NcMP - *Neospora caninum* membrane proteins

NcT - *Neospora caninum* tachyzoites

NcS - *Neospora caninum* sonicate

NK - Natural killer

OAT - Omental adipose tissue

PBS - Phosphate-buffered saline

PE - Phycoerythrin

PerCP - Peridin-chlorophyll protein

PCR - Polymerase chain reaction

PMA - Phorbol myristate acetate

qPCR - quantitative PCR

RT-PCR - Real-time PCR

SAT - Subcutaneous adipose tissue

SDS - Sodium dodecyl sulphate

sIgA - Secretory IgA

SVF - Stromal vascular fraction

Th -T helper

TGF-β - Transforming growth factor beta

TLR - Toll-like receptor

TNF-α - Tumor necrosis factor alpha

Treg - T regulatory

WAT - White adipose tissue

WT - Wild-type

1. Introduction

Neospora caninum is an apicomplexan protozoan capable of infecting a wide range of animal hosts, including dogs and cattle [1]. The main clinical and economically relevant effect resulting from neosporosis, the disease caused by *N. caninum* is abortion occurring in cattle. Therefore, industries dependent on cattle such as the dairy and beef industries are negatively impacted by this infection. The annual global loss due to *N. caninum* associated abortions was estimated around 2,380 million USD, with 1,739 million USD corresponding to the dairy industries and 641 million USD being associated with the beef industries [2].

Immunization has been demonstrated to be a promising and cost-effective control method against neosporosis [3]. Therefore, a more complete understanding of the host immune response to *N. caninum* is necessary to more adequately design studies on immunization, which could lead to the development of an effective and safe vaccine allowing the control of neosporosis, currently not available.

The immune response elicited in *N caninum* infected hosts may occur in several tissues that the parasite can reach and even persist in, such as neural and muscular tissues (brain, skeletal muscle) [4]. Recently, it was demonstrated that this parasite might also colonize the adipose tissue. While previously considered only a depot for lipids, the adipose tissue has been increasingly recognized as also affecting the immune response [5]. Despite this acknowledged role, only a few studies have detailed the impact of infection in the adipose tissue immune cell composition and function.

1.1 *Neospora caninum*

N. caninum, is an obligatory intracellular parasite, of the genus *Neospora* belonging to the Phylum *Apicomplexa*, family *Sarcocystidae*. It was first described in a case of encephalomyelitis in dogs [6], with its adverse effects on cattle being described afterward [7]. Other described animal hosts having presented clinical signs of neosporosis include sheep, deer, horses, water buffaloes or bison [4]. Presently, no reported evidence exists of successful infection by *N. caninum* in humans [1].

1.1.1 Life cycle

In Figure 1, the *N. caninum* life cycle is represented. During its life cycle *N. caninum* may present different parasitic stages. Depending on the hosts in which it is

present and the tissues in which the parasite is found, the three main infectious stages of the parasite are tachyzoites, tissue cysts, and oocysts [1].

In intermediate hosts, such as sheep, horses or cattle, tachyzoites and tissue cysts are both found within host cells. Tachyzoites are 6 × 2 μm in size, while tissue cysts are usually found with a round or oval shape, with a length of up to 107 μm. Cysts are encountered primarily in the central nervous system (CNS). Inside the tissue cysts bradyzoites can be found enclosed within the tissues' walls, which present a thickness of 4 μm. They are sized 7-8 × 2 μm [1]. Finally, unsporulated oocytes present a size around 11.7 × 11.3 μm [1].

Unsporulated oocysts can be found in definitive hosts of *N. caninum*. Dogs, Australian dingoes, coyotes, and wolves have been demonstrated to be definitive hosts of this protozoan [1, 8]. After the uptake of tissue cysts by a definitive host, the parasite forms unsporulated oocysts. These may be shed to the environment, where they can sporulate and be ingested by the intermediate hosts through contaminated sources (food, water or soil, for example) [1]. Sporozoites, when entering the intestinal tract, will penetrate the cells and develop into tachyzoites. These are able to divide and spread to other cells at a very fast pace, invading and possibly destroying them. When neural cells are invaded by tachyzoites, these are able to differentiate into bradyzoites. This process may occur in case a strong immune response against the parasitic infection is developed [1]. This stage of the microorganism will then develop into tissue cysts as a form of parasite protection. The tissue cysts may then be ingested by a definitive host such as the dog, and complete the cycle.

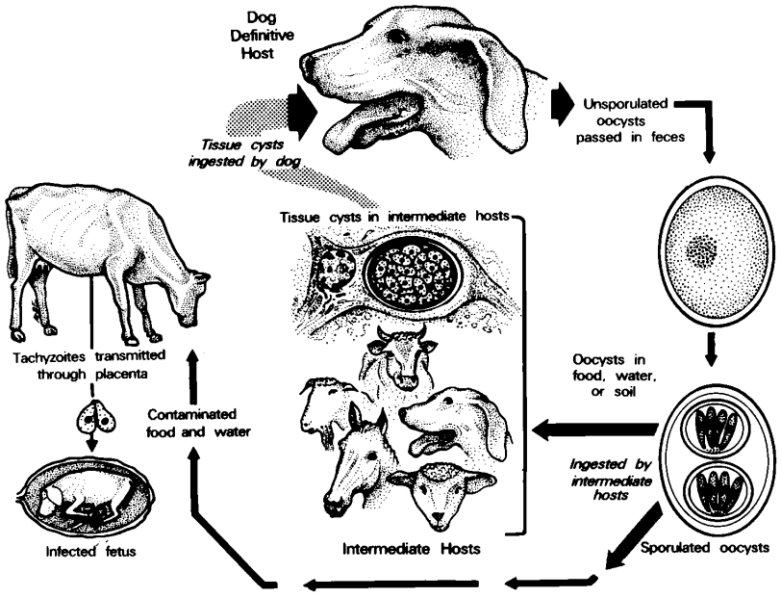


Figure 1 *N. caninum* life cycle [1].

1.1.2 Transmission

In dogs, the more frequent mode of *N. caninum* transmission likely occurs by carnivorism. However, studies have demonstrated that the parasite can be transferred transplancetally, from the dam to the neonates during terminal stages of gestation or post-natally via milk [4].

In cattle, transmission is possible both through the ingestion of *N. caninum,* and transport through the placenta (vertical mode of transmission). Cows are able to transmit the infection into their offspring (Figure 1) [4], with transplacental transmission occurring in an exogenous manner, with a primary, oocyst-derived, infection of a pregnant dam, or in an endogenous manner, with the reactivation of infection in a chronically infected dam, with the rate of transmission varying in these two scenarios [9]. Cow-to-cow transfers have not been described for this parasite [10].

1.2 Neosporosis

1.2.1 Clinical signs

In dogs, the main clinical signs due to *N. caninum* infection can be found in congenitally infected pups, displaying hind limb paresis that can progress into full paralysis. Neurologic signs are dependent on the site of infection, with the hind limbs being more severely affected than the front limbs, often presenting a rigid hyperextention [1].

Other dysfunctions include difficulty in swallowing, paralysis of the jaw, muscle flaccidity, muscle atrophy and heart failure, demonstrating the great adverse effects of the infection on the dogs' nervous system [1]. Generally, the disease may be localized or generalized, with virtually all organs involved. Fatal neosporosis has been reported in eight to 15-year-old dogs [1].

In cattle, the principal effect encountered in infected animals is abortion, occurring in cows of any age from 3 month gestation to term, however most abortions occurring in five to six month gestation [4].

Fetuses infected with *N. caninum* may present several manifestations of the disease, either dying *in utero*, being resorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or born clinically normal but persistently infected [4]. Other clinical signs found in cattle include neurologic signs, an inability to rise and below average birth weight [4]. In terms of possible neurological effects, besides occasionally developing hydrocephalus or narrowing of the spinal cord, the hind limbs or forelimbs

or both may be flexed or hyperextended. Other effects include ataxia, decreased patellar reflexes, and loss of conscious proprioception. Exophthalmia or asymmetrical appearance in the eyes has also been described in calves [4].

1.2.2 Diagnosis

Current methods for the diagnosis of neosporosis have demonstrated to be both difficult and expensive [4]. Serologic examination of the dam and the fetus, and the detection of lesions and *N. caninum* in a fetus by immunohistology and PCR have been utilized to diagnose this disease [11]. Despite this, the fact that asymptomatic congenital *N. caninum* infections are common, as well as the presence of the parasite not signifying that the abortion was caused by it, can lead to potential misdiagnosis [4].

Other tests may determine the occurrence of different features of the disease. Avidity tests have been used for the differentiation between acute and chronic infection. Low avidity values are related with acute infection, but the window for analysis of acute infection is short, lasting lasts several week post infection [4]. ELISA tests based on recombinant proteins specific for each stage of the parasite during infection have also been utilized [4].

1.2.3 Control

N. caninum infection control methods include embryo transfer, artificial insemination of seropositive dams with semen from beef bulls, culling, replacement heifers, chemotherapy, and vaccination. Annual serological screenings may also help control the potential infection by *N. caninum* [4].

Current chemotherapy treatments have not demonstrated a safe and effective alternative as a control measure. Chemotherapy should also not affect the cattle production, in terms of quality of milk or beef produced. Despite this, studies have been developed acting on a later stage of infection [4].

1.3 Immune response to *Neospora caninum*

1.3.1 Host immune response

Cattle

As mentioned before, the parasite may be transmitted to cattle either through ingestion of oocysts in contaminated food or water or it may be passed vertically from mother to fetus across the placenta [11]. Once within the host body, as *N. caninum* is an obligate intracellular pathogen, cell-mediated immune (CMI) responses are essential in defending the host. Namely interferon (IFN)-γ production, as well as antibody responses have been identified as protective to the host [11].

In non-pregnant animals, infection with *N. caninum* does not usually cause clinical disease. Various types of cells are involved in this response. Natural Killer (NK) cells have been demonstrated to help the immune response through the production of IFN-γ [12]. As a first line of defense, these cells promote an environment that can lead the immune system to mount a T helper 1 (Th1)-type response. $CD4^+$ T cells are also able to act on naturally infected cattle, limiting multiplication of intracellular parasites through the production of IFN-γ [13]. However, in that host, the parasite can still persist as bradyzoites within tissue cysts in the CNS [14].

In pregnant cattle, the infection can lead to more hazardous consequences, due to an exacerbated immune response. The fetus is presented to the immune system as a semi-allogeneic graft [11]. The Th1-type immune response that demonstrates a protective capacity against *N. caninum* and other intracellular pathogens has been proven to be detrimental to pregnancy [15]. Other pro-inflammatory cytokines such as Interleukin (IL)-2, IL-12 and IFN-γ can have an adverse effect on the fetus, potentially damaging or causing fetal rejection or abortion [14]. Progesterone, a hormone whose levels are increased from early to mid-gestation in pregnant cattle, has been shown to induce a T helper 2 (Th2)-type response bias [15]. Th2 cells are associated with the downregulation of IFN-γ-mediated immune responses leading to a possible persistence of the infection.

An important factor in the immune response of pregnant cattle is antibody production. Serum antibody titres specific for *N. caninum* in pregnant cattle fluctuate throughout gestation, with different values representing different numbers of parasites present in the organism. The timing of an increase in such titres during pregnancy is useful in predicting disease outcome [11]. An increase in circulating antibodies around mid-gestation is more likely to be linked to abortion than a rising antibody titre towards the end of gestation [16].

Mice

Several studies have been developed using the murine model. Advantages such as its small size and quick reproduction allow studies with a high number of

animals, with significant results [17]. Due to these advantages, the immune response presented by this animal model is also of importance to understand how a *N. caninum* infection is developed.

Different strains of mice may present different immune responses to the same stimulus. Outbred mice are more resistant to *N. caninum* infection, with a Th1 type immune response being encountered. This response is characterized by high IFN-γ and low IL-4 production [18], and IgG2a antibody production [19]. If infected during pregnancy, their immune response then switches to a Th2 type with lower IFN-γ and higher IL-4 expression, similar to the response found in cattle [20]. On the other hand, with inbred strains the response can vary, due to their specific relative haplotype [21].

Protection against *N. caninum* infection in mice is mainly attributed to a Th1 type immune response characterized by production of IFN-γ and IL-12 [22]. Cytokines such as TNF-α and TGF-β have also been demonstrated to be involved in the control of parasite proliferation [23]. Macrophage activation by IFN-γ is also crucial for the immune response against *N. caninum*. Activated macrophages may kill the parasite, in association with an increase in the levels of nitric oxide [24]. Macrophages are also antigen-presenting cells (APC), by displaying pathogen-derived antigens in association with major histocompatibility complex (MHC) molecules, being thus able to activate T lymphocytes [24]. Dendritic cells are also powerful APC capable of activating T cells [25].

While the cell-mediated immunity is crucial for protection of the host, the humoral immune response can help resolve the infection. It was previously found that in infected hosts a B-cell stimulatory effect is induced with increased numbers of total and immunoglobulin-secreting B cells, as well as serum levels of *N. caninum*-specific immunoglobulins, largely of the immunoglobulin G2a (IgG2a) and IgM isotypes [26].

1.4 Mucosal Immunity

Immune cells present in mucosal tissues are usually transported and accumulated in mucosa-associated lymphoid tissues (MALT). The main function of the mucosal immune system includes the protection of membranes against colonization and invasion of dangerous organisms, and prevention of the uptake of either dangerous microbes or undegraded antigens which include foreign proteins [27]. It can also prevent the overexertion of the immune response against foreign matter, possibly harmful to the host organism.

The MALT is composed of compartments such as Peyer's patches, mesenteric lymph nodes, appendix, isolated follicles in the intestine, the tonsils and adenoids [27].

The MALT also presents a high amount of lymphoid cells in the parenchyma of mucosal organs and exocrine glands, forming the mucosal effector sites where the immune response is developed. These tissues contain B cells, T cells and other cell subpopulations [27].

Antigens taken up by epithelial cells in mucosal inductive sites can be transported to or captured by APC (including dendritic cells, macrophages, and B cells) and then presented to both $CD4^+$ and $CD8^+$ T cells, also present in the mucosal inductive site [27]. Immune responses are affected by the nature of antigen, the type of APC presenting the antigen, and the local microenvironment [27]. Nonpathogenic antigens usually lead to Th2-type response, as well as a response by regulatory T cells, which can lead to a suppression of systemic immunity [28]. On the other hand, pathogen antigens and adjuvants, which can present motifs sensed by APCs as being dangerous to the host (Toll-like receptor (TLR) ligands, for example) can lead to a stronger immune response, with both the humoral and cell mediated immune response being involved in the immune reaction [28].

B and T cells from the mucosal inductive sites, after the encounter with the antigen can be moved through the lymph, settling in different mucosal sites, where they are able to differentiate into memory or effector cells [27]. Finally, chemokines present in the local micro-environment also affect the immune response through the regulation of cell chemotaxis [29]. Studies have indicated that mucosal DCs are able to influence T cells homing properties, controlling its migration [27]. Herewith, a common mucosal immune system can be envisioned, with immunocytes activated at one site disseminating to remote mucosal tissues rather than to systemic sites, extending the immune response in terms of range. Also, since chemokines, integrins and cytokines are differentially expressed among mucosal tissues, this might lead to the compartmentalization observed with specific mucosal inductive sites linking with particular effector sites [27].

1.4.1 Protection mechanisms

Several pathways can be considered when planning an immunization strategy that could more suitably lead to the activation of the desired immune response. Among the various body tissues available, the mucous membranes covering the aerodigestive and urogenital tracts, as well as other areas such as the eye conjunctiva, inner ear and exocrine glands, can be an effective route of immunization [27]. Since these tissues present several mechanisms that allow the protection of the host against potential pathogens and other foreign matter, including developed innate and adaptive immune

systems, an improvement in its response against these insults through an immunization process might effectively protect the organism [27].

The adaptive humoral immune defense at mucosal surfaces is essentially mediated by secretory IgA (SIgA) antibodies [27]. The resistance of SIgA to proteases makes these antibodies advantageous for functioning in mucosal secretions, leading to its effectiveness. The induction of IgA against mucosal pathogens and soluble protein antigens is majorly dependent on T helper cells [30]. Despite this, locally produced IgM and IgG, and in the lower respiratory tract and in the genitourinary mucosa, serum-derived IgG can also contribute significantly to immune defense [27].

Mucosal cytotoxic T lymphocyte (CTL) responses have been described in several types of immunizations, having been demonstrated to help the immune clearance of pathogens in several animal models of infection with enteric or respiratory viruses and intracellular parasites [27]. Also, IFN-y producing $CD4^+$ T cells have been found to be important for mucosal immune defense to both viral and bacterial infections [27].

While the mucosal tissues possess several immune mechanisms that allow the protection against pathogens hazardous to the host, the mucosal immune system demonstrates also a capacity to tolerate self-antigens and other environmental antigens present in the microbiota, in food and airborne. These different mechanisms include activation-induced cell death, anergy, and most important, the induction of regulatory T cells [31].

1.5 Vaccination strategies

1.5.1 Mucosal vaccination

Mucosal surfaces seem to be the more accessible anatomical location for the induction of an immune response. Also of importance, various studies have demonstrated that local mucosal immune responses are important for protection against disease, principally for diseases starting on mucosal surfaces such as the respiratory, gastrointestinal or urogenital mucosae. Mucosal immune responses are most efficiently induced by the administration of vaccines within the mucosal surface than with injected vaccines, which usually perform poorly in terms of mucosal immunity [32].

Various challenges affect the efficiency of mucosal vaccination. Vaccines administered mucosally encounter mucosal barriers much like pathogens and other foreign macromolecules, being also prone to attack by proteases and nucleases and

stopped by epithelial barriers. These obstacles would lead for a need for large doses of antigen [32]. In addition to this, soluble non-adherent antigens are rarely taken up, inducing immune tolerance in the intestine [32]. As such, particulate delivery systems that adhere to mucosal surfaces or are able to selectively target mucosal cells are likely to be the most effective [32]. Also important for the effectiveness of a mucosal vaccine is the use of an adjuvant that will effectively stimulate the immune response in a manner that will help protect the host [32].

1.5.2 Vaccination against neosporosis

An effective immunization strategy against *N. caninum*, should consider several targets such as prevention of tachyzoite proliferation and dissemination in pregnant cattle to avoid transplacental transmission to the fetus, avoidance or reduction of oocyst shedding in dogs or other definitive hosts, and prevention of tissue cyst formation in animals that have been infected with oocysts or tissue cysts [33]. A vaccine targeting mechanisms that would stimulate both protective cellular immune responses and antibody responses at mucosal sites and even the whole organism could be crucial to prevent the infection [34]. Considering specifically neosporosis, several vaccines have been under evaluation, such as live vaccines which include less virulent isolates of *N. caninum*, attenuated strains generated by irradiation or chemical means, or genetically modified transgenic strains [33]. Live *N. caninum* vaccines were shown to be effective in cattle [33, 35] and mice [36, 37], however problems involving safety, costs of production, and stability of the final product still exist.

Other alternative, subunit vaccines, presents several advantages such as reduced costs in production, processing and storage, increased stability and shelf life [33]. The parasite antigens related to adhesion and invasion of host cell represent interesting targets for subunit vaccines. They have been applied as bacterially expressed recombinant antigens or as DNA vaccines [33]. In addition to monovalent vaccines, also polyvalent combinations of different antigens have been used, increasing protection. Vaccines have been combined with immune-stimulating carriers, and chimeric vaccines incorporating immune-relevant domains of several antigens to form a single protein are under development [33]. DNA vaccines against *N. caninum* in animal models have not been as successful as vaccines based on antigen delivery [33].

Cattle

A vaccine against bovine neosporosis (Bovilis Neoguard™) had been available in selected countries, being sold commercially [33]. It was based on killed tachyzoite lysates, demonstrating moderate protection in field trials [38]. More recent trials revealed however vast differences in efficacy at the farm level, suggesting that vaccination itself may increase the risk of early embryonic death [39]. This then lead to the withdrawal of the vaccine from the market. A live-vaccine using low virulence strains such as Nc-Nowra is now being considered a better alternative [34]. This type of vaccine has demonstrated to be able to reduce the vertical transmission after challenge with a virulent isolate, with great safety [40]. A reduction of pregnancy rate after artificial insemination was observed in some vaccinated groups compared to non-vaccinated controls, representing a possible disadvantage.

Mice

A vaccine mixture of inactivated tachyzoites and bradyzoites from *in vitro* cultures has been developed and assessed in mice [41]. When compared to an inactivated whole tachyzoite vaccine in a pregnant mouse model, while immunization with tachyzoite extracts reduced vertical transmission and cerebral infection in the dams, the tachyzoite–bradyzoite mixture exacerbated congenital and cerebral neosporosis, leading to problems associated with the infection. A strong Th1-type immune response was observed after vaccination and an increased IL-4 expression, as well as high levels of antibodies directed to bradyzoite antigens were observed after challenge infection. Another study showed increases in immunogenicity and protection against acute infection once killed tachyzoite lysates were used together with ArtinM, a D-mannose-specific lectin from *Artocarpus integrifolia* seeds that is capable of inducing several inflammatory processes, such as neutrophil migration and activation, degranulation of mast cells as well as induction of IL-12 production by macrophages and dendritic cells [42].

In terms of live vaccines, a vaccine that utilizes transgenic *N. caninum* tachyzoites expressing NcSAG4, a bradyzoite antigen, was produced, presenting low persistence in mice [43]. Also, immunization with a low virulent strain (Nc-Spain 1H), inducing mainly a Th1-type immune response, conferred protection against cerebral infection, with a reduction of congenital infection reaching up to 86% with low immunization dose [37].

One other interesting type of vaccines also under investigation is subunit vaccines. Immunization studies were carried out in mice, with the i.n. administration of *N. caninum* membrane antigens introduced with a CpG adjuvant. The results demonstrated a protective effect of the mucosal immunization against i.g. established neosporosis [3]. Another study presented a vaccine based on i.n. vaccination of non-pregnant mice with protein disulfide isomerase. While the vaccine previously demonstrated a protective Th2-type immune response, with survivors displaying decreased cerebral parasite burden [44], in newer studies, this vaccine in a pregnant mouse model induced a Th2-type immune response, not demonstrating the same results [45]. While in non-pregnant mice the vaccines conferred a mixed Th1/Th2 response that helped protect against infection, in pregnant mice a Th2-type response was encountered, reducing the protection.

1.5.3 Factors affecting vaccination

Adjuvants and carriers are usually crucial for the success of a vaccine, improving the vaccines' effectiveness by boosting the immune response. For example, immunization of mice against neosporosis with recombinant NcSRS2-ISCOM formulations, capable of stimulating the immune system, demonstrated an induction of specific antibody responses to native NcSRS2, a *N. caninum* surface protein, and a significant reduction of cerebral parasite load in immunized mice [46].

Concerning adjuvants specifically, their choice is of great importance in order to induce the required effects, since different adjuvants can stimulate the immune system through different ways. For example, utilization of saponin adjuvant in the i.p. vaccination of mice with recNcPDI led to a strong humoral immune response, although not protective against *N. caninum* infection [44]. Nevertheless, a protective effect was found when the same antigen was suspended with cholera toxin adjuvant, with an i.n. administration [45]. New adjuvants were tested in combination with *N. caninum* tachyzoite extract and ArtinM, demonstrating protective Th1-type immune stimulation in mice [42].

The route through which the vaccine is delivered is also of importance towards vaccination efficacy. One study demonstrated that three different recombinant proteins conferred different levels of protection when injected i.p. or i.n., with the protective effect of vaccination depending largely on the route of antigen delivery, but also on the type of antigen utilized [44]. This shows that a different way of administration is important, but it is not the only factor affecting the vaccines efficacy.

The route of infection is another factor that may affect a vaccine's success. Experimental studies present a wider choice of infection, leading to more variable results when treating the infection [33]. However, different studies might use different routes hindering the ability to compare the obtained results [33]. Oral administration of oocysts mimics the conditions of a natural infection, but the number of oocysts needed to perform these studies is usually too difficult to obtain [33]. Despite this, other ways of inoculation are available. Cattle have been infected with tachyzoites by a combined intravenous/intramuscular inoculation later replaced by intravenous injection alone or subcutaneous inoculation [33]. Results have nonetheless demonstrated that intravenous injection provokes a more severe reaction than subcutaneous inoculation [47]. Another alternative is the use of naturally infected animals for vaccination studies [39], limited by the costs of such studies, as well as other obstacles, that prevent the use of experimentally infected cattle prior to clinical studies [33]. In mice, oral ingestion of oocyst has functioned, as well as the administration of tachyzoites orally, i.g., i.p., or subcutaneously [33]. The infection route may interfere with the induced immune response, having a certain effect on the efficacy of the vaccine.

The host, specifically its genetic background or immune status, and also the genetic background of the strain of parasite used for infection can alter the efficacy of a vaccine. No clear evidence that some breeds are more susceptible than others has been found, with some wild species of cattle demonstrating a natural capacity to be a host for *N. caninum* [48]. Differences in the abortion rate and immune response were nevertheless found [49]. Concerning the parasite, different strains present significant differences in the pathogenicity and virulence, with several studies utilizing genetic and protein analysis to demonstrate a difference between the expression patterns of the different strains [33]. There have been found both similarities and differences between the infection patterns of these strains, leading to different immune responses [50]. Consequently, different strains may lead to a different outcome of infection.

1.6 Adipose tissue

When considering the adipose tissue as a whole, two main types of adipose tissue are known: white adipose tissue (WAT) and brown adipose tissue (BAT). While WAT acts as storage for excess energy as triglycerides, BAT has been demonstrated to act through the dissipation of energy by producing of heat [51]. BAT can be easily found in small mammals and newborns, helping them survive cold temperatures. Despite this, it has been shown that adults also have metabolically active BAT, possibly being important in energy homeostasis [51].

Concerning morphology and physiology of the adipose tissue, white adipocytes are presented as spherical cells. A single lipid droplet, comprised of triglycerides, is deposited within the cell, representing more than 90% of the cell volume (52). Mitochondria in white adipocytes demonstrate a thin and elongated morphology, varying in amount [51].

Brown adipocytes, on the other hand, present small vacuoles containing triglycerides, with a polygonal shape changing in diameter (52). Also contrary to white adipocytes, mitochondria of brown adipocytes are numerous, possessing a large size, with a spherical structure, packed with laminar cristae [51]. BAT exhibit more capillaries than WAT due to its higher oxygen demand, as well as a denser nerve supply [51]. This, accompanied by the high number of mitochondria leads to the brown color of BAT.

Anatomically, WAT can be found throughout the body as visceral WAT (vWAT) and subcutaneous WAT (sWAT) [52]. Visceral adipose tissue is distributed around internal organs (stomach, liver, for example), acting as a padding mechanism [52]. It can be sub-classified into mesenteric, retroperitoneal, perigonadal and omental adipose tissue [52]. Subcutaneous adipose tissue is located under the skin, insulating the body from heat or cold. The main locations of sWAT include the abdominal cavity and underneath the skin. Intramuscular fat that is lodged amongst skeletal muscles is also considered subcutaneous adipose tissue [52].

In terms of the functions of adipose tissue, while previously thought to be only crucial in the regulation of whole-body fatty acid homeostasis, recent developments have uncovered new ways through which this tissue is able to affect life. Nowadays, it is widely accepted that adipose tissue is an active endocrine organ, influencing metabolism in both health and disease, which in itself led to the subsequent discovery of many adipocyte-derived secreted proteins (adipokines) such as adiponectin, resistin, retinol binding protein-4 (RBP4) and IL-6 [53]. These are bioactive peptides, with important endocrine function, being able to affect the endocrine system. Also, adipose tissue presents various receptors that allow the interaction with traditional hormone systems as well as the CNS [53]. This network allows for the adipose tissue to affect various biological processes including energy metabolism, neuroendocrine and immune functions [53].

1.6.1 Immune response in adipose tissue

In the adipose tissue, its expansion is accompanied by chronic low-grade inflammation that primes target organs for the development of obesity-associated

chronic inflammatory diseases [54]. Different immune cells present in the tissue are important to induce, as well as regulate obesity-induced systemic inflammation. These include proinflammatory immune cells along with anti-inflammatory immune cells. Although most types of immune cells are already present in the adipose tissue, their number increases dramatically with the progression of obesity [54].

1.6.2.1 Innate immune response in adipose tissue

Innate immune cells, which include neutrophils, dendritic cells, macrophages, mast cells, and eosinophils, when activated are able to affect not only how the adipose tissue is structured, but also how it functions.

Neutrophils can act through or upon several processes, such as opsonization, agglutination or complement activation, being active in the first parts of the immune response [54]. Activated neutrophils are able to infiltrate adipose tissue early during diet-induced obesity in mice in an attempt to limit the local inflammatory process, demonstrating its capacity in controlling the immune response [55].

Adipose tissue macrophages comprise 40–60 % of all adipose tissue immune cells [54]. Two major macrophage phenotypes have been described: classically activated or M1, which trigger a proinflammatory, type 1 immune response, and alternatively activated or M2, which promote anti-inflammatory, type 2 responses during the healing process [54]. M1 cells are characterized by expression of CD11c, and are activated when stimulated with IFN-γ or LPS [56]. Cells of this subtype are producers of reactive oxygen species and nitric oxide that are hazardous to pathogens internalized by the macrophage. Alternatively, M2 macrophages, characterized by expression of CD206, result from induction by IL-4 and IL-13 [57], and are related to tissue repair.

Eosinophils have also been demonstrated to affect local macrophage activity and polarization in the adipose tissue. Contrary to neutrophils, eosinophils are important mediators of Th2 immunity, producing a vast array of cytokines, like IL-4 or IL10, crucial in anti-inflammatory immune responses, M2 polarization of macrophages, and differentiation of Th2 cells. Alternative (M2) activation of macrophages is driven by IL-4. In absence of eosinophils, M2 macrophage number is greatly decreased, with diminished glucose tolerance and insulin resistance [58]. Herewith, there may be a connection, with eosinophils being able to lead macrophage polarization towards a M2 phenotype.

1.6.2.2 Adaptive immune response in the adipose tissue

The adaptive immune system allows not only an effective response to an immediate threat, but also can help develop an immunological memory [59].

Adaptive immunity can influence the immune response, not only through the stimulation of innate immunity by B and T lymphocytes, but also by suppressing the immune response with regulatory T (Treg) cells presenting a suppressive function [60].

Regarding B cells, B1 type B cells have been demonstrated to respond to pathogen-associated molecules more rapidly than B2 B cells, which are more commonly found [61]. B1 cells have a less-diverse but more polyreactive antibody repertoire than B2 cells [61]. B1 cells confer resistance to some bacterial and viral infections, through antibody production, mainly of the IgM isotype [62]. Despite this, B cells can lead as well to the generation of IgA in the gut mucosa. B cells, specifically B2 cells, are able to produce IgG2c antibodies, important in adipose tissue inflammation and in the immune response [63]. Among its potential effects, due to the recruitment of B cells into adipose tissue preceding M1 polarization of macrophages, IgG2c antibodies are thought to focus macrophage polarization toward a M1 phenotype, enhancing the proinflammatory response in this tissue [64].

In adipose tissue, T cells are the second largest immune cell population after macrophages. They can be divided in different subtypes. $CD4^+$ T cells can be classified into Th1, Th2 and Th17 cells [63]. Th1 cells main secretory product, IFN-γ, promotes M1 polarization of macrophages, demonstrating the significant role of Th1 cells and IFN-γ in the mediation of inflammation [63]. Conversely, Th2 cells present a suppressive role in the development of obesity-related inflammation and immune response, producing the cytokines IL-4 and IL-13 [63]. $CD8^+$ T cells are cytotoxic immune cells involved mainly in the antiviral response, producing cytolitic molecules as well as IFN-γ [63]. $CD8^+$ T cells have also been described to influence the immune response against infectious pathogens, not only responding to infection by intracellular bacteria, but also protozoal pathogens [65]. $CD8^+$ T cell infiltration is followed by the infiltration of macrophages, being able to stimulate M1 macrophage polarization [66]. Therefore, $CD8^+$ T cells may be involved in early phases of macrophage recruitment and M1 polarization.

Th17 cells are involved in autoimmune disorders and infectious diseases, producing Th17-specific cytokines IL-17 and IL-23 [63]. Th17 cells are able to protect the host against extracellular bacterial and fungal infections [67]. As Th17 cells functions are still not completely understood. While the cytokines produced by Th17 cells, IL-17 and IL-23, are supposed to interfere with pathogenic inflammation, IL-17 is

also thought to be involved in the development of atherosclerosis and cardiovascular diseases [68], contributing to chronic inflammation [67].

$CD4^+$ $CD25^+$ $Foxp3^+$ Treg are mainly suppressors of inflammatory response, maintaining self-tolerance and regulating the proinflammatory Th1 and Th17 responses. Thus, they prevent exaggerated immune responses, preserving an anti-inflammatory environment with the release of IL-10, among other anti-inflammatory cytokines [63].

1.6.3 Leptin

Leptin is a 16-kDa protein hormone secreted mainly by the adipose tissue [69]. Other tissues or cells able to produce this hormone include the placenta, mucosa of the gastric fundus, skeletal muscle, and mammary gland epithelial cells [70]. Leptin participates in growth regulation, metabolism, and behavior [69], acting in the hypothalamus and affecting regulation of body weight, food intake, and lipid storage. Besides these functions, leptin has also been demonstrated to affect other processes such as angiogenesis, osteogenesis, haematopoiesis and reproduction [71]. Its secretion has been observed to be increasingly higher in obese individuals when compared with lean subjects, with plasma levels of leptin presenting a linear correlation with adipose tissue mass [69]. One other important process that can be affected by this hormone is insulin sensitivity, since high plasma levels of leptin in obesity have been connected to insulin resistance. Leptin has also been shown to inhibit insulin secretion by pancreatic beta cells [69].

Leptin is also able to modulate the immune response, through the regulation of monocyte and macrophage function [72]. Mice deficient in both leptin and its receptor showed defective cell-mediated immunity and lymphoid atrophy with enhanced susceptibility to infection and injuries [73]. Also, leptin may act as a mediator in regulating the innate immune response, as leptin expression can be induced rapidly by inflammatory stimuli such as LPS, IL-1 and TNF-α during the acute phase of immune response [73]. Regarding the humoral response, leptin, besides affecting it indirectly through the cytokines released during the innate immune response, can also play a direct role in adaptive immunity by modulating T cell-mediated immune responses [73]. This is indicated through the expression of the functional leptin receptor OB-Rb in different T cell subpopulations and B lymphocytes. As mentioned previously, mice deficient in leptin and its receptors have demonstrated immunity impairments, including

T cell impairment, suggesting a possible protective role of this hormone by enhancing T cell survival [73].

Decreases in function of leptin receptors may diminish the inhibitory effect on food intake, reducing loss of energy and leptin deficiency. This, in turn, may result in various diseases, ranging from severe obesity to hypogonadism, with immune deficiency mediated by T-lymphocytes being also possible. These illnesses may be treated with hormonal replacement [69].

2. Aims of the thesis

This thesis will consist in two different experimental parts. The first one concerns the mucosal immunization developed by our group. As a protective effect of i.n. immunization was already demonstrated for WT mice, in this work we will assess whether the protective effect may also be achieved in immunocompromised IL-12/IL-23 p40-deficient ($p40^{-/-}$) mice, which lack an intact cell-mediated immune response. Alongside this, we will also determine whether the immune response elicited by i.n. immunization could be detected in the long term.

Considering the capacity of *N. caninum* to infect the adipose tissue, in a second part of this thesis, we will analyze the production of host protective cytokine IFN-γ in the adipose tissue of infected mice. Also in this study we will investigate whether the immune cells of adipose tissue may present a memory response upon re-exposure to the parasite. Finally, since leptin serum levels have been shown to be elevated in *N. caninum* infected mice, and considering that no differences in leptin expression was found between adipose tissue of infected and non-infected mice, the tissue responsible for the rise in leptin serum levels will be tentatively identified in order to understand its' possible role in the immune response against *N. caninum*,

3. Materials and Methods

3.1 Animals

Female wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories (Barcelona, Spain) and kept at the ICBAS animal facilities (Porto, Portugal) during the experiments. Female interleukin-12 (IL-12)/IL-23 p40-deficient ($p40^{-/-}$) C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, USA) and housed and bred also at ICBAS in individual ventilated cages. Hiding and nesting materials were provided. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of the animals used for scientific purposes, and Portuguese rules (DL 113/2013). Authorization to perform the experiments was issued by competent national board authority, Direção-Geral de Alimentação e Veterinária (0420/000/000/2012).

3.2 Parasites

Neospora caninum tachyzoites (NcT) (Nc-1, ATCC® (50843)) were serially cultured in VERO cells. Cell cultures were maintained at 37º C in Minimum Essential Medium (MEM), containing Earle's salts (Sigma-Aldrich, St Louis, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Biowest, France), 2 mM L-Glutamine, penicillin (200 IU/ml) and streptomycin (200 µg/ml) (all from Sigma-Aldrich) in a humidified atmosphere with 5% CO_3. For parasite isolation infected VERO cells were cultured until the host cell monolayer was 80% destroyed. Culture supernatants and adherent cells were recovered and centrifuged at 1500 g, for 15 minutes, at 4 °C. After discarding the supernatant, the pellet was then passed through a 25g needle, and washed three times with Phosphate Buffered Saline (PBS). Each wash was performed with centrifugation of the parasitic suspension at 1500 g for 15 minutes, at 4 °C. The final pellet was ressuspended in 3 mL of PBS and passed through a Sephadex ™ G-25M-filled PD-10 column (Amersham Biosciences Europe GmbH, Freiburg, Germany). Parasite concentration was determined with a hemocytometer.

3.3 Tachyzoite lysate and cell-membrane proteins preparation

For *N. caninum* tachyzoite membrane protein (NcMP) extraction free tachyzoites were resuspended in PBS with 0,75% Triton X-114 (Sigma-Aldrich), incubated for 10 minutes in ice and centrifuged at 10 000 g for 30 minutes at 4°C. The resulting supernatant was recovered and then placed in a water bath at 30°C for 3 minutes. The procedure was repeated and the resulting supernatant was centrifuged at 1000 g for 3 minutes at room temperature. The aqueous phase was discarded and the NcMP were precipitated with the addition of absolute ethanol, mixed vigorously for 15 seconds and incubated for 60 minutes on ice. The samples were then centrifuged at 12 000 g for 20 minutes at 4 °C and the resulting pellet was dried, resuspended in PBS and stored at -20°C. *N. caninum* sonicates were obtained by disruption of tachyzoites following sonication (26 cycles of 15 seconds at 100 W) with a Branson cell disrupter model W 185 D in an ice bath. The obtained *N. caninum* sonicates (NcS) were sequentially filtered through a 0,2 µm pore-size filter and stored at -20°C. Quantification of NcMP and NcS was performed using the Lowry protein assay.

Protein migration profile of the extracted proteins was confirmed through SDS–PAGE followed by silver nitrate staining. First, a discontinuous SDS–PAGE gel (4–10% acrylamide) was loaded with 10 µg NcMP or NcS samples, which were previously heated at 95 °C for 5 minutes, and electrophoresis was realized with a 25 mA constant current. After electrophoresis, the gel was placed in a fixation solution (50% ethanol, 12% acetic acid and 0,05% formalin) overnight. After removal of the fixation solution, the gel was washed in 20% ethanol, followed by incubation with sensitizing solution (0,02% (w/v) sodium thiosulfate), for 2 minutes with gentle agitation. The gel was then washed twice with deionized water and protein staining was carried out by addition of a cold silver staining solution (0,2% (w/v) silver nitrate, 0,076% formalin) with incubation for 20 minutes with shaking. Subsequently, after removal of the staining solution the gel was rinsed with deionized water twice for removal of excess silver ions. After discarding the water, the gel was rinsed shortly with the developing solution (6% (w/v) sodium carbonate, 0,0004% (w/v) sodium thiosulfate, 0,05% formalin). A new portion of the developing solution was added to gel, and incubated until protein visualization was possible. To stop the reaction, terminating solution (12% acetic acid) was added.

3.4 Immunization protocol and challenge infections

For analysis of long term immunization, WT mice were immunized i.n. at day zero under isoflurane anaesthesia with 20 µl of PBS containing 30 µg NcMP and 10 µg CpG 1826 VacciGrade (Invivogen, San Diego, USA) (NcMP/CpG group, n=4). Sham-immunized mice were treated with PBS alone (PBS group, n=4) or with PBS containing

10 µg CpG 1826 VacciGrade (Invivogen, San Diego, USA) (CpG group, n=4). The immunization procedure was repeated, with the boost being administered 3 weeks after the first immunization. At 18 weeks after the boosting immunization, all mice were challenged i.g. with 5 x 10^7 *N. caninum* tachyzoites, as previously described [74]. Mice were euthanized 19 weeks after the boosting immunization.

Interleukin-12 (IL-12)/IL-23 p40-deficient C57BL/6 mice were similarly immunized i.n. at day zero under isoflurane anaesthesia with 20 µl of PBS with 30 µg NcMP and 10 µg CpG (NcMP/CpG group, n=7). Sham-immunized mice were treated with PBS containing 10 µg CpG (CpG group, n=7). The immunization procedure was repeated, with the boost being administered 3 weeks after the first immunization. At 3 weeks after the boosting immunization, all mice were challenged i.p. with 1 x 10^4 *N. caninum* tachyzoites. The mice were euthanized 4 weeks after the boosting immunization.

For the assessment of IFN-γ production by adipose tissue cells and leptin distribution in different tissues/organs, WT mice were infected i.p. with 1 x 10^7 *N. caninum* tachyzoites or administered with PBS. The animals were euthanized at different time-points after challenge (24 hours, 2 months and 1 year).

3.5 Collection of Biological Samples

In the immunization experiments, spleens were recovered in Hanks' Balanced Salt Solution (HBSS, Sigma-Aldrich) supplemented with 2% FBS, for further analysis. Liver, lungs and brain were also removed and divided in two similar pieces, being either formalin-fixed for histological analysis or stored at –80 ºC for DNA extraction.

Different collections were performed during the experimentation for antibody detection. Regarding the experiment analyzing long term immunization, at 1, 7 and 13 weeks after the boosting immunization, vaginal lavages were performed, for detection of *N. caninum*-specific IgA. Also in these time points, blood was collected from the submandibular vein, for the detection of *N. caninum* specific IgG. Blood was collected from all mice 1 week after infection, from the retro-orbital pathway for detection of *N. caninum*-specific IgG, and intestinal lavages were performed for the detection of *N. caninum*-specific IgA. For the experiment concerning $p40^{-/-}$ mice, both serum collection and lavages were performed in the same manner, only differing in the serum collection and vaginal lavages before infection being performed at 1 week1 after the boosting immunization only. Blood collected was allowed to clot overnight for serum extraction.

For assessment of IFN-γ production by cells resident/recruited to the adipose tissue, mesenteric adipose tissue (MAT) and subcutaneous adipose tissue (SAT), were removed and placed in Hanks' balanced salt solution supplemented with 4% BSA, 10 mM HEPES buffer (Sigma- Aldrich, St Louis, MO) for further analysis. Mesenteric lymph nodes (MLN) were also removed and placed in the same solution. Liver, lung, brain and gonadal adipose tissue (GAT) were also collected, divided in two similar pieces, being either formalin-fixed for histological analysis or stored at –80 ºC for DNA extraction.

In another set of experiments, the liver, kidneys, lungs, brain, stomach, intestine muscle, SAT and MAT were collected and a portion stored in TriReagent™ (Sigma-Aldrich) for RNA extraction or at -80ºC for protein analysis or DNA extraction.

3.7 Cell culture

For spleen cell stimulation, spleens were aseptically removed from the infected animals, homogenized in HBSS (Sigma-Aldrich) supplemented with 2% FBS, with red blood cells being lysed. The samples were centrifuged at 300 g, for 10 minutes. The supernatant was discarded and the pellet was ressuspended in RPMI-1640 complete medium: RPMI-1640 supplemented with 10% FBS (Biowest), HEPES (10 mM), 2 mM L-Glutamine, penicillin (200 IU/ml) and streptomycin (200 µg/ml) (all from Sigma-Aldrich). For cell stimulation, the samples were place in 96-well plates, with final number of cells in each well being 5 x 10^5 cells. Cells were then stimulated with 60 µg/ml of NcS, for 3 days, at 37 ºC, in a humidified atmosphere with 5% CO_2.

For intracellular cytokine detection by flow cytometry, 1 X 10^6 splenic cells per well were cultured and incubated in 5% CO2 at 37 °C for 4 hours and 30 minutes, with stimulation of 20 ng/ml PMA (Sigma-Aldrich), 200 ng/ ml ionomycin (Merck, Darmstadt, Germany) and 10 ng/ml brefeldin A (Epicentre Biotechologies, Madison, USA).

Regarding adipose tissue, samples from MAT, SAT, and MLN, which were used as control, were collected, placed in HBSS, supplemented with 4% BSA, 10mM HEPES Buffer Solution and 2mg/mL collagenase (all from Sigma-Aldrich), with an incubation in a water bath (37 ºC), for 60 minutes, with mixing every 10 minutes. MLN was incubated only 30 minutes in the water bath, also with mixing. Then, digested samples were homogenized to single-cell suspensions, passed through 100 µm cell strainers (BD Biosciences Pharmingen, San Diego, USA), counted and centrifuged at 216 g, 10 minutes, 4 °C. The supernatant was discarded, with the cell-containing pellet (stromal vascular fraction (SVF) cells) being resuspended in RPMI-1640,

supplemented with 10% FBS, 1% penicillin/streptomycin, 10mM HEPES Buffer, 0.1M β-mercaptoethanol and 0,5% Antibiotic Antimycotic Solution (all from Sigma-Aldrich). Cells were then cultured in 96-well plates, with final number of cells in each well being 5 x 10^5 cells. For the analysis of cytokine production by MAT, SAT and MLN, cells either were not stimulated, or were stimulated with dead *N. caninum* tachyzoites (2,5 x 10^6 NcT per well). The cell culture was then incubated for 2 days, at 37 ºC, in 5% CO_2. For the analysis of cytokine production, the plates were first centrifuged at 485 g, during 10 minutes, at 4 ºC. The supernatants were then used for the analysis of cytokine production.

3.8 Flow Cytometry

For extracellular staining, the cells were pre-incubated in FACS buffer (PBS 1x, 1% BSA, 2mM azide) with Fc block, and then incubated with Peridinin-Chlorophyll protein -cychrome 5.5 (PerCP-Cy5.5) -conjugate anti-mouse CD4 (clone RM4- 5), and Phycoerythrin (PE) -conjugate anti-mouse CD8 (clone 53-6.7) (both from BD Biosciences). After extracellular staining, cells were fixed with 2% formalin (Panreac Applichem, Barcelona, Spain), washed and permeabilized with 0.05% saponin (Sigma-Aldrich) in FACS buffer. Cells were then incubated again in saponin with FITC anti-mouse IFN-γ (clone XMG1.2,) and PE-Cy7 anti-mouse IL-4 (clone BVD4-1D11) or respective isotype controls (all from BD Bioscience) monoclonal antibodies according to manufacturer's instructions after pre-incubation of the cells with Fc block. Data was acquired in an EPICS XL flow cytometer using the EXPO32ADC software (Beckman Coulter, Brea, USA), with at least 300 000 events being acquired per sample. Data was analyzed in FlowJo v9.7.5. (Tree Star, Inc., Ashland, USA).

3.9 Cytospin preparation

For the preparation of cytospins of the MAT, SAT and MLN cells, these were centrifuged at 195 g for 5 minutes using Cytospin3 Cytocentrifuge (Shandon, Cheshire, UK). The cytospin sections were then fixed in ice-cold methanol, air dried and stored at -20 °C, for immunohistochemical analysis.

3.10 Immunohistochemistry

Immunohistochemistry was performed for leptin, in order to detect its presence in adipose tissue samples and organs. First, deparaffinized and rehydrated sections

were exposed to heat-induced antigen retrieval with 1M citrate buffer (pH=6.0), for 3 minutes, with the incubation afterwards of the section with 0.3% hydrogen peroxide in methanol (Merck) for 20 minutes, in order to block endogenous peroxidase. Sections were then incubated in a moist chamber for 20 minutes with normal goat serum (Dako, Glostrup, Denmark) diluted 1:5 in 10% BSA (Sigma-Aldrich), for leptin detection, in order to prevent non-specific staining. After discarding the serum, sections were incubated with rabbit anti-mouse leptin (1:750, Abcam, Cambridge, UK), overnight (O. N.), at 4 C for leptin staining. After this, the leptin-stained sections were incubated with HRP-labelled anti-rabbit secondary antibody (1:1000, Abcam) for 60 minutes, at room temperature. Immunohistochemical reaction was developed with 3, 3'-diamino-benzidine (DAB + Substrate System; Dako). Finally, all samples were counterstained with hematoxylin and permanently mounted with Entellan ® (Merck).

Cytospins of MAT, SAT and MLN cells prepared previously were also analyzed through immunohistochemistry, for the detection of *Neospora caninum.* First the cytospins were incubated at room temperature for 1 hour, and then incubated with TBS 1x. Subsequently, the cytospins were incubated with 0.3% hydrogen peroxide in methanol (Merck) for 20 minutes, incubated in a moist chamber for 20 minutes with normal rabbit serum (Dako) diluted 1:300 in 10% BSA (Sigma-Aldrich). After discarding the serum, the cytospins were incubated with goat anti-*N. caninum* polyclonal serum (1:1500, VMRD, Pullman, USA), for 2 hours at room temperature. After this, the cytospins were incubated for 45 minutes with HRP-labelled anti-goat secondary antibody (1:500, Millipore, Billerica, USA). Immunohistochemical reaction was developed with 3, 3'-diamino-benzidine (DAB + Substrate System; Dako). Lastly, the cytospins were counterstained with hematoxylin and permanently mounted with Entellan ® (Merck).

The images taken for the analysis of the stained region for both adipose tissue samples and organ samples were obtained with 200x magnification field. Regarding the analysis of the cytospins, the images were taken with 1000x magnification field. For the analysis of adipose tissue, only the stained area within the adipose tissue was considered. This was possible through the manual selection of the area of interest in the images through ImageJ v1.7.0 software (National Institute of Mental Health, Bethesda, USA).

3.11 DNA extraction

DNA extraction was performed in brain, liver, lung and gonadal adipose tissue samples as previously described in detail [75]. Briefly, samples were first homogenized, with a subsequent digestion overnight with 1% sodium dodecyl sulphate (SDS) solution containing 1mg/mL Proteinase K, in a water bath at 55 ºC. DNA was then isolated using the phenol-choroform method, with the DNA being precipitated using an ammonium acetate/ethanol mix. Isolated DNA was recovered in an adequate volume of DNA/RNAse-free water (Sigma-Aldrich) and quantified using Nanodrop ND1000 apparatus (Thermo Scientific).

3.12 Detection of *Neospora caninum*

Neospora caninum DNA in brain samples collected during the experiments was detected through a Quantitative PCR. For this, primers and a TaqMan probe designed for a 103-bp Nc5 gene of *N. caninum* were used. The Real Time PCR was performed using the Kappa Master Mix (Grisp, Portugal) in a Rotor-Gene 6000 Corbett apparatus (Qiagen, Sydney, Australia). DNA amplification was performed using 1µl of DNA corresponding to 1000 ng in a final volume of 10 µl, when performing DNA amplification with the samples from the immunization experiments. For the adipose tissue experiments, the maximum amount of DNA used for DNA amplification was 2000 ng, also in 1 µl. A Master mix was first prepared containing 0,2 µM of the forward primer (Neo S: 5'- GTTGCTCTGCTGACGTGTCG) and 0,2 µM of the reverse primer (Neo A: 5'- GCTACCAACTCCCTCGGTT), 0,1 µM of Taqman® probe Neo TM (6FAM-CCCGTTCACACACTATAGTCACAAACAAAA-BBQ) and 1× Master Mix. The following PCR program run was utilized: an initial activation step (95ºC, 3 minutes) and amplification in 60 cycles, with denaturation (95°C, 5 seconds) and a combined annealing/extension steps (60°C, 20 seconds). Samples containing DNA corresponding to 2 x 10^5 to 2 x 10^0 *N. caninum* tachyzoites were included as standards. Quantitative evaluation of fluorescence signals from the PCR products was performed with Rotor-Gene software (version 1.7.75).

3.13 RNA isolation and quantitative Real-Time Polymerase Chain Reaction (RT-PCR) analysis

Total RNA was extracted from brain, muscle and stomach of C57BL/6 mice, two months post-infection, using TriReagent™ (Sigma-Aldrich) according to manufacturer's

instructions. All RNA samples were recovered in 20 µl of DNA/RNAse-free water (Sigma-Aldrich) and quantified using Nanodrop ND-1000 apparatus (Thermo Scientific). Synthesis of cDNA was then performed from 2,5 µg of total RNA in a 10 µl final volume using Maxima® First Strand cDNA Synthesis kit for RT-qPCR (Fermentas, Thermo Scientific), according to manufacturer's instructions. The PCR program run was as follows (25ºC, 10 min.; 50ºC, 30 min.; 85ºC, 5 min) using TProfessional Basic Thermocycler (Biometra GmbH, Goettingen, Germany). Real Time PCR was then used for the quantification of leptin (*Lep*) mRNA expression levels with the Kapa SYBR Fast qPCR Kit (Kapa Biosystems Inc, Wilmington, USA) in a Rotor Gene 6000 Corbett (Qiagen, Sydney, Australia). Hypoxanthine phosphoribosyl-transferase I (HPRT) was used as a reference gene. For the quantification of mRNA expression levels, the reaction was performed in a final volume of 10 µL containing 0.2µM of each specific primer: *Hprt* forward: ACA TTG TGG CCC TCT GTG TG, *Hprt* reverse: TTA TGT CCC CCG TTG ACT GA, Arg1 Forward: CTC CAAG CCA AAG TCC TTA GAG, *Lep* forward: TCAAGCAGTGCCTATCCAGA, *Lep* reverse: AAGCCCAGGAATGAAGTCCA (all from Tib Molbiol, Berlin, Germany) and 1× Master Mix plus 1 µL of the newly-synthesized cDNA. The RT-PCR program was the following: an initial activation step (95ºC, 5 minutes), followed by amplification in 40 cycles, with denaturation (95ºC, 10s) and annealing (62ºC, 20s). Quantitative evaluation of fluorescence signals from the PCR products was performed with the Rotor-Gene software (version 1.7.75) and was determined by plotting the fluorescence signals vs the cycle numbers at which the signals crossed the baseline. The correlation coefficient among the standard reactions assured linearity. An analysis of the specificity of the RT-PCR products was realized through the melting curve.

Analysis of real-time PCR data was made by the comparative CT method. Individual relative gene expression values were calculated using the formula: $2^{-(\text{Ct gene of interest- Ct constitutive gene})}$ [76].

3.14 Cytokine Detection

The concentrations of IFN-γ and IL-4 in cell culture supernatants were quantified with the Mouse IFN-γ ELISA Ready-Set-Go!® (eBioscience, San Diego, USA) and the IL-4 ELISA Ready-Set-Go!® (eBioscience) kits, respectively, both according to the manufacturer's instructions.

3.15 Antibody Detection

Serum IgG1 and IgG2a antibodies specific for NcMP were quantified by ELISA. First 96-well plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with NcMP diluted in PBS at a concentration of 5 µg/ml, being incubated overnight at 4° C. All the wells were blocked with 2% BSA (Sigma-Aldrich) in TST buffer (150 mM NaCl, 10 mM EDTA and 0,05% Tween 20, pH 8) for 60 minutes at room temperature. The serum samples were then serially diluted in 1% BSA TST buffer and incubated for 60 minutes at room temperature, followed by washing and addition of alkaline phosphatase-coupled goat anti-mouse IgG1 or IgG2a monoclonal antibodies (mAb) (Southern Biotechnology Associates, Birmingham, USA), with an incubation for 60 minutes at room temperature. After washing, the specifically bound antibodies were detected by adding p-nitrophenyl phosphate (Sigma-Aldrich) substrate solution. The reaction was stopped after significant development by the addition of 0,1M EDTA, pH 8. IgA antibodies specific for NcMP were quantified by ELISA as described above, however alkaline phosphatase-coupled goat anti-mouse IgA mAb (Southern Biotechnology Associates) was used for the detection. The absorbance was measured at 405 nm and 570 nm. For determination of titres, first the values of 507 nm measurements were subtracted to the 405 nm measurements. After logarithmic transformation of these values, a linear regression was performed for all values of dilution of each antibody. With this, the titres were expressed as the $\log_{10}$ values of the control well (no sample) measurement.

3.16 Statistical analysis

Statistical analyses were performed utilizing GRAPHPAD Prism software (Version 5.0, GraphPad Software, Inc. La Jolla, CA). Statistical significance of results concerning leptin production experiments was determined by nonparametric Mann–Whitney U-test (*P ≤ 0,05; **P ≤ 0,01; ***P ≤ 0,001). For analysis of cytokine production and antibody production, one-way analysis of variance was performed (*P ≤ 0,05; **P ≤ 0,01; ***P ≤ 0,001). Regarding the colonization analysis, and study of the frequency of splenic T-cells, a unpaired t test was performed (*P ≤ 0,05; **P ≤ 0,01; ***P ≤ 0,001). In the scatter dot graphs the mean for each group was displayed as a horizontal line. Column graphs are represented showing the mean plus one standard deviation.

4. Results

4.1 Immunization studies against *N. caninum*

4.1.1 Immunization of p40$^{-/-}$ mice

In previous studies it was demonstrated that mucosal immunization with *N. caninum* membrane proteins (NcMP) plus CpG adjuvant protected WT mice against i.g. established neosporosis [3]. p40$^{-/-}$ mice were previously shown to be lethally susceptible to i.p. infection with *N. caninum* [77]. Consequently, investigating whether these immunocompromised mice could still be effectively immunized against the parasite would be of interest in order to elucidate the immunization mechanism.

In order to perform the i.n. immunization *N. caninum* membrane proteins were extracted and *N. caninum* sonicates were prepared to be used for stimulation of spleen cell cultures. The protein migration profile of these preparations was determined through electrophoretic analysis.

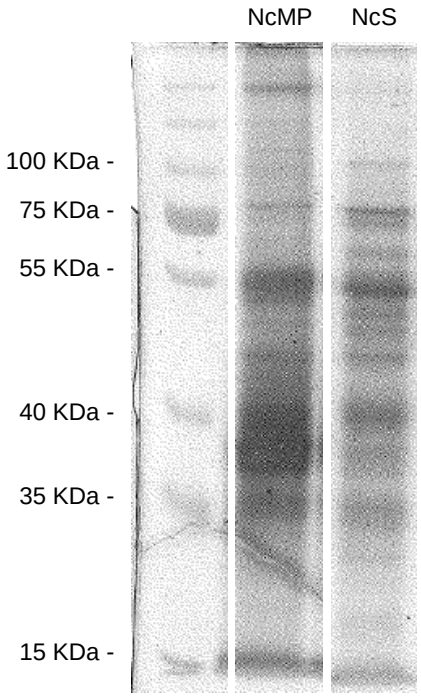


Figure 2 Electrophoretic migration profiles of *N. caninum* membrane protein extracts (NcMP) and *N. caninum* sonicates (NcS). Staining was performed using silver nitrate staining.

In figure 2, it is possible to observe a denser molecular band is present at 55 and 37 KDa, when comparing the protein migration profile of NcMP to that of *N.*

caninum sonicates. This indicates an enrichment of the proteins with these molecular weights.

4.1.1.1 Immunization confers protection in p40$^{-/-}$ mice

In order to assess whether i.n. immunization with NcMP plus CpG adjuvant could have a protective effect in p40$^{-/-}$ mice that were infected i.p., the parasitic burden in the brain of immunized and sham-immunized infected mice was determined by quantitative PCR specific for parasite DNA, since the brain is one of the organs were *N. caninum* is able to persist [4].

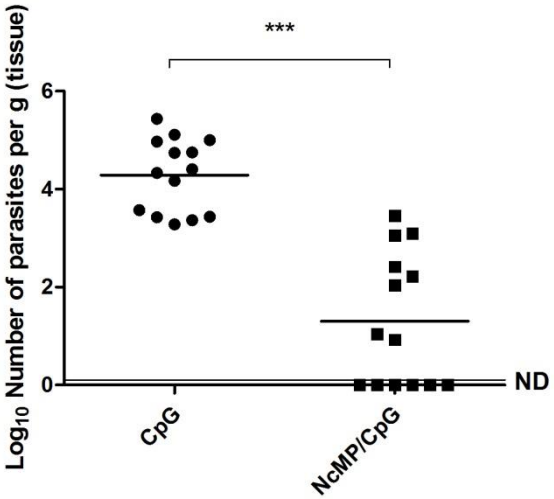


Figure 3 Brain parasitic load assessed by quantitative PCR 1 week after intraperitoneal infection with 1 x 10^4 NcT in mice previously sham-immunized with CpG adjuvant alone (CpG) or immunized intranasally with NcMP plus CpG adjuvant (NcMP/CpG). Results correspond to pooled data of two independent experiments. Each symbol represents an individual mouse (CpG n = 14; NcMP/CpG n = 14). Horizontal lines correspond to the mean value in each group (***P < 0,001). Not detected levels (ND) are indicated by a horizontal line.

As presented in figure 3, the group of animals that had been immunized presented a much lower parasite load than CpG-treated controls. In fact, in several of the immunized animals no parasite DNA could be detected in brain samples. Thus, the immunization procedure used induced a protective effect on p40$^{-/-}$ mice.

4.1.1.2 The proportions of IFN-γ producing T-cells upon infection were not altered by immunization

As demonstrated before, T cells are crucial for the immune response against *N. caninum*, with the release of IFN-γ being important in the establishment of a protective immune response [22]. Consequently, flow cytometry was used to assess the

frequencies of IFN-γ producing T cells in the immunized animals and respective CpG-treated controls.

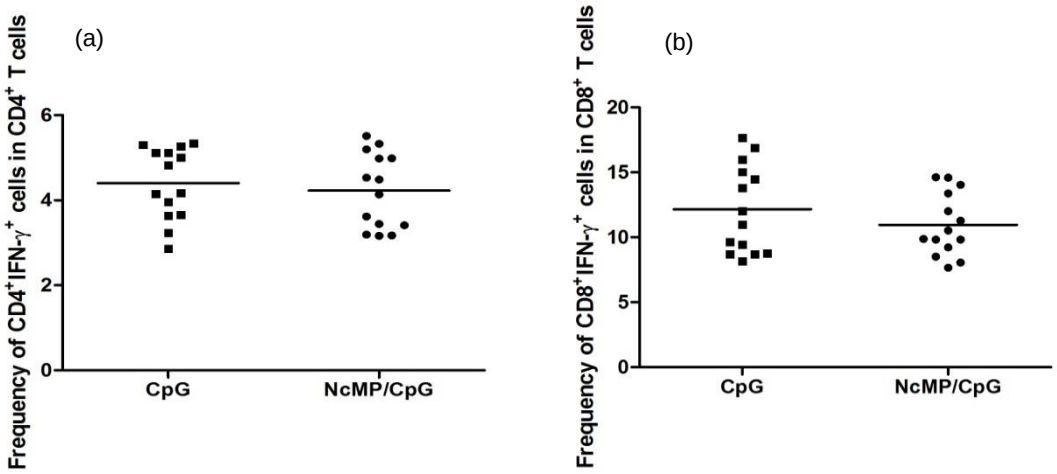


Figure 4 Frequency of splenic IFN-γ^+ $CD4^+$ T cells in total $CD4^+$ T cells (a), and frequency of splenic IFN-γ^+ $CD8^+$ T cells in total $CD8^+$ T cells (b), detected 1 week after intraperitoneal infection with 1×10^4 NcT in mice sham-immunized with CpG adjuvant alone (CpG) or previously immunized with NcMP with CpG adjuvant (NcMP/CpG). Results correspond to pooled data of two independent experiments (CpG n = 14; NcMP/CpG n = 14). Horizontal lines correspond to the mean value in each group.

In figure 4 is presented the frequency of splenic IFN-γ-positive (IFN-γ^+) $CD4^+$ T cells in total $CD4^+$ T cells, and of splenic IFN-γ^+ $CD8^+$ T cells in total $CD8^+$ T cells of the previously mentioned groups. No difference was found in the frequency of IFN-γ producing $CD4^+$ or $CD8^+$ T cells between sham-immunized and immunized mice.

4.1.1.3 IFN-γ and IL-4 production by $p40^{-/-}$ mice is not altered by the immunization

Production of IFN-γ is associated with resistance to *N. caninum* infection [18] while production of IL-4 and IL-10 has been associated with susceptibility to this infection [23]. In order to determine whether the immunization influenced the production of IFN-γ and IL-4 by $p40^{-/-}$ mice, cytokine production by NcS antigen-stimulated splenocytes was assessed *in vitro* in 3-day cultures. Cytokine production was then determined by ELISA.

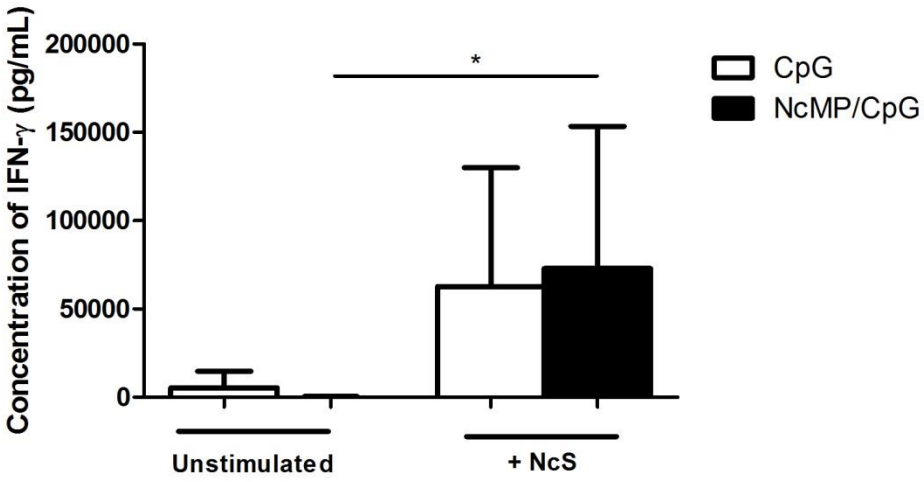


Figure 5 Concentration of IFN-γ detected in spleen cell culture supernatants. Splenocytes were collected 1 week after intraperitoneal infection with 1 x 10^4 NcT from mice sham-immunized with CpG adjuvant alone (CpG) or immunized with NcMP plus CpG adjuvant (NcMP/CpG). Spleen samples were either unstimulated or stimulated with 60 µg/µL of *N. caninum* sonicates (+ Ncs) for 3 days. Results correspond to pooled data of two independent experiments. Unstimulated samples presented significant differences with stimulated samples. Each bar represents the mean value for each group. Error bar = SD. One-way analysis of variance was performed (*P ≤ 0,05).

Regarding IFN-γ production, significant differences could be found between unstimulated and stimulated samples of immunized mice, as shown in Figure 5. On the other hand, no significant differences were observed between stimulated splenocytes of sham-immunized and immunized mice. This was also found for both groups of unstimulated splenocytes.

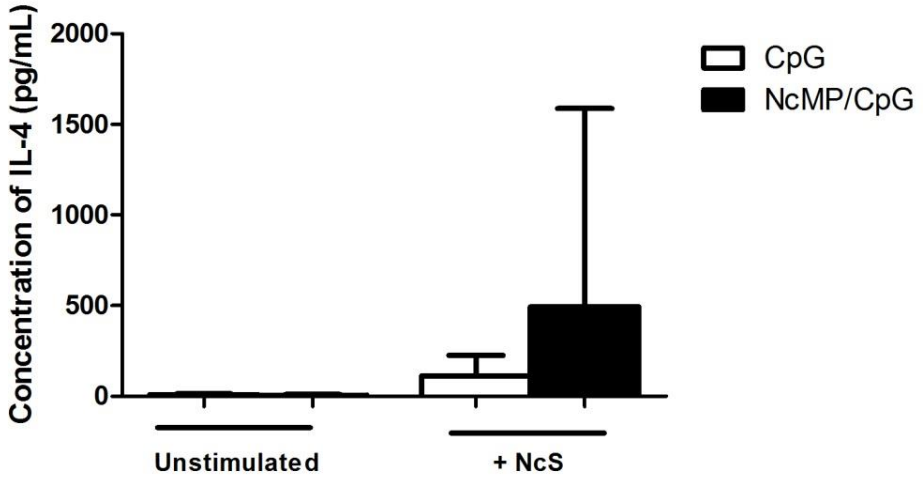


Figure 6 Concentration of IL-4 detected in spleen cell culture supernatants. Splenocytes were collected 1 week after intraperitoneal infection with 1 x 10^4 NcT from mice sham-immunized with CpG adjuvant alone (CpG) or immunized with NcMP plus CpG adjuvant (NcMP/CpG). Spleen samples were either unstimulated or stimulated with 60 µg/µL of *N. caninum* sonicates (+ Ncs) for 3 days. Results correspond

to pooled data of two independent experiments. Unstimulated samples presented significant differences with stimulated samples. Each bar represents the mean value for each group. Error bar = SD.

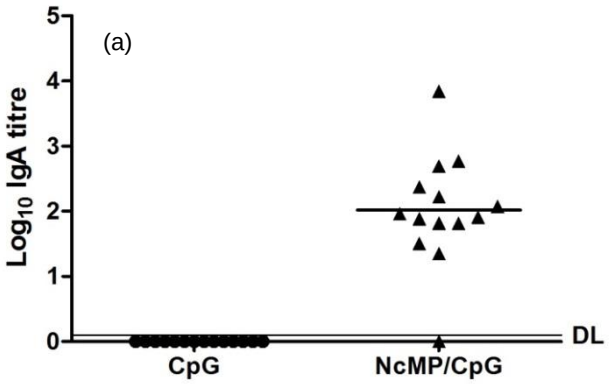
As represented in figure 6, no significant differences were found between unstimulated and stimulated samples regarding IL-4 production, likely due to the high variability detected in the concentration of this cytokine in the supernants of cultured splenocytes recovered from immunized mice and stimulated *in vitro* with *N. caninum* sonicates.

The above results indicate that immunized mice did not present a significantly different IFN-γ and IL-4 production as compared to controls treated with adjuvant alone.

4.1.1.4 Antibody production is induced by immunization in p40$^{-/-}$ mice

Antibody production remains an important part of the immune response against neosporosis. Production of IgA is characteristic of specific immune responses at the mucosa [32]. Therefore, an analysis of the antibody response at mucosal sites is of interest, in order to monitor whether the immune response was progressing well in the immunized mice. Moreover, parasite-specific IgG antibodies have also been shown to be induced by i.n. immunization [3].

Therefore, IgA and IgG (IgG1 and IgG2a) NcMP-specific antibody titres were determined by ELISA in the immunized mice and controls. IgA titres were measured in vaginal lavage fluids (VLF) and intestinal lavage fluids (ILF) samples, while IgG titres were determined in serum samples.



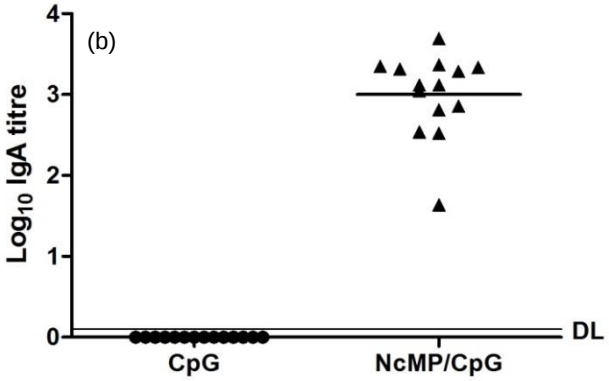
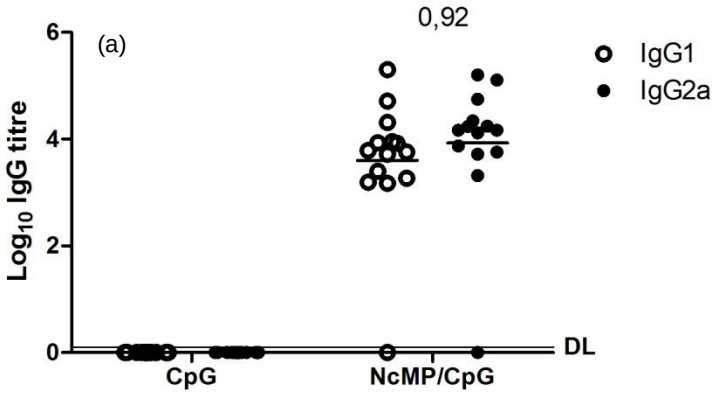


Figure 7 *N. caninum* membrane proteins (NcMP)-specific IgA titres detected by ELISA in vaginal and intestinal lavage fluids (VLF and ILF, respectively). IgA titres were determined in VLF collected 1 week after the boosting immunization from mice immunized intranasally with NcMP plus CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG) (a). IgA titres were determined in ILF collected from the same groups 1 week after intraperitoneal infection with 1 x 10^4 *N. caninum* tachyzoites (NcT) performed 3 weeks after the boosting immunization (b). Results correspond to pooled data of two independent experiments. Each symbol represents an individual mouse (CpG n = 14; NcMP/CpG n = 14). Horizontal lines correspond to the mean value in each group. Detection limit (DL) is indicated by a horizontal line.

Concerning *N. caninum* NcMP-specific IgA titres before the exposure of the mice to the parasite, it can be observed in figure 7 (a) that immunized mice presented higher IgA levels when compared to mice to which only CpG adjuvant was administered. This was also shown in figure 7 (b), where the measurement of IgA titres after the infection with *N. caninum* is represented. Therefore, the immunization with NcMP/CpG was able to induce an increase in the production of IgA.



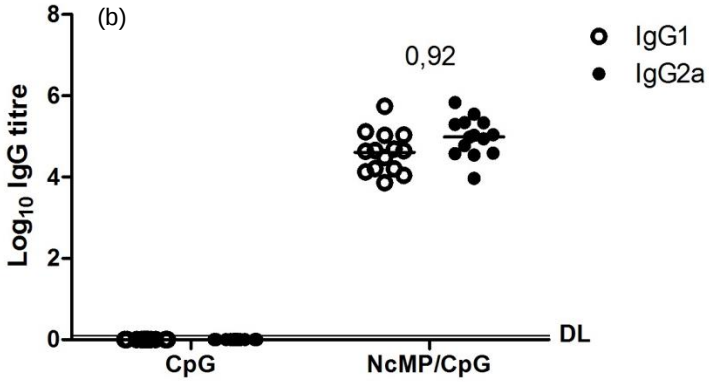


Figure 8 *N. caninum* membrane proteins (NcMP)-specific IgG1(open circles) and IgG2a (closed circles) titres detected by ELISA in serum. IgG titres were determined in serum collected 1 week after the boosting immunization from mice immunized intranasally with NcMP plus CpG adjuvant (NcMP/CpG) or sham-immunized with CpG adjuvant alone (CpG) (a), and serum collected from the same groups 1 week after intraperitoneal infection with 1 x 10^4 *N. caninum* tachyzoites (NcT) performed 6 weeks after the boosting immunization (b). Results correspond to pooled data of two independent experiments. Each symbol represents an individual mouse (CpG n = 14; NcMP/CpG n = 14). Numbers above each group represent the IgG1:IgG2a ratio, calculated with the mean $\log_{10}$ titres for the correspondent IgG isotype. Horizontal lines correspond to the mean value in each group. Detection limit (DL) is indicated by a horizontal line.

Concerning NcMP-specific IgG antibody titres before infection, as shown in figure 8 (a), a mixed IgG1 and IgG2a response in the NcMP/CpG groups was found, with a IgG1/IgG2a ratio of 0,92. Despite this, no significant difference was determined between the titres of IgG2a and IgG1 in immunized mice. Also, significant differences were encountered when comparing both IgG subtypes of CpG treated and immunized mice.

After infection, as shown in figure 8 (b), a mixed response was found once again in immunized mice, with the IgG1/IgG2a ratio being 0,92. Also, as observed before the infection, CpG treated mice did not present NcMP-specific IgG titres, differing significantly from the immunized mice. Consequently, the immunization process was able to prime an IgG antibody response, more biased towards the production of IgG2a.

4.1.2 Long term immunization of WT mice

As a protective effect was observed when WT mice were infected i. g. for 1 week, 4 weeks after an i.n. boosting immunization, there was interest in determining whether the protection was still maintained at a longer term. Herewith, an immunization study was developed in which WT mice were immunized i.n. and infected i.g. 18 weeks

after the boosting immunization. These mice were then euthanized 1 week upon infection for analysis.

A colonization analysis of brain samples from these animals was performed, in order to determine whether the immunization was able to protect WT mice in a long-term manner. Parasitic DNA was not detect, however. Therefore, it was not possible to determine the long-term protective effects of the immunization.

4.1.2.1 Cytokine production by WT mice is not increased after infection in long term immunized mice

As before, cytokine production of splenocytes NcS antigen-stimulated *in vitro* in 3-day cultures was determined by ELISA so as to determine if long term immunization led to differences in cytokine production by WT mice.

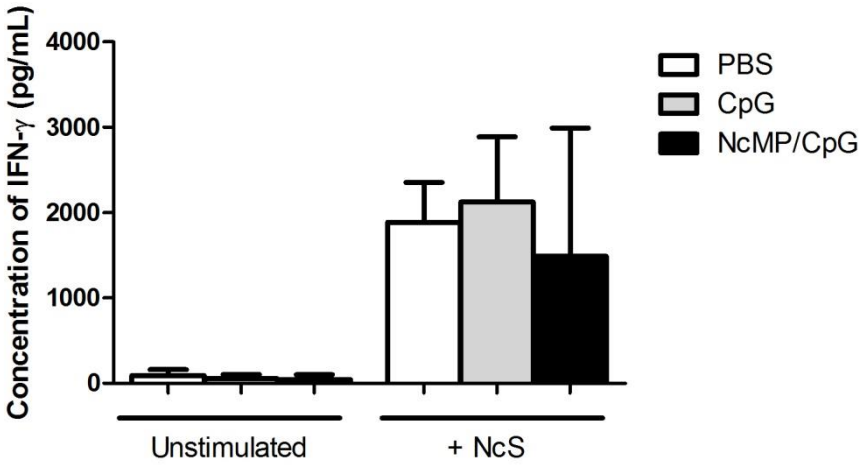


Figure 9 Concentration of IFN-γ detected in spleen cell culture supernatants. Splenocytes were collected 1 week after intragastric infection with 5 x 10^7 NcT from mice sham-immunized with CpG adjuvant alone (CpG) or immunized with NcMP plus CpG adjuvant (NcMP/CpG). Spleen samples were either unstimulated or stimulated with 60 µg/µL of *N. caninum* sonicates (+ Ncs) for 3 days. Unstimulated samples presented significant differences with stimulated samples. Each bar represents the mean value for each group. Error bar = SD.

As demonstrated in figure 9, significant differences in IFN-γ production were found between unstimulated and stimulated cell cultures of spleen samples. No differences were found, however, between the sham-immunized mice, which include mice administered with PBS (PBS) or mice administered with CpG adjuvant alone (CpG), and the immunized mice (NcMP/CpG). This was observed for either the stimulated or unstimulated cell cultures.

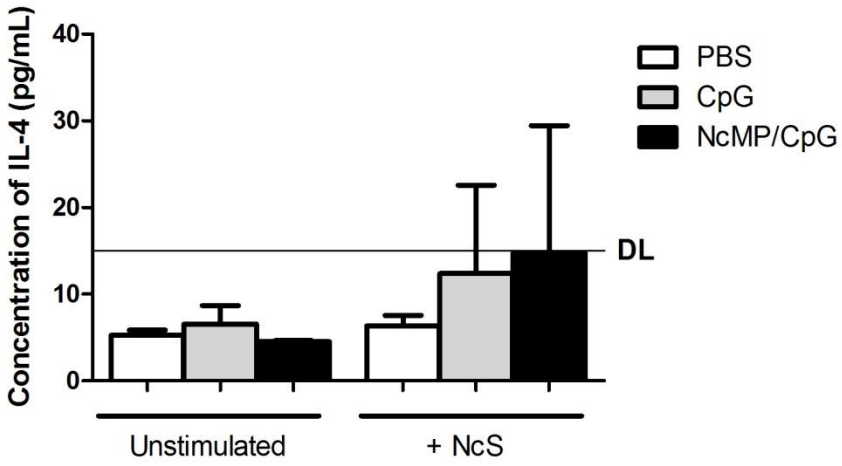


Figure 10 Concentration of IFN-γ detected in spleen cell culture supernatants. Splenocytes were collected 1 week after intragastric infection with 5 x 10^7 NcT from mice sham-immunized with CpG adjuvant alone (CpG) or immunized with NcMP plus CpG adjuvant (NcMP/CpG). Spleen samples were either unstimulated or stimulated with 60 µg/µL of *N. caninum* sonicates (+ Ncs) for 3 days. Each bar represents the mean value for each group. Error bar = SD. Detection limit (DL) is indicated by a horizontal line.

The concentration of IL-4, shown in figure 10, demonstrated no differences between unstimulated and stimulated cell cultures of spleen samples. Concurrently, no differences in IL-4 production were found between the sham-immunized mice, and the immunized mice, for either stimulated or unstimulated samples. Despite this, IL-4 levels measured were not higher than the detection limit established by the ELISA kit. Hence, no differences in IFN-γ and IL-4 production were found due to immunization of the mice.

4.1.2.2 Antibody titres remain elevated after long term immunization in WT mice

Antibody titres were also measured in order to understand whether antibody levels of both IgA and IgG remained elevated 19 weeks after the boosting immunization.

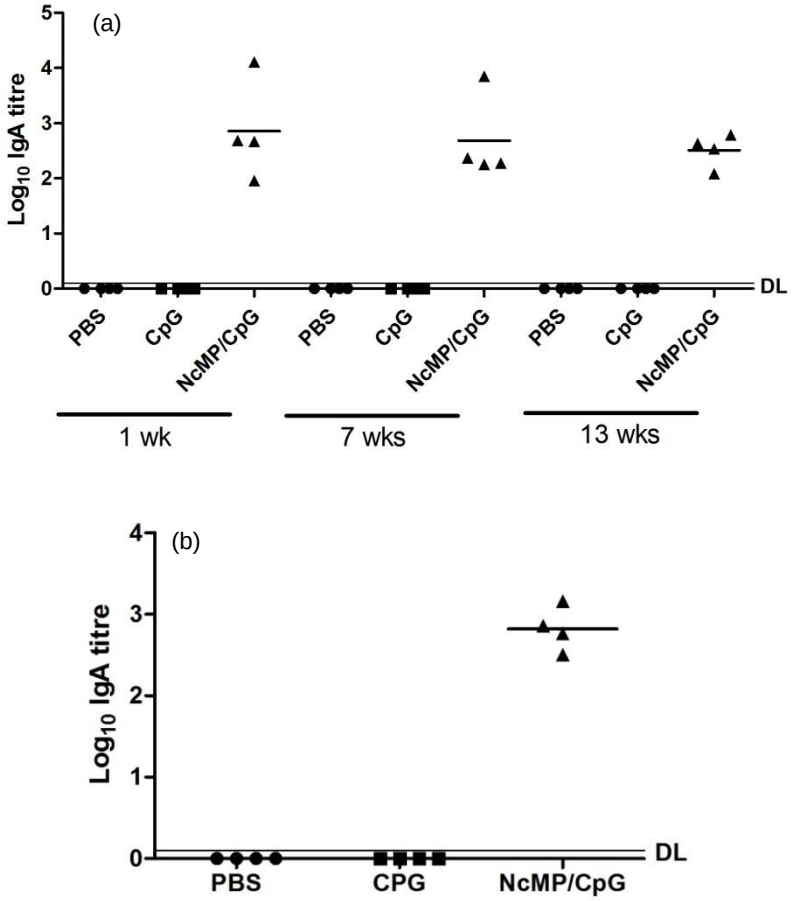


Figure 11 *N. caninum* membrane proteins (NcMP)-specific IgA titres detected by ELISA in vaginal and intestinal lavage fluids (VLF and ILF, respectively). IgA titres were determined in VLF collected 1, 7 and 13 weeks after the boostingf immunization from mice immunized twice intranasally with NcMP with CpG adjuvant (NcMP/CpG) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG) (a). IgA titres were determined in ILF collected from the same groups 19 weeks after the boosting immunization, with the intragastric challenge with 5 x 10^7 *N. caninum* tachyzoites being performed 18 weeks after the boosting immunization (b). Each symbol represents an individual mouse (PBS n = 4; CpG n = 4; NcMP/CpG n = 4). Horizontal lines correspond to the mean value in each group. Detection limit (DL) is indicated by a horizontal line.

NcMP-specific IgA titres before infection, as presented in figure 11 (a), were significantly higher in immunized animals (NcMP/CpG), when compared to both sham-immunized mice (PBS and CpG). This was observed for the three different time-points analyzed before the infection.

IgA titres after infection, shown in figure 11 (b), were significantly higher in immunized mice, when compared to both sham-immunized mouse groups. Therefore, the immunization process was able to increase IgA titres, which remained elevated by 19 weeks after the boosting immunization.

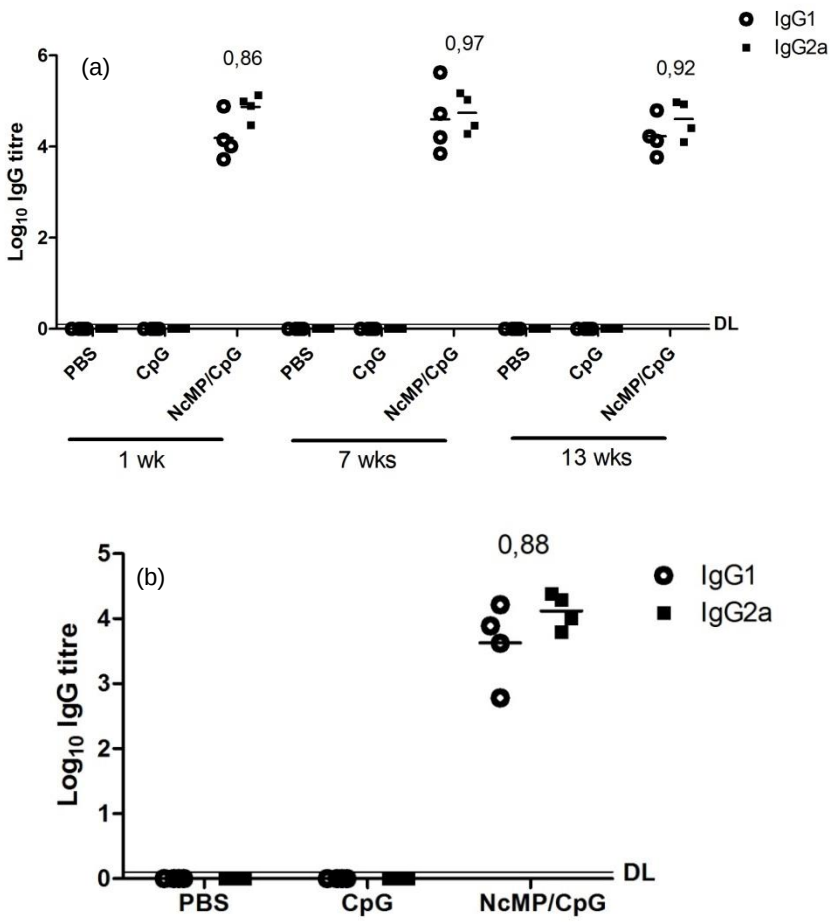


Figure 12 *N. caninum* membrane proteins (NcMP)-specific IgG1 (open circles) and IgG2a (closed circles) titres detected by ELISA in serum. IgG titres were determined in serum collected 1, 7 and 13 weeks after the boosting immunization from mice immunized intranasally with NcMP plus CpG adjuvant (NcMP/CpG) or sham-immunized with PBS (PBS) or CpG adjuvant alone (CpG) (a), and serum collected from the same groups 19 weeks after the boosting immunization, with the intragastric challenge with 5 x 10^7 *N. caninum* tachyzoites being performed 18 weeks after the boosting immunization (b). Each symbol represents an individual mouse (PBS n = 4; CpG n = 4; NcMP/CpG n = 4). Numbers above each group represent the IgG1:IgG2a ratio, calculated with the mean log_{10} titres for the correspondent IgG isotype. Horizontal lines correspond to the mean value in each group. Detection limit (DL) is indicated by a horizontal line.

In terms of NcMP-specific IgG antibody titres before infection, observed in figure 12 (a), it is possible to discern that, in all the time-points evaluated before infection, a mixed response was found in the NcMP/CpG group, regarding IgG1 and IgG2a. Ratios of IgG1/IgG2a before infection are 0,86 for 1 week after the boosting immunization, 0,97 for 7 weeks after the boosting immunization and 0,92 at 13 weeks after the boosting immunization.

After infection, as shown in figure 12 (b), the same mixed response was encountered, with the IgG1/IgG2a ratio being 0,88. Hence, the immunization process induced an IgG antibody response, more balanced towards an IgG2a type immune

response, with this response being maintained in long term, still visible at 19 weeks after the boosting immunization.

4.2 Immune response in the adipose tissue

As previously mentioned, it has been demonstrated that *N. caninum* is able to transiently colonize the adipose tissue, presenting also a capacity to influence the immune response [5]. With this, the immune response in the adipose tissue was analyzed, with the study of IFN-γ production by the immune cell population of this tissue.

4.2.1 IFN-γ production by adipose tissue immune cell population

IFN-γ, as seen before, remains a crucial cytokine in terms of immune response, leading to the activation of macrophages, among other important functions. Consequently, the levels of IFN-γ produced by immune cells from both mesenteric and subcutaneous adipose tissue were measured, as well as cells recovered from mesenteric lymph nodes as a control group.

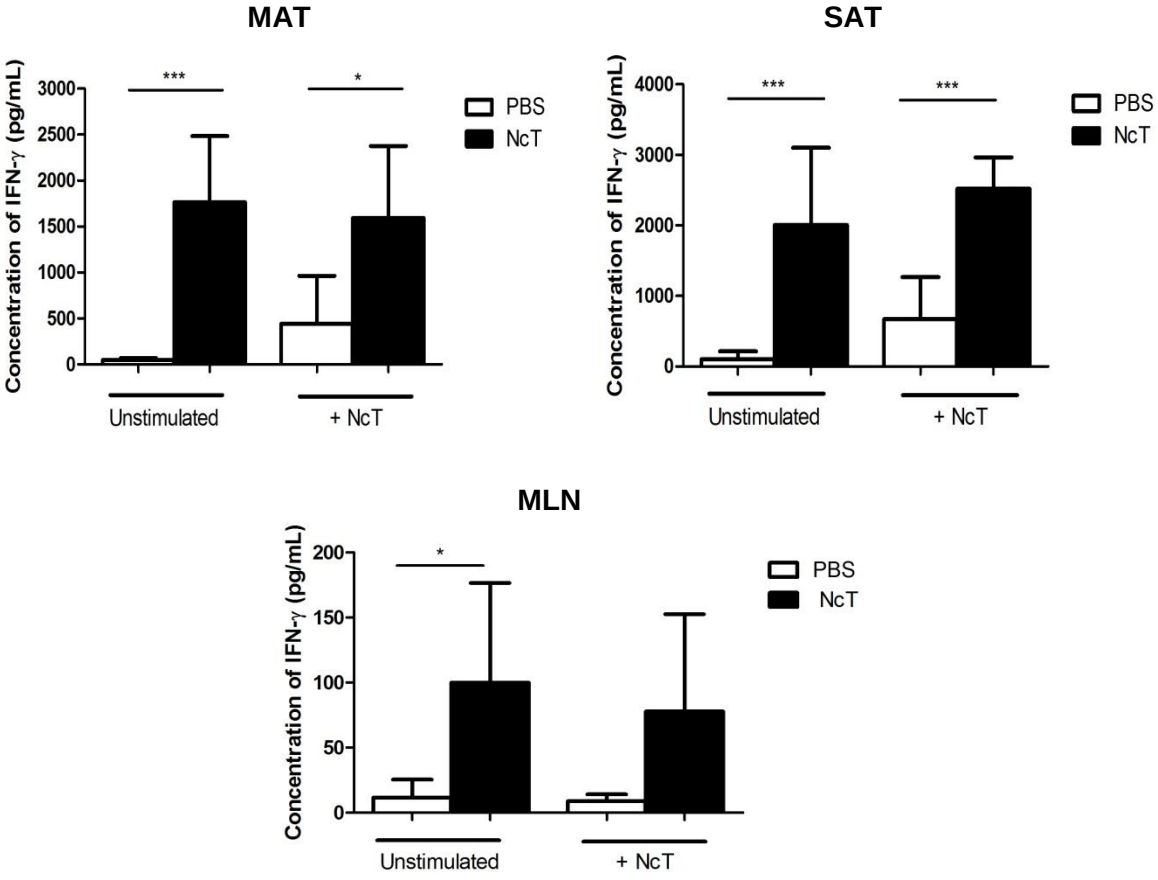


Figure 13 Concentration of IFN-γ in the supernatants of mesenteric adipose tissue (MAT) or subcutaneous adipose tissue (SAT) stromal vascular fraction cells, and mesenteric lymph nodes (MLN) cells of WT mice treated with PBS (PBS) or mice infected for 24 hours with NcT (NcT). Samples were either unstimulated or stimulated with 1 x 10^6 dead NcT (+ NcT) for 2 days. Results correspond to pooled data of two independent experiments. Statistical significances are represented between groups (One-way ANOVA; *P ≤ 0,05, ***P ≤ 0,001).

In figure 13 is presented the IFN- γ production of SVF cells removed from MAT, SAT and cells from MLN of mice euthanized 24 hours post-infection. Cells were either unstimulated or stimulated with 1 x 10^6 dead *N. caninum* tachyzoites for 2 days (+ NcT). As demonstrated in the graphs, in adipose tissue, cells from infected mice showed higher amounts of IFN-γ produced, when compared to samples obtained from PBS treated mice, in either stimulated and unstimulated groups. On the other hand, unstimulated cells of infected mice did not present significant differences when comparing with the samples from infected mice stimulated with NcT. In MLN, only unstimulated cells of PBS treated mice presented significant differences with unstimulated samples from infected mice. Therefore, despite the stimulation not being able to effectively increase IFN-γ production, cells from mice infected with the parasite demonstrate a significantly higher production of IFN-γ than samples from control mice. This was observed with unstimulated and stimulated samples.

So as to ascertain whether the differences observed in IFN-γ production in cell samples from adipose tissue of infected mice were due to the presence of the parasite in the tissue, an immunohistochemistry analysis was performed to detect *N. caninum*, parasites in samples from both MAT and SAT.

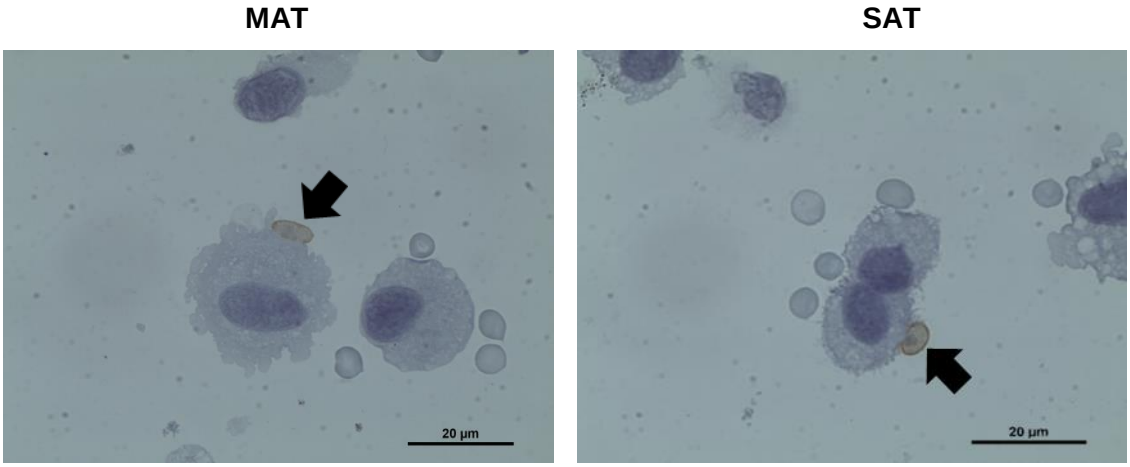


Figure 14 Representative images of *N. caninum* immunohistochemistry staining in stromal vascular fraction cells isolated from both mesenteric adipose tissue (MAT) and subcutaneous adipose tissue (SAT) of infected C57BL/6 mice, euthanized 24 hours after infection. *N. caninum* tachyzoites were specific stained (brown coloration) with anti-NcT antibody (black arrow).

As demonstrated in Figure 14 it is possible to observe the presence of *N. caninum* within the tissues, indicating that the parasite may have primed the immune response in these tissues. MLN samples were also analyzed however the parasite was not found therein.

In the murine model, high numbers of the parasite are found in both the lungs and liver, during the earliest stages of infection [78]. Accordingly, we also detected parasitic DNA in lungs and liver in mice 24h after infection (n=3) with parasitic concentration of 7900,81 ± 7676,305 NcT/ µg DNA and 2823,33 ± 972,1154 NcT/ µg DNA, respectively.

Cytokine production was also evaluated in samples from mice infected for 1 year, in order to understand if the mice, even after a long period of time, remained prepared for a possible re-exposure to the parasite.

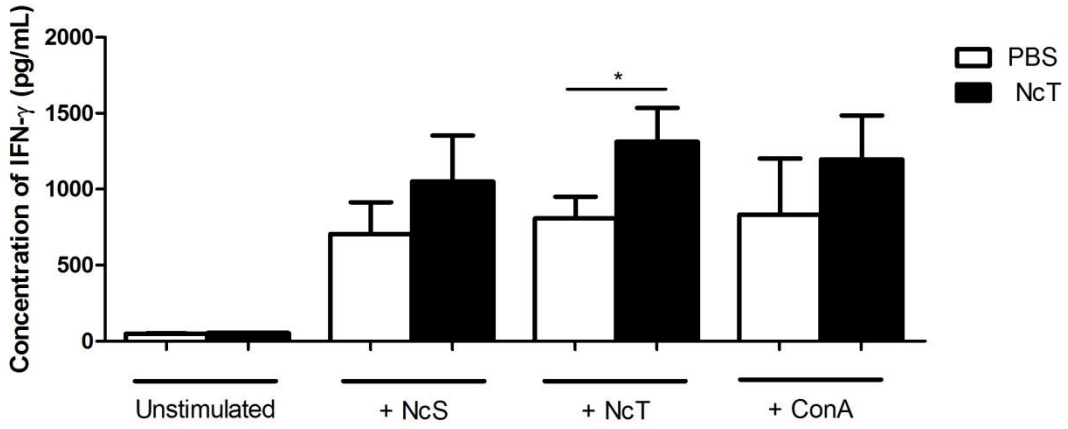


Figure 15 Concentration of IFN-γ from cell cultures of mesenteric adipose tissue (MAT) samples of WT mice treated with PBS (PBS) or mice infected for 1 year with NcT (NcT). Samples were either unstimulated or stimulated with 1 x 10^6 dead *N. caninum* tachyzoites (+ NcT), with 30 µg/µL of *N. caninum* sonicates (+ Ncs), or with 2,5 µg/mL of concanavalin A for 2 days (+ConA). Unstimulated samples presented significant differences with all groups of stimulated samples. Statistical significances are represented between groups (One-way ANOVA; *P<0,05).

Figure 15 presents the analysis of IFN-γ by ELISA of cell cultures from MAT samples of infected and non-infected mice. Cells removed from mice infected for 1 year were unstimulated, or stimulated either with sonicates of *N. caninum* (+ NcS), NcT (+ NcT) or, as a positive control, Concanavalin A (+ ConA). As seen in the graph, there were significant differences between the unstimulated samples and the stimulated samples. Also of importance, among the stimulated samples, it is possible to observe that only the stimulation with NcT led to significant differences between the sham-infected and infected mice.

Parasite colonization of the brain, lung and GAT was analyzed through RT-PCR with primers specific for *N. caninum*, so as to determine whether the parasite persisted

in infected mice 1 year after infection. Parasitic DNA was detected in the brain of two out of 5 infected mice, with the lungs and GAT samples not presenting any sign of *N. caninum* presence. Despite this, in the presence of *N. caninum* stimulus the MAT cells from mice infected for 1 year produced higher levels of IFN-γ than non-infected mice, indicating that an immunological memory due to previous exposure to the parasite was induced in the adipose tissue.

4.3 Leptin analysis in *N. caninum* infected mice

Leptin has shown an ability to affect the immune response. As this protein is capable of influencing the development and function of immune cells that are critical for this response, such as T cells, macrophages and monocytes [73], understanding its' role in the response to *N. caninum* infection may prove relevant in determining a possible treatment course.

4.3.1 *N. caninum* infection influences leptin serum levels in mice

It has been previously shown that leptin levels are increased in the serum of two month *N. caninum*-infected mice [5]. However, leptin mRNA expression in the different adipose tissues analyzed did not present significant differences between infected and PBS treated mice [5]. Since adipose tissue is the main source of this adipokine, this result could indicate that leptin synthesis by adipocytes could be affected. Therefore, in this work, total leptin levels were quantified by ELISA, so as to observe if there were differences in protein production in mesenteric adipose tissue (MAT) between PBS treated and infected mice.

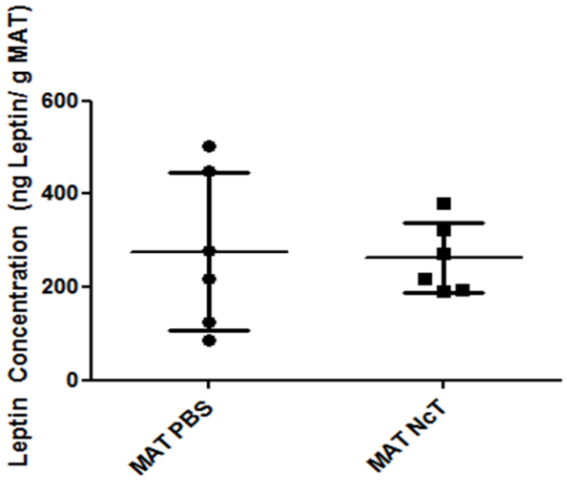
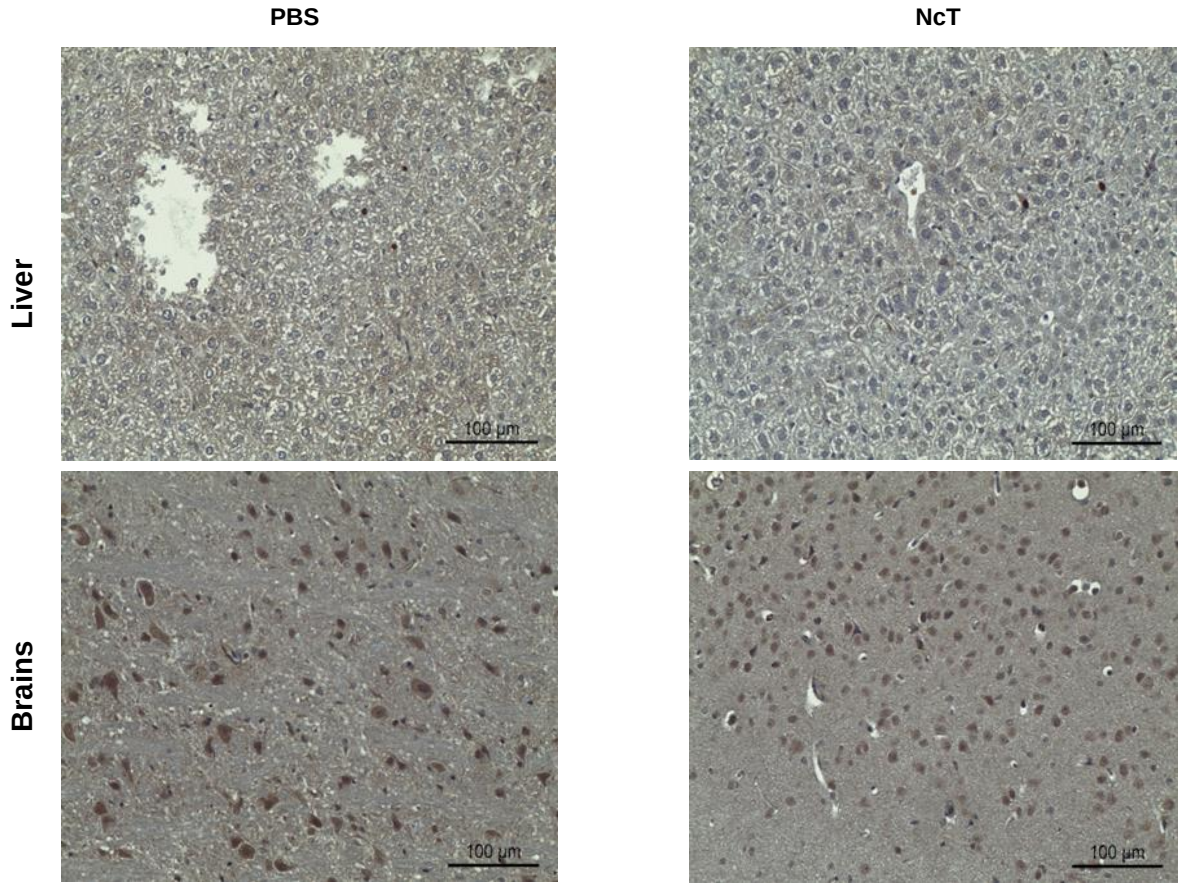
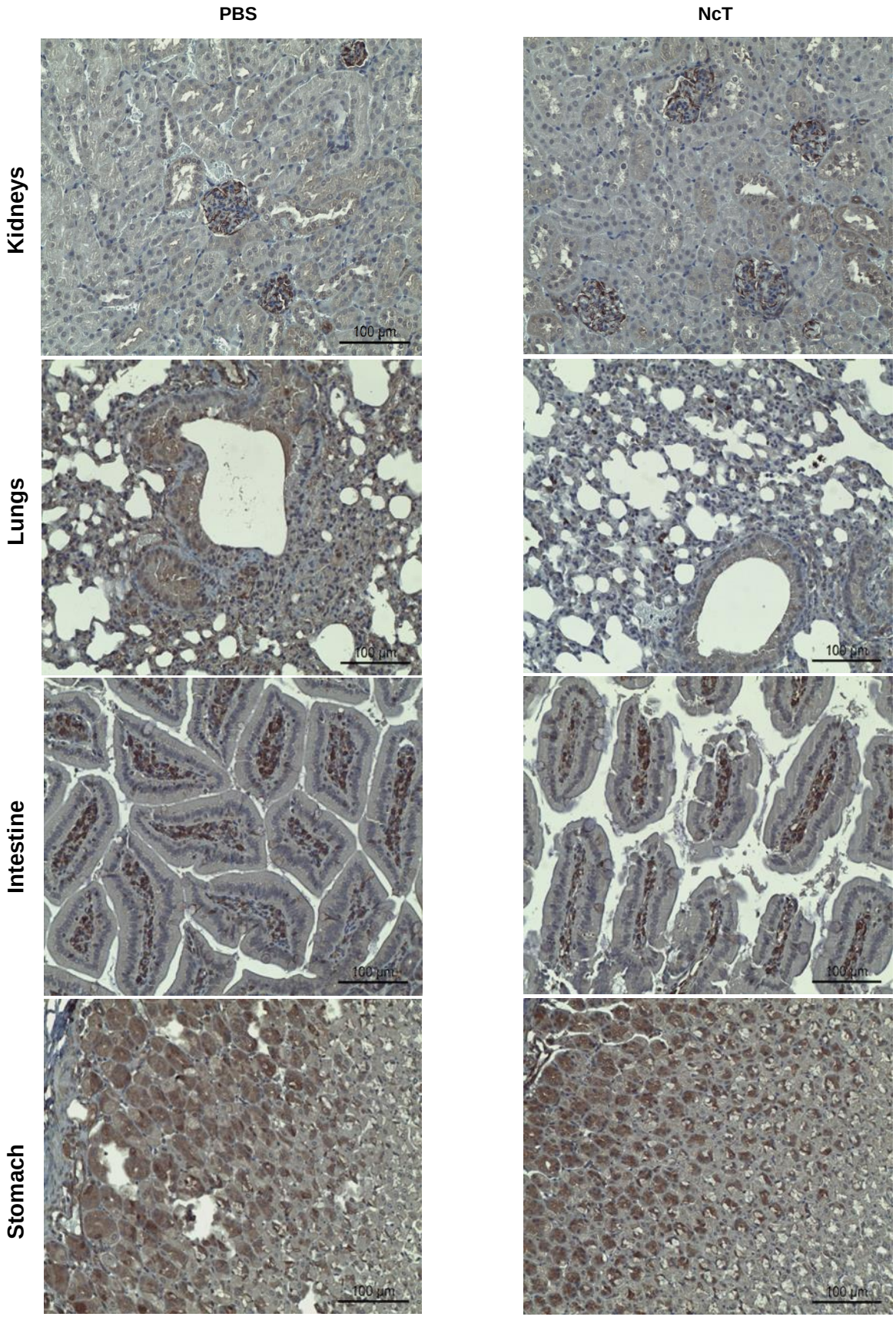


Figure 16 Leptin levels per gram of mesenteric adipose tissue (MAT) of C57BL/6 mice, 2 months after intraperitoneal infection of 1 x 10^7 *N. caninum* tachyzoites (NcT) or administration of PBS. Horizontal lines

represent the mean values of the respective group (± SD). Each symbol represents an individual mouse (PBS n = 6; NcT n = 6).

No differences were found regarding leptin production in MAT, as shown in figure 16. Consequently, other tissues and organs were analyzed so that a possible source of leptin production could be found. Therefore, immunohistochemistry was performed in order to detect possible differences in leptin presence in the different organs.




PBS
NcT
Kidneys
Lungs
Intestine
Stomach
100 µm

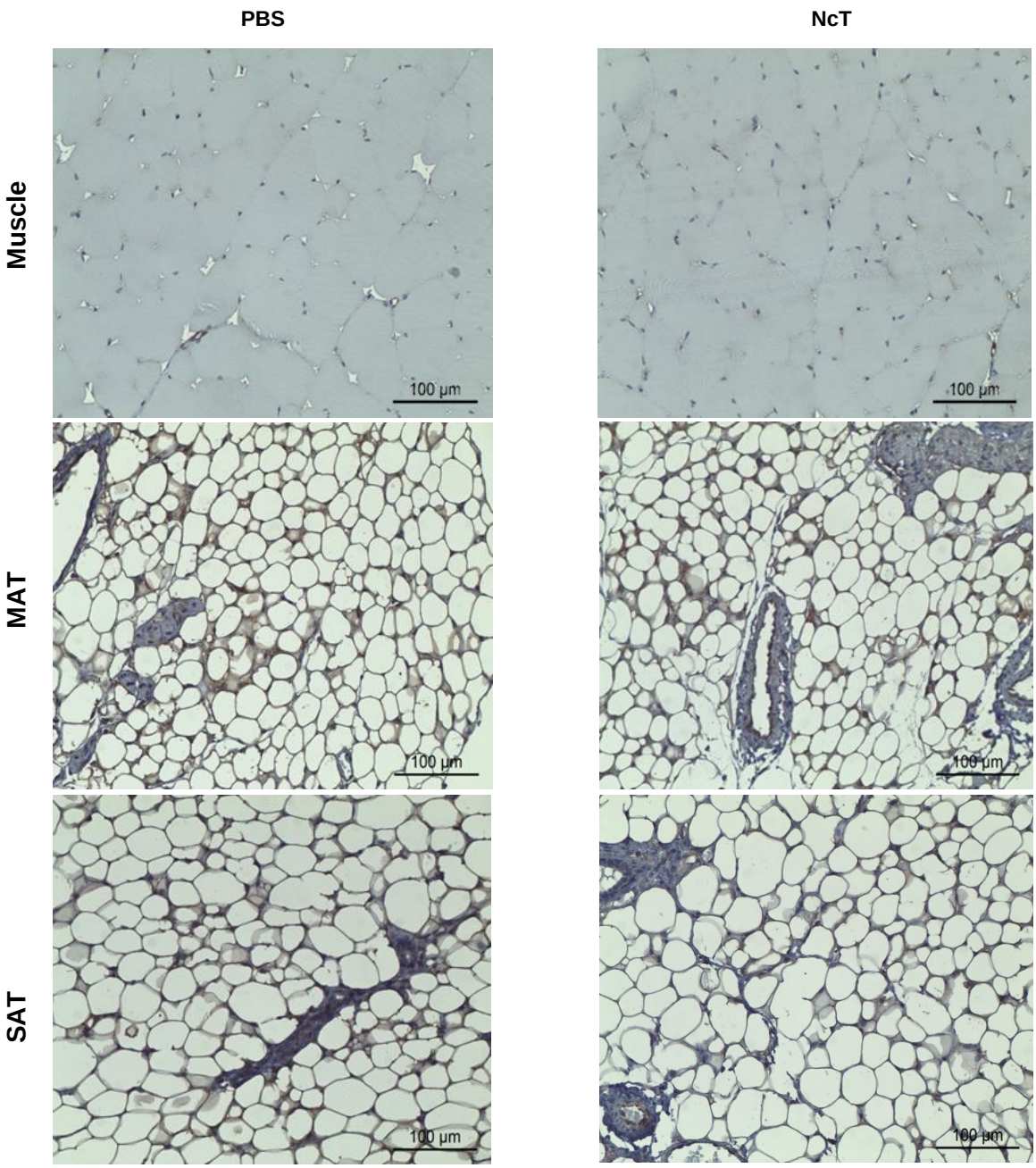
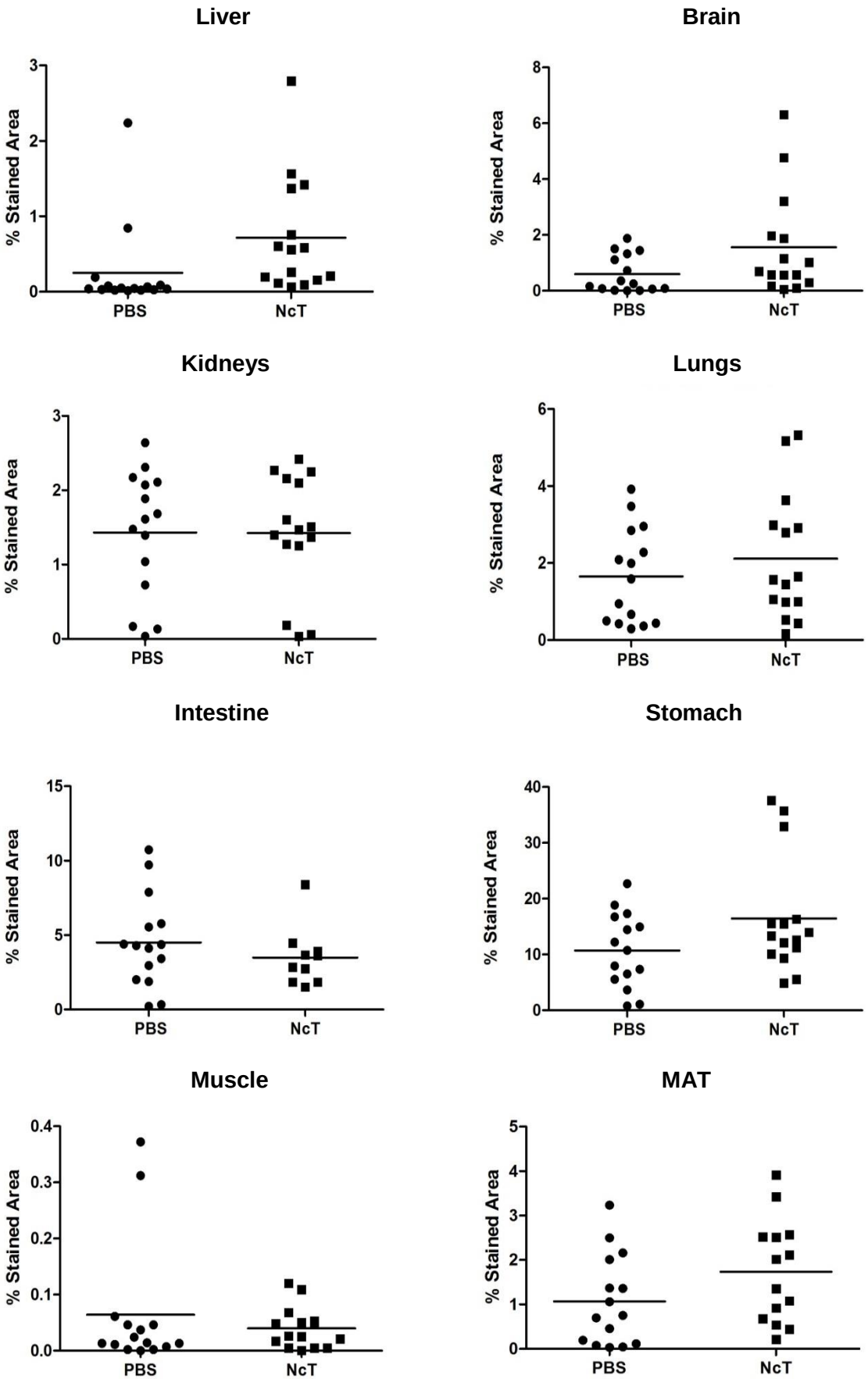


Figure 17 Representative images of immunohistochemistry analysis of leptin staining, colored brown. Liver, brain, kidneys, lungs, intestine, stomach, mesenteric adipose tissue (MAT), and subcutaneous adipose tissue (SAT) of C57BL/6 mice euthanized 2 months after i.p. infection with *N. caninum* Tachyzoites (NcT) or administration of PBS (PBS) were stained with a polyclonal anti-leptin antibody.

As shown in figures 17 and 18, no differences were found between infected and non-infected mice in the percentage of stained area for leptin in any tissue or organ analyzed.


Liver
% Stained Area
PBS
NcT
Brain
% Stained Area
PBS
NcT
Kidneys
% Stained Area
PBS
NcT
Lungs
% Stained Area
PBS
NcT
Intestine
% Stained Area
PBS
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PBS
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MAT
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PBS
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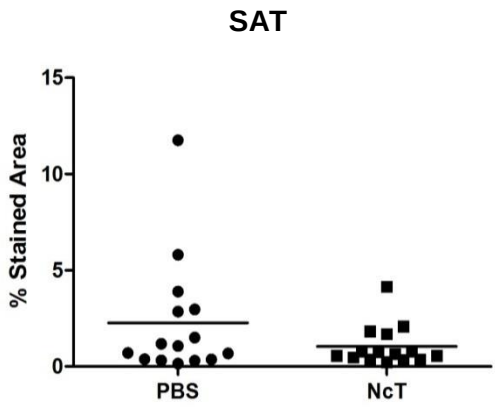
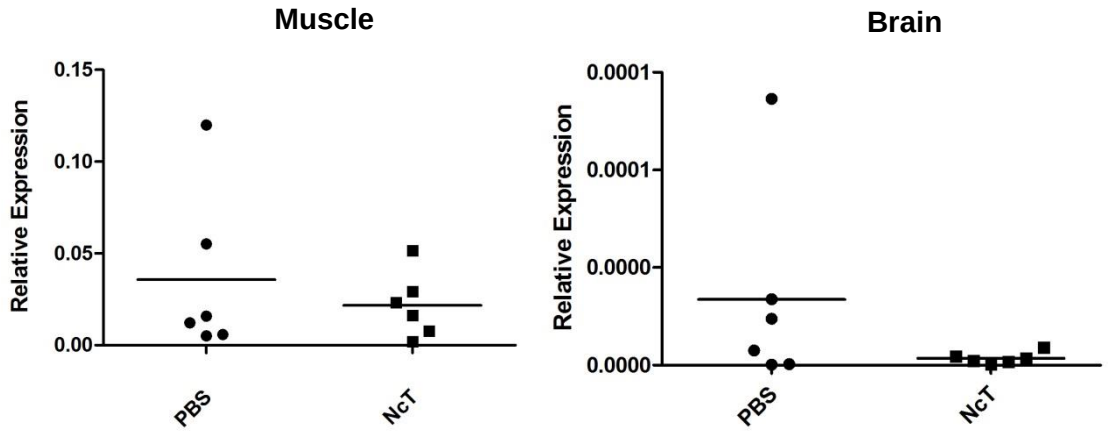


Figure 18 Percentage of leptin - stained area of analyzed liver, brain, kidneys, lungs, intestine, stomach, mesenteric adipose tissue (MAT) and subcutaneous adipose tissue (SAT) 2 months after infection with *N. caninum* tachyzoites (NcT) or administration of PBS (PBS). Horizontal lines represent the mean values of the respective group. Each symbol is a representative image of the tissue being analyzed, for either control (PBS) or infected (NcT) mice.

As the immunohistochemistry method did not reveal significant differences in terms of leptin presence in the tissues and organs analyzed, leptin gene expression was analyzed in select tissues, in order to observe if there were differences in terms of expressions between infected and control mice. Herewith, a Real Time (RT)-PCR was performed with samples of cDNA from muscle, stomach and brain. Muscle and brain were analyzed as these organs are known to be sites of persistence of *N. caninum* in mice [1], and stomach was chosen as an organ of interest due to gastric chief cells being also a source of leptin [73].



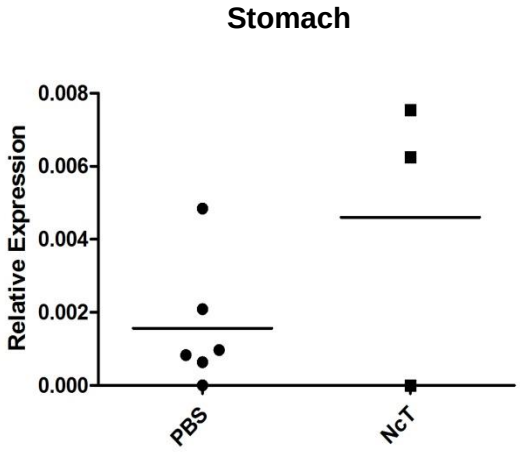


Figure 19 Relative levels of leptin mRNA (*Lep* gene) normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA, detected by real-time PCR in brain, muscle and stomach samples of mice two months after i.p. infection with 1×10^7 *N. caninum* tachyzoites (NcT) or PBS (PBS). Each symbol represents an individual mouse. Horizontal lines represent the mean values of the respective group (PBS n = 6; NcT n = 6).

According to figure 19, no differences were found when analyzing the mRNA expression of the *Lep* gene, in muscle, brain or stomach. Thus no organ was yet found to be responsible for the observed rise in serum leptin levels.

While leptin production has not yet been demonstrated to be altered due to the infection with *N. caninum,* leptin levels have been found to be increased in the serum of chronically (two month) infected mice [5]. To determine if leptin levels remained altered at later time points after *N. caninum* infection, serum leptin levels were measured 1 year after infection.

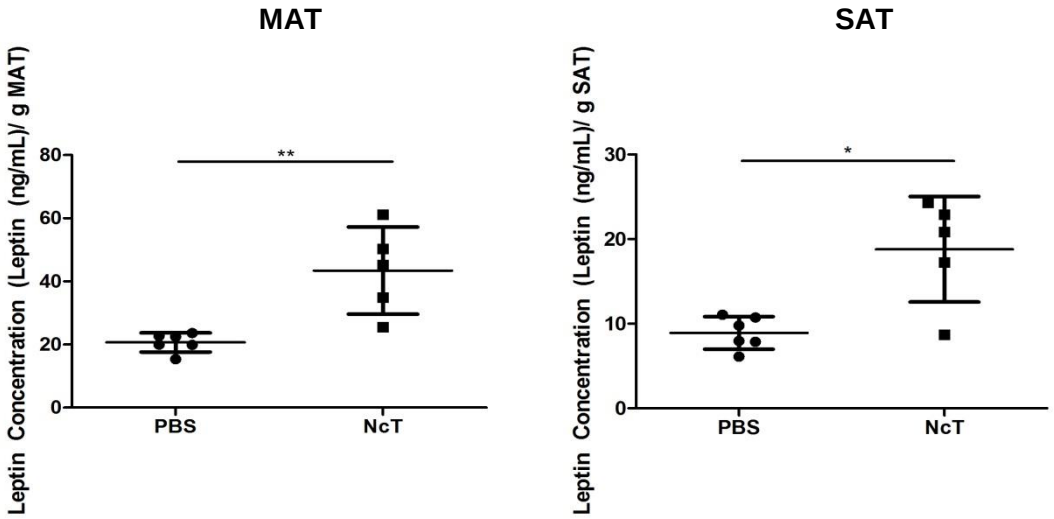


Figure 20 Serum concentration levels of Leptin (ng/mL) per mesenteric adipose tissue (MAT) and subcutaneous adipose tissue (SAT) weight of WT mice treated with PBS (PBS) or mice infected 1 year

with NcT (NcT). Horizontal lines represent the mean values of the respective group (± SD). Each symbol represents an individual mouse (PBS n = 6; NcT n = 5). Statistical significances are represented between groups (Mann-Whitney U test; *P ≤ 0,05, **P ≤ 0,01).

As shown in figure 20, mice infected for 1 year presented significant higher levels of leptin in the serum, when compared to PBS treated mice. This was observed for leptin levels normalized to either MAT or SAT weight. This results show that *N. caninum* infection still affects adipokine production even at long time points after infection.

5. Discussion

Understanding how the immune response develops in *N. caninum* infected hosts is still a matter of importance, not only in order to comprehend how the parasite is able to protect itself from this response, but also so as to be able to mount an effective protective strategy against the parasite.

As previously discussed, vaccination strategies remain the most effective and safe choice for protection of the host against neosporosis. Previous reports have shown the protective effect of i.n. immunization with *N. caninum* antigens in mice infected i.p. [44, 45]. The main mechanism through which this protective effect is induced is however not yet understood. In this work, it was demonstrated that, in IL-12/IL-23 p40-deficient ($p40^{-/-}$) mice immunized i.n. with membrane proteins of *N. caninum* tachyzoites plus CpG adjuvant presented a lower parasitic burden in the brain when infected i.p. with *N. caninum* tachyzoites than sham-immunized mice treated only with CpG adjuvant. As $p40^{-/-}$ mice are deficient in the p40 subunit of IL-12 and IL-23, their functions are absent in these mice. These include the promotion of Th1-type cytokine responses, among which induction of IFN-γ production by T and NK cells [79], which are important mechanisms for the control of parasite proliferation and survival. Thus, despite a deficiency in the proinflammatory response, a protective effect was still obtained with immunization in the $p40^{-/-}$ mice.

Alongside this, the frequency of splenic IFN-γ-positive (IFN-γ^+) $CD4^+$ T cells in total $CD4^+$ T cells, and of splenic IFN-γ^+ $CD8^+$ T cells in total $CD8^+$ T cells was also analyzed through flow cytometry. T cells remain an important part of the immune response against *N. caninum*, influencing cell mediated immunity through the release of IFN-γ. $CD4^+$ T cells demonstrate a significant response against the parasite, acting through the production of IFN-γ, with $CD8^+$ T cells presenting an ability to induct IFN-γ production when a host is infected. This proinflammatory activity of $CD8^+$ T cells is generally helped by the activity of $CD4^+$ T cells [65]. Therefore, the contribution of these cells to the protective effect is also of interest. With this, the immunization did not alter the frequency of both IFN-γ^+ $CD4^+$ T cells and IFN-γ^+ $CD8^+$ T cells, indicating that the protective effect induced by the immunization is not critically dependent on IFN-γ producing T cells.

Concurrently, IFN-γ and IL-4 production by spleen cells of both sham-immunized and immunized mice was also analyzed. IFN-γ, specially, is crucial for the response against intracellular parasites, activating macrophages [80]. Results demonstrated that immunization does not influence the production of any of these cytokines. As IFN-γ is usually important in mediating the protection of the host against

the parasite, an alternative mechanism may be involved in this protective effect of the immunization.

Antibody responses have been demonstrated to be an active part in the protection of the host against infection. Previous reports showed that, in WT mice, i.n. immunization with *N. caninum* membrane proteins plus CpG adjuvant led to increases in both IgG1 and IgG2a antibody titres [3]. In this work, this response was also observed, with the ratio of IgG1 and IgG2a tending towards higher titres of IgG2a. Considering IgG2a has been associated with Th1-type responses and IgG1 has been related to a Th2-type response [81], it is possible to discern that, with immunization, the immune response is more directed towards a Th1-type. As this type of response can be linked to the production of proinflammatory cytokines such as IFN-γ, increased levels of these cytokines would be observed. Despite this, and as seen before, no differences in IFN-γ production were found in immunized mice. IgG2a production has been shown to be, in some cases of infection with parasites, independent of IFN-γ induction [82]. As demonstrated in this previous study, IFN-γ receptor-knockout mice were able to produce IgG2a after infection with *T. gondii*, an apicomplexan parasite related *to N. caninum*. This would signal that production of IgG2a is not completely dependent of IFN-γ. Thus, despite the possible bias towards a Th1-type response due to higher IgG2a titres, no differences in IFN-γ production allude to a protective mechanism independent of IFN-γ.

Nevertheless, IgG1 titres remained elevated also, demonstrating a balanced Th1/Th2 response. Despite the importance that a proinflammatory response has in the protection of the host against the parasite, an exaggerated reaction may lead to hazardous consequences to the host. Therefore, a Th2-type response may help counterbalance a potential adverse effect. Thus, a protective effect based on both a Th1- and Th2-type response may be important for an effective defense against the parasite, without being harmful to the host.

IgA has also been shown to be important in the control of the infection as an important mediator of mucosal immunity, being able to agglutinate the parasite [3]. Here, it was possible to observe that immunized mice presented higher titres of IgA, when compared to control mice, either before or after infection. These results indicate that the immunization is able to induce an increase in the strength of the immune response, possibly helping protect the host. Despite this result, in this experiment, as the infection was established i.p., the protective effect of IgA was not important for this infection since IgA acts on the intestinal mucosa.

Bearing these results in mind, a protective effect was found in immunocompromised $p40^{-/-}$ mice due to the immunization. This was possible, however,

without a marked Th1-type response or a protective function of IgA. The mechanism through which the protective effect of the immunization is produced is still yet to be found.

Despite this short-term protective effect, understanding whether this response could be maintained in a longer period of time was also important, in order to create a protective immunization that would have long term effects. So as to determine whether this long term effect was found, a colonization analysis of WT mice immunized i.n. with membrane proteins of *N. caninum* tachyzoites plus CpG adjuvant that were i.g. infected with *N. caninum* tachyzoites 18 weeks after the boosting immunization, and sham-immunized mice treated only with PBS or CpG adjuvant was performed. This analysis, however, did not present discernible results. A possible reason was that no viable parasites were present in the inoculum and therefore no infection was established in the host.

Cytokine production, however, was analyzed for these mice, with the long term immunization not leading to differences in IL-4 and IFN-γ production, when comparing immunized and sham-immunized mice. As seen before, no significant differences were encountered for either cytokine when considering a shorter term. As such, the protective effect, even in a long-term capacity, is not critically dependent on the Th1-type response.

One key difference to the short-term experiment is the route of infection. As the mice were infected i.g., the potential protective effect that IgA may have against *N. caninum* becomes significant as the parasite has to surpass the intestinal mucosa. IgA titres remained elevated even 19 weeks after the boosting immunization, and as IgA is able to agglutinate the parasite, it is possible that the capacity of the parasite to invade the intestinal mucosa and proliferate within the host is diminished due to the activity of IgA. Thus, as the parasite is not able to activate a stronger proinflammatory response due its lower proliferation, IFN-γ production, among other mechanisms related to a Th1-type response would not be significant. Nonetheless, as the short term experiment demonstrated, no differences in IFN-γ production were found when the infection was i.p., removing the protective effect of IgA in the mucosa. As such, the main protective effect against *N. caninum* produced by the immunization may be independent of IFN-γ and IgA.

Meanwhile, both IgG1 and IgG2a remained elevated in a long term immunization, both before and after infection, with IgG2a presenting once again higher titres. Therefore, while the balanced Th1/Th2 response was still observed, a Th1-type response was to be more pronounced.

Considering this, while the protective effect of long term immunization was not possible to be determined, antibody titres remained elevated 19 weeks after the boosting immunization, indicating that, at least in terms of antibody response, a protective effect could be expected. On the other, once again no differences were found for IFN-γ and IL-4 production, suggesting that even in long term IFN-γ does not play a critical function in the protective effect produced by the immunization. Nevertheless, a colonization analysis would effectively demonstrate whether the immunization is able to protect the host in a long-term manner against the parasite.

As seen before, knowing how the immune response to *N. caninum* infection is developed is crucial, allowing for the construction of a protective mechanism that helps control the infection. Thus, analyzing how the response is affected by the infection in lesser known to be affected organs may be of interested so that a more comprehensive analysis of the infection is available. Among the less studied tissues, in terms of immune response, adipose tissue presents an intriguing target. Due to its function as an endocrine organ, it is able to affect different processes within the organism. Even though few reports have investigated the immune response elicited in adipose tissue upon infection, it has been established, however, that *N. caninum* is able to transiently colonize the adipose tissue when the host is infected i.p., affecting the immune response [5]. In this work, the acute immune response in adipose tissue to *N. caninum* infection was analyzed, with mice being euthanized 24 hours after i.p. infection with *N. caninum* tachyzoites.

IFN-γ production of samples from adipose tissue of different anatomical locations was under evaluation, regarding differences in production between infected and control mice. As in an acute phase of the infection, the protection is largely mediated by Th1-type response, IFN-γ production by NK cells, $CD4^+$ and $CD8^+$ lymphocytes is very important, in order to control parasite replication and prevent a latent infection through formation of tissue cysts [83]. Here, it was demonstrated that infection with the parasite, already at 24 hours, led to a significant increase in IFN-γ production by adipose tissue stromal vascular fraction cells, indicative that a protective immune response was elicited. In order to confirm that this difference in production of IFN-γ was due to the presence of the parasite in the tissue, an immunohistochemical analysis of both mesenteric and subcutaneous adipose tissue was realized. It was possible to observe the parasite present in the tissue samples removed from both adipose tissues. Therefore, because of the infection of the parasite, an enhanced immune response was observed, with higher amounts of IFN-γ being produced when the mice were infected with *N. caninum*.

Besides the analysis of the acute immune response to the infection, an exploration of how the host may response in case of a long term infection is also important for the understanding of possible long term mechanisms available for the host, such as immunological memory. Thus, mice were infected i.p. with *N. caninum* tachyzoites and euthanized 1 year after infection for analysis. As before, IFN-γ production of samples from mesenteric adipose tissue was evaluated. It was possible to observe that MAT cells recovered from infected mice produced higher amounts of IFN- γ upon stimulation with dead *N. caninum* tachyzoites when compared to cells from non-infected mice. Nonetheless, when colonization analysis was performed on these mice, no traces of the parasite were encountered, possibly explaining the fact that no differences in IFN-γ production were found between the unstimulated samples of infected and non-infected mice. Results regarding *T. gondii*, also an apicomplexa parasite related to *N. caninum*, have demonstrated that immunological memory is crucial, in order to protect the host against reactivation of a chronic infection with this parasite. With mouse models of chronic infection, it has been demonstrated that common protection mechanisms, such as T cells and IFN-γ, are critical in the prevention of a possible reactivation of the infection of *T. gondii* [84]. Herewith, these results indicate that there may be an immunological memory, with immune cells present in the adipose tissue, when re-exposed to *N. caninum*, increasing the production of IFN-γ. Analysis of other mechanisms and production of other cytokines involved in the immune response to the infection may be of interest, in order to unveil possible new ways through which protection may be conferred.

Among the hormones produced by the adipose tissue, leptin is able to affect the immune response in an organism, influencing T lymphocyte survival and development [73], as well as macrophage and monocyte function [85]. Then, its' role in the host response to *N. caninum* infection would be relevant to explore. Previously, it was demonstrated that mice infected i.p. with *N. caninum* tachyzoites presented significantly higher levels of leptin in the serum, at 2 months after infection [5]. Despite this, in the tissues mostly related to leptin production, the adipose tissue, leptin expression did not present significant differences between infected and non-infected mice [5].

Nevertheless, other tissues have been described to produce leptin, such as skeletal muscle, stomach mucosa and placenta [70]. Therefore, in this work we assessed leptin presence in other tissues/organs, so as to identify which could be responsible for the higher serum leptin levels detected in *N. caninum*-chronically infected mice. As demonstrated through immunohistochemical analysis, no differences were found in terms of leptin stained area between infected and non-infected mice.

Also, leptin expression was analyzed in certain tissues that were studied through immunohistochemistry, such as the brain, the muscle and stomach. As previously mentioned, the brain and muscle are known sites of *N. caninum* persistence, and gastric cells have shown a capacity to produce leptin, so these tissues were selected for leptin expression analysis. As this work demonstrates, no differences were found in these tissues regarding leptin mRNA expression between infected and control mice. Therefore, the organs responsible for the rise in leptin levels in serum, at 2 months after infection, are still unknown. Nevertheless, the evaluation of leptin production in these tissues is a possible interesting analysis going forward, as well as the analysis of leptin expression in other tissues. More specifically, the placenta has also been demonstrated to be able to produce leptin, and so the analysis of leptin expression and production in reproductive organs may be of interest.

As 2 months after infection mice demonstrated elevated levels of leptin in serum, when compared with control mice treated with PBS [5], despite no parasitic DNA being detected in the infected mice, in order to understand if the same effects were observed in mice infected for 1 year, leptin serum levels were measured. Results demonstrated that, despite the parasite not being detected at both 2 months and 1 year after infection, infected mice, when compared to control mice, presented significantly higher amounts of leptin in serum. A study regarding *T. gondii* demonstrated significantly higher plasma leptin levels in groups of rats infected with this parasite during a period of 4 weeks [86]. Therefore, these results represent the possible effect that infection with apicomplexa parasites may have on leptin, and general hormonal production. This effect may be observed not only within weeks or months of infection, but even after 1 year, indicating a long term influence of the parasite on the host hormonal production. As leptin has been demonstrated to influence the immune response, a possible link may exist between the immune reaction mounted when the host is infected and the amount of leptin that is produced by the infected host.

6. Conclusion

With this work, it was possible to demonstrate that i.n. immunization with *N. caninum* membrane antigens was protective in a host with an impaired cellular immune response. Nevertheless, the mechanism responsible for this protection is still under study. Moreover, it was also shown here that long-term memory was induced in WT mice, indicating that this immunization procedure is robust.

On the other hand, it was determined that in the adipose tissue of *N. caninum*-infected mice, a proinflammatory response was induced by the parasite, helping to control the infection locally. Also, the immune cells present in the adipose tissue also demonstrated immunological memory, by responding to re-exposure to the parasite antigens through the increased production of IFN-γ.

Finally, it was observed that, while leptin serum levels of infected mice remained increased two months and even 1 year after infection, no differences were encountered in terms of leptin production in the adipose tissue when comparing infected and control mice. As other tissues have also not presented a response into which tissue or organ is responsible for the rises in leptin levels in serum, this source is yet to be found.

7. References

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