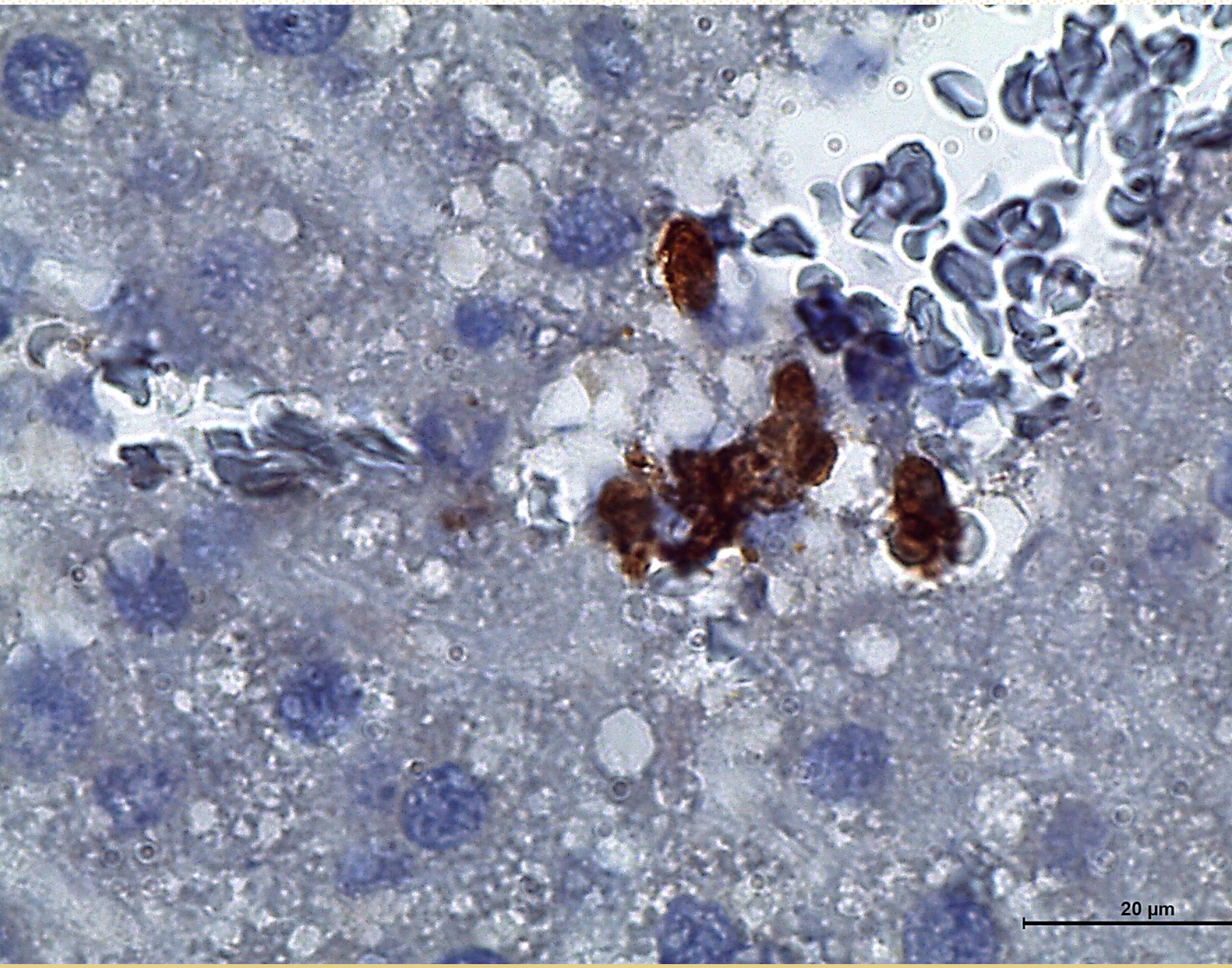

Ricardo Fróis-Martins

ANALYSIS OF THE IMMUNE RESPONSE IN MICE IMMUNIZED WITH ANTIGENS OF THE PROTOZOAN PARASITE NEOSPORA CANINUM

Tese de mestrado em Investigação Biomédica

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ANALYSIS OF THE IMMUNE RESPONSE IN MICE IMMUNIZED WITH ANTIGENS OF THE PROTOZOAN PARASITE NEOSPORA CANINUM

Dissertation for applying to Master´s Degree in Biomedical Research – Specialization in Immunity and Infection submitted to the Faculty of Medicine – University of Coimbra

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Abstract

Neospora caninum is an obligate intracellular parasite that causes neosporosis, a disease that inflicts heavy economic losses in dairy and beef cattle farms worldwide. No commercial vaccine is currently available for neosporosis. An intranasal immunization approach, using *N. caninum* antigen extracts enriched in membrane proteins plus CpG adjuvant was previously shown to confer protection against intragastrically established neosporosis. The obtained results indicated that mucosal-produced IgA mediated the protective effect. To challenge this hypothesis, the elicited immune response and protective effect conferred by the i.n. immunization was assessed here in mice infected by the intraperitoneal route, thus overcoming the mucosal barrier. In the immunized mice, the parasitic burden was found significantly reduced as early as 24 h post i.p. infection. Leukocytes obtained from lymphoid and parenchymal organs of the immunized mice produced high levels of interferon-γ and interleukin-17A in response to *in vitro* antigen recall stimulation. Furthermore, mRNA transcripts encoding immunity-related GTPases, IRGm1, IRGm3 and IRGb6, were found elevated in the lungs and IRGm3 mRNA expression was increased in the liver of immunized mice. The protective effect of GTPases was confirmed in mice harboring macrophages insensitive to IFN-γ. These mice showed higher parasite burdens than WT counterparts with no differences found between immunized and control groups. mRNA transcripts encoding GTPases were significantly decreased in the mice with impaired IFN-γ responsiveness. Altogether, our results indicate that the intranasal immunization approach induces Th1/Th17-type immune responses and is also effective in protecting mice when infection was systemically established, possibly through IFN-γ-induced GTPases.

Key words: *Neospora caninum*; Immunization; Mucosa; Antibodies; T cells

Resumo

Neospora caninum é um parasita intracelular obrigatório que causa neosporose, uma doença que inflige enormes perdas económicas na produção bovina de leite e de carne, em todo o mundo. Até ao momento, não se encontra disponível nenhuma vacina comercial para a neosporose. Foi previamente desenvolvida uma estratégia de imunização intranasal, usando um extrato antigénico rico em proteínas de membrana de *N. caninum* e utilizando CpG como adjuvante, que mostrou conferir proteção contra a neosporose estabelecida pela via intragástrica. Os resultados previamente obtidos indicaram que a IgA produzida em mucosas poderia mediar a proteção observada. De forma a explorar esta hipótese, avaliámos a resposta imunológica e o efeito protetor conferido pela imunização intranasal em murganhos infetados pela via intraperitoneal, ultrapassando assim a barreira da mucosa. Os murganhos imunizados apresentaram uma redução significativa na carga parasitária 24 h após o desafio com parasitas pela via intraperitoneal. Leucócitos provenientes de órgãos linfoides ou parenquimatosos de murganhos imunizados produziram elevados níveis de interferão-γ e interleucina-17A em resposta a uma reestimulação *in vitro* com antigénios parasitários. Além disso, transcritos de mRNA que codificam as GTPases induzidas por IFN-γ, IRGm1, IRGm3 e IRGb6 foram detetados em níveis elevados nos pulmões e, no caso da expressão de IRGm3, no fígado de murganhos imunizados. O possível efeito protetor das GTPases foi também sugerido utilizando murganhos com macrófagos insensíveis ao IFN-γ. Comparados com os murganhos da estirpe selvagem estes ratinhos possuíam maiores cargas parasitárias, sem apresentarem, contudo, diferenças significativas entre os grupos imunizado e controlo. No seu conjunto, os nossos resultados indicam que a estratégia de imunização intranasal desenvolvida, induz uma resposta do tipo Th1/Th17 e confere também proteção contra a infeção estabelecida de forma sistémica, possivelmente através de GTPases induzidas por IFN-γ.

Palavras Chave: *Neospora caninum*; Imunização; Mucosa; Anticorpos; Células T

Index

Abbreviations

Ab	Antibody
Ag	Antigen
APCs	Antigen-presenting cells
BALT	Bronchus-associated Lymph tissue
BSA	Bovine serum albumin
CD	Cluster of differentiation
CpG-ODN	CpG-oligodeoxynucleotides
DNA	Deoxyribonucleic acid
DCs	Dendritic cells
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FcR	Fc receptor
GALT	Gut-associated lymph tissue
γδ T cells	Gamma-delta T cells
GBP	Guanylate binding proteins
GRA	Dense granule
GrzB	Granzyme B
IFN-γ	Interferon-γ
IFN-γ-NR	Macrophages non-responsive to IFN-γ
Ig	Immunoglobulins
IL	Interleukin
ILF	Isolated lymphoid follicles
IRGs	Immunity-related GTPases
LN	Lymph nodes
MALT	Mucosa-associated lymphoid tissue
MIC	Microneme
MLN	Mesenteric lymph nodes
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
N. caninum	*Neospora caninum*
NcCyP	*N. caninum* cyclophilin
NADPH oxidase	Nicotinamide Adenine Dinucleotide Phosphate-oxidase
NALT	Nasopharynx-associated lymphoid tissues
NcHsp70	*N. caninum* heat shock protein 70
NcMIC3	*N. caninum* microneme protein 3
NcMP	*Neospora caninum* membrane proteins
NcSAG1	*Neospora caninum* surface antigen 1
NcSRS2	*Neospora caninum* SAG-related sequence 2
NcT	*Neospora caninum* tachyzoites
NET	Neutrophil extracellular traps
NK	Natural Killer
PAMP	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors

PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
qPCR	Quantitive real-time polymerase chain reaction
rec	Recombinant
ROP	Rhoptries
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNF-α	Tumour necrosis factor α
TLR	Toll-like receptor
T. gondii	*Toxoplasma gondii*

Introduction

Neospora caninum is an obligated intracellular parasite that was recognized in 1984 as a causative agent of neuromuscular disorder in dogs. Classified as a species in 1988, *N. caninum* can infect a wide range of animal hosts including some of veterinary interest, such as cattle. The parasite transmission in cattle can occur via the gastrointestinal tract or transplacentally, being the last route the most frequent and effective. The common outcome of this transmission is a seropositive born fetus. However, neosporosis also induces a high abortion rate which leads to huge economic losses in beef and milk industries. Although several vaccine candidates are currently being studied, to date, no commercial vaccine is available to prevent neosporosis. Our group successfully developed an experimental vaccination approach that proved successful in the murine host. This approach was based on mucosal vaccination using membrane proteins from *N. caninum* tachyzoites as target antigens and CpG-OND as adjuvant. However, the mechanisms underlying the observed protection were not yet completely characterized.

Life cycle, infection transmission and economic impact

N. caninum presents a heteroxenous life cycle which implies infection of more than one host in the life cycle (Fig. 1). The definitive hosts are wild dogs, coyotes, gray wolves and dingoes, and the intermediate hosts can be diverse and include chicken and cattle[1,2]. During the life cycle the parasite displays three known infectious stages: sporozoites, tachyzoites and bradyzoites[3]. Parasite sexual replication occurs inside of acutely infected definitive hosts, generating oocysts in intestinal epithelial cells which are expelled through feces in an unsporulated non-infectious form[4,5]. Oocyst are an environmental resistant form of the parasite that may undergo sporulation 24 h to 72h after excretion[2]. Intermediate hosts ingesting food or water contaminated with sporocysts may then become infected - horizontal transmission[2]. Once inside of gastrointestinal tract, sporozitoides released by sporocysts parasitize the intestine and differentiate into tachyzoites[2]. *N. caninum* tachyzoites (NcT) are able to infect a wide range of nucleated host cells[2]. Under host immune pressure, tachyzoites can

differentiate into bradyzoites, a tissue cyst quiescent life stage, usually located in the central nervous system and skeletal muscles[6]. This quiescent stage causes a chronic assymptomatic infection[6]. Ingestion of infected tissues, containing tachyzoites or bradyzoites, by dogs or other definitive hosts complete the *N. caninum* life cycle.

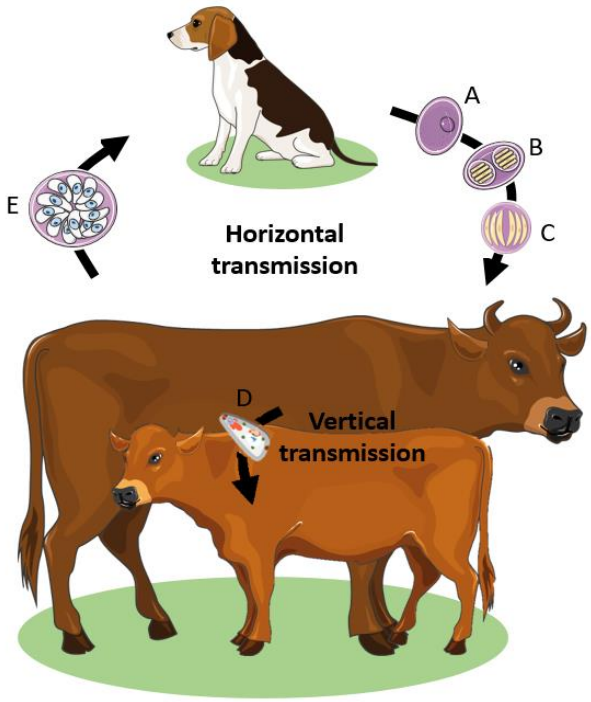

Figure 1- *N. caninum* heteroxenous life cycle. The definitive host are canids and the intermediate host are herbivores, such as cattle and sheep. Canids shed to the environment *N. caninum* oocysts (A). The oocysts undergo sporulation (B) originating sporocysts (C). Intermediate hosts, such as cows, ingest the sporocysts and become infected. Inside the host, sporocysts differentiate in tachyzoites, a fast replicative stage of parasite. Tachyzoites can cross the placenta and infect the fetus (D). Under host immune pressure, tachyzoites differentiate into bradyzoites, a quiescent stage of the parasite which forms cysts in brain and muscle. To complete the life cycle, canids should eat infected tissues containing *N. caninum* bradyzoite cysts. This figure was adapted using a template on the Servier Medical Art website (www.servier.com) licensed underthe Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/).

Vertical transmission, the most frequent and efficient mode of *N. caninum* transmission, can be divided in two different mechanisms depending on the time point of infection, i.e. exogenous and endogenous transplacental transmission[7]. Endogenous transplacental transmission occurs when infection in chronic infected cattle is reactivated during pregnancy. In this case, immune suppression caused by pregnancy allows bradyzoites to recrudescence into the tachyzoite stage leading to a new spread in tissues including uterus, placenta and, hence, fetus[8,9]. Exogenous transplacental transmission, on the other hand, occurs with infection of naïve cattle during pregnancy by horizontal transmission. *N. caninum* will then spread to dam tissues and pass to the fetus through the placenta. Since the fetus is only immunocompetent in the third

trimester of gestation, the pregnancy stage at which infection occurs can dictate the outcome of it. The higher percentage of unborn fetuses occurs in the second gestational trimester[8]. The median losses due to abortions induced by *N. caninum* were estimated to be more than $1,298,3 million per *annum*[10]. Approximately two thirds of the losses occur in dairy industries and the other third in beef industries[10].

Host cell invasion and temporal distribution

N. caninum invasion of host cells is dependent on the host cell cytoskeleton and it is a process that requires parasite metabolic energy, as well as the parasite actin/myosin system[11,12,13,14]**.** The parasite host cell´s invasion starts with an initial low affinity, not orientated, parasite-host contact mediated by two major immunodominant *N. caninum* surface protein receptors, *N. caninum* SAG related sequence 2 (NcSRS2) and *N. caninum* surface antigen 1 (NcSAG1)[15,16]. Not all NcT that interact with the host cell membrane will invade the cell[17]**.** The parasite reorientates itself and discharges proteins from microneme (MIC), dense granule (GRA) and rhoptry (ROP) organelles at the apical end[8]. MIC proteins are membrane bound molecules, such as *N. caninum* microneme protein 3 (NcMIC3), or soluble molecules capable of forming protein complexes. NcMIC3 has exposed conservative adhesion domains, epidermal growth factor (EGF)-like domains, that interact with sulfated glycans of putative host cell receptors, such as sulfated glycosaminoglycan, establishing a strong physical parasite-host interaction[11,18].

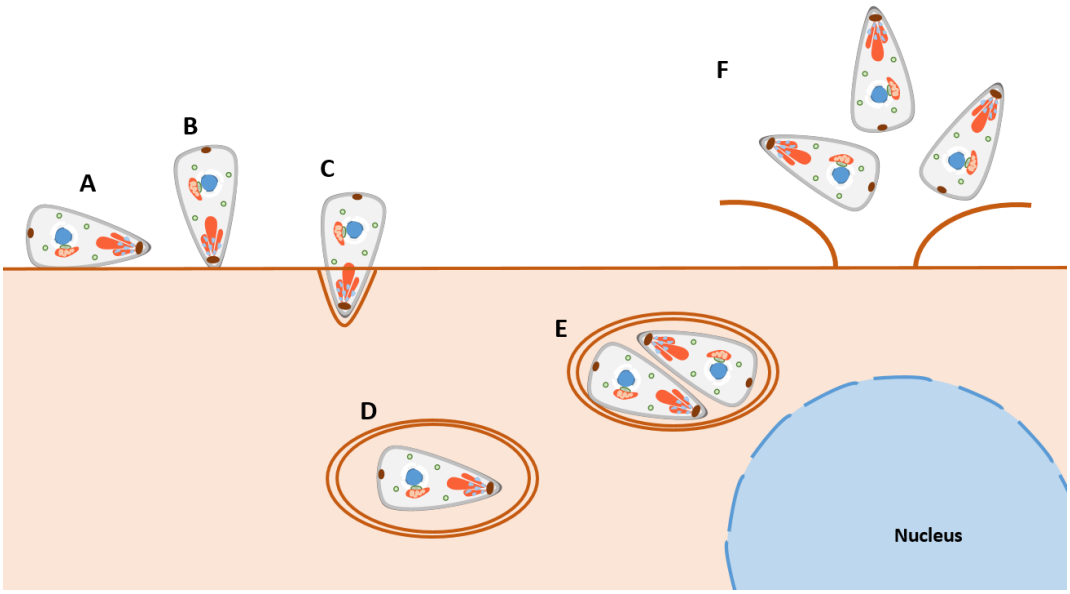

Figure 2- Host cell invasion. NcT attach to the host cell in a low-affinity and not orientated contact (A). NcT reorientate themselves and discharge proteins at the apical end. MIC proteins allow a strong parasite-host adhesion (B). NcT invade the host cell (C) and create a parasitophorous vacuole membrane (D) within which NcT will replicate (E). After several NcT replications, the host cell lyses and parasites are released (F) and infect surrounding cells.

The formation, installation and maturation of the parasitophorous vacuole (PV), in which the parasites will replicate is mediated by ROP and GRA components released during the invasion process (Fig. 2)[8,19]. The active protozoan replication causes host cell lysis and NcT are released, promoting infection of surrounding cells. This progressive cycle can lead to lesion formation and further clinical disease[2,20]. In mice, the clinical signs of neosporosis are associated with parasite load in the brain and consist of development of rough hair coats, weight loss, and neurological signs, such as head tilting, pelvic limb weakness, and impairment of movement[21,22,23].

Acute infection in intermediate hosts is considered to occur in the first thirteen days upon the parasitic invasion and can be divided in a first period (up to 7 days) and a second period (5-7 days to 13 days)[21]. The first period consists of parasite spread to blood and organs such as the spleen, liver and lungs (Fig. 3) [21]. After reaching the peak of parasite burden at day 1 to 3, these organs and blood start losing gradually the parasite burden until 5 to 7 days coinciding with host immune response development[21]. In the second period, almost all host tissues are clear of parasites, except the lungs and the brain[21]. NcT is only cleared from the lungs at 11 to 13 days, therefore this parasitic infection has the lungs as a target organ in acute phase while in the chronic phase the target organ is the brain[21]. *N. caninum* is detected in brain from 5 to 7 days post infection[21] and this organ has high parasite burdens up to day 28 post infection[21]. After this time the parasite burden starts to decrease and low levels are maintained over time[21].

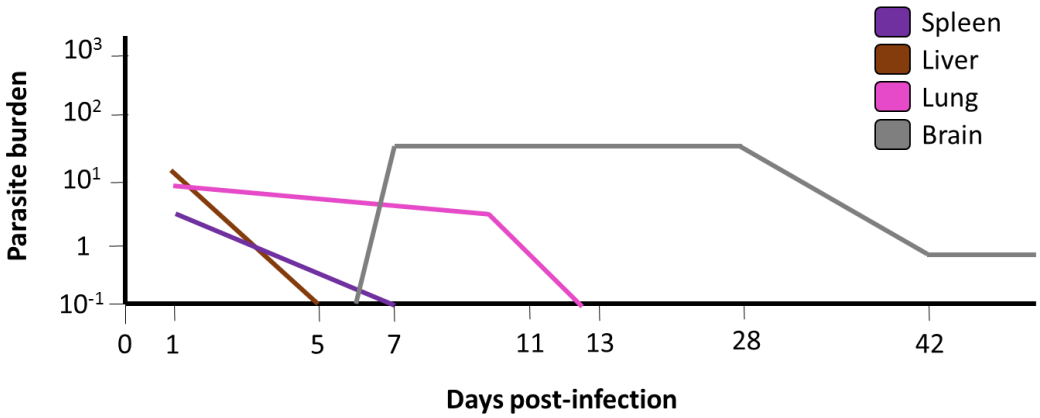

Figure 3- Temporal distribuition of *N. caninum* burdens in experimentally infected mice. At day 1 of infection, lungs, spleen and liver present the highest levels of parasite burden. Parasite clearence in the spleen and liver occurs at 5 and 7 days, respectively, while clearence in the lungs only occur after day 11. From day 6, brain presents increasing parasite burden, which is maintained detectable over time. This figure was based on data from Esther Collantes-Fernández, et al, 2006.[21].

Immune response at the intestinal mucosa

The intestinal mucosa is largely exposed to the external environment. Organ homeostasis is maintained by an epithelial cell layer, a physical barrier which allows selective uptake of nutrients and protects the intestine against pathogens, such as *N. caninum,* which are acquired by oocyst ingestion[24]. *N. caninum* infects the gastrointestinal tract and disseminates throughout the body leading to an acute infection. The resistance to the acute phase is mainly mediated by pro-inflammatory cytokines characteristic of Th1-type adaptive immunity, such as IFN-γ[25,26]. The hosts that resist an acute infection may develop a latent chronic infection[27].

Innate immunity

The first step for intestinal parasite colonization is the attachment to and invasion of host epithelial cells. Besides their function as a physical barrier, epithelial cells can initiate a non-specific immune response when exposed to pathogens[24]. Enterocytes recognize highly conserved molecules of pathogens, generally designated as pathogen-associated molecular patterns (PAMPs), and secrete cytokines and chemokines, such

as IL-8[24]. This recognition is majorly mediated by Toll-like receptors (TLRs), a family of pattern recognition receptors (PPRs)[28]. IL-8 is a chemokine that attracts several immune cell types such as neutrophils, antigen-presenting cells (APCs), monocytes and T cells, resulting in the establishment of a potentially protective immune response and pathogen clearance[24].

Neutrophils are the most abundant leukocytes circulating in the blood and one of the first cell types to arrive at the site of infection[29,24]. The trafficking can be mediated by diverse chemokines and chemokine receptors[24]. Upon contact with pathogens, neutrophils can phagocytose them and produce reactive oxygen species (ROS) through the NADPH oxidase complex or degranulate[29]. In addition, neutrophils can produce antimicrobial extracellular networks (NETs) which immobilize the pathogens[29,30,31]. In mice, depletion of neutrophils at the time of infection led to the impairment of Th1-type cytokine production and increased susceptibility to *Toxoplasma gondii*, a protozoan closely related to *N. caninum*, indicating an important role of neutrophils in parasite replication control[32].

Infected epithelial cells secrete IL-15, a cytokine that, as well as IL-12, IL-18 and IL-2, stimulates natural killer (NK) cell activation[33,24]. NK cells are early producers of IFN-γ and exhibit cytotoxic activity by a perforin-dependent mechanism. Therefore, NK cells help on parasite clearence.[34,35]

IFN-γ is a key pro-inflammatory cytokine that contributes to the stimulation of dendritic cells (DCs) and macrophages[28,36]. DCs and macrophages can recognize *N. caninum* through TLRs, i.e, TLR2, TLR9 and TLR11, and produce IL-12 early upon infection eliciting a host protective immune response[37–43]. Macrophages can control *N. caninum* infection via nitrate and nitrite production and can promote inflammation by activation of vascular endothelium and recruitment of neutrophils through tumor necrosis factor α (TNF-α) and IL-8 secretion[36,44,45]. DCs are important to bridge innate and adaptive immunity[46]. DCs capture antigens (Ag) at the site of infection (gut) and migrate to the T cell area of mesenteric lymph nodes (MLNs) to activate T cells[28,43].

Adaptive immunity

Activation of innate immune cells at the site of infection is followed by Ag presentation and activation of Ag-specific T and B cell in inductive sites (Fig. 4). The mucosal inductive sites are organized mucosal-associated lymphoid tissue (MALT) and draining lymph nodes[47]. MALT comprises several anatomical compartments such as gut-associated lymphoid tissues (GALT), bronchus-associated lymphoid tissues (BALT) and nasopharynx- associated Lymphoid tissues (NALT) diffused along the mucosal tissue surface[47,48]. GALT is constituted by Peyer´s patches (PP) and isolated lymphoid follicles (ILF), two important inductive sites for mucosal B and T cells[47]. Primed by DCs, naïve T cells may become memory and effector cells that migrate to infection sites.

The important role of T cells in *N. caninum* infection scenario was highlighted by Tanaka *et al.*[26]. These authors have shown that mice depleted of $CD4^+$ and $CD8^+$ T cells, using monoclonal antibodies (mAb), succumbed within 30 days to neosporosis. Both T cell populations are producers of IFN-γ in early stages of infection. However $CD4^+$ T cells dominate IFN-γ cytokine production later on[49,50]. IFN-γ, as well as type I interferons, IFN-α and IFN-β, elicit gene expression via JAK/STAT dependent and independent signaling in immune and non-immune cells such as macrophages, epithelial cells and fibroblasts, resulting in a diversity of activities mediating host defense[51]. IFN-γ induces expression of Immunity-related GTPases (IRGs), a family of proteins important to resistance against intracellular bacteria and protozoa[53]. IRGs can interact with guanylate-binding proteins (GBPs), also belonging to interferon-inducible GTPases family, and together accumulate in PVM, promoting its disruption and pathogen destruction[52,53]. Additionally, IRGs can enhance phagosome maturation and also induce autophagy[54]. Upregulated expression of IRGs was detected in the spleen and brain of *N. caninum*-infected mice 7 days upon-infection[55]. In the absence of $CD8^+$ T cells the levels of IRGs mRNA in infected mice were significantly lower indicating an important contribution of IFN-γ produced by $CD8^+$ T cells in IRGs-mediated protective mechanisms in the acute phase of infection[55]. $CD8^+$ T cells may

also have cytotoxic function in the infected host that seemed nevertheless irrelevant in *N. caninum* acutely infected mice[55].

Although $CD8^+$ T cells were shown to have a protective role in *N. caninum* infection, $CD4^+$ T cell subsets confer greater immune protection[26]. $CD4^+$ T cells are the main source of IFN-γ produced after the early stages of infection[34]. *N. caninum* cyclophilin (NcCyP), a microbial protein present in NcT, appears to act as a major component of the parasite in inducing IFN-γ production[56]. In addition, in *N. caninum* infected cattle, cytotoxic $CD4^+$ T cells were described, which operate via perforin/granzyme degranulation[57]. In bovines, macrophages primed with soluble *N. caninum* Ag can induce polarization of bovine $CD4^+$ T cells into IL-$17A^+$ $CD4^+$ T cells in an IL-6-dependent manner[58]. Higher levels of IL-22 and IL-23R mRNA indicate development and stabilization of Th17 cell phenotype[58]. IL-17A is a pro-inflammatory cytokine that mediates Th17-type immunity[59]. This cytokine has a pleiotropic function in the immune response depending on the target cell populations[59]. IL-17A can enhance tight junction synthesis in epithelial cells, promoting integrity of intestinal barrier during homeostasis or infection, as well as inducing the production of antimicrobial agents by epithelial cells, neutrophils and macrophages[60]. Gamma-delta (γδ) T cells are another source of IL-17A[60]. Although γδ T cells are present at low frequencies in mice, bovines have high proportions of this type of T cells[61]. γδ T cells can express WC1, a cluster of proteins which act as a PRRs recognizing PAMPs potentiating a specific response through TCR[62]. WC1 proteins can be expressed in different combinations that allow γδ T cells to recognize different pathogens and become active, therefore γδ T cells are independent of MHC/TCR interaction[62,63]. *N. caninum* infected fibroblasts are capable of inducing polarization of bovine γδ T cells in to IL-$17A^+$ γδ T cells via IL-6 and TGF-β1[64]. IL-$17A^+$ γδ T cells have a protective role against the parasite which is mediated by cell-cell contact with infected fibroblasts[64]. Thus, IL-17A may have a role in controlling of *N. caninum* infection.

Humoral immunity

Humoral immunity has been implicated in protection against of all kind of pathogens, including parasites[65]. This type of immune response has B cells as effector cells, and is characterized by antibodies (Ab) production and secretion[65]. Ab are soluble proteins also designated immunoglobulins (Ig) that present, in the one hand, affinity to small sequences of pathogen proteins and, in the other hand, affinity to immune cell receptors, namely Fc Receptors (FcR) [65]. The Ab-FcR interaction can induce many protective effects in the immune response, such as to (a) promote activation of DCs and increase of their ability to activate T cells; (b) induce release of cytokines by APCs; (c) promote phagocytosis of the parasite and target them to the lysosome pathway through opsonization[65]. In *N. caninum* infection, antibodies can have the ability to interfere with parasite-host interaction due to their affinity to *N. caninum* surface proteins or MIC secreted proteins, hence impairing host cell invasion. *N. caninum* infected animals present antibodies specific for the parasite[21,66–69]. BALB/c mice infected via the intraperitoneal route with NcT show an acute response characterized by early and rapid development of immunoglobulin-secreting splenic B cells, as well as a significant increase of serum *N. caninum*-specific antibodies, namely, IgM, IgG2a and IgG1 isotypes[21,70]. IgG2a are the predominant antibodies in the serum at the second and third week of *N. caninum* infection and IgG1 antibodies predominate afterwards[21]. IgG2a and IgG1 are respectively associated with Th1- and Th2-type immune responses[71]. Mouse IgG2a showed to be superior in complement activation and more efficient in interaction with Fc receptors when compared with IgG1 [72]. Despite this evidence, the role of immunoglobulins in protozoan protection is not fully understood.

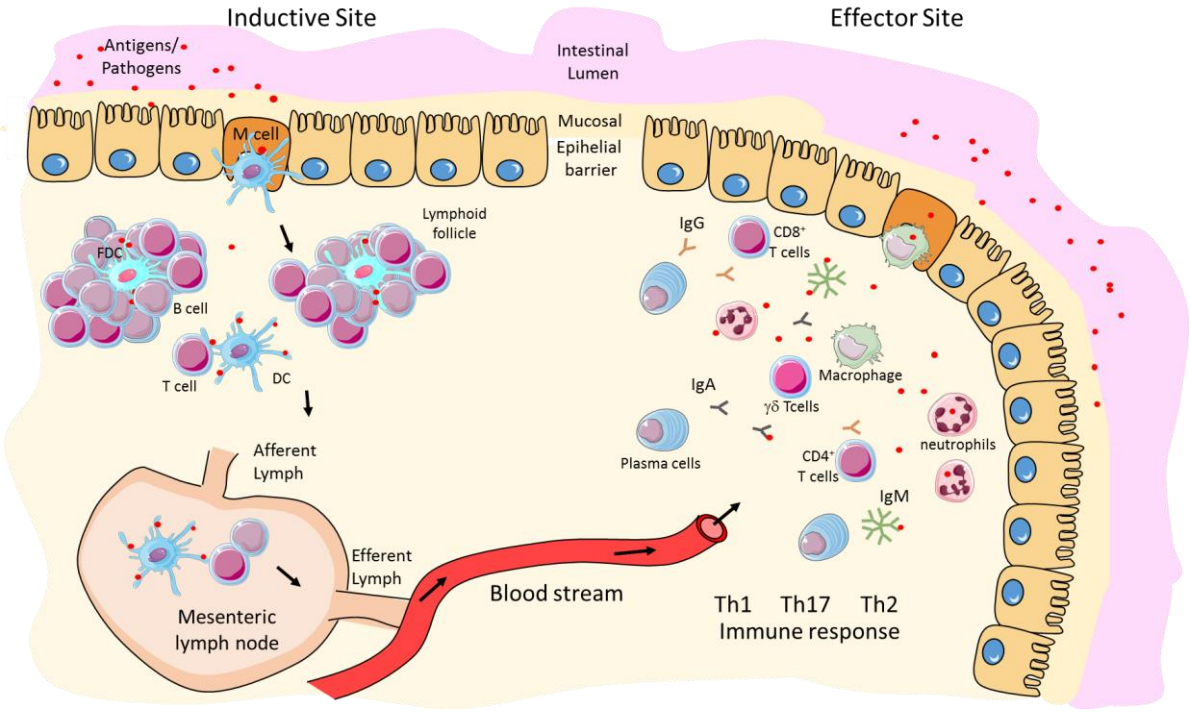

Figure 4- Description of intestinal immune system. Inductive sites are composed by Peyer's patches and isolated follicles (region rich in B cells and T cells). M cells allow antigens present in intestinal lumen to cross the epithelial barrier into the lamina propria and contact with immune cells. DCs and Follicular DCs uptake the antigens and prime T and B cells in Mesenteric Lymph nodes (MLN) or in isolated follicles. Activated T and B cells express specific receptors allowing the homing to effector sites. In effector sites, innate immune cells, such as macrophages and neutrophils, and adaptive immune cells come together to kill pathogens. This Figure was adapted using a template on the Servier Medical Art website (www.servier.com) licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/).

Mucosal vaccination

The great economic impact caused by *N. caninum* is the main reason for the development of a vaccine that could prevent parasite transmission and infection[10]. *N. caninum* has the gastrointestinal tract as an entry point and uses gut mucosa to replicate and disseminate throughout the body, resulting in acute infection. Therefore, a vaccine that confers protection in the mucosa and systemically is thought to be required to prevent parasite infection. Mucosal immunization, unlike the parenteral vaccination, can induce both protective mucosal and systemic immunity[73,74,75].

Adjuvants

Antigen delivery in mucosal routes may present some limitations, such as the antigen uptake and presentation, or even lower antigen immunogenicity [75]. To overcome this difficulties, adjuvants can be used to achieve an effective and protective immune response[76]. These are molecules or formulations that can have immunostimulatory or delivery system properties[75,77]. Immunostimulatory adjuvants, such as lipopolysaccharides, bacterial enterotoxins and bacterial DNA, can modulate and guide the immune response through TLR recognition[75,77]. CpG-oligodeoxynucleotides (ODN), an unmethylated "CpG motif" characteristic of bacterial DNA and not present in mammals, is a very potent adjuvant by interacting with TLR9 expressed by specific immune cells, such as DCs and B cells, promoting both innate and adaptive immune responses[75,78]. The choice of adequate adjuvants is crucial in increasing vaccination efficiency.

Types of vaccines

Prevention of NcT replication and dissemination in cattle, tissue cyst formation and vertical and horizontal transmission are the requirements for an efficient, complete and powerful vaccine against neosporosis. To this purpose, different strategies have been studied that include live-attenuated vaccines, killed NcT lysate-based vaccines, and recombinant proteins vaccines[19].

Live-attenuated vaccines can use attenuated *N. caninum* parasite obtained by several approaches, namely extended number of passages in tissue culture, temperature-sensitive mutants or irradiation in order to suppress parasite proliferation and virulence[79,80]. A vaccine that confers bradyzoite stage protection using a strain of *N. caninum* genetically manipulated to express constitutively a bradyzoite specific-Ag, NcSAG4 protein, is another approach to prevent the establishment of chronic infection[81]. Despite promising results, the possibility of regression to a virulence phenotype, the limited shelf-life, as well as logistical issues, are limiting factors that make live attenuated vaccines of limited feasibility[19].

Killed NcT Lysate is another strategy of immunization. Bovilis Neoguard™ was the first registered and commercialized vaccine[19]. This vaccination which contained tachyzoite lysate as target antigen showed low efficiency in reducing abortion and in vertical transmission protection in dairy cattle[82]. In mouse models, NcT crude lysate had different protective effects, depending on the of different adjuvants, in an antigen dose-dependent manner[83] or even increased the susceptibility to neosporosis[70]. Immunization with NcT lysate antigens when associated with CpG ODN conferred protection against the parasite, whereas NcT excreted-secreted antigens, despite the higher induction of pro-inflammatory cytokines, showed a non-efficient protective response[84]. Thus, parasite lysates have a multitude of proteins with immunogenic properties. Some may induce a protective immune response, while others could have the opposite effect, as shown for the MIC protein NcMIC4[85].

Recombinant (rec) Proteins are another antigen type assayed for vaccination. This strategy has been increasingly investigated in recent years. These proteins are mostly immune-dominant antigens involved in parasite-host cell invasion or are the major surface components of the parasite[19]. Recombinant protein antigens are being studied alone or in antigen combinations aiming at increasing not only the protective response but also the effectiveness of that protection[86–88]. NcMIC1 is a protein implicated in *N. caninum* cell adhesion and invasion[89]. Mice immunized with *N. caninum* recombinant protein NcMIC1, associated with RIBI Adjuvant System, showed lower levels of parasite burden in the brain, specific antibodies to NcMIC1 protein, and did not show clinical neosporosis symptoms 21 days post infection[89]. In combination with recNcROP2 and recNcMIC3, recNcMIC1 immunization induced protection against the parasite in pregnant and non-pregnant mouse models[90]. *N. caninum* recombinant proteins, recNcPDI, recNcROP2 and recNcMAG1 induced different levels of protection dependent on protein administration route[91]. Applied intraperitoneally, recNcROP2 conferred 70% survival rate in parasite challenged mice and recNcPDI and recNcMAG1 respectively conferred 20 and 50% survival rates[91]. When the delivery route was intranasal, the protective effect of recNcPDI immunization was amplified, reaching 90% survival rate, whereas recNcMAG1 lost its protective effect[91]. Protection induced

by recNcROP2 immunization was maintained independently of the administration route[91].

Our group developed an innovative approach to vaccination by using a *N. caninum* extract enriched in membrane proteins (NcMP) that was administered with CpG-ODN adjuvant via the intranasal route[73]. NcMP is an extract enriched in hydrophobic proteins, characterized by a molecular weight below 75 kDa and a neutral or acidic isoelectric point[92]. The immunodominant proteins of NcMP extract were identified as NcMIC1, NcGRA7 and NcHsp70[92]. Intestinal and vaginal lavage fluids from mice immunized with NcMP plus CpG adjuvant (CpG NcMP) showed high titres of parasite-specific IgA[73]. Moreover, immunized mice presented high parasite-specific IgG1 and IgG2a levels in the serum[92]. Elevated NcMP-specific IgA was still detected 4 months after immunization boost[92]. These antibodies were capable of agglutinating NcT in large bodies, presumably preventing parasite-host interaction and hence invasion and progressive infection[73, 92]. NcMP-specific IgG isotype ratio (IgG1/IgG2a) was lower than one in CpG NcMP immunized mice, indicating that this immunization approach promoted a predominant Th1-type immune response bias[73]. In macrophage cultures challenged with NcT, these antibodies reduced parasitic load in a dose dependent-manner, due to their opsonizing ability[73]. *In vivo*, the parasitic load in the brain of mice infected intragastically with NcT, assessed 7 days upon the infectious challenge, was significantly low in CpG NcMP immunized mice comparatively with control groups[73]. Therefore, the developed mucosal immunization approach was shown to protect mice intragastrically challenged with *N. caninum*, a route better mimicking the natural route of infection. The induced protection was maintained in the long-term and was associated with: (a) high level of NCMP-specific immunoglobulins; (b) high production of IFN-γ by spleen and MLN cells of immunized mice when stimulated with *N. caninum* antigens; and (c) low levels of parasitic burden in the brain[92].

Dissertation objectives

Previous results have shown the effectiveness of intranasal vaccination with NcMP plus CpG adjuvant in conferring protection against neosporosis. Although antibodies were implicated in the protective mechanism, the immune response in vaccinated mice was not yet fully characterized. Taking this into account, the present work aimed to:

- Characterize the immune response induced by immunization at an early time point upon infection (24 hours);

- Assess if the intranasal immunization protocol, which confers protection against intragastric infection with *N. caninum*, could also confer protection against an infection established by the intraperitoneal (i.p.) route;

-Identify further immune effector mechanisms that could be contributing for protection induced by the intranasal immunization.

Experimental design

The experimental vaccination approach consisted in immunizing mice twice intranasally followed by an i.p. challenge with 1×10^7 NcT (Fig. 5). Two control groups were used: a non-immunized group treated with vehicle alone, Phosphate Buffered Saline (PBS), and a sham-immunized group treated with adjuvant alone (CpG). The immunized group received NcMP and CpG adjuvant (CpG NcMP). The immunization was performed on days 0 and 21 (boost). *N. caninum* infection was carried out three weeks after the second immunization. The blood, small intestine, lungs, liver, and spleen were collected to evaluate several parameters, such as antigen-specific antibodies levels, cytokine production, gene expression and parasite burden. Cytokine production was also measured in *in vitro* culture supernatants of cells obtained from lungs, liver and spleen and challenged with parasite antigens.

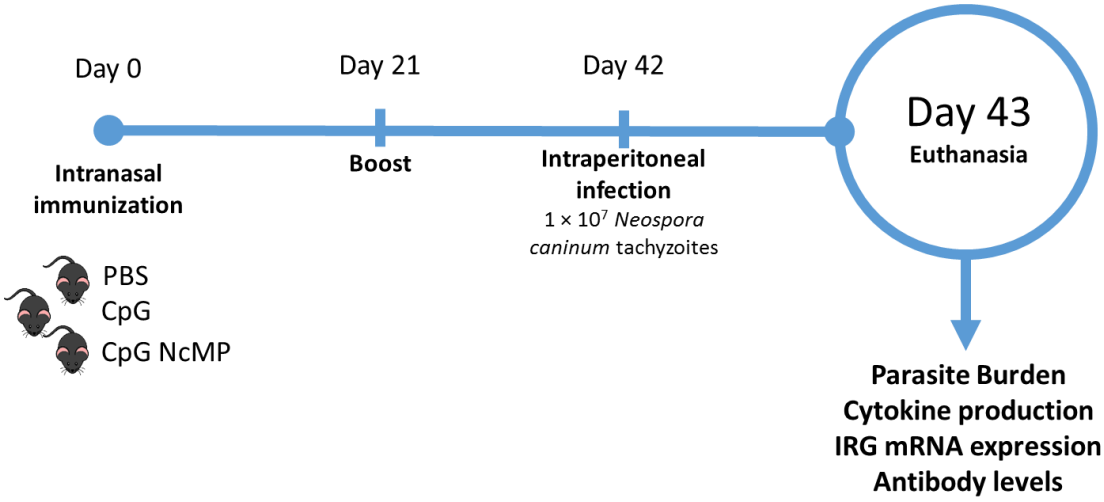

Figure 5- Immunization scheme. Three groups were used: a non-immunized group (PBS), a CpG immunized group (CpG) and the third group, immunized with CpG-adjuvant and NcMP (CpG NcMP). The immunization was performed at day 0 and day 21 (boost). Three weeks after the boost immunization, mice were infected i.p. with 1×10^7 NcT. One day post infection, mice were euthanized and blood, small intestine, liver, lungs, and spleen were collected for further analysis.

Materials and methods

Mice

Female C57BL/6 (B6) mice were obtained from Charles River (Barcelona, Spain). Animals were kept at the animal facilities of the Institute Abel Salazar during the experiments. B6 background mice harboring macrophages non-responsive to IFN-γ [93], (IFN-γ-NR) mice, were bred at the animal facilities of i3S. In these mice, the other cells are able to respond and produce cytokines normally[93]. Animals were kept at the animal facilities of the Instituto de Investigação e Inovação em Saúde (i3S) during the experiments. Mice were kept in pathogen-free conditions at 22 °C on standard 12/12 light dark cycles. *Ad libitum* food and water were provided in individually ventilated cages. Procedures involving mice were performed according to *"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 animals used for scientific purposes" and by Portuguese law (Decreto-Lei113/2013)**.** Experiments were approved by the institutional board responsible for animal welfare (ORBEA) at ICBAS (document 109/2015) and by the competent national board (document 0421/000/000/2016).

Parasites

N. caninum tachyzoites (NC-1 isolate) were cultured and propagated in VERO cells maintained at 37 °C in Minimum Essential Medium (MEM) containing Earle's salts (Gibco: Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (200 IU/ml) and streptomycin (200 µg/ml) (all from Sigma, St Louis, USA) in a humidified atmosphere of 5% CO_2 in air.

Free parasitic forms of *N. caninum* were obtained when infected the host cell monolayer was 80% destroyed. Briefly, using a cell scraper, the culture supernatants and adherent cells were harvested and then centrifuged at 1500 g for 15 min. The pellet was passed 10 times through a 25G needle and washed three times in PBS. Then, the obtained pellet was resuspended with a 25G needle in 3 ml of PBS and passed through a PD-10 column filled with Sephadex™ G-25 M (Amersham Biosciences

Europe GmbH, Freiburg, Germany). Parasite concentration was determined with a hemocytometer.

Extraction of cell-membrane proteins from *N. caninum* tachyzoites

The NcMP were extracted by using a modification of a previously described method[94,95]. Briefly, free tachyzoites were suspended in PBS containing 0.75% Triton X-114 (Sigma) and incubated for 10 min on ice. Then, the preparation was centrifuged at 10000 g for 30 min at 4 °C. The supernatant was recovered, incubated at 30 °C for 3 min, cooled on ice for 10 min and centrifuged at 10000 g for 30 min at 4 °C. The resulting supernatant was placed in a water bath at 30 °C for 3 min and centrifuged at 1000 g for 3 min at room temperature. The aqueous phase was discarded and the NcMP were precipitated with four volumes of absolute ethanol, vortexed vigorously for 15 s and incubated for 1 h on ice. The samples were centrifuged at 12000 g for 20 min at 4 °C. The resulting pellet was air dried and suspended in PBS and stored at -20 °C.

Electrophoretic analysis of *N. caninum* membrane proteins

NcMP were prepared for their use as target antigens in mucosal immunization. After extraction, they were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and the protein migration profile was determined (Fig. 6). Briefly, 10 μg of NcMP were heated for 5 min in loading buffer to denature proteins, prior to electrophoresis. Then, the membrane proteins were loaded on a 4% polyacrylamide stacking gel (pH 6,8) and constant current was applied (25 mA). The separation was made in a 10% polyacrylamide resolving gel (pH 8,8) for approximately 1 h. Gels were then silver stained. Briefly, the gel was fixed with a fixation solution (12% acetic acid; 50% ethanol; 0,05% formalin) for 2 h to restrict protein movement. The gel was washed with 20% ethanol three times to remove remaining detergent and interfering ions, sensitized with 0,02% sodium thiosulfate and briefly washed twice with water before being stained with 0,2% silver nitrate. To reveal the electrophoretic profile of NcMP, the gel was exposed to developing solution (6% sodium carbonate; 0,0004% sodium thiosulfate; 0,05% formalin). The reaction was terminated with 12% acetic acid[96].

N. caninum antigen preparation

NcT were disrupted by sonication (30 cycles of 15 s at 100 W) with a Branson cell disruptor model W 185 D (Emerson Electric Co., St. Louis, MO, USA) in an ice bath to prevent sample heating. The obtained sonicates (NcS) were centrifuged at 5000 g for 15 min at 4 °C, 0,2 μm pore-size filtered, and stored at -20 °C.

Protein quantification

Prepared NcS and NcMP were quantified using the Lowry assay. This method is a biochemical assay that use colorimetric techniques to quantify proteins. The protein concentration is deduced from the concentration of tryptophan and tyrosine residues that reduced Folin reagent. A solution of 0,05% bovine serum albumin (BSA; Sigma) in PBS was used to prepare standard curve samples. Briefly, BSA, NcS and NcMP were each mixed with deionized water and a solution of copper (II) sulfate, sodium carbonate, sodium hydroxide and potassium sodium tartarate. Samples were incubated 10 min in the dark, and then Folin reagent was added. Upon a further 30 min incubation in the dark, the absorbance was measured at 750 nm.

Immunization and infection procedures

Mice were immunized intranasally twice with 3 weeks of intervals (day 0 and 21). Each animal received 20 μl of the immunizing preparation containing 30 μg of NcMP and 10 μg of GpG 1826 VacciGrade (Invitrogen, Carlsbad, CA, USA) (CpG NcMP group). Control animals received 20 μl of PBS alone (PBS group) or PBS with 10 μg of CpG (CpG group). At day 42, mice were infected i.p. with 1×10^7 NcT.

Sample collection

At day 43, 24 h after infection, animals were anaesthetized with isoflurane and blood was collected by retro-orbital bleeding to measure NcMP-specfic IgG in serum. The animals were then euthanized by cervical dislocation. Spleen, lungs and liver were aseptically removed and sectioned. Half of the liver, lungs and spleen were used to isolate immune cells. Brain and the other half of organs were divided and stored at -80 °C with or without 400 μl of NZYol™ (NZYTech, Lisbon, Portugal) (for RNA and DNA isolation, respectively) or formalin-fixed (for histology and immunohistochemistry). To

collect intestinal NcMP-specific IgA, the small Intestine was removed and washed with 2 ml of PBS containing protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Mannheim, Germany). The intestinal fluids were centrifuged twice, first at 4500 g for 15 min at 4 °C and then at 4500 g for 1 h at 4 °C, and stored at -20 °C. Blood samples were collected and allowed to clot at 4 °C overnight. Then, serum was obtained after centrifugation at 9500 g for 15 min at 4 °C and stored at -20 °C.

Antibody detection

The Immunoglobulins IgG1, IgG2a and IgA, were measured by ELISA. Briefly, 96 well plates (Maxisorp, Nunc, Rosklilde, Denmark) were coated with NcMP diluted in PBS (5 μg/ml) overnight at 4°C. Plates were washed three times with TST solution (10 mM Tris, 150 mM NaCl, and 0,05% Tween-20, pH 8), followed by blocking for 1 h at room temperature with 2% BSA TST solution. Plates were always washed three times between steps with TST solution. The samples were successively diluted in 1% BSA TST solution, added to the plates, and incubated for 2 h at room temperature. Diluted secondary antibody conjugated with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL, USA) was added to the plates and incubated for 1 h at room temperature. Immune conjugates were detected and quantified with p-nitrophenyl phosphate (Sigma) diluted in AP Buffer (1 mg/ml). The reaction was stopped with EDTA (0,1M; pH 8) and the absorbance at 405 nm and 570 nm was determined using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) using the Gen5^{TM} software (BioTek). The detection limit was defined with serum from naïve mice. The antibody titres were expressed as the $\log_{10}$ value of the reciprocal highest dilution with an absorbance higher than the value of the naïve serum samples.

DNA isolation and detection of *N. caninum* tachyzoites by quantitative real-time PCR

DNA from the liver and lungs was extracted by using a previous described methodology[25]. Briefly, the organs were homogenized in SE buffer (75 mM NaCl; 25 mM EDTA) and digested overnight at 55 °C after the addition of 1% SDS and 1 mg/ml Proteinase K (USB Corporation, Cleveland, OH, USA). Nucleic acids were then isolated

by the phenol (Sigma)-chloroform (Merck) method, followed by precipitation with 2,5 M ammonium acetate in absolute ethanol. After two washes with 70% ethanol, DNA was air dried and suspended in ultra-pure water. DNA concentration was determined in a Nanodrop ND-1000 apparatus (Thermo Scientific, Waltham, MA, USA)

The parasite burden in the Liver and lungs of infected mice was assessed by a quantitative real-time PCR (qPCR) analysis of the parasite DNA performed in a Corbett rotor gene 6000 system (Corbett Life Science, Sydney, Australia). Product amplification was performed with 500 ng of template DNA using the NZY qPCR Green Master Mix (NZYTech) for the amplification of a 103 bp sequence of the Nc5 region of *N. caninum* genome using the primers NcA 5' GCTACCAACTCCCTCGGTT 3' and NcS 5' GTTGCTCTGCTGACGTGTCG 3' both at a final concentration of 0,2 µM and the florescent probe FAM-CCCGTTCACACACTATAGTCACAAACAAAA-BBQ (all designed and obtained from TIB-Molbiol, Berlin, Germany). The DNA samples were amplified using the following program: 95 °C for 3 min followed by 45 cycles of 95 °C for 5 s and 60 °C for 20 s with green fluorescence acquisition. Results were expressed as log10 mg parasitic DNA per mg of total DNA.

RNA isolation and real-time PCR analysis.

Total RNA was extracted from homogenized lung and liver tissue samples. The homogenates were incubated with clorophorm and centrifuged at 4500 g for 15 min at 4 °C. The aqueous phase was collected and the RNA precipitated with an equal volume of absolute isopropanol for 2 h at -20 °C. After centrifugation at 4500 g for 15 min at 4 °C, the pellet was washed with 70% ethanol and air dried. All RNA samples were recovered in 20-25 µL of nuclease-free H2O and quantified using Nanodrop ND-1000 apparatus (Thermo Scientific). Synthesis of cDNA was performed from 1.5 – 4 µg of total RNA, prepared as described above, in a 20 µl final volume using NZY First-Srand cDNA Synthesis Kit (NZYTech), according to manufacturer's instructions. The PCR program run (25 °C, 10 min; 50 °C, 30 min; 85 °C, 5 min) was performed in a TProfessional Basic Thermocycler (Biometra GmbH, Goettingen, Germany). Real-time PCR was then used for the semi-quantification of Irgm1, Irgm3, Irgb6 mRNA expression with the NZY qPCR Green Master Mix (2x) (NZYTech) in a Rotor-Gene 6000 (Corbett Life Science). Hypoxanthine guanine phosphoribosyl transferase (Hprt) was used as

reference gene. The reaction was performed in a final volume of 10 µl containing 0,2 µM of each specific primer: Hprt forward: ACA TTG TGG CCC TCT GTG TG, Hprt reverse: TTA TGT CCC CCG TTG ACT GA; lrgm1 forward: CTC TGG ATC AGG GTT TGA GGA GTA, lrgm1 reverse: GGA ACT GTG TGA TGG TTT CAT GAT A; Irgm3forward: CTG AGC CTG GAT TGC AGC TT, Irgm3 reverse: GTC TAT GTC TGT GGG CCT GA; Irgb6 forward: TTG CCA CCA GAT CAA GG TCA C, Irgb6 reverse: CAA GGT GAT GTC ATA TTC AGA GAT G (all from Tib Molbiol) and 1× Master Mix plus 1 µL of the newly-synthesized cDNA diluted 1/10. The cDNA amplification was done as follows: denaturation at 95 °C for 5 min, followed by amplification in 35 cycles (95 °C, 10 s; 62 °C, 20 s). Real-time PCR data was analyzed by the comparative threshold cycle (CT) method. Individual relative gene expression values were calculated using the following formula: $2^{-(\text{CT gene of interest} - \text{CT constitutive gene})}$ [97].

In vitro cell cultures

Spleens were homogenized in sterile HBSS (Sigma) and erythrocytes were lysed using ACK solution (0,15 M ammonium chloride, 10 mM potassium carbonate, 0,1 mM EDTA). Lungs and liver were treated with 1 mg/ml of Collagenase D (Roche Diagnostics GmbH), incubated in shaking water bath at 37°C for 30 min and then homogenized and filtered through 100 µm pore-size cell strainers. Homogenized liver samples were treated with Percoll 33% to remove hepatocytes. Both organs were treated with ACK to eliminate erythrocytes. At the end of the homogenizing procedures, splenocytes and cells from the lungs and liver were recovered in RPMI-1640 (Sigma), supplemented with 10% of fetal bovine serum (Biowest, Nuaillé, France), HEPES (10 mM), Penicillin (200 IU/ml) and Streptomycin (200 µg/ml) (all for Sigma), β-mercaptoethanol (0,05 mM) (Merk, Darmstad, Germany). Cells were plated (2×10^5 cells/well) in round-bottom 96 well plates with medium or NcS (25 µg/ml) for 72 h at 37 °C and 5% CO_2. At the end of the incubation period, the plates were centrifuged at 300 g for 10 min at 4 °C, supernatants were collected and kept frozen at -80 °C until further use.

To assess the early producers of cytokines, splenocytes were cultured (1×10^6 cells/well) for 16 h in the presence of NcS (25 µg/ml). 10 µg/ml Brefeldin A (Sigma) were added for the last 5 h. Cells washed in PBS before antibody staining.

Flow cytometry

The expression of cytokines by splenic T cells was evaluated by flow cytometry. Ag stimulated splenocytes were incubated for 30 min on ice with Fixable viability dye (FVD) APC-Cy7-conjugated (eBioscience, San Diego, CA, USA) and washed with PBS. FVD reacts with cellular proteins (amines) staining both Live and Dead cells. Since Dead cells have a damaged membrane, FVD can penetrate the cell and react with intracellular proteins resulting in a more intense staining. After pre-incubation with anti-mouse CD16/CD32 (eBioscience) for Fcγ R blocking for 15 min on ice, splenocytes were surface stained with the following mAb (at previously determined optimal dilution): anti-mouse CD3 Brilliant Violet (BV) 510- conjugated (clone 17A2; BioLegend, San Diego, CA, USA) or Phycoerythrin (PE)-conjugated (clone 145-2c11; BD Biosciences, San Jose, CA, USA), anti-mouse CD4 eFluor 450 conjugated (clone RM4-5; eBioscience), anti-mouse CD8 Fuorescein isothiocyanate- or PE-conjugated (clone 53-6.7; BD Biosciences), anti-mouse CD107a (Lamp-1; BD Biosciences) PE-conjugated, anti-mouse CD44 (clone IM7) Peridinin-chlorophyll protein-cychrome (PerCP-Cy5.5)- or PE-Cy7-conjugated (both from BioLegend), anti-mouse CD62L Allophycocyanin (APC)-conjugated (clone MEL-14; BioLegend). Cytokine production was evaluated by intracytoplasmic staining of splenocytes stimulated with NcS in the presence of Brefeldin A, that were incubated with FVD and surface stained with anti-CD3, anti-CD44 and anti-CD4 or anti-CD8. Following extracellular staining, cells were washed and fixed with 2% formaldehyde in PBS for 25 min at room temperature. Cells were washed with PBS and permeabilized with permeabilization buffer (0,05% saponin, Sigma, in PBS). Intracytoplasmic staining was performed with anti-mouse IFN-γ APC-conjugated (clone XMG1.2; BD Biosciences), anti-mouse TNF-α PerCP-Cy5.5 or FITC-conjugated (clone MP6-XT22; both from BioLegend), anti-mouse IL-17A FITC-conjugated (clone TC11-18H10.1; BioLegend), anti-mouse IL-10 PE-conjugated (JES5-16E3; BD Biosciences), and anti-mouse Granzyme B PE-Cy7-conjugated (clone NGZB; eBioscience) for 30 min at room temperature, after a pre-incubation with anti-mouse CD16/CD32 for 15 min. Cells were washed twice with permeabilization buffer before analysis.

Cytokine assessment in culture supernatants by ELISA

The concentration of the cytokines IFN-γ, IL-17A, IL-10 and IL-4 was determined by ELISA in the supernatants from 3 days-cell cultures of lungs, liver and spleens with or without NcS stimulus. IFN-γ, IL-17A and IL-4 levels were assessed by using Ready-Set-Go! (eBioscience) ELISA kits and IL-10 was quantified using a Mouse IL-10 Duo Set ELISA Development System (R&D Systems, Minneapolis, MN, USA), according to manufacturers' instructions. Briefly, plates were coated overnight with the capture antibodies in PBS. Then, plates were washed and blocked with 1× ELISA/ELISPOT Diluent for 1 h. Standards and samples were added to the plates and incubated for 2 h at room temperature. Afterwards, plates were washed, incubated for 1 h with detection antibodies, followed by a 30 min incubation with Avidin horseradish peroxidase (HRP)-conjugated. At the end, TMB substrate was added and the reaction was stopped with H_2SO_4. Plates were read at 450 nm and 570 nm.

Immunohistochemistry

In order to prepare the samples for histological analysis, each portion of the lungs, liver and brain preserved in formalin were placed in a cassette and immersed in 70% ethanol. Cassettes were placed in a tissue processor, where tissues were immersed in solutions of: 70% ethanol, 80% ethanol, 90% ethanol, 2× in 100% ethanol, 3× in Clear Rite for 1 h each and in 2× Paraffin for 1 h and 20 min each. 4 µm tissue sections of paraffin-embedded organs were mounted on amino-propyl-tri-ethoxy-silane (Sigma) coated slides and incubated at 60 °C for 30 min. To assess the parasite burden, immunohistochemistry of lung and liver tissue sections was performed using a *N. caninum* antiserum (VMRD, Pullman, WA, USA). Slides were dewaxed twice in xylene, 5 min each, and hydrated with sequential passages in 100%, 95%, 70% ethanol, 2 minutes each and 5 minutes in running water. For antigen retrieval, slides were boiled in 10 mM citrate buffer (pH 6) for 3 min, under pressure, in a pressure cooker. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Slides were washed three times with TST and the tissues were delimitated using a hydrophobic pen. Slides were blocked with normal rabbit serum (Dako, Glostrup, Denmark) diluted 1:5 in 10% BSA TST solution for 30 min. Excess serum was removed before incubation with the primary antibody diluted 1:500 in 5% BSA TST for

90 min at room temperature. Slides were washed three times and then incubated for 30 min with the secondary antibody (rabbit anti-goat IgG HRP-conjugated; Millipore, Billerica, MA, USA) diluted 1:500 in 5% BSA TST. All the incubations were done in a humidified chamber to prevent the slides from drying. Slides were incubated for 5 min with diaminobenzidinetetrahydrochloride (DAB) substrate (Dako) and immediately washed in running water for at least 10 min. The activity of HPR forms a colored and insoluble precipitate upon addition of DAB substrate. Finally, slides were lightly counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany), followed by a 15 min wash in running water. Tissues were then dehydrated with ethanol (70%, 95%, 100% ethanol, 2 min each) and diaphanized twice with xylene, 5 min each and mounted in Entellan® mounting medium (Merck). Slides were analyzed under light microscopy. A positive reaction was indicated by the presence of brown staining.

Statistical analysis

Statistical analysis was performed using GraphPad prism, Version 6.0 (GraphPad Software, Inc, La Jolla, CA, USA). Statistical analysis between groups was done by using one-way ANOVA followed by the Tuckey's post hoc test or unpaired T-student test depending on the number of groups compared.

Results

Electrophoretic profile of N. caninum membrane proteins

The electrophoretic profile of the extracted NcMP was assessed by SDS-PAGE and compared with other NcMP samples available in the laboratory. As shown in Figure 6, the obtained protein band profile of the extracted NcMP was similar to the ones used for comparison and with the profile previously reported by Ferreirinha et al. 2013[73]. As can be observed in Figure 6, the electrophoretic profile of these hydrophobic proteins is clearly different from the one exhibited by the hydrophilic proteins separated during NcMP extraction.

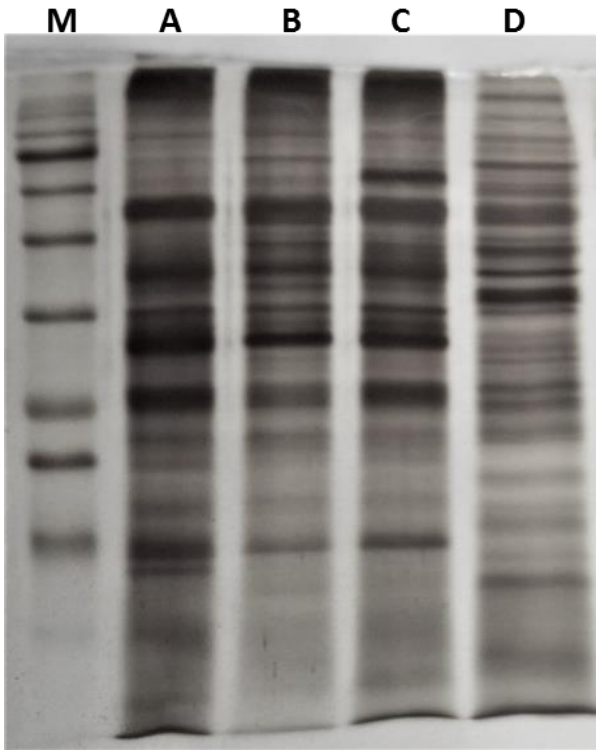

Figure 6- Electrophoretic profile of *N. caninum* membrane proteins. The extract enriched in *N. caninum* membrane proteins (NcMP) was obtained from *N. caninum* tachyzoites. The electrophoretic profile of the prepared NcMP (A) was compared with two different NcMP samples previously prepared (B and C) and with a substrate enriched in hydrophilic proteins collected during NcMP extraction (D). M- molecular weight marker.

Higher levels of NcMP-specific antibodies in immunized mice

Production of antibodies is the hallmark of specific immune responses[98]. Therefore, the levels of IgG1 and IgG2a in the serum of immunized and sham-immunized and IgA in the intestinal lavage fluids were assessed by ELISA. As shown in Figure 7, elevated levels of NcMP-specific IgG1, IgG2a and IgA were detected in immunized mice. Control groups showed lower levels of all NcMP-specific antibodies (Fig. 7).

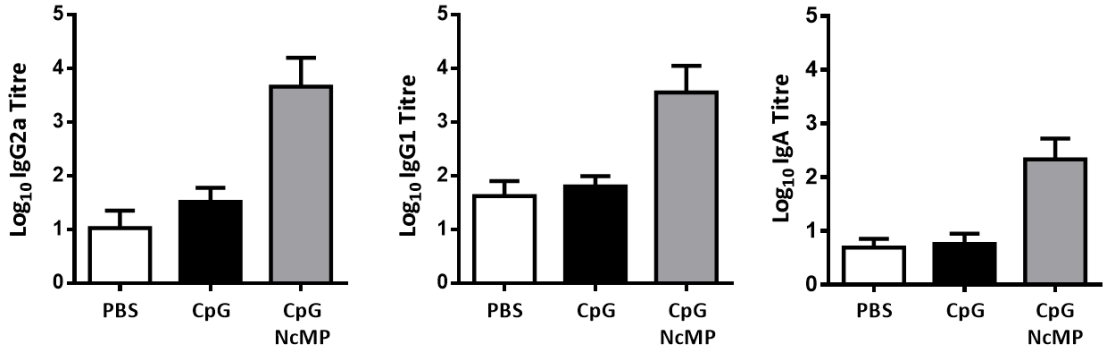

Figure 7 - Higher levels of NcMP-specific antibodies in the serum and intestinal lavage fluids of immunized mice. Levels of NcMP-specific serum IgG2a and IgG1 and of intestinal lavage fluids IgA measured by ELISA. Data is presented as log10 of the antibody titres. n=One way-ANOVA followed by the Tuckey's post hoc test. Results correspond to pooled data from two independent experiments.

Increased antigen-specific CD4⁺ and CD8⁺ T cells cytokine producers and CD8⁺ T cells Grz B producers in the spleens of immunized mice

$CD4^+$ and $CD8^+$ T cells play a critical role in the protection against *N. caninum*[26,99]. Therefore, *in vitro* antigen-stimulated production of pro-inflammatory cytokines was assessed in splenic $CD4^+$ and $CD8^+$ T cells collected from immunized mice and controls. Splenocytes were isolated and cultured in the presence or absence of NcS for 16 h, the last 5 h with added brefeldin A. The T cell populations were gated as shown in Figure 8.

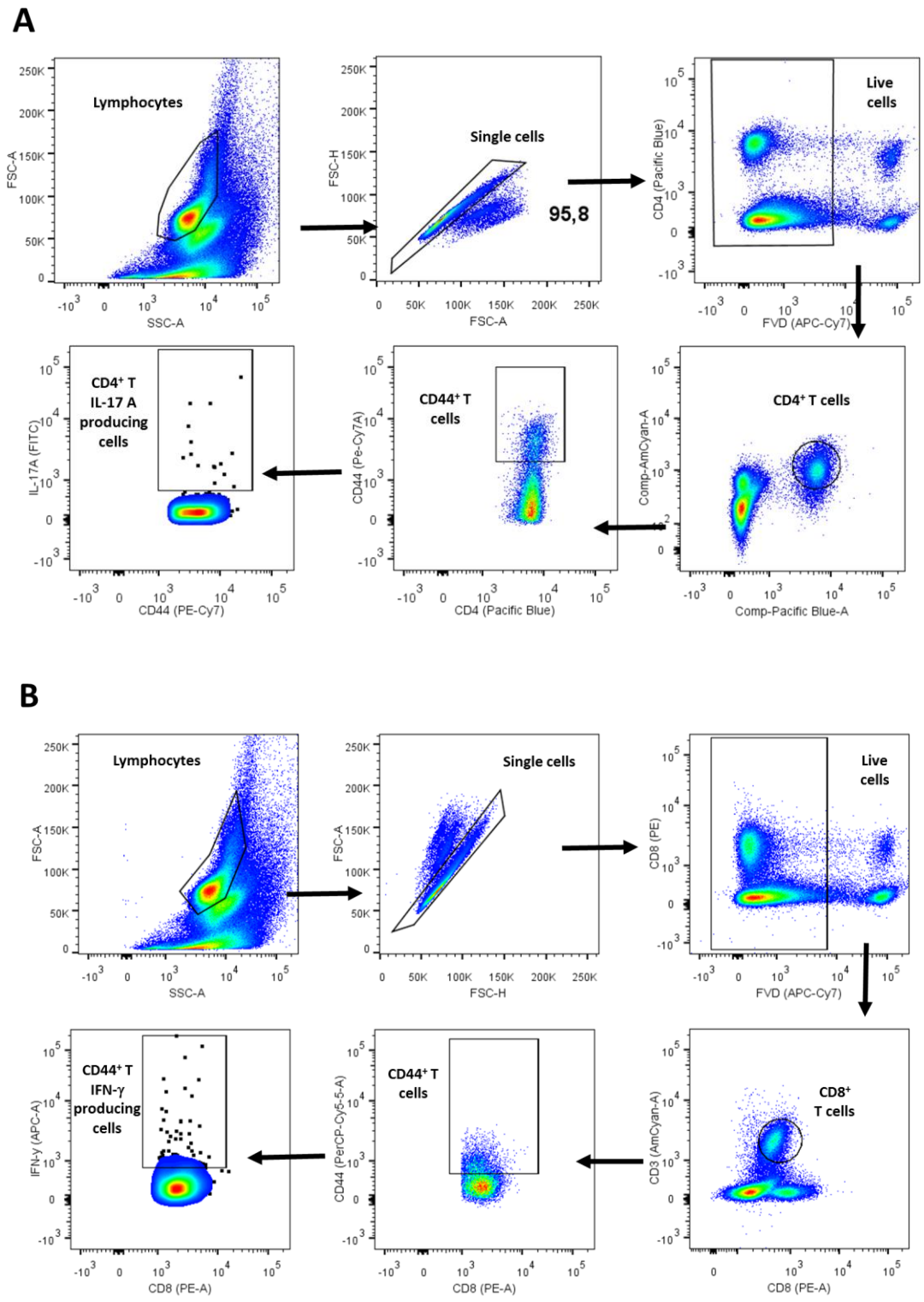

Figure 8 - Gating strategy used to analyze populations producing pro-inflammatory cytokines. Representative example showing the sequential gating strategy used to identify (A) T $CD4^+CD44^+$ IL-17A-producing cells or (B) T $CD8^+CD44^+$ IFN-γ-producers from the spleen of an immunized mice (CpG NcMP), 24 h upon infection, cultured with NcS for 16 h with addition of brefeldin A at the last 5 h. Lymphocytes were gated according to forward scatter (FSC) and side scatter (SSC) parameters. Live cells were gated in the FVD negative cells.

As can be observed in Figure 9A, higher proportions of $CD4^+CD44^+IFN\text{-}\gamma^+$, $CD4^+CD44^+IL\text{-}17A^+$, and $CD4^+CD44^+TNF\text{-}\alpha^+$ T cells were detected in the spleens of immunized mice when compared to those of sham-immunized mice. Also, $CD8^+CD44^+IFN\text{-}\gamma^+$ and $CD8^+CD44^+GrzB^+$ T cells were found in higher proportions in the spleens of immunized mice than in sham-immunized mice (Fig. 9B). Accordingly, cell numbers of these populations were higher in the spleens of immunized mice, comparatively to those of controls. Namely, $CD4^+CD44^+IFN\text{-}\gamma^+$ (CpG NcMP: $17{,}237\times10^3 \pm 8{,}388\times10^3$ *vs* CpG: $6{,}044\times10^3 \pm 4{,}071\times10^3$), $CD4^+CD44^+IL\text{-}17A^+$ (CpG NcMP: $6{,}374\times10^3 \pm 3{,}224\times10^3$ *vs* CpG: $1{,}317\times10^3 \pm 1{,}075\times10^3$; $P< 0{,}05$), $CD4^+CD44^+TNF\text{-}\alpha^+$ T cells (CpG NcMP: $13{,}833\times10^3 \pm 6{,}937\times10^3$ *vs* CpG: $5{,}448\times10^3 \pm 3{,}818\times10^3$), and cell numbers of $CD8^+CD44^+IFN\text{-}\gamma^+$ (CpG NcMP: $94{,}772\times10^3 \pm 121{,}172\times10^3$ *vs* CpG: $5{,}570\times10^3 \pm 5{,}195\times10^3$) and $CD8^+CD44^+GrzB^+$ T cells (CpG NcMP: $197{,}279\times10^3 \pm 116{,}052\times10^3$ *vs* CpG: $18{,}283\times10^3 \pm 8{,}630\times10^3$; $P< 0{,}05$).

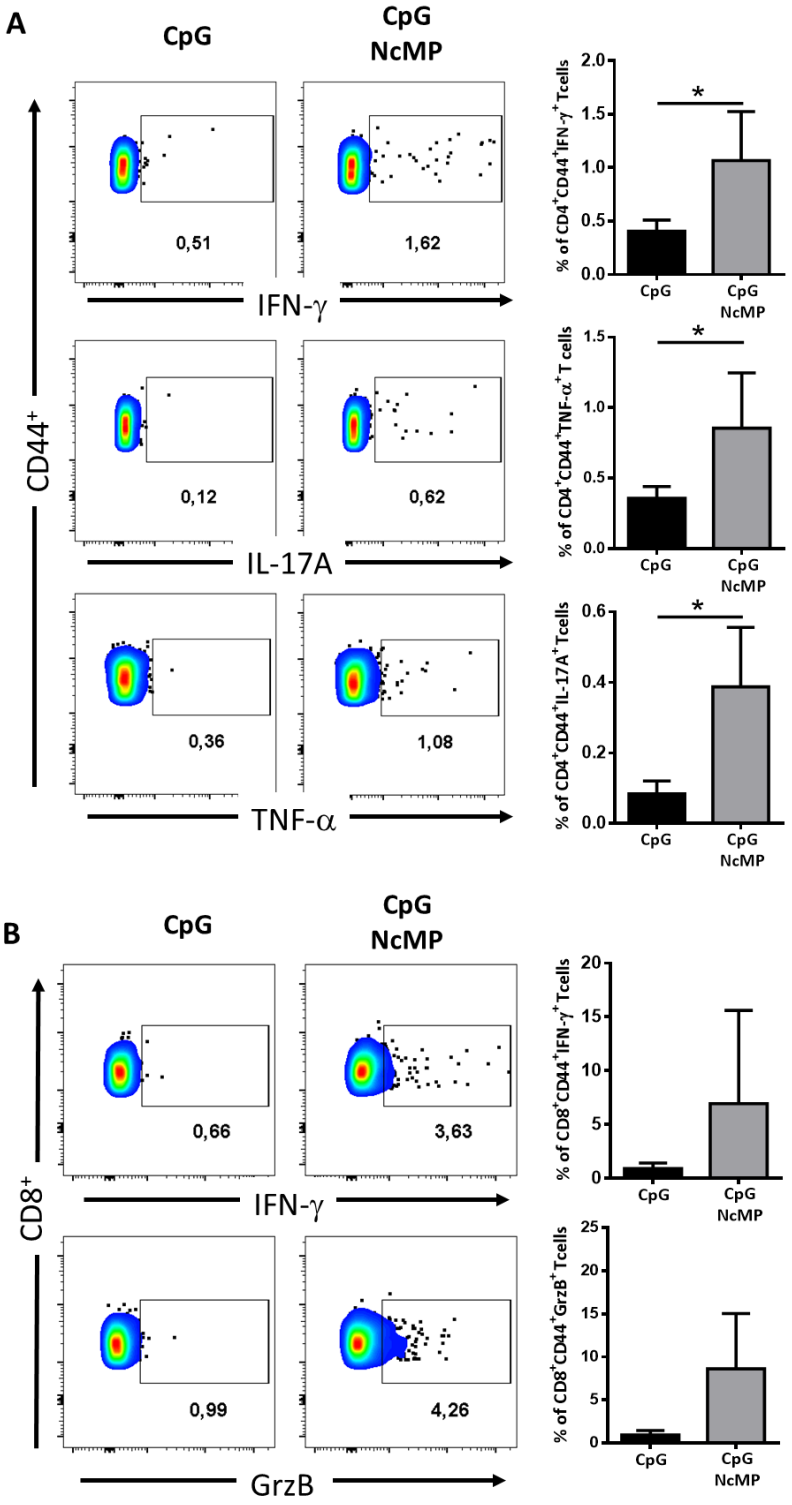

Figure 9 - Increased percentage of CD4⁺CD44⁺T cells expressing IFN-γ, IL-17A or TNF-α and CD8⁺CD44⁺T cells expressing IFN-γ or Granzyme B in the spleens of immunized mice (A) Percentage of splenic CD4⁺CD44⁺-gated T cells expressing IFN-γ, IL-17A or TNF-α and (B) percentage of splenic CD8⁺CD44⁺-gated T cells expressing IFN-γ and GrzB in sham-immunized (CpG) or immunized (CpG NcMP) mice, detected by intracellular staining after stimulation with *N. caninum* sonicates for 16 h, under brefeldin A treatment for the last 5 h. n=4 for both groups; Bars represent means plus one SD; Unpaired T-student test was used to compare CpG vs CpG NcMP groups (* *P*< 0.05). These results are from one representative experiment out of two independent experiments.

Increased IFN-γ, IL-17A and IL-10 production upon antigen recall stimulation of immunized mice splenocyte cultures

IFN-γ plays a critical role in host resistance to *N. caninum* infection[26]. Since increased frequencies of IFN-γ- and IL-17A-producing T cells were detected in the spleens of immunized mice, the splenocytes' cytokine profile was further evaluated by measuring the amount of IFN-γ, IL-17A, IL-10 and IL-4 in the supernatants of 3-day splenocyte cultures. IFN-γ and IL-10 levels were low in non-stimulated splenocytes from all mouse groups, whereas upon antigen stimulation, the supernatants of splenocytes from immunized mice showed a marked increase in the levels of IFN-γ and, to a lesser extent, of IL-10 (Fig. 10A and 10D). As shown in Figure 10B, IL-17A was present only in supernatants of cells obtained from immunized mice. The levels of IL-4 were below detection limit (DL) in all groups with or without stimulation (Fig. 10C).

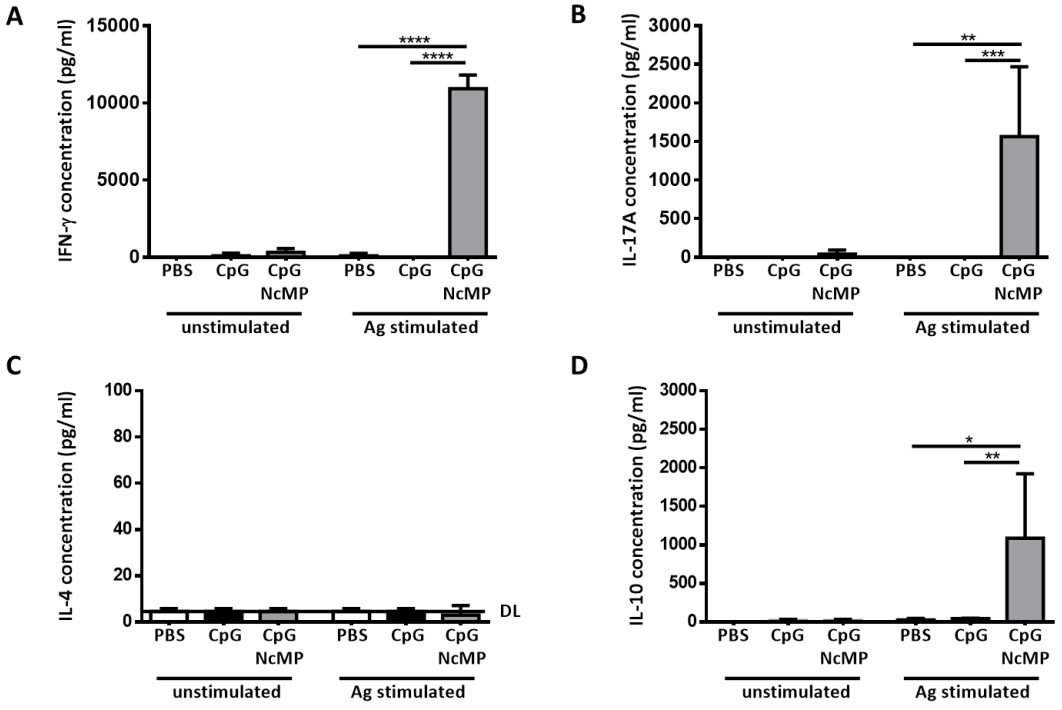

Figure 10 - Increase of IFN-γ, IL-17A and IL-10 levels in the supernatants of isolated splenocytes from immunized mice. Levels of (A) IFN-γ, (B) IL-17A, (C) IL-4 and (D) IL-10 in the supernatants of isolated splenocytes from non-immunized, sham-immunized and immunized mice cultured with or without *N. caninum* antigens for 3 days. n=3 for PBS group and n=4 for CpG and CpG NcMP groups; DL means Detection limit; Bars represent means plus one SD; One way-ANOVA followed by the Tuckey's post hoc test (* P< 0.05; ** P< 0.01; *** P<0,001; **** P<0,0001). Results are from a representative experiment out of three independent experiments.

Increased proportions of memory/activated CD8⁺ and CD4⁺ T cells in the lungs of immunized mice and increased IFN-γ and IL-17A production by the lungs and liver cells from immunized mice following parasite-specific antigen recall

Flow cytometry was used to assess the populations of tissue activated/memory $CD4^+$ or $CD8^+$ T cells in the lungs of immunized and sham-immunized mice (Fig. 11A), two T cell subsets that are efficient producers of IFN-γ in the course of *N. caninum* infection[49]. Both subsets of T cells were found at increased proportions in the lungs of immunized mice, 24 h upon infection (Fig. 11B).

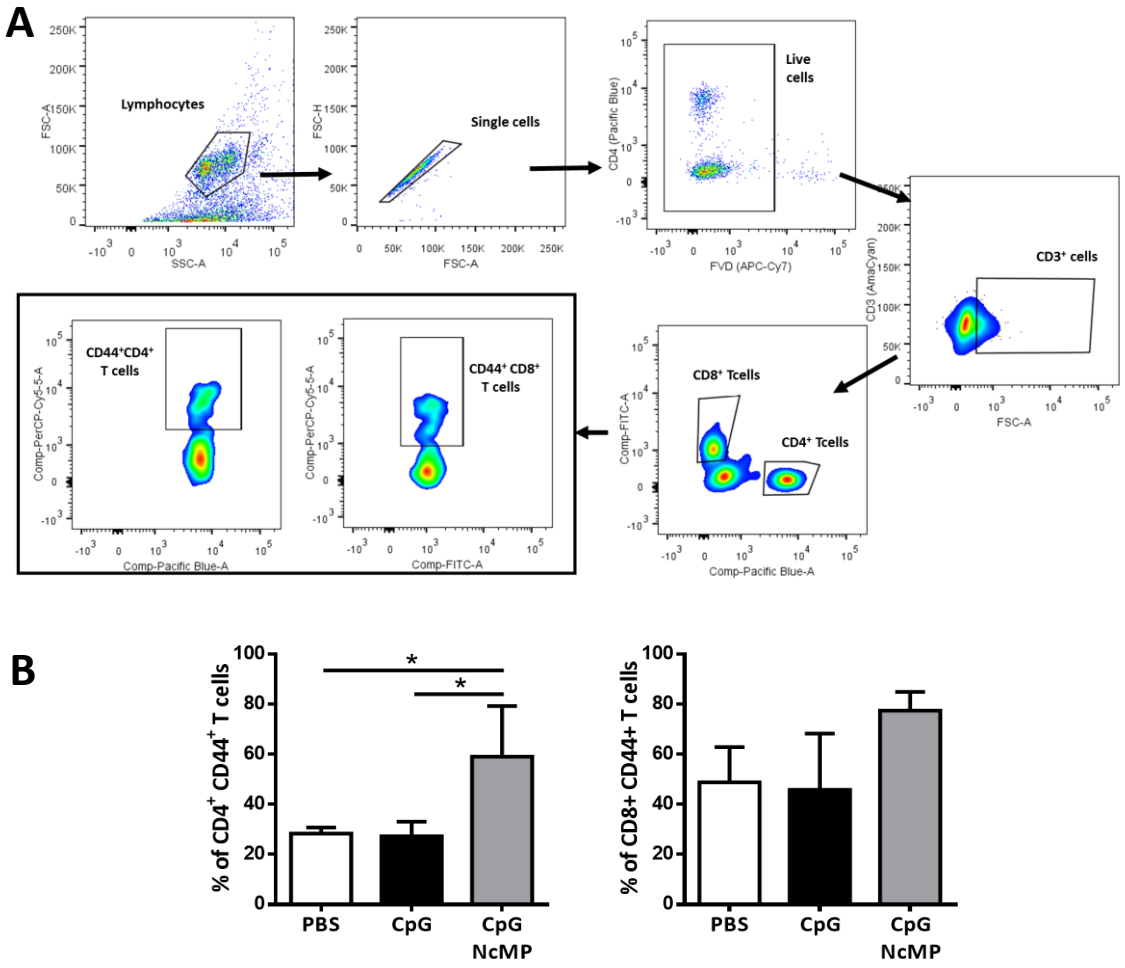

Figure 11 - Increased frequencies of CD8 and CD4 tissue memory/activated T cells in the lungs of immunized mice. (A) Representative example of the gating strategy used to identify memory/activation populations, based on CD44 expression; (B) Percentage of $CD4^+CD44^+$-gated and $CD8^+CD44^+$-gated T cells isolated from the lungs of non-immunized (PBS), sham-immunized (CpG) or immunized mice (CpG NcMP), 24 h upon infection. Bars represent means plus one SD; One way-ANOVA followed by the Tuckey's post hoc test (* $P < 0.05$).

Since the levels of IFN-γ and IL-17A were much higher in splenocyte cultures from immunized mice, we assessed the liver and lungs's cytokine profile 24 h after infection. Immune cells of both organs were isolated and cultured for 3 days with or without *N. caninum* antigen stimulation. The results showed that antigen re-stimulated cells from immunized mice produced higher levels of IFN-γ than those of sham-immunized or non-immunized mice (Fig. 12A and 12B). In the lung cells, IFN-γ production was only detected in the immunized group following specific antigen recall (Fig. 12A). In cells obtained from the liver, all groups showed production of IFN-γ regardless of the presence or absence of Ag stimulation. Nevertheless, immunized mice cells presented higher cytokine levels in both conditions (Fig. 12B). Antigen recall had no effect in IFN-γ production in cells from mice of CpG and PBS groups, but enhanced IFN-γ production in cells from immunized mice (Fig. 12B). For both organs, IL-17A production was only detected in cells from the immunized mice (Fig. 12C and 12D). The IL-17A levels were very low in the unstimulated condition, whereas in Ag stimulation condition, IL-17A levels were markedly high (Fig. 12C and 12D). In the Ag-stimulated cultures of lung cells, IL-10 was only detected in the supernatants of immunized mice cells, while in the liver, IL-10 was detected in all groups of unstimulated cells (Fig. 12E and 12F). Ag stimulation induced a high production of IL-10 in liver cells isolated from immunized mice and a reduced production in both control groups (Fig. 12F). As shown in Figures 12G and 12H, IL-4 was not detected in any group, whereas in the liver it was detected only in the non-immunized group.

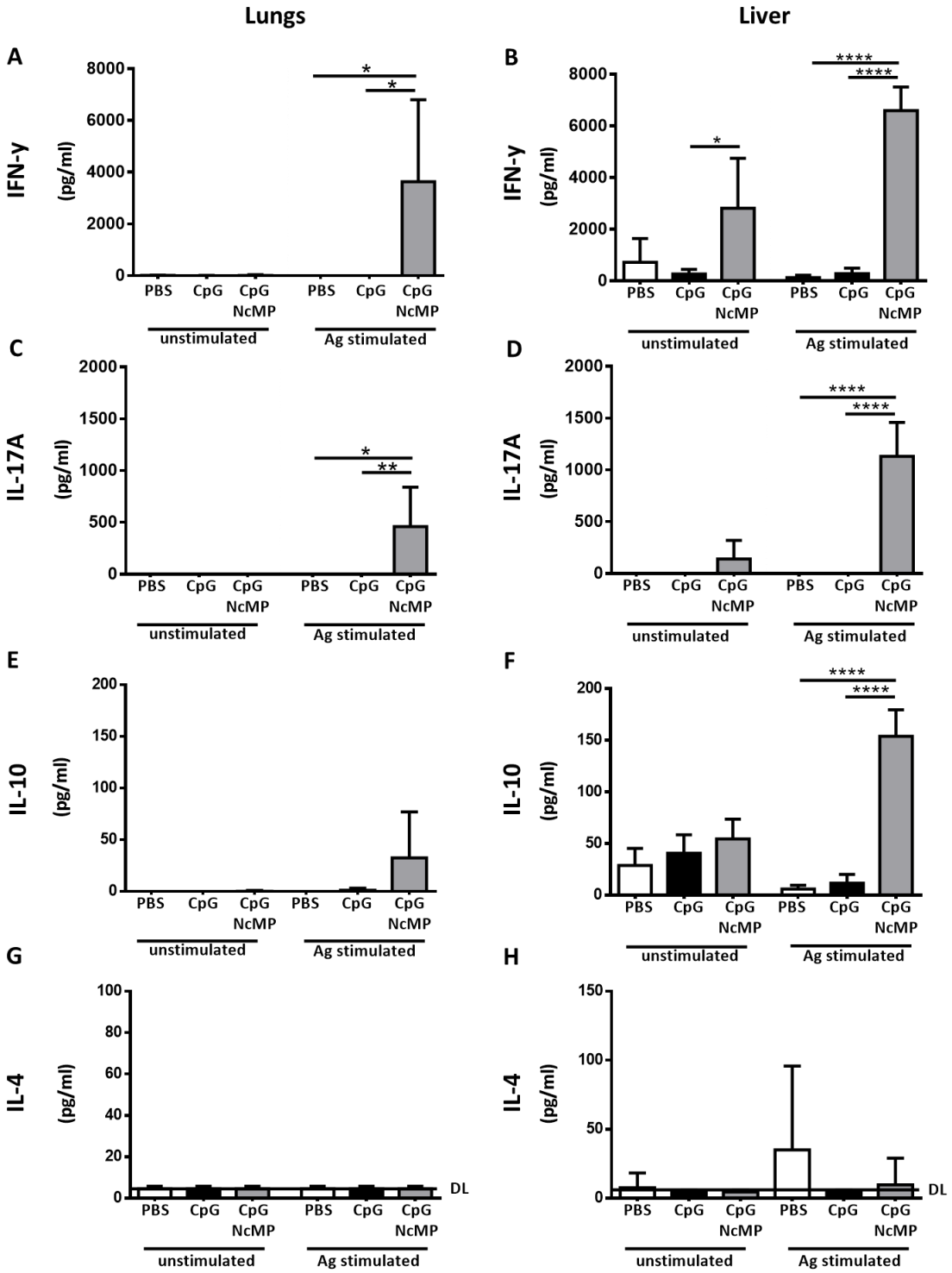

Figure 12 - **Increased production of IFN-γ and IL-17A in liver and lung leukocyte cells and increased IL-10 production in liver leukocyte cells of immunized mice, collected 24 h upon infection.** Cytokine profile detected by ELISA in the supernatants of cells cultured for 3 days with or without *N. caninum* antigen stimulation. Cells isolated from (A, C, E, and G) lungs or (B, D, F, and H) liver of non-immunized (PBS), sham-immunized (CpG) or immunized (CpG NcMP) mice. Bars represent means plus one SD. n=3-4. DL means Detection limit. One way-ANOVA followed by the Tuckey's post hoc test (* P< 0.05; ** P< 0.01; ***P< 0.001; ****P<0.0001). Results correspond to a representative experiment out of two independent experiments.

Increased expression of interferon-γ inducible GTPases in the lungs and liver

Expression of IRGs is induced by IFN-γ signalling[100]. IRGs are an important immune effector mechanism against intracellular parasites, which was shown to be able to disrupt *T. gondii* PV membrane, thus killing the parasite. However, in *N. caninum* infection only co-localization of these proteins with the PV was proved[52,53]. Having detected high levels of IFN-γ in the supernatants of cells isolated from the lungs and liver of immunized mice when stimulated with *N. caninum* antigens, we assessed the expression levels of several IRGs mRNA. Our results showed an increase in the expression of IRGm1, IRGm3 and IRGb6 mRNA in the lungs of immunized mice (Fig. 13A) and increased expression levels of IRGm3 mRNA in the liver of immunized mice, when compared to PBS and CpG counterparts. This augment was nevertheless not statistically significant, most probably due to the reduce number of samples analysed (n=3 per group). No differences were detected among groups regarding the liver expression of IRGm1 and IRGb6 (Fig. 13B).

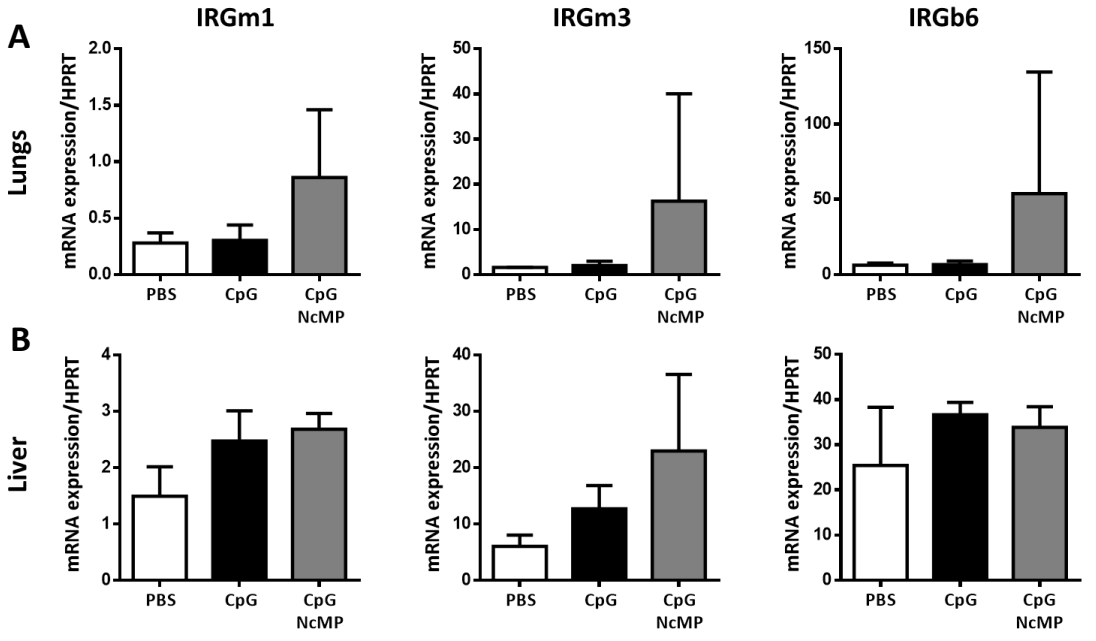

Figure 13 - Increased expression of IRGs in immunized mice. Relative levels of IRGm1, IRGm3, IRGb6 mRNA normalized to hypoxanthine guanine phosphoribosyl transferase (Hprt) mRNA. mRNA was extracted from (A) lung and (B) liver of PBS, CpG and CpG NcMP mice, 24 h after i.p infection with 1×10^7 NcT. n=3 per group. Bars represent mean values of the respective group plus one SD. Statistical significance between immunized mice and other groups is indicated above bars. (one-way ANOVA and Turkey's post-hoc test).

Reduced parasitic burden in the lungs of immunized mice

Parasitic DNA in the lungs was previously detected until the end of the acute phase (13th day of *N. caninum* infection), while liver parasite burden was detected until day 5 after infection[21]. Therefore, lungs and liver are considered target organs in the acute phase[21]. Parasite burdens were measured by qPCR in both organs one day after intraperitoneal infection with 1 × 10^7 NcT. As shown in Figure 14, the lungs of immunized mice showed the lowest levels of parasite burden, although liver parasite burden of immunized mice was not significantly decreased when compared to animals from the CpG group (Fig. 14).

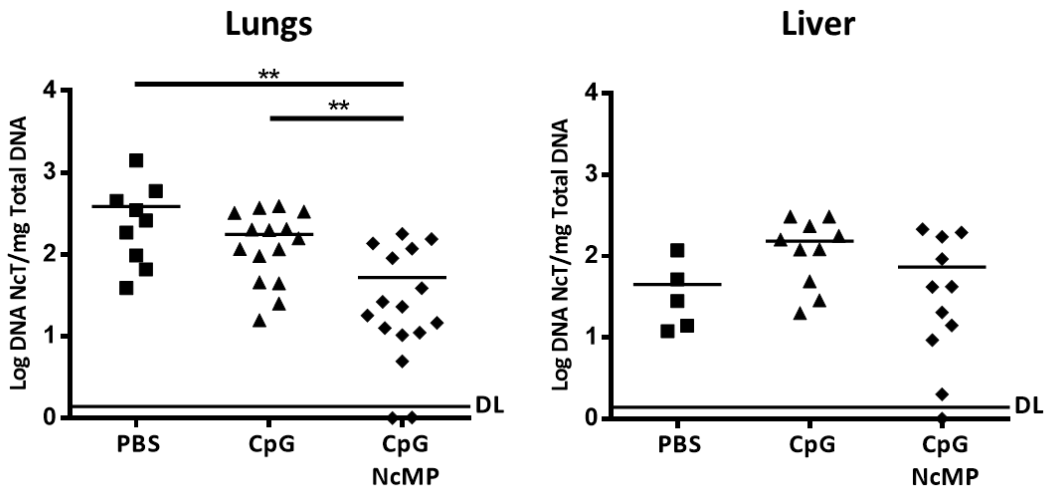

Figure 14 - Decreased susceptibility in the lungs of immunized mice. Parasite burden in the lungs and liver of non-immunized (PBS), sham-immunized (CpG) and immunized mice (CpG NcMP) 24 h upon infection with 1×10^7 NcT. Each dot represents an individual mouse. Horizontal lines correspond to means in each group. DL means Detection limit. Results correspond to pooled data of 3-4 independent experiments.

IFN-γ-NR mice are more susceptible to N. caninum infection.

Macrophages are innate immune cells that mediate the first line of defense against pathogens. IFN-γ activates macrophages and induces an increased expression of IRGs[101]. Due to the higher levels of IFN-γ and the increased expression of IRGs mRNA detected in the lungs, we used the IFN-γ-NR mouse model to understand if the early protection detected in the infected immunized mice was mediated by macrophages through enhanced expression of IFN-γ inducible proteins, such as the expression of IRGs. Parasite burdens in the lungs and liver of wild-type and mutant mice were assessed by using qPCR. As shown in Figure 15A, our results showed an increase of the parasite burdens in both organs of all IFN-γ-NR mouse groups compared with all WT mouse groups. The differences between sham-immunized mice and immunized mice were no longer observed in the lungs when using the IFN-γ-NR mouse model (Fig. 15A). In the liver, no differences in parasite burdens were observed between immunized and sham-immunized WT mice, while immunized IFN-γ-NR mice showed higher parasite burden than sham-immunized IFN-γ-NR mice (Fig. 15A).

Besides qPCR parasite quantification, the presence and relative abundance of NcT in the liver and lungs of IFN-γ-NR and WT mice was evaluated by immunohistochemistry. Using this technique, NcT were detected in all mouse groups (Fig. 15B). The visualization of the parasites in the lung sections was difficult, in part due to the histological structure of the tissue, and no NcT were visualized in WT mice (Fig. 15B). In the liver sections of IFN-γ-NR mice, free tachyzoites and several grouped zoite foci, in general of small dimension, were observed (most likely tachyzoites, given the early time point of infection). These results were in accordance with the qPCR data, no clear differences were seen in the liver sections of sham-immunized and immunized mice. In contrast, in the liver sections of WT mice rare but larger zoite foci were found, though no differences were detected between mouse groups (Fig. 15B). No inflammatory infiltrates of considerable size were observed, what would be expected due to the reduced time upon infection (Fig. 15B).

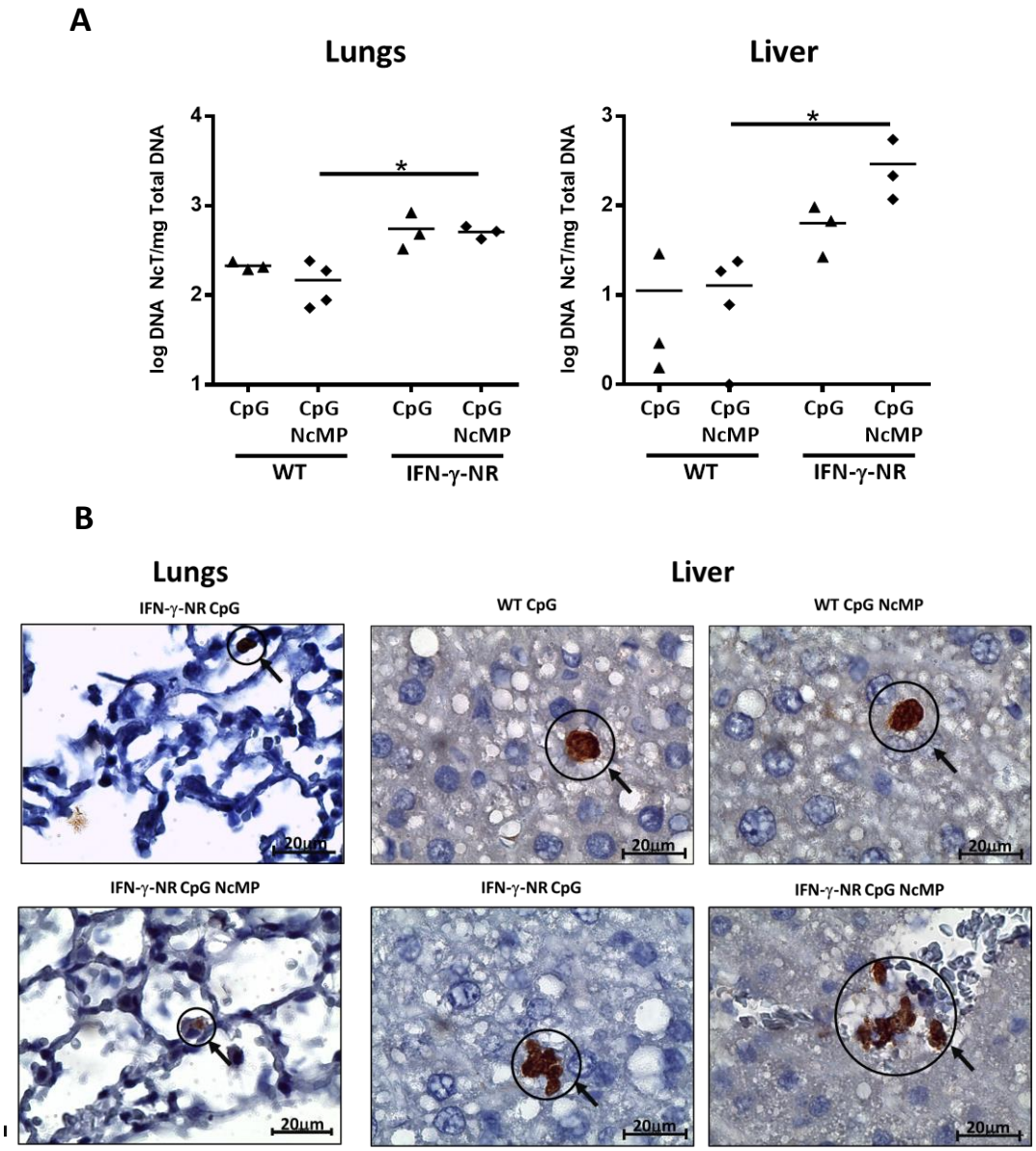

Figure 15 - Increased parasite burden in IFN-γ-NR mice. (A) Parasite burden in the lungs and liver of sham-immunized (CpG) and immunized mice (CpG NcMP) of WT or IFN-γ-NR mice 24 h upon infection with 1×10^7 NcT. (B) Representative micrographs of *N. caninum* tachyzoites (brown colour, denoted by circles plus arrows) detected by immunohistochemistry in lung and liver sections from WT and IFN-γ-NR mice, as indicated.

Splenocytes from immunized IFN-γ-NR mice produced higher levels of IFN-γ, IL-17A and IL-10

Since parasite burden in sham-immunized and immunized IFN-γ-NR mice was increased when compared with wild-type mice, it was mandatory to evaluate if the cytokine profile of isolated splenocytes from mutant mice was different from the one observed in WT mice. As shown in Figure 16A, splenocytes from IFN-γ-NR CpG NcMP immunized mice produced cytokine amounts equivalent to the ones of WT counterparts. Despite having macrophages not responding to IFN-γ, these mice had no defects in IFN-γ, IL-17A or IL-10 production. In fact, supernatants from splenocyte cultures of immunized IFN-γ-NR mice had slightly higher IFN-γ levels than WT counterparts in the unstimulated condition. However, the difference was not detected after Ag stimulation (Fig. 16B). As reported above, isolated splenocytes from immunized groups (both WT or IFN-γ-NR) stimulated with *N. caninum* antigens for 3 days had higher production of all cytokines when compared to the corresponding sham-immunized WT or IFN-γ-NR mice (Fig. 16B). The supernatants of splenocytes isolated from immunized IFN-γ-NR mice had higher levels of IL-17A and IL-10, with or without *N. caninum* Ag stimulation, than the WT counterparts (Fig. 16A and 16B). As before, the cytokine IL-4 was not detected in any of the cell cultures.

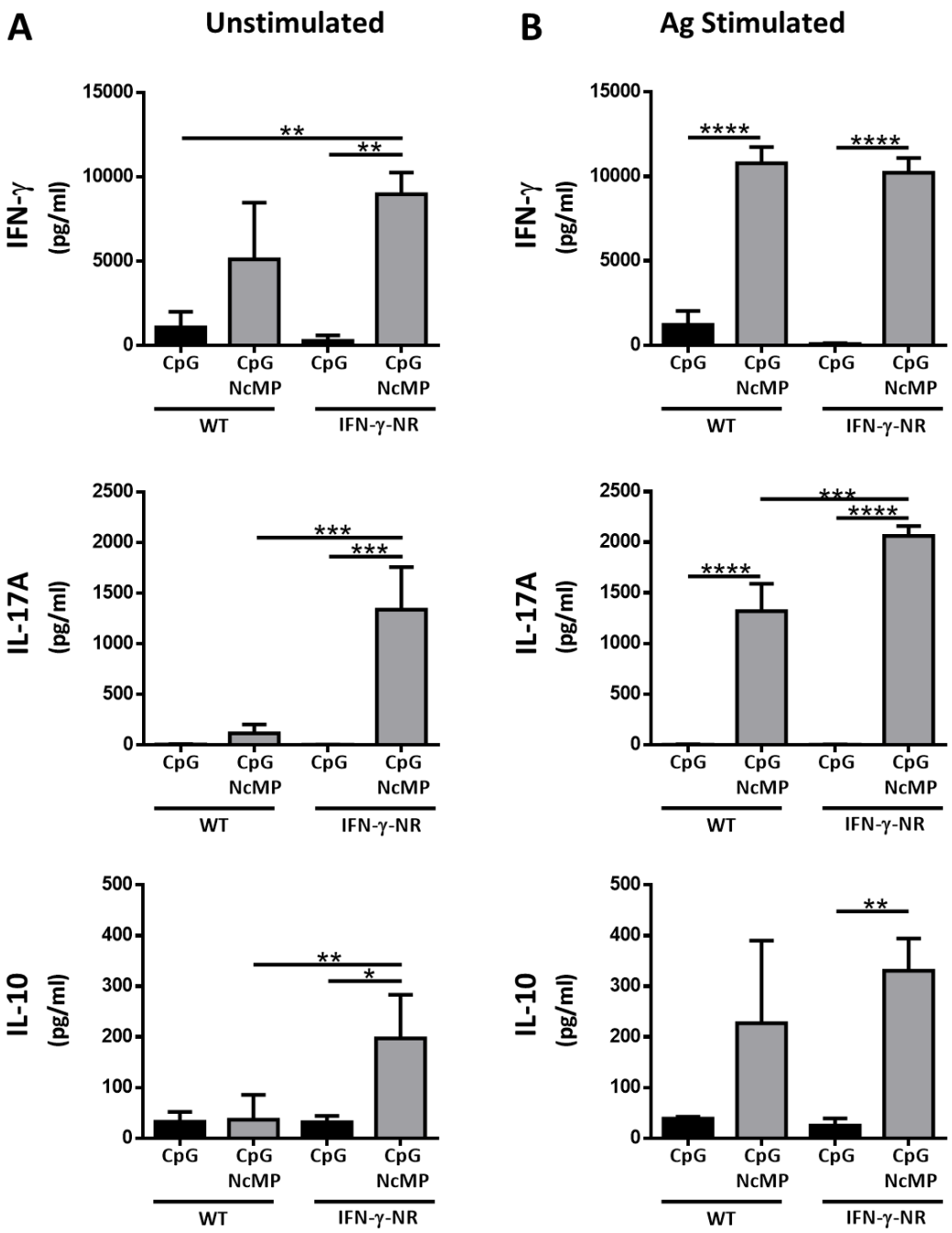

Figure 16 - Increased production of IFN-γ, IL-17A and IL-10 by splenocytes of IFN-γ-NR immunized mice 24 h upon infection. Cytokine profile detected by ELISA in the supernatants of cells isolated from sham-immunized (CpG) or immunized (CpG NcMP) mice and cultured with or without *N. caninum* antigen stimulus for 3 days. Bars represent means plus one SD. n=3-4 per group. One way-ANOVA followed by the Tuckey's post hoc test (* P< 0.05; ** P< 0.01; ***P< 0.001; ****P<0.0001).

Decreased expression of Immunity-related GTPases in the lungs of 24 h-infected IFN-γ-NR mice

Due to the observed higher production of cytokines by IFN-γ-NR mouse splenocytes, as well as higher susceptibility of these mice to *N. caninum* infection, we evaluated the expression of mRNA encoding IRGs of different steps of the mechanism, such as IRGm1, IRGm3 and IRGb6. Both groups of IFN-γ-NR mice presented a significant decrease of expression in all measured IRGs when compared with WT counterparts (Fig. 17). Immunized IFN-γ-NR mice showed higher levels of expression of IRGs mRNA than CpG sham-immunized counterparts, but no differences in IRGs mRNA expression were detected between immunized and sham-immunized WT mice (Fig. 18).

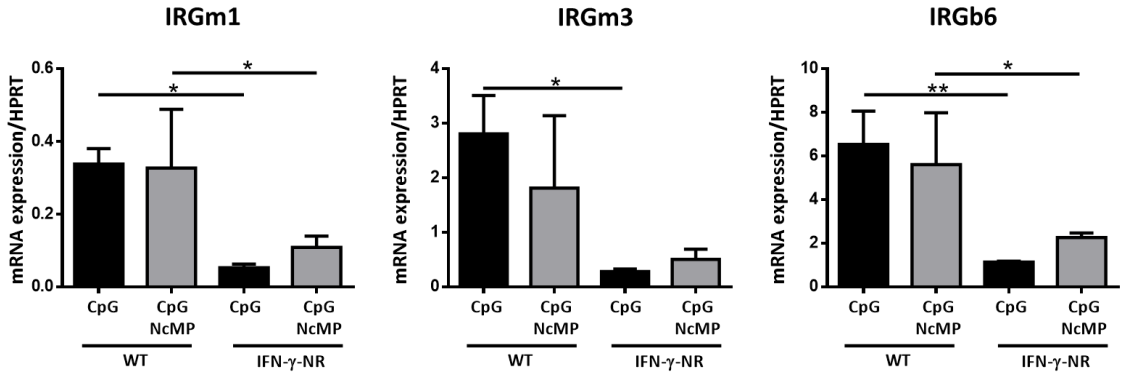

Figure 17 - Decreased IRGs expression in the lungs of IFN-γ-NR mice. Relative levels of Irgm1, Irgm3 and Irgb6 mRNA, normalized to hypoxanthine guanine phosphoribosyl transferase (Hprt) mRNA, detected by real-time PCR in the lungs of sham-immunized or immunized of WT or IFN-γ-NR mice 24 h upon infection; n= 3-4 per group. Bars represent means plus one SD; One way-ANOVA followed by the Tuckey's post hoc test (* P< 0.05; ** P< 0.01).

Discussion

Different approaches have been explored to induce a protective response against *N. caninum*, thereby preventing its dissemination and transmission. Our group previously developed a candidate vaccine composed by an extract enriched in membrane proteins of the parasite and CpG as adjuvant that proved to confer long term protection against *N. caninum* in immunized mice[73,92]. Although IFN-γ is known to be crucial for the control of neosporosis, and despite protection achieved with the intranasal CpG NcMP immunization, the numbers and frequencies of splenic T cells producing IFN-γ in immunized mice, 7 days upon intragastrically challenged with *N. caninum*, were found reduced[73]. On the opposite, in these mice high levels of *N. caninum*-specific serum IgGs and vaginal and intestinal fluids IgA were reported. This indicated that in this particular model of infection parasite-specific mucosal IgA production could be a candidate mechanism of protection[73]. Nevertheless, the presence of high titres of parasite-specific IgG2a hinted that IFN-γ could be induced by immunization even though this cytokine and the respective producing cells were not detected in increased levels in the immunized mice[73]. One of the suggested reasons for this result was the evaluation of the immune response at a quite late time point upon infection. Therefore, analysis of the immune response elicited by intranasal immunization in an earlier time point upon infection could allow the clarification of the protective mechanisms induced by the vaccine. Also, using a different route of infection that could overcome the intestinal barrier would help elucidating the role of mucosal IgA in the observed protection. Here, using the same immunization protocol and an i.p. infection, thus overcoming the mucosal barrier, we show that, immunization was also protective and that protection was fast observed (24 hours upon infection).

As reported before[73,92], serum and intestinal lavage fluids of immunized mice had high levels of NcMP-specific Immunoglobulins indicating the development of a parasite-specific immune response driven by the used intranasal immunization. Supernatants of immunized mice splenocyte cultures stimulated with parasite antigens presented higher levels of IFN-γ and IL-17A than in controls. These results were in accordance

with the flow cytometry analysis of Ag-stimulated splenocytes, which showed higher proportions of antigen-specific $CD4^+CD44^+$ T cells producing IFN-γ, TNF-α or IL-17A and $CD8^+CD44^+$ T cells producing IFN-γ. This indicated that memory/activated $CD4^+$ and $CD8^+$ T cells could be possible candidates responsible for the increased production of IFN-γ and IL-17A. Since the lungs and the liver are two target organs in the acute phase of *N. caninum* infection, the cytokine profile was assessed in those organs. Lungs and liver of immunized mice showed the same type of immune response polarization, characterized by high levels of pro-inflammatory cytokines IFN-γ and IL-17A. The increased proportions of $CD4^+$ and $CD8^+$ T cells with a memory/activation phenotype observed in the lungs of immunized mice argues in favor of the generation of specific memory $CD8^+$ or $CD4^+$ T cells that migrated to this target parenchymal organ or tissue-resident memory T cells promoted by the immunization. Tissue-resident memory T cells of both subsets were previously shown in the lungs[102]. These cells could be the producers of the pro-inflammatory cytokines IFN-γ and IL-17A detected elevated in lung cell supernatants from immunized mice, upon antigen recall. Supernatants of cells from the liver of immunized mice presented higher levels of the anti-inflammatory cytokine IL-10 than controls after Ag stimulation. This was not seen in the supernatants of the other organs analyzed. The liver has multiple functions, namely detoxification[103]. Due to anatomical features and localization, the liver is constantly in contact with gut-derived molecules from dietary or commensal bacteria, which have ability to induce inflammation[103]. Thus, to maintain a healthy liver homeostasis, myeloid-derived suppressor cells produce IL-10, TGF-β and arginase to suppress inflammation[104]. Despite this environment prone to induce immune tolerance, the liver can develop fast and robust immune responses what could explain the high levels of pro-inflammatory cytokines detected in the supernatants of liver leukocyte cells of immunized mice.

IFN-γ and IL-17A are pro-inflammatory cytokines characteristics of Th1- and Th17-type immune responses. Our results have shown a fast and robust production of IFN-γ and IL-17A in the spleen, liver and lungs of immunized mice, 24 h upon infection. The role of IL-17A in *N. caninum* infection is largely unknown. A recent report showed that IL-17A receptor deficiency or neutralization of endogenous IL-17A lead to the reduction of mortality in mice infected with cysts of the related protozoan *T. gondii*, indicating a

non-protective role for IL-17A[105]. However, in another study, the obtained results indicated a protective role of IL-17A mediated by neutrophil recruitment, since deficient IL-17R$^{-/-}$ mice showed increased susceptibility to *T. gondii*[106]. Thus, the role of IL-17A in protection against coccidian parasites is still unclear**.** The high levels of NcMP-specific IgA detected in immunized mice could be linked to high levels of IL-17A produced in these mice 24 h upon infection, since Th17-type immune response was recently associated with the production of high affinity antigen-specific IgA in germinal centers[107]. Early production of IL-17A could be inducing the activation of antigen presenting cells and contribute to increased IFN-γ levels, as well as to the recruitment of neutrophils to sites of infection. This could contribute to protection since neutrophils have shown to be an important host protective population in early stages of intraperitoneal established *T. gondii* infection[32,108,109]. The protective role of IFN-γ in *N. caninum* infection is well known[26,35,36,55]. Inhibition of IFN-γ-dependent mechanisms led to host increased susceptibility to *N. caninum* infection[26,99]. IFN-γ interacts with its receptor expressed in macrophages, monocytes, and other cells, and induce downstream effects culminating in the expression of genes, such as those encoding IRGs[110]. Since immunized mice showed high levels of IFN-γ, we looked for IRGs mRNA expression, that was shown to be protective against intracellular pathogens[101]. Our results showed an increase of mRNA expression of IRGm1, IRGm3 and IRGb6 in the lungs and IRGm3 in the liver of immunized mice. Unlike *T. gondii*, *N. caninum* does not express ROP18 protein[111]. ROP18 originates a protein complex with two other proteins that inactivates IRGs, preventing its accumulation in the parasitophorous vacuole and hence its disruption[111,112]. Therefore, IRGs could be immune effector proteins mediating the early protection observed in immunized mice. Macrophages are innate immune cells that play a role in the early immune response against pathogens. Nishikawa et al. previously showed that macrophages failed to control *N. caninum* infection in the absence of IFN-γ, whereas the treatment of IFN-γ-deficient mice with recombinant IFN-γ delayed mouse death due to neosporosis[25]. Therefore, macrophages could be the effector cells that are mediating intracellular parasite control through IRGs[113,114]. Other IFN-γ–inducible mechanisms are the production of nitric oxide and reactive oxygen species. However, these mechanisms do not seem to

be responsible for protection against neosporosis, since *Nos2* $^{-/-}$ mice and *p47phox* $^{-/-}$ mice harbored parasite burdens similar to the ones of WT mice[55]. To further investigate this hypothesis, we used a mouse model in which macrophages do not respond to IFN-γ [93]. These mice showed higher parasite burdens than WT counterparts in the lungs and in the liver. This is the first evidence showing that control of *N. caninum* burden by macrophages is directly dependent on IFN-γ signaling. Immunized IFN-γ-NR mice showed the same or higher parasite burdens in the lungs and liver, respectively, than sham-immunized mice. Although the results are from a single experiment with reduced numbers of mice per group, this indicates that protection conferred by the immunization could be lost in these deficient mice. It would be interesting to further explore this subject using higher numbers of animals and additional time points. Supernatants of splenocytes obtained from immunized IFN-γ-NR mice showed higher levels of cytokine production than the WT counterparts. The differences between IFN-γ-NR and WT mice in IFN-γ and IL-10 production were abolished with antigen stimulation of splenocytes, while the differences in IL-17A production were not. The higher parasite burden in the lungs and liver of deficient mice could be the reason for the heavy production of cytokines in these animals. The similar or even higher parasitic loads in immunized when compared with control IFN-γ-NR counterparts, despite the high levels of cytokines indicate that IFN-γ rather than IL-17A could be contributing to protection against *N. caninum* infection. We further analyzed the IRGs mRNA expression and our results showed a significant decrease in the expression of IRGs mRNA when compared to WT counterparts. This indicates that production of IRGs by IFN-γ-primed macrophages could be mediating parasite control at early times of infection. Despite the impaired IFN-γ signalling in macrophages, IFN-γ-NR mice still presented some expression of IRGs mRNA. This could be resulting from some non-hematopoietic cells that respond to IFN-γ and/or from activation of macrophages by type I interferons, such as IFN-α or IFN-β[115,116]. Thus, a higher pro-inflammatory environment in immunized IFN-γ-NR mice could be the reason why lung IRGs mRNA expression was higher in these mice than in the lungs of sham-immunized controls. The differences seen in lung IRGs mRNA expression observed between immunized and sham-immunized WT mice in the first experiments were no longer

observed in WT mice of this particular experiment. This could explain the absence of protection conferred by immunization in these mice. This could be due to technical reasons affecting the successful immunization of the WT animals with CpG plus NcMP or due to the immune response kinetics since the 24 h upon infection is a very early time point and slight differences in infection kinetics could affect the overall outcome. Moreover, we cannot exclude a contribution of the animal facilities' environment and of the animals' microbiota, once the mice from this and the other experiments had different proveniences and were housed in distinct animal facilities.

To conclude, our intranasal immunization strategy induced a Th1/Th17-type response and conferred protection at early stages of i.p.-established *N. caninum* infection. Increased expression of IRGs mRNA in macrophages, mediated by IFN-γ induced by vaccination, could be the mechanism behind that early protection. The results of this study provide evidence that support our previous studies indicating that this vaccination strategy is a promising one to prevent *N. caninum* infection. Given that IFN-γ also mediates protection to neosporosis in cattle[117,118], it is likely that this immunization strategy could confer protection in this economic relevant host.

Future perspectives

Considerable work has begun to reveal the mechanisms that confer protection against *N. caninum* infection. The role of IL-17A in early protection, as well as the mechanism behind protection conferred by IRGs, are still unclear. Therefore, further studies have to be done such as:

- Neutralization of IL-17A in immunized mice before and during infection or immunization of IL-17A receptor knock-out mice in order to assess several parameters such as IgA production, neutrophil recruitment, inflammation and parasite burden;
- *In vitro* parasite control by specific IRGs' silencing in IFN-γ stimulated macrophages;
- Immunization of specific IRGs knock-out mice to assess the role of these proteins on vaccine protection;
- Transference of immune cells such as $CD8^+$ or $CD4^+$ T cells isolated from immunized mice to non-immunized mice to understand their role in parasite control.

References

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