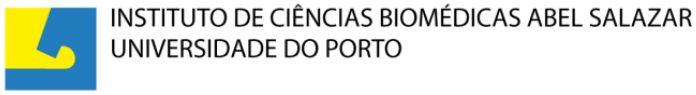

Refinement of a mucosal vaccination strategy against neosporosis

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Resumo

Neospora caninum é um protozoário intracelular e que se assemelha ao patógeno *Toxoplasma gondii*, sendo este capaz de infectar humanos. A neosporose é a doença causada pelo parasita *N. caninum*, estando associada à ocorrência de abortos em gado bovino que se traduzem em grandes perdas económicas para as indústrias de lacticínios e carne. Medidas preventivas como "testar e eliminar" têm sido incapazes de restringir novas epidemias. A única vacina comercial contra a neosporose, Bovilis(®) Neoguard, foi recentemente retirada do Mercado e portanto o desenvolvimento de vacinas ainda decorre. Uma vacina subunitária experimental composta por proteínas membranares do parasita (NcMP) e CpG como adjuvante, demonstrou conferir protecção contra neosporose estabelecida por ambas vias intragástrica e intraperitoneal em ratinhos. A protecção alcançada foi mantida a longo-prazo. Apesar de ser eficaz, o adjuvante CpG é caro, sendo necessário procurar alternativas mais baratas, mantendo o nível de eficácia da vacina, para viabilizar o seu uso futuro na veterinária. Carbigen™ é um adjuvante derivado de carbómeros usado na veterinária e, sendo barato, é adequado para vacinação em larga escala. Consequentemente, aqui procuramos testar se o Carbigen™ poderia ser utilizado como adjuvante, substituindo o CpG, numa vacinação intranasal de NcMP contra a neosporose. O efeito de adjuvante do CpG induz uma polarização da resposta imune típica de células Th1, caracterizada por níveis elevados da citocina interferon-γ (IFN-γ) e de uma razão de anticorpos IgG1/IgG2a específicos para o parasita inferior a um. Neste trabalho, ratinhos vacinados com NcMP e Carbigen™ não desenvolveram imunidade protectora contra a infecção peritoneal de *N. caninum*, como demonstra a análise da carga parasitária dos cérebros de ratinhos imunizados e controlo (pseudo-imunizados com Carbigen™). Houve, no entanto, protecção nos pulmões, o que indica que esta estratégia de imunização poderá ser eficaz no controlo da infecção ao nível da mucosa. Apesar desta vacina induzir uma produção preferencial de IFN-γ, comparativamente à IL-4, a produção de anticorpos do isotipo IgG1, associado a uma resposta imunológica Th2, prevaleceu em ratinhos imunizados. A vacinação induziu a expansão de ambas populações $CD4^+$ e $CD8^+$ de células T de memória, mas as diferenças significativas encontradas na expressão de factores de transcrição característicos de linhagens celulares de células Th $CD4^+$ não foram consistentes em todos os orgãos. Em células T $CD8^+$ não se verificaram

alterações na expressão de Granzima B, mas a expressão do factor de transcrição Tbet estava aumentado nas células T $CD8^+$ de animais imunizados. Adicionalmente, IL-17A e IgA intestinais específicas para *N. caninum*, ambos mediadores da resposta imunológica na mucosa intestinal, foram detectados em níveis mais elevados em animais imunizados do que nos animais controlo. Estes resultados evidenciam o potencial da vacina em impedir a penetração do parasita através da barreira intestinal e consequente disseminação pelo hospedeiro.

Abstract

Neospora caninum is an obligate intracellular protozoan closely related to the human pathogen *Toxoplasma gondii*. Neosporosis, the disease caused by *N. caninum,* is associated with abortions occurring in cattle which translate into heavy economic losses in beef and dairy industries. Preventive measures such as test and cull have failed to restrain further epidemics. The only commercialized vaccine against neosporosis, Bovilis(®) Neoguard, was recently withdrawn from the market and therefore vaccine development is still underway. An experimental subunit vaccine composed of *N. caninum* membrane proteins (NcMP) and CpG adjuvant administered intranasally was previously shown to confer protection against intragastrically and intraperitoneally established neosporosis in mice. The achieved protection was effective in the long term. Although effective, CpG adjuvant is expensive and therefore searching for alternative and cheaper adjuvants that still could maintain vaccination efficacy may be important towards future use of the vaccine in veterinary clinics in cattle. Carbigen™ is a cheap carbomer-based adjuvant already under use in the veterinary field suitable to be used in large scale vaccination. Thus, we sought here to test if Carbigen™ could be used as adjuvant, instead of CpG, for the NcMP-based intranasal vaccination against neosporosis. The adjuvant effect of CpG encompasses induction of a Th1-type immune response, characterized by high interferon-γ (IFN-γ) production and parasite-specific IgG1/IgG2a ratios lower than one. Herein, mice immunized with NcMP plus Carbigen™ adjuvant did not develop protective immunity to *N. caninum* infection established intraperitoneally, as shown by comparatively assessing parasite burden in the brain of immunized mice and controls. There was nevertheless protection in the lungs of Carbigen™ immunized mice, indicating that this strategy may control infection at the mucosa level. Although the tested immunizing preparation induced the preferential production of IFN-γ as compared to IL-4, production of Th2-associated antibody isotype IgG1 prevailed in the immunized mice. Immunization induced the expansion of $CD4^+$ and $CD8^+$ T cell populations though significant differences on the expression $CD4^+$ T cell lineage-specific Tbet, and RORγt transcription factors were not consistent for all organs analyzed. The expression of the transcription factor Gata3, associated with Th2-type cells was not different among mouse groups. Also, expression of Granzyme B in $CD8^+$ T cells remained unchanged with immunization

but Tbet expression by these cells was found increased in $CD8^+$ T cells from immunized mice. In addition, higher levels of IL-17A and *N. caninum*-specific intestinal IgA, both mediators of gut mucosal immunity, were detected in the immunized animals than in sham-immunized controls. This highlights the potential of this vaccine to impair parasite penetrance into the host through the intestinal barrier and further dissemination.

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Rita.

Família.

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List of Abbreviations

ACK	Ammonium-chloride-potassium
ADCC	Antibody-dependent cell-mediated cytotoxicity
AP	Alkaline phosphatase
APC	Allophycocyanin
APC	Antigen-presenting cell
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	Bovine serum albumin
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
cDC	Conventional dendritic cell
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FVD	Fixable viability dye
GAGs	Glycosaminoglycans
HBSS	Hank's balanced salt solution
HSV	Herpes simplex virus
i.n	Intranasal
i.p	intraperitoneal
IBK	Infectious bovine keratoconjunctivitis
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ISCOM	Immune stimulating complexes
MadCAM	Mucosal vascular addressin cell adhesion molecule
MEM	Minimum essential medium

MJ	Moving junction
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
NALT	Nasal-associated lymphoid tissue
NBT	Nitro blue tetrazolium
NcMP	*Neospora caninum* membrane proteins
NcS	*Neospora caninum* sonicate
NK	Natural killer
p.i	post-infection
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cells
PDI	Protein disulfide isomerase
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PV	Parasitophorous vacuole
qPCR	Quantitative polymerase chain reaction
Qs	Quackenbush
RORyt	RAR-related orphan receptor gamma t
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
sIgA	Secretory Immunoglobulin A
TBS	Tris-buffered saline
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TST	Tris-buffered saline, Tween-20
TVN	Tubovesicular network

1. Introduction

Neospora caninum is an apicomplexan protozoan mostly associated with abortion in cattle (Dubey *et al.*, 1996). It is an obligate intracellular parasite closely related to *Toxoplasma gondii*. However, *N. caninum* does not infect humans as *T. gondii* does (Petersen *et al.*, 1999). From a historical perspective, *N. caninum* was consecutively misidentified as *T.gondii* long until the first report of an unknown parasite causing encephalomyelitis and myositis in dogs shed light into the misconception (Bjerkas *et al.*, 1984). The authors firstly observed that serum from infected dogs did not react with *T.gondii* trophozoites. In 1987, four calves displaying ataxia were euthanized and histopathological examination showed protozoal cysts in association with multifocal myelitis, meningitis and encephalitis (Parish *et al.*, 1987). Later in 1988, the unknown parasite was finally classified as *Neospora caninum* (Dubey *et al.*, 1988). In order to close the gap between *N.caninum* and newborn infection in cattle, a histological reexamination of the sections previously obtained (Parish *et al.*, 1987) finally confirmed cattle as a host for the parasite. In parallel, detection of *N.caninum* in the brain, heart, liver, kidney and placenta of aborted fetus indicated for the first time the abortive potential the parasitic infection had in pregnant cows (Thilsted & Dubey, 1989). Over the years, neosporosis was increasingly detected throughout the world and is now considered one of the major causes of abortion in cattle worldwide. In humans, despite some serological evidence on immunocompromised individuals, neosporosis is not considered to be a zoonotic disease. Thus, its relevance is mainly due to the economic burden it has on both dairy and beef industries adding up to yearly losses of roughly 1.3 billion US dollars (Reichel *et al.*, 2013). The seroprevalence of the protozoan is virtually global and abortion in herds may occur sporadically or in form of “storm” where up to a third of the breeding herd may abort within few months. To date, there is no effective control measure for neosporosis. The most common method employed by farmers is selective breeding, achieved by culling infected cattle. The so-called test and cull strategy is very expensive since infected cattle show no symptoms of the disease prior to abortion and undertaking periodic serologic screenings is not economically feasible for the industry. Despite being seemingly efficient on controlling *N. caninum* pathogenesis, the use of coccidiostatic drugs draws concern on the effect of such long treatments on the quality of milk and beef (Dubey *et al.*, 2007). In contrast, vaccination in the context of neosporosis is predicted to be cost-effective and there are no concerns regarding the quality of cattle derived products when vaccinated (Reichel *et al.*, 2006). To date, no protective vaccine is yet available for use in cattle, motivating further research on

the topic comprising different approaches in respect of vaccine formulation but also the mechanisms underlying the immune response to neosporosis.

1.1 *Neospora caninum*

Structurally, *N. caninum* tachyzoites have specialized secreting organelles conferring it the ability to adhere and invade host cells, namely micronemes, rhoptries and dense granules. Of all the structural similarities with *T. gondii*, the organization and number of these organelles throughout the protozoan differs significantly between them, being portrayed by the first electron microscopy images taken (Speer *et al.*, 1999). Apart from the secretory products and their relevance in host cell interaction, the surface proteins present on the parasite membrane are evidently key-players for the anchoring necessary and consequent series of events culminating on the establishment of the parasite in the host cytoplasm. NcSAG1 and NcSRS2 are two major immunodominant surface proteins and its neutralization with monoclonal antibodies inhibited invasion (Nishikawa *et al.*, 2000). Micronemes are cigar-shaped organelles located in the most apical part of the tachyzoite, which are thought to secrete proteins essential to the initial adhesion of the parasite. Conversely, the parasite must have specific targets on the host cell membrane that integrate this mechanism. NcMIC3 protein, for instance, has EGF-like domains in its structure that are known binding sites for Glycosaminoglycans (GAGs). GAGs residues are widely distributed in cell membranes and the depletion of chondroitin sulphate, *in vitro*, prevented the adhesion of tachyzoites to Vero cells (Naguleswaran *et al.*, 2003). Rhoptries are club-shaped organelles and their roles ranges from parasite ingression to the development of the cytoplasmic compartment where the protozoan establishes, the parasitophorous vacuole (PV), which does also contain fragments of the host cell membrane. As in every other Apicomplexan parasite, invasion is an active process differing from phagocytosis where host mediated internalization through cytoskeleton reorganization plays a major role. Once rhoptry proteins are secreted to the host-parasite tight junction, a multiprotein complex comprising both rhoptry and microneme proteins named moving junction (MJ) is formed. The moving junction is a ring-shaped structure formed initially at the apical end which then moves throughout the parasite until the posterior bulb, providing a tight connection between the parasite and host cell plasma membrane during invasion (Besteiro *et al.*, 2011). Also, MJ functions as a filter for host plasma membrane that will be added to the PVM, protecting the vacuole from host lysosomes fusogenic activity (Straub *et al.*, 2009). The ROP2 family of rhoptry proteins are commonly associated with the formation of the vacuole and modulation of its surface and, to date, only NcROP1, NcROP2Fam-1, NcROP4, NcROP5, NcROP9, NcROP30 and NcROP40 have been identified. NcROP2Fam-1 was shown to be co-localized with the PVM, with

no apparent catalytic activity (Alaeddine *et al.*, 2013), but influence on invasion and egress of the parasite (Pastor-Fernández, 2016. The ROP18 kinase is essential to *T.gondii* pathogenesis through the inhibition of the disruptive action of host GTPases on the PVM, but has no orthologue in *N.caninum* (Reid *et al.*, 2012). Examples like the latter are key to a better understanding of the still largely unknown lytic cycle and the constraints surrounding translated knowledge from one apicomplexan to another, as similar modes of action not always are carried out by the same effector proteins. Lastly, dense granules are located mostly in the basal side of the parasite Its proteins are likely to be involved in the maintenance of the PVM and the tubulovesicular network (TVN), which mediates the mobilization of the host endomembrane system (Mercier *et al.*, 2005). Vaccination with recombinant NcGRA7, tested in mice, has shown partial protection on vertical transmission of the parasite in pregnant mice (Nishikawa *et al.*, 2009). Thus, efforts on proteomic studies are essential for a better understanding of target proteins that may prove useful for vaccine development.

1.2 Life cycle and transmission

N. caninum has a heteroxenous life cycle considering that it needs at least two different hosts to complete the cycle. Dogs and other canids are definitive hosts that can shed unsporulated oocysts in their feces (Basso *et al.*, 2001). The oocysts resistant wall allows them to persist in the environment for a considerable time before the occurrence of sporulation which confers the oocyst two sporocysts each containing four sporozoites capable of infecting the intermediate host (Figure 1). After infection, oocysts appear to resist the harsh environment found in the stomach before arriving to the intestine where sporozoites are released to colonize the gut epithelium and differentiate into tachyzoites. They are globular or ovoid in shape, measuring around 6 x 2 µm and characteristically motile (Dubey *et al.*, 1996). As tachyzoites undergo asexual development, termed endodyogeny, a wide dissemination through the host tissues is inevitable, hence its ability to infect neural cells, liver cells, macrophages, endothelial cells, skeletal muscle cells and fibroblasts (Dubey *et al.*, 2002). It is assumed that tachyzoites undergo 20 rounds of replication before converting to bradyzoites and accumulate within tissues forming tissue cysts where they remain latent (Donahoe *et al.*, 2015). These thick-walled structures are commonly found in skeletal muscles, brain or spinal cord and may even establish themselves there for the rest of the intermediate host life. In the context of abortion, tachyzoites efficiently cross the placenta and infect the fetus. Thus, vertical transmission may occur either during primo-infection or parasite reactivation from bradyzoite to tachyzoite.

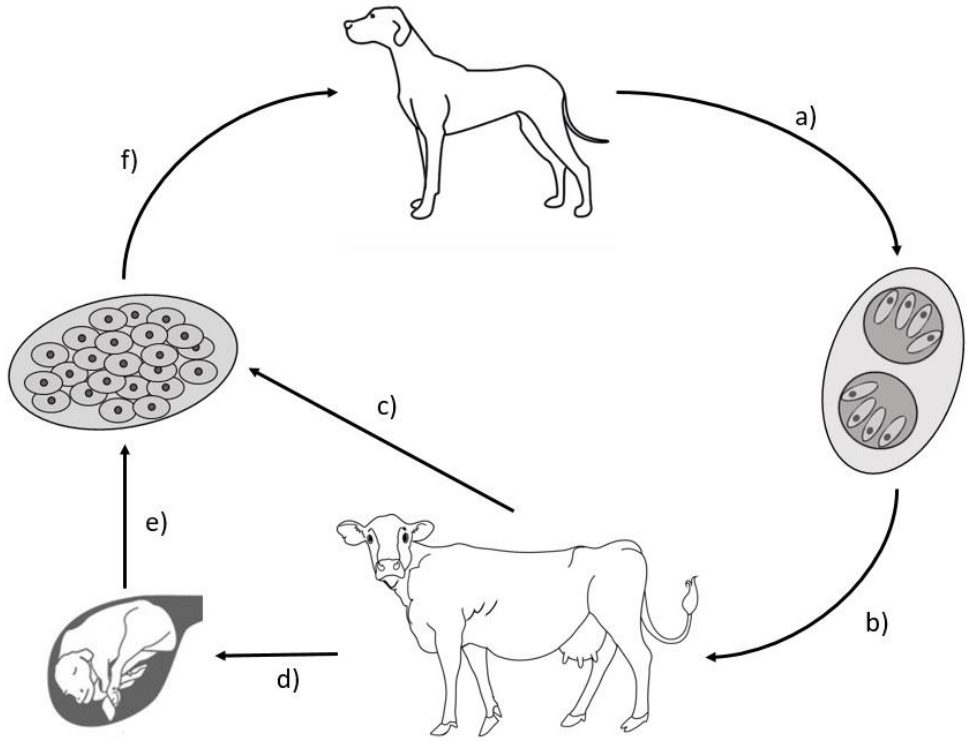

Figure 1. Life cycle of *Neospora caninum*. a) Unsporulated oocysts shed in dogs' feces sporulate within 24 hours in the environment; b) Cattle, as an intermediate host, ingest oocysts and become infected; c) After dissemination, parasite tachyzoites accumulate within tissues and form tissue cysts; d) Vertical transmission to the fetus; e) tissue cysts are present in the placenta; f) dogs may ingest placentas left unattended by the farm keepers and complete the life cycle. Sometimes dogs may have the chance to eat infected remains of dead calves.

The life cycle is complete when the definitive host ingests parasite laden tissues (McAllister *et al.*, 1998). In a farm where cattle and dogs coexist, this happens most commonly through the ingestion of placentas left unattended by farm keepers (Dijkstra *et al.*, 2002). In the definitive host, tissue cysts are likely to circulate past the stomach and colonize the intestine where sexual development of the parasite takes place (Dubey *et al.*, 1970; Kul *et al.*, 2015). The formation of an immature oocyst in the intestine then precedes its excretion into the environment. Evidence on the inexistent oocyst shedding on the feces of cows points out the lack of horizontal transmission between cows, leaving vertical transmission to the newborn calves as the only cow-to-cow route of transmission. Farms inflicted with epidemic events of neosporosis are frequently associated with horizontal transmission, such that the presence of oocyst laden dog feces in the farm is the most probable cause of the epidemic (Dubey *et al.*, 2007). Alternatively, considering that after an epidemic episode the infected cows remain in the herd, one may infer that the disease becomes endemic and its dissemination occurs mainly due to vertical transmission. Importantly, with the definition of coyotes (Gondim *et al.*, 2004) and white-tailed deer (Vianna

et al., 2005) as definitive and intermediate hosts in North America, the propagation of *N. caninum* through a sylvatic cycle makes culling and other preventive measures taken up by farm keepers even harder.

1.3 Neosporosis

Between the two hosts of the common life cycle, the most striking difference is that dogs are affected by the disease at any age while disease in cattle is mostly associated with congenitally infected calves. In fact, adult cows infected through horizontal transmission are asymptomatic and do only display signs of infection when they either fail to give birth or give birth to an ataxic calf, unable to stand or move. Further analysis on the serologic basis is key to determine if an adult cow is infected and histopathological analysis of either aborted fetus or tissues from euthanized calves confirms vertical transmission of the parasite. The clinical outcome of infected calves is dictated by the gestational stage at which infection occurs. Up to 3 months of gestation, early embryonic death is the most likely consequence as the fetal lymphoid tissues are still under development (Dubey, 2003). From 3 to 7 months, either abortion occurs or calves are born with neuromuscular disorders. Infection during late gestation is thought to be counteracted by a more competent fetal immune system (Bartley *et al.*, 2013) and often leads to the birth of normal though seropositive calves (Innes *et al.*, 2002). Up to 95% of calves born from seropositive cows remain clinically normal whereas the rest is born with clinical signs such as underweight, ataxia, hyperextended limbs and occasionally narrowed spinal cord (Dubey *et al.*, 2003). In the first semester of pregnancy, observed fetal lesions were more severe and parasitic loads in brain, heart, kidney and lung decreased over time, though remaining detectable in the brain (Collantes-Fernandéz *et al.*, 2006). Experimental infection of pregnant cows, subcutaneously, at either 70 or 140 days of gestation (Maley *et al.*, 2003; Macaldowie *et al.*, 2004), showed that parasitic DNA is detected at 14 days post-infection (pi) in the placenta, whereas infection at 210 days of gestation led to an earlier vertical transmission around 28 days pi with necrotic lesions until 42 days pi and resolution of inflammation by 56 days pi, denoted by the proliferation of connective tissue (Benavides *et al.*, 2012). These findings illustrate that pathogenesis of *N. caninum* is strongly dependent on the stage of gestation which in its turn relates to the degree of fetal immunocompetence.

1.4 Immune response

1.4.1 Cell-mediated Immunity

Protection against neosporosis is mostly related with a Th1-type response, mediated by interferon-γ (IFN-γ). In mice, production of IgG2a antibodies is also characteristic of Th1-type

immunity. In contrast, Th2-polarized responses which are characterized by preferential production of interleukin-4 (IL-4) and IgG1 antibodies, are associated with increased susceptibility to infection and thus, the balance between these types of response may dictate the disease outcome. In cattle, the establishment of a chronic infection may be behind a protective cell-mediated immunity elicited soon after infection as protozoan cysts accumulate preferentially in the spinal cord and brain. In pregnant dams infected at mid-gestation, fetal lymph node cells showed cell proliferation and IFN-γ responses at day 28 pi, followed by a decreased response by day 42 pi, when coincidently IgM and IgG antibodies were detected (Bartley *et al.*, 2004). IFN-γ is involved in the activation of genes coding for GTPases and nitric oxide synthases, both mediators of the inhibition the intracytoplasmic parasite growth through the disruption of the PVM. Nevertheless, this pro-inflammatory cytokine requires a balanced regulation to prevent excessive inflammation and cellular damage. Strong IFN-γ mediated responses may lead to placental lesions and consequent abortion (Almeria *et al.*, 2010). Maley *et al.* (Maley *et al.*, 2006) have shown that cattle challenged intravenously at day 70 of gestation completely aborted until day 28 pi. Subcutaneous infection, even at early gestation, did not elicit abortion in all animals as no parasite and expected inflammatory response was detected in the surviving fetuses. Resorting to immunohistochemistry techniques for cell phenotyping purposes, these authors found that the placenta inflammatory infiltrate comprised mainly cells positive for CD3, CD4, γδ TCR, NKp46 and fewer $CD8^+$ cells. Moreover, IFN-γ mRNA was detectable in the cytoplasm of mononuclear cells. Early studies in mice have shown that treating $CD4^+$ cells with anti-CD4 antibodies induced higher susceptibility to infection than blocking CD8 receptors (Tanaka *et al.*, 2000), underlining the influence of helper T cells for a protective immune response. Later into gestation and aided by the almost fully developed fetal immune system, dams are much less likely to abort. Infection at day 210 and analysis further until day 56 pi only resulted in congenitally infected fetuses (Bartley *et al.*, 2013). By day 14 pi, cellular mediated immunity was supported by the production of IFN-γ and IL-4 cytokines and splenocyte proliferation in response to *N.caninum* antigen recall (Bartley *et al.*, 2013). Unlike primates, ruminants have a cotyledonary placenta that blocks immunoglobulins and cytokines transport to the fetus. Thus, any occurring fetal immune responses are due to an active infection taking place in the uterus of the mother (Osburn *et al.*, 1982). To prevent rejection of the semi-allogenic fetus, the immunological environment in the placenta favors the production of rather anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β (Entrican, 2002). This may explain the occurrence of a more severe disease and abortion in dams, where an immunosuppressive phenotype would allow the parasite proliferation and dissemination through developing fetal tissues. A cellular environment with IL-4 would be prone to the development of a Th2-type

immune response, antagonizing the otherwise protective Th1-type response. A Th2-type biased response was observed in pregnant mice challenged with the Nc-Liverpool strain, where IL-4 and IL-10 levels surpassed those of IFN-γ, TNF-α and IL-12. One cannot rule out the importance of each type of immune response as fetal survival in pregnant dams seems to be depend on the tight balance between both in respect of gestational stages. Furthermore, Darwich *et al.* (Darwich *et al.*, 2016) could not correlate IFN-γ levels alone with a protective immune response but rather the ratio between IL-4/IFN-γ seemed to dictate fetal survival in infected dams. Also, in the early stages of infection in calves, NK cells and $CD8^+$ T cells were found to be the most important producers of IFN-γ whereas $CD4^+$ T cells emerged over time as the major IFN-γ producing cells (Boysen *et al.*, 2006). It was recently shown that IFN-γ production is a major protective mechanism conferred by $CD8^+$ T cells against *N. caninum* infection in mice (Correia *et al.*, 2015). Importantly, adoptive transfer experiments with $CD8^+$ T cells showed only partial protection conferred by this population in the infected mice, highlighting that other protective mechanisms may be necessary for full protection. Still in the murine model, macrophage depletion led to an increased sensitivity to infection and severe disease (Abe *et al.*, 2014). Further work from the same authors demonstrated that CCR5-deficient mice were also more susceptible to infection leading to increased mortality and neurological impairment (Abe *et al.*, 2015). Along with dendritic cells, other types of antigen-presenting cells (APCs) such as macrophages secrete IL-12 which is detected earlier in infection (Khan *et al.*, 1997; Teixeira *et al.*, 2010). This cytokine drives the differentiation of Th1 cells (Macatonia *et al.*, 1995). An important subset of dendritic cells is the plasmocytoid dendritic cells (pDCs). These cells are not $CD11c^{hi}$, unlike myeloid or conventional dendritic cells (cDCs). Their role in infection is mostly based on significant secretion of type I interferon (IFN-α and -β) whose functions range from T cell activation, APC activity and inhibition of viral replication. Only more recently, did the relevance of type I interferon in parasite infection start to be discussed (Silva-Barrios & Stäger, 2017). Interferon-α/β receptor (IFNAR) is tightly related with the STAT signaling and downstream activation of interferon stimulated genes (Ivashkiv & Donlin, 2014). Interestingly, IFNAR phosphorylation by p38 kinase promotes the degradation of the receptor, hindering interferon inducible signaling. Interestingly, *N.caninum* infection has been shown to correlate with p38 activation and consequent decrease in IL-12 levels (Mota *et al.*, 2016). The importance of pDCs was demonstrated in a report in which detection of IL-12, as soon as 12 hours pi, was mostly accountable of pDC activity than of cDC (Teixeira *et al.*, 2010). The production of IL-12 by pDCs fell into control levels 2 days after the infection but the number of pDCs did not vary significantly throughout the 5-day course of experiment, whereas cDCs did. Mice deficient in Interleukin-17 Receptor A (IL-17RA) are highly susceptible to *T. gondii*. Despite that neutrophil

function in IL-17RA mice remains normal, reduced chemokine expression and consequent neutrophil recruitment may account for the increased susceptibility to infection (Kelly *et al.*, 2005). As *N. caninum* and *T. gondii* mechanism of infection are similar, IL-17A may also have an important role in the early immune response mounted against the former parasite.

1.4.2 Humoral Immunity

The systemic immune response to *N. caninum*, orchestrated by Th1 and Th2 cells is characterized by promoting IgG2a and IgG1, respectively (Murphy *et al.*, 2016). In the murine model, a report has shown a slight predominance of IgG2a antibodies during the first three weeks of infection, before being surpassed by IgG1 until at least 64 days post infection (García-Melo *et al.*, 2010). Intra-species pathogenicity of *N. caninum* resulted only in more or less rapid antibody response though maintaining the same tendency between the two subclasses (García-Melo *et al.*, 2010). IgG antibodies can mediate antibody-dependent cell-mediated cytotoxicity

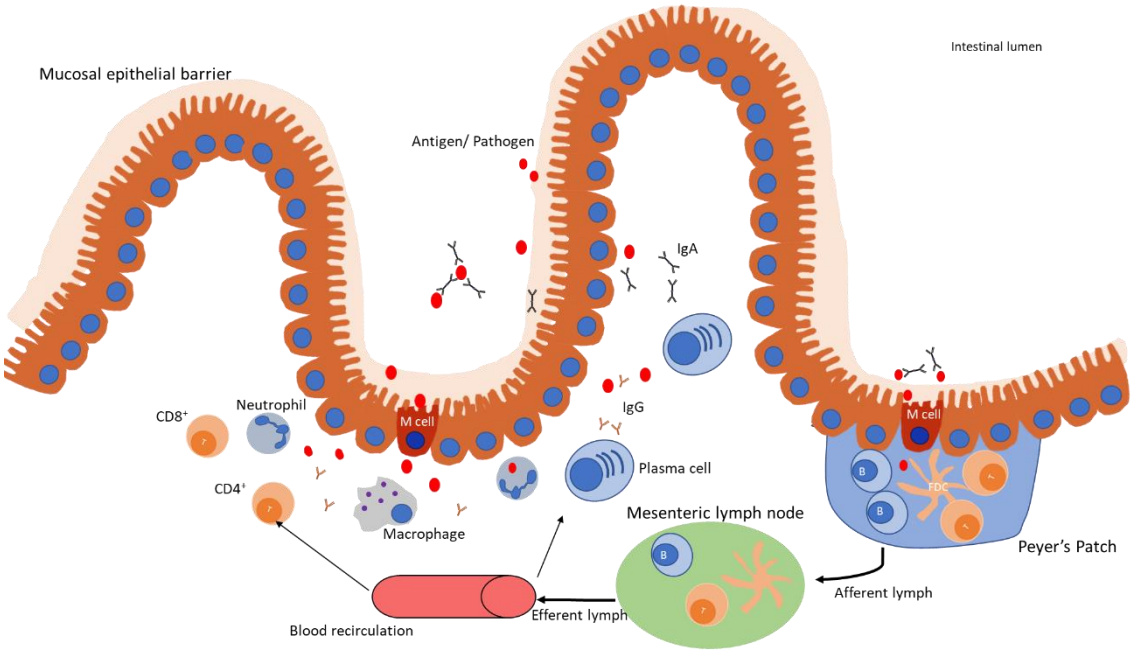

Figure 2. Overview of the gut immune response. Antigen presentation takes place in gut secondary lymphoid organs, from where primed lymphocytes enter the blood circulation. Homing of these cells to the gut provides both humoral and cellular types of immune response. Neutrophils and later macrophages are a first line of defense against the parasite and may become infected themselves. Cytotoxic $CD8^+$ T cells release their granule contents and aid in the clearance of infected cells. $CD4^+$ T cells mediate Th1, Th2 and Th17 immune responses. Th1 cells release IFN-γ that stimulates macrophages phagocytic activity. Plasma cells produce IgGs and secretory IgAs. The latter is released into the intestinal lumen and prevents parasites from crossing the mucosal intestinal barrier.

(ADCC) events involving NK cells (Vivier *et al.*, 2004), while also playing a key role in the neutralization of pathogens and opsonization prior to phagocytosis (Murphy *et al.*, 2016). Although the role of IgG during *N. caninum* infection is not yet completely understood, IgG2b antibodies against vesicular structures present in the cytoplasm of the closely related protozoan

T. gondii inhibited asexual replication in mice (Mineo *et al.*, 1994). Moreover, the incubation of opsonizing IgG antibodies decreased parasite invasion, *in vitro*, under a dose-dependent manner (Ferreirinha *et al.*, 2014).

In contrast with systemic immunity, mucosal immunity is dominated by the main effector immunoglobulin IgA, that predominates in mucosal secretions where it is referred as secreted IgA (SIgA). In response to parasites released from ingested cysts in the gastrointestinal tract, SIgA is portrayed as a first line of defense and its functions have been reviewed (Mantis *et al.*, 2011). Blocking of epithelial receptors and immune exclusion are examples of the host protective role of SIgA. Immune exclusion is a process where several antibodies bind to the pathogen through polyvalent surface antigens. The agglutination later leads to entrapment in the mucus and the pathogen is excreted aided by peristaltic movements. Such effect may be behind the reported protective effect of a mucosal vaccine, which conjugated high and persistent titres of IgA with abolished parasite burden in the brain though without significant IFN-γ secretion following 7 days pi. (Ferreirinha *et al.*, 2014; Ferreirinha *et al.*, 2016). Despite the absence of a long-lasting cellular immune response, early secretion of IFN-γ is not ruled out while mucosal immunoglobulins may indeed hinder gut epithelium invasion by the protozoan. In addition, IgA immune complexes can cross the epithelial membrane via selective adhesion to Peyer's Patch M cells and target dendritic cells, triggering both mucosal and systemic immune responses (Favre *et al.*, 2005). The protein disulfide isomerase (PDI) is a chaperone involved in protein folding and post-translational modification in the endoplasmic reticulum (Wilkinson & Gilbert, 2004). It is believed that that PDI plays a role in tachyzoite-host cell adhesion/invasion (Naguleswaran *et al.*, 2005) and since anti-PDI IgA was consistently detected in bovine tear fluid (Liao *et al.*, 2006), this may be one mechanism of host defense against *N. caninum*. Mice immunized with an extract of membrane proteins produced mucosal IgA that were mainly specific to microneme protein NcMIC1 (Ferreirinha *et al.*, 2016), which is involved in host cell invasion by the parasite. Interestingly, mice deficient in Th17 cells have failed to generate antigen-specific IgA responses. Thus, it is thought that Th17 cells acquire a follicular helper T cell phenotype in Peyer's Patches where they orchestrate IgA-producing B cells expansion (Hirota *et al.*, 2013). Taken together, these findings suggest that IgA agglutinating and neutralizing effector functions are carried out in a specific manner and under tight regulation from Th17 cells, which may constitute a primal and very important immune response to infection.

1.5 Experimental Models

1.5.1 *In vitro*

Different kinds of primary cells and primary cell lines have been used for culturing *N. caninum*. Initially isolated from bovine monocytes and cardiopulmonary endothelial cells (Dubey *et al.*, 1988), the parasite is most commonly grown in green monkey kidney epithelial (Vero) cells, MARC-145 monkey kidney cells and human foreskin fibroblasts (HFF). Tachyzoite to bradyzoite differentiation, using Vero cells as host, was induced with nitroprusside (Vonlaufen *et al.*, 2004), allowing for the characterization of the antigenic composition of each parasite stage. To date, no *in vitro* model has succeeded in delivering oocyst generation. This has restrained models of intragastric infections to the use of tachyzoite stages of the parasite combined with previous neutralization of the acidic pH found in the stomach (Teixeira *et al.*, 2007). *In vitro* studies are useful to determine the dynamics of parasite attachment and invasion of host cells. In (Naguleswaran *et al.*, 2003), monolayers of Vero cells were cultured and treated with proteolytic enzymes demonstrating a crucial role of chondroitin sulphate residues for parasite adhesion. In addition, drug and antibodies efficacy assessment are also performed using *in vitro* models, namely HFF cells (Muller *et al.*, 2016; Coceres *et al.*, 2012). Differences in parasite strain virulence have been demonstrated (Atkinson *et al.*, 1999; Collantes-Fernández *et al.*, 2006; Regidor-Cerrillo *et al.*, 2010), which also pointed out expected variability following Nc-1 or Nc-Liv challenge. In addition, consensus surrounding the optimal or rather maximum number of parasite culture passages is still lacking. While 20 passages may be considered a turning point for the parasite virulence, other researchers consider 37 as minimal for number of passages, reaching as far as 74 before regarding them as less virulent (Bartley *et al.*, 2006).

1.5.2 *In vivo*

Mice have been extensively used as experimental models to study neosporosis. The outbred Quackenbush (Qs) strain (Quinn *et al.*, 2002) is suitable for pregnant mice testing, as it shows less pathogenicity in response to the parasite and increased litters than other inbred strains. Either immune response or vaccination studies frequently used the BALB/c and C57BL7/6 inbred mouse strains, where the protection criteria typically comprise mouse survival; clinical signs and parasite burden measured by Polymerase Chain Reaction (PCR) and Immunohistochemistry (IHC). Vertical transmission is often assessed by PCR detection in pups' brain and lungs or mortality. Studies regarding the basis of the immune response against *N. caninum* have utilized immune-deficient mouse strains. Namely, T-cell deficient nude mice (Ammann *et al.*, 2004), B-cell deficiency µMT mice (Eperon *et al.*, 1999) or the C57BL/10ScCr strain simultaneously lacking TLR-4 and a functional IL-12 receptor (Botelho *et al.*,2007) as well

as others have been used as models. The main constraints that limit the extensive use of cattle as an *in vivo* model are space and cost requirements (Monney *et al.*, 2014). Due to the lack of model standardization, different *N. caninum* isolates, different breeds of cattle, doses and time of inoculation have led to variability in the results obtained which may bring harm to the better understanding of pathogenesis in cattle (Benavides *et al.*, 2014). As reviewed by Benavides, in the cattle abortion model, fetal viability is monitored by means of trans-rectal ultrasonography during gestation. In addition, parasite dissemination and severity is tested through immunohistochemistry, qPCR and histopathological analysis of target organs (brain, lung, heart and liver). In non-abortion models, vertical transmission is based on serologic analysis. In ruminants, maternal immunoglobulins do not cross the placenta (Black *et al.*, 1995) thus any antibody detected in pre-colostral serum from calves indicates the occurrence of a fetal immune response against the parasite (Innes *et al.*, 2001; Davison *et al.*, 1999).

1.6 Vaccines

The natural route of infection is the gastrointestinal tract so mucosal immunity plays a major role in the containment of the infection. In addition to enterocytes and goblet cells that produce large quantities of mucus, the mucosal epithelium homes an important secondary lymphoid tissue, the Peyer's patches. Peyer's patches are covered by microfold cells (M cells) that provide an entry point for antigens through the epithelium and also for dendritic cell screening through its dendrites expansion further to the intestinal lumen. Here APCs converge to the presentation of antigens to B- and T-cells, which then become activated and enter the lymphatic circulation until being drained to the bloodstream in the thoracic duct (Murphy *et al.*, 2016). Once their recirculation starts, cells must have the ability to home to the specific tissue where they were activated and evidently where infection is taking place. There is a specific subset of dendritic cells (DCs) in the lamina propria of the gut termed $CD103^+$ DCs. During APC presentation to the T cells, they release retinoic acid that induces the expression of homing markers on the surface of the T cell, $\alpha4\beta7^+$ $CCR9^+$. Moreover, depletion of vitamin A in mice resulted in reduced numbers of activated/memory T cells in the gut mucosa, exclusively (Iwata *et al.*, 2004). The integrin $\alpha4\beta7^+$ that binds MAdCAM-1 residues typically present in the gut epithelium whereas the latter is a receptor for chemokine CXCL25, also released by gut epithelial cells. Interestingly, MadCAM-1 residues are not only confined to the gut and are indeed present in the respiratory and urogenital tracts. Such redundancy favors an immunization strategy, against horizontal transmission, targeting a rather easy and safe route of administration that is the nasal cavity in order to confer protection in the gut as it represents the first line of defense against infection. The nasal-associated lymphoid tissue (NALT) is covered by M cells, similarly to

the gut Peyer's Patches, providing an entry-point for antigens delivered at these surfaces. Previous studies on intranasal immunization of mice against Herpes simplex virus (HSV) infection have shown the distal migration of HSV-specific T cells to the vaginal mucosa. Furthermore, they showed how cervical but not iliac lymph node-derived DCs were capable of inducing IFN-γ secretion by $CD4^+$ T cells (Sato *et al.*, 2014), proving its importance as a draining lymph node in the context of intranasal immunizations. How lymphocyte imprinting and recirculation contributes to the immunological cross-talk between different mucosa is still unclear. Nevertheless, this route of immunization has already produced promising results, ensuring long-lasting immunological memory and it will be further discussed below. Evidence on the influence of DCs origin towards the lymphocyte homing fate have established Peyer's Patches and MLN derived DCs as inducers of α4β7 and CCR9 expression and consequent gut-homing phenotype, whereas DCs from peripheral lymph nodes induce higher levels of E- and P-selectin ligands and CCR4 expression, in what is believed to be a default mechanism of imprinting (Mora *et al.*, 2005). A different paradigm dominates the literature concerning vaccines against neosporosis. Congenital transmission is a consequence of the systemic burden of the parasite so that one would hypothesize a vaccine administered through a systemic route (i.e intraperitoneal, subcutaneous and intramuscular) to protect against infection. The administration of a vaccine through this route employs a rather central mechanism of immunization allowing APCs to converge in other secondary lymphoid organs, generating memory cells prone to be activated in the event of a systemic infection of the parasite. Naturally, prevention of horizontal transmission does prevent further systemic dissemination and consequent congenital transmission but only few reports focused on mucosa directed vaccines. In this section, advances on vaccine development will be described with respect to each type of vaccine: subunit, live attenuated and killed.

To date, the only commercialized vaccine against neosporosis, Bovilis® Neoguard (Merck), comprised a killed *N.caninum* tachyzoite formulation, adjuvanted by Havlogen. Initially, the vaccination effect was shown to be protective as abortion incidence was decreased (Romero *et al.*, 2004). This study, conducted in Costa Rica, demonstrated that results were farm-specific. Of the 20 farms tested, 15 showed a positive effect, no effect was observed on 4 and negative effect was reported on 6 farms. Another study in New Zealand (Weston *et al.*, 2012) registered the same farm-specific effect concluding that vaccination was not efficient on reducing abortion and that it did not prevent vertical transmission. No vaccine has been further commercialized and future perspectives on the most viable vaccine strategy are controversial.

1.6.1 Live attenuated

The use of attenuated live strains of pathogens have a considerable history of usage in humans as well as in animals such as cattle and sheep. It proves advantageous as its pathogenicity is sufficient to induce immunological memory in the host without causing disease. The observation of asymptomatic infections in cattle led to the possibility of isolating a *N. caninum* strain with attenuated virulence. In fact, Nc-Nowra strain was isolated from an asymptomatic calf and tested in mice for its diminished ability to cause neurological disorders (Miller *et al.*, 2002). Further testing consisted of immunizing pregnant mice with Nc-Nowra live tachyzoites, at day 5 of gestation, before Nc-Liverpool challenge (Miller *et al.*, 2005). A significant decrease on transplacental transmission, from 75% to 0.8% highlighted the protective potential that a live vaccine had on congenital transmission of the parasite. Similar results were obtained in cattle (Williams *et al.*, 2007). A group of 6 cows, previously immunized and challenged with Nc-Liv virulent strain at day 70 of gestation were protected from abortion. Similarly, Rojo-Montejo *et al.* (Rojo-Montejo *et al.*, 2009), reported a low virulent strain Nc-Spain 1H suitable for live vaccine strategies. Heifers were challenged at day 70 of gestation, and only mild histopathological lesions consistent with *N. caninum* infection were detected in the brain and heart of the dam. Furthermore, the efficacy of such strategy was assessed in a later report (Rojo-Montejo *et al.*, 2013) and protection against fetal loss was only partial, as well vertical transmission was also not prevented following a mid-gestation challenge. Employing live vaccines for veterinary uses has significant drawbacks such as reversion of virulence and limited shelf-time. For instance, vaccine storage at -80ºC did not decrease parasite viability while immunogenicity was indeed lost (Weber *et al.*, 2013). Indeed, Toxovax®, a live vaccine against toxoplasmosis in sheep, has an on-demand production to ensure the viability of the parasite when inoculated in the host.

1.6.2 Subunit

Subunit vaccines make use of specific proteins extracted from the pathogen or produced recombinant. The latter option accounts for a limitation that is the absence of post-translation modifications that are restricted to eukaryotes, when producing recombinant proteins in prokaryote clones, and that may or may not result in loss of antigenic epitopes. Nevertheless, this strategy is well supported by numerous reports using extracted proteins, recombinant proteins or a mixture of the latter with the respective DNA to overcome the previously stated limitation. Target proteins are often involved in the attachment of the parasite and invasion, where microneme and surface proteins have an important role. Vaccines combining both recombinant proteins and DNA have also been tested where NcSAG1 and NcSRS2 recombinant

proteins were combined with their DNA counterparts. Recombinant proteins alone did not have such protective effect as when combined with DNA, in terms of reducing the parasite burden in the brain (Cannas *et al.*, 2003). It is important to notice that some antigens or immunizing strategies may be counter-protective. For instance, mice immunized with recombinant, native or DNA encoding microneme protein NcMIC4 showed higher parasitic burden and increased mortality (Srinivasan *et al.*, 2007). Reports on vaccine efficacy using bovine experimental models usually fall under live or killed tachyzoites extracts strategies. A combination of NcHSP20, NcSAG1 and NcGRA7 was used to vaccinate pregnant cows, but failed to prevent vertical transmission (Hecker *et al*, 2014). To this day, this was the only report of subunit vaccination against neosporosis, using the pregnant bovine model. Protection in non-pregnant cows was reported using oligomannose-coated liposomes containing NcGRA7 (Nishimura *et al.*, 2013). Subunit vaccines are advantageous due to the reduced costs of production, storage and increased shell-life. In clear contrast with live attenuated vaccines, they lack virulence which in turn calls for the addition of an adjuvant to the vaccine formulation. Adjuvants can induce an immunomodulatory effect or even an enhancement of antigen bioavailability. Thus, their action acts as a booster of the immune response and they will be further discussed later. In mice, intranasal mucosal immunization using membrane protein extracts adjuvanted by CpG protected against intragastric infection (Ferreirinha *et al.*, 2014). In this report, intestinal parasite-agglutinating IgA were shown to be produced after immunization, thus providing a first line of defense against horizontally transmitted parasites in the gastrointestinal mucosa. Furthermore, this vaccine formulation elicited a long-term protective effect as IgG1/IgG2a ratio remained below 1, denoting a Th1- type bias, for as far as 13 weeks (Ferreirinha *et al.*, 2016). In accordance, *in vitro* studies with stimulated spleen and mesenteric lymph nodes (MLN) cells derived from immunized mice showed increased IFN-γ production whereas IL-4 levels remained low (Ferreirinha *et al.*, 2016). Future studies should address the suitability of the vaccine in cattle and pregnant hosts, considering both strong humoral and cell-mediated responses using murine *in vivo* and *in vitro* models. In addition to what was previously mentioned, few studies have approached mucosal immunizations (Debache *et al.*, 2011; Debache *et al.*, 2013). It is difficult to draw conclusions on the most promising vaccine based on the reported results as there is no established model to study neosporosis in both bovine and mice experimental models. Typically, three inbred strains of mice are employed as experimental models of neosporosis, with differing responses to challenge (Mols-Vorstermans *et al.*, 2013). Also, most studies have developed vaccine formulations based on non-pregnant mouse models (Monney & Hemphill, 2014), passing evident immunological alterations surrounding pregnancy and its impact on neosporosis outcome. In fact, using the non-pregnant model is only justified when assessing vaccine efficacy

against horizontally transmitted neosporosis (Ferreirinha *et al.*, 2014; Ferreirinha *et al.*, 2016). Moreover, efforts put into *in vitro* differentiation of oocysts have proven unsuccessful, which may account for the reduced follow-up on mucosal vaccines. Even so, tachyzoite differentiation from oocysts occurs in the gastrointestinal tract, and such model of infection is still valid. Vaccine efficiency is dictated for its ability to: prevent tachyzoite proliferation and dissemination to avoid vertical transmission; prevent tissue cyst formation in intermediate hosts or reduction of oocyst shedding in dogs (Monney *et al.*, 2011). In other words, a vaccine that stimulates both cellular and humoral immune responses with antibody response at mucosal and systemic sites.

1.6.3 Adjuvants

Adjuvants are commonly added to vaccine formulations to enhance the immune response required for protective immunity. Their use is mostly important on inactivated and subunit vaccines, where the virulence cues provided by the pathogen over the course of infection are not present. Their influence on the enhancement of the immune response may be due to an increase in antigen half-life, improvement of either delivery systems or antigen presenting mechanisms by APCs and the production of immunomodulatory cytokines. Under these effects, the required amount of antigen and additional immunizations are reduced thus improving vaccine cost-effectiveness. Recently developed vaccines have focused on the use of agonists for TLR (Toussi & Massari, 2014). TLRs are present on the surface of APCs or intracellularly, and recognize specific pathogen-associated molecular patterns (PAMPs), triggering signal pathways involved in inflammation and later adaptive immunity (Kabelitz, 2007). TLR9 recognizes unmethylated CpG dinucleotides, most commonly found in bacterial and viral genomes, leading to the secretion of inflammatory cytokines IL-6, IL-12 and IFN-γ (Klinman *et al.*, 1996) and increased $CD8^+$ T cell responses (Overstreet *et al.*, 2010). In fact, mice immunized with *N. caninum* membrane proteins developed both systemic and mucosal immunity which was enhanced depending on CpG inclusion as adjuvant (Ferreirinha *et al.*, 2014). CpG like other adjuvants of bacterial nature, monophosphoryl lipid A, Freund's adjuvant and cholera toxin, may have limited safety due to the potential exacerbated inflammation elicited (Vogel, 2000). Alternatively, antigen delivery based on biodegradable polymeric microparticles is also a valid strategy, regarding the slow release and depot effect of the antigen. In fact, their depot effect exceeds by far that provided by aluminum salts or water in oil emulsions (Sivakumar *et al.*, 2011). One example of this class of adjuvants is Carbigen™, carbomer-based, and widely used in veterinary vaccines considering the above-mentioned advantages (Peters *et al.*, 2012). Also, it was reported its use in another vaccine against Infectious bovine keratoconjunctivitis (IBK), showing great promise in the stimulation of ocular mucosal IgA titres (Angelos *et al.*, 2014).

Carbomer polymers have a high percentage of carboxylic acids, allowing hydrogen bonds to form with mucin proteins, highly abundant in nasal cavities, which in turn confers them bioadhesive properties in mucosal surfaces. Additionally, their quelating effect on Ca^{2+} is associated with a loosening of the epithelial tight junctions and consequent increased molecular uptake through a paracellular route (Clausen *et al.*, 2002). Their release mechanism is mostly due to a growing osmotic pressure inside the gel that destabilizes the structure and starts breaking off pieces of the matrix (Singla *et al.*, 2000). Interestingly, sterile inflammation with carbopol via intraperitoneal (i.p) has been correlated with increased IFN-γ secretion and increased uptake of APCs during sterile inflammation (Gartlan *et al.*, 2016). Nevertheless, IFN-γ stimulation conjugated with increased IgA titres have never been reported unanimously in a single vaccination attempt with this specific adjuvant.

2. Objectives and dissertation outline

The main objective of the present work was to evaluate the efficacy of Carbigen™ as adjuvant in an intranasal immunization approach to prevent *N. caninum* infection using NcMP as target antigen. Previous reports have shown that a combination of CpG with NcMP provides a protective effect against intragastrically established neosporosis. Carbigen™ is not as expensive as CpG and would facilitate scaling up vaccine testing in bovines and eventually commercializing it.

This dissertation contains four chapters: Introduction, Materials and Methods, Results and Discussion, Conclusions and future perspectives. In Chapter 1, the context of the subject of the dissertation is presented. Chapter 3 provides a description of the materials and methods used to carry out the specified procedures. In Chapter 4, a description of the obtained results and their discussion is presented combined. The main conclusions drawn from this work, as well as perspectives for future work are presented in Chapter 5.

3. Materials and Methods

3.1 Mice

Animal experiments were approved by the animal ethics committee (Organismo Responsável pelo Bem Estar Animal) of ICBAS and authorized by the animal welfare section of the competent national board, Direcção Geral de Alimentação e Veterinária (document 0421/000/000/2016).

3.2 Parasites

Neospora caninum tachyzoites (Nc-1 isolate) were cultured and serially passaged in VERO cells, using Minimum Essential Medium (MEM; Sigma Aldrich, St Louis, MO, USA) with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), L-glutamine (2 mM), penicillin (200 IU/mL) and streptomycin (200 µg/mL) (all from Sigma). Cells were incubated at 37 °C under a 5% CO_2 humified atmosphere until 70% of the host cell monolayer was destroyed. For parasite isolation purposes, cells were harvested using a cell scraper and the obtained supernatants were then centrifuged at 1500 g for 15 min at 4 °C. The pellet was washed thrice in Phosphate Buffered Saline (PBS), with identical centrifugations. Finally, the pellet was resuspended in 3 mL of PBS and passed through a PD-10 column filled with Sephadex™ G-25M (Amersham Biosciences Europe GmbH, Freiburg, Germany). Total parasite count was achieved using a haemocytometer.

3.3 Cell-membrane proteins and tachyzoite lysates extraction

Neospora caninum Membrane Proteins (NcMP) were extracted from cultured tachyzoites. Firstly, they were resuspended in 0.75% Triton X-114 (Sigma), distributed into eppendorf tubes and incubated on ice for 10 min with a subsequent 10000 g centrifugation for 30 min at 4 °C. The supernatant was then transferred to new tubes and incubated for 3 min at 30 °C following incubation on ice for 10 min. The process was repeated once again and the recovered supernatants were incubated at 30 °C, and centrifuged at 1000 g, for 3 min at room temperature. The latter step was to facilitate phase separation, as the aqueous phase was discarded and the hydrophobic phase precipitated with the addition of absolute ethanol and incubated for 1 hour on ice. Finally, the samples were centrifuged at 12000 g for 20 min at 4 °C and the respective pellet dried, resuspended in PBS and stored at -20 °C. *Neospora caninum* Sonicates (NcS) were obtained through the disruption of tachyzoites following sonication (26 cycles of 15 seconds at 100 W) using a Branson cell disrupter model W 185 D. Samples were kept on ice bath to prevent overheating. Then, NcS were sequentially passed through 0.45 µm and

0.2 µm pore-size filters and stored at -20 °C. Both extracts were quantified, on the protein basis, using the Lowry protein assay (Lowry *et al.*, 1951).

3.4 Immunizations, challenges and tissue sample collection

Mice were distributed randomly by the different experimental groups. Immunizing formulations were administered via the intranasal route in a final volume of 15 µL in PBS. Formulations contained 30 µg of NcMP adjuvanted by 10% Carbigen™ or 10 µg CpG 1826 VacciGrade (Invivogen, San Diego, CA). Control groups consisted of mice sham-immunized with PBS, Carbigen™ 10% and 10 µg CpG. Boost immunizations were done three weeks afterwards. At six weeks, the animals were infected with 1 x 10^7 freshly isolated *N. caninum* tachyzoites. One week following infection, mice were anesthetized with isoflurane and euthanized through cervical dislocation. Brain, lungs and liver were collected and stored at -20 °C. Spleens were aseptically removed for cell culture purposes. To obtain intestinal lavages, PBS with protease inhibitors (Mini Complete, Roche, Basel, Switzerland) was consecutively passed through the small intestine and then centrifuged at 4500 g, for 15 min at 4 °C, having its supernatant collected and again centrifuged at 10000 g, for 1 hour at 4 °C. Blood samples were allowed to clot, then serum was removed, centrifuged at 13000 rpm for 15 min at 4 °C, transferred to new tubes and stored at -20 °C.

3.5 *In vitro* cell cultures and cytokine detection

Splenocytes were isolated from the spleens of control and immunized mice. Briefly, spleens were homogenized in Hanks' balanced salt solution (HBSS) (Sigma) and passed through 100 µm cell strainers (BD Falcon, Franklin Lakes, NJ, USA). ACK Buffer was added to lyse red-blood cells and cells were washed in HBSS before being resuspended in complete RPMI-1640 medium (Sigma), with 10% FBS, HEPES (10 mM), penicillin (200 IU/mL) and streptomycin (200 µg/mL), (all from Sigma), and β-mercaptoethanol (0.05 µM) (Merk, Darmstad, Germany). The lungs and liver were cut in small pieces and placed in RPMI containing 2 mg/mL Collagenase D (Roche) for 45 min at 37 °C in a water bath with agitation. Then, samples were homogenized, passed through 100 µm cell strainers. Liver leukocytes were isolated through density gradient centrifugation using 33% Percoll (GE Healthcare, Chicago, IL, USA) solution in PBS for 12 min at room temperature, 750 g with minimal brake. Cells from lungs and liver were washed twice with HBSS 2% FBS before being resuspended in 500 µL of complete RPMI-1640 medium. In between washes, red-blood cell lysis was achieved with Ammonium-Chloride-Potassium (ACK) Lysing Buffer. Spleen, liver and lung cell concentration was adjusted to 2x10^6 cells/mL. Cells were plated (2x10^5/well) in round bottom 96-well plates (Nunc, Roskilde, Denmark) and were left unstimulated, or either stimulated with NcMP (25 µg/mL) or NcS (25 µg/mL) for 3 days at 37 °C

and 5% CO_2, when supernatants were collected. IFN-γ, IL-4, and IL-17A cytokines present in culture supernatants were quantified by ELISA using eBioscience™ Mouse ELISA Ready-SET-Go!™ kits respective to each cytokine. The reaction was stopped with the addition of 1 M H_2SO_4 and plates were read in BioteK™ µQuant Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA) using Biotek™ Gen5™ Data Collection and Analysis Software (Thermo Fisher Scientific). Absorbance was measured at 450 nm, and corrected with the respective values at 570 nm. Standards for each cytokine were serially diluted. Cytokine concentration was calculated from the respective standard curve.

3.6 Flow cytometry

Lymphocyte lineage and activation was assessed using flow cytometry analysis. Data were acquired in a BD FACSCanto™ II cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and processed using FlowJo version 10 (Tree Star inc., Ashland, OR, USA). Single-staining controls for each antibody and unstained samples were used for compensation. Fluorescence minus one (FMO) controls for each antibody aided in the gating strategy employed. Each well had 10^6 cells which were stained with the following anti-mouse monoclonal antibodies: eFluor 506 CD3 (17A2), eFluor 450 CD4 (RM4-5), PE-Cy7 CD44 (IM7), PE CD62L (MEL-14), PerCP Tbet (eBio4B10), APC RORγt (APC), AlexaFluor488 Gata3 (TWAJ), PE-Cy7 Granzyme B (NGZB) (all from eBioscience, San Diego, CA, USA); PE CD44 (IM7), APC CD62L (MEL-14) and FITC CD8 (53-6.7) (Biolegend, San Diego, CA, USA). Cell viability was assessed using APCeFluor 780 Fixable viability dye (FVD; eBioscience). Each sample was stained for detection of both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations. Both populations were stained for detection of CD44 and CD62L activation/effector memory surface markers detection. $CD4^+$ T cells lineage was assessed through Tbet, RORyt and Gata3, being $CD8^+$ stained for Tbet and Granzyme B expression. Firstly, cells were stained with FVD and incubated 30 min on ice. After washing with PBS, antibodies specific for surface markers were added to the cells and incubated for 25 min on ice, protected from light. After washing with FACS buffer (10 mM Sodium Azide, 2% FBS in PBS), Foxp3 Fixation/ Permeabilization solution (eBioscience) was added and cells were incubated overnight at 4 °C. Cells were washed using Permeabilization Buffer (as provided by the manufacturer). To block non-specific binding, 2% rat serum was added and incubated 15 min at room temperature. Antibodies for intracellular staining were added directly to the wells and incubated 30 min at room temperature, protected from light. Cells were washed twice and resuspended in 150 µL of FACS buffer. Washing refers to the addition of buffer solution followed by centrifugation at 500 g for 5 min at room temperature.

3.7 Antibody detection

Intestinal IgA, serum IgG1 and IgG2a were quantified by ELISA. Flat-bottomed 96-well plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4 °C with NcS diluted in PBS at a concentration of 5 µg/mL. Plates were washed and blocked for 1 hour at room temperature with 2% BSA in TST (150 mM NaCl, 10 mM Tris, 0.05% Tween-20, pH 8.0) buffer solution. Samples were diluted in 1% BSA in TST and added and incubated for 2 hours at room temperature. The detection of bound antibodies was achieved using alkaline phosphatase coupled to secondary antibodies specific to IgA, IgG1 or IgG2a (Southern Biotech, Birmingham, AL, USA) and p-nitrophenyl phosphate (Sigma) as a substrate. The reaction was stopped with the addition of 0.1 M EDTA (pH=8.0) and plates were read in BioteK™ µQuant Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA) using Biotek™ Gen5™ Data Collection and Analysis Software (Thermo Fisher Scientific). Absorbance was measured at 405 nm, and corrected with the respective values at 570 nm. Serum from non-immunized and non-infected mice was used as a threshold value to determine the reciprocal antibody titre for every other sample.

3.8 DNA Extraction

Organs were weighted and homogenized in 1 mL of SE buffer (75mM NaCl; 25mM Na_2 EDTA; pH 8.0), and then added to a solution containing 1% SDS and 0.5 mg/mL Proteinase K (Sigma). Samples were then incubated overnight and DNA was extracted following the phenol-clorophorm (from Sigma and Merk, respectively) method followed by ammonium acetate/ethanol precipitation. DNA was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). DNA concentration was then adjusted to 500 ng/mL.

3.9 Parasite burden quantification

DNA from the brain and lungs of infected mice was used in a quantitative polymerase chain reaction (qPCR) directed to the amplification of a 103-bp sequence of the conserved Nc5 region of the *N. caninum* genome, using the NZY qPCR Green Master Mix (NZyTech, Lisbon, Portugal). The primers used were NcA 5'-GCTACCAACTCCCTCGGTT-3' and NcS 5'-GTTGCTCTGCTGACGTGTCG-3', having both a final concentration of 0.2 µM. The TaqMan fluorescent probe, FAM-CCCGTTCACACACTATAGTCACAAACAAAA-BBQ, was used at 0.1 µM. For each sample reaction, a total 1000 ng of template DNA was used. Samples were loaded into a Corbett rotor gene 6000 system (Corbett Life Science, Sydney, NSW, Australia). The DNA samples were amplified with the following thermal cycle: 95 °C for 3 min and 60 cycles of 5 seconds at 95 °C and 20 seconds at 60 °C. Data were analyzed with the Rotor Gene 6000 software v1.7 (Corbett Life Science).

3.10 SDS-Page and Western Blot

Extracted NcMP and NcS proteins were boiled for 5 min in loading buffer to denature them. NZYColour Protein Marker II was used as a molecular weight marker (NZyTech, Lisbon, Portugal). Each polyacrylamide gel electrophoresis ran at 25 mA followed by silver nitrate staining (Gromova *&* Celis, 2006). Gels were transferred onto PVDF membranes and blocked with 2% (w/v) BSA (Sigma) in TST buffer. Serum antibodies obtained from mice immunized with NcMP+CpG and NcMP+Carbigen™ were diluted at 1:200 in 0.1% (w/v) BSA (Sigma) TST Buffer. After washing with 0.1% BSA (Sigma) TST Buffer, alkaline phosphatase-coupled goat anti-mouse IgG (Southern Biotech) diluted at 1:2000 was added. Detection of bound antibodies was achieved after the addition of NBT/BCIP substrate (Roche).

3.11 Statistical analysis

Statistical analysis was performed with GraphPad software (Version 5.0, Graphpad Software, Inc. La Jolla, CA). Column graphs display columns with vertical lines representative of mean and corresponding standard deviation (SD) on each tested group. Scatter dot graphs represent mean for each group as a horizontal bar. Statistical analysis was performed using one-way ANOVA with Sidak *post-hoc* test or unpaired two-tailed t-test, as specified in the legends.

4. Results and Discussion

4.1 Preparation of Neospora caninum membrane proteins extract (NcMP)

The antigens used for immunization were prepared by using *N. caninum* tachyzoites grown *in vitro*. The protein migration profile of the NcMP extract was analyzed by SDS-PAGE. As shown in Figure 3, the migration profile of NcMP shares most of the bands detected for NcS. Nevertheless, the band intensity corresponding to proteins of approximately 65 and 46 kDa differs between the two extracts (Figure 3, black arrows). Extraction with the non-ionic detergent Triton X-114, used to prepare NcMP, provides a separation between an aqueous phase and hydrophobic phase, which proves advantageous to the enrichment of membrane proteins (Taguchi & Schätzl, 2014).

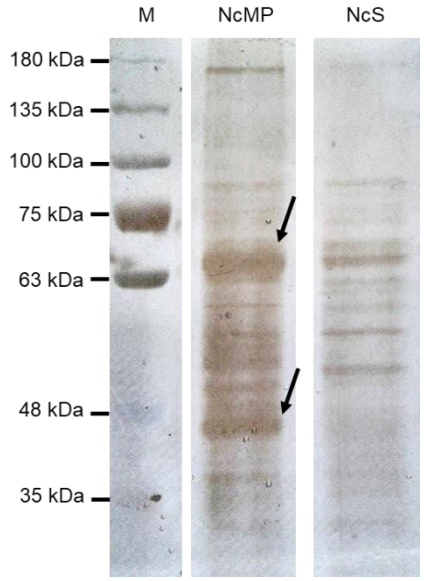

Figure 3 SDS-Page analysis of extracted NcMP and NcS proteins. Gels were stained using silver nitrate. The first lane contains NZYColour Protein Marker II (M). Numbers correspond to bands' molecular weight. NcMP and NcS are in the second and third lanes, as indicated. Black arrows indicate proteins with approximately 65 and 46 kDa.

The initial attachment of parasites to host cells is mediated by membrane proteins, which provide an anchoring effect that is further enhanced with the secretion of microneme proteins (Besteiro *et al.*, 2011). This mechanism of host cells' infection may thus be targeted with the use of NcMP as vaccine antigen.

4.2 Cytokine production in mice immunized with NcMP and Carbigen

To assess the protective effect of intranasal immunization using NcMP antigen plus Carbigen^{TM} adjuvant, C57BL/6 mice were challenged i.p with 1 x 10^7 tachyzoites 3 weeks upon the last immunizing administration. Splenocytes, liver and lungs mononuclear cells were collected from the immunized mice and sham-immunized controls, prior to or after infection, and stimulated *in vitro* with NcS and NcMP antigens. The levels of IFN-γ, IL-4 and IL-17A were assessed by ELISA in the culture supernatants 3 days upon the antigen stimulation. The levels of IFN-γ were detected elevated in the culture supernatants of splenocytes from immunized mice, collected either before or after infection (Figure 4), reaching similar levels in both cases. Cell culture supernatants of splenocytes collected from infected sham-immunized mice also presented similarly elevated levels of this cytokine following NcS stimulation, indicating that infection majorly contributed to the production of this cytokine, irrespective of prior antigen immunization. Nevertheless, lower levels of IFN-γ were detected in the supernatants of NcMP-stimulated cultures than in NcS-stimulated counterparts. In a previous report, an antigen recall assay using splenocyte cultures was used to test the efficacy of transformed *Mycobacterium bovis* strains with antigens from *M. tuberculosis*, as vaccines (Rao *et al.*, 2003). In that study, IFN-γ levels in the cultures were dependent on the antigens used to immunize the mice, highlighting the role of antigens in the response. Differences in the stimulation observed here may be also due to the presence of PAMPs in the whole-parasite sonicates that may possibly be absent in the membrane antigen extracts used in immunizations. The membrane proteins fraction prepared herein include mostly hydrophobic proteins thought to be key-players on the parasite attachment and adhesion to host cells. A co-stimulation with PAMPs present in the NcS may be a possible explanation to this differential response to antigen stimulation. Another possible explanation could be the hydrophobic nature of the NcMP that leads to protein aggregation and may hinder protein access and processing by antigen-presenting cells during the limited incubation time. A differential effect of the antigen used for stimulation was also observed for IL-4 and IL-17A (Figures 4, 5 and 6). The levels of IL-4 in the supernatants of lungs and liver cells were very low in general and the highest levels of this cytokine were measured in the

supernatants from non-infected immunized mice stimulated with NcS (Figures 5 and 6).

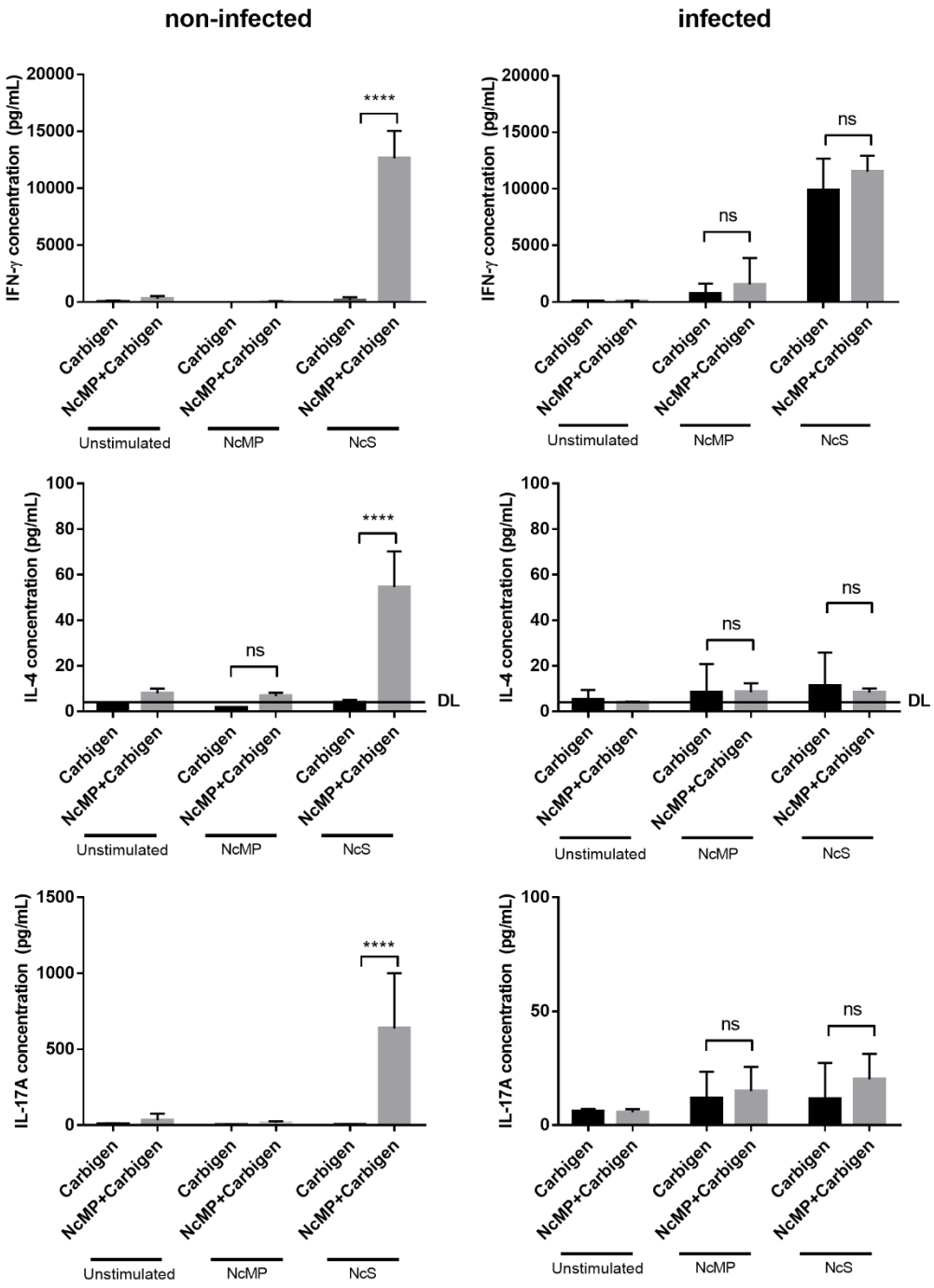

Figure 4. Detection by ELISA of cytokine levels in the supernatants of cultured splenocytes unstimulated or stimulated with NcMP or NcS for 3 days. Cells were collected 3 weeks upon the last of two i.n immunizations (non-infected) with NcMP and Carbigen™ (NcMP+Carbigen) or sham-immunization with Carbigen™ (Carbigen) or 7 days upon the infectious challenge (infected). Bars represent means plus SD. Number of mice per group in each experiment non-infected/infected: Carbigen (n=4/6), NcMP+Carbigen (n=4/7). Each condition was set in duplicate. Detection limit DL- (4 pg/mL) is indicated by a horizontal line. Statistical significances between groups were calculated using one-way ANOVA and Sidak *post-hoc* test (*** $p < 0.001$; **** $p < 0.0001$; ns – not significant).

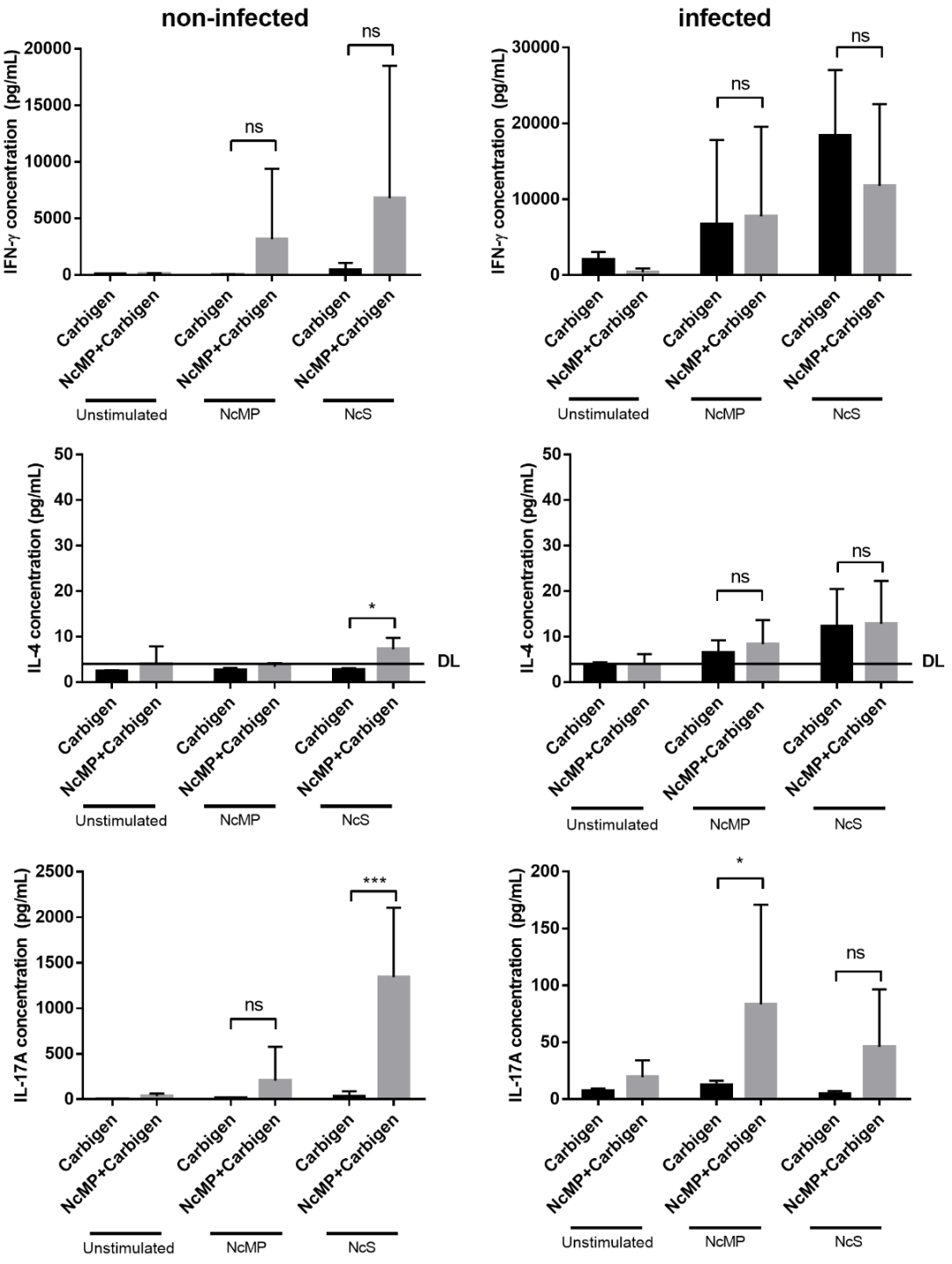

Figure 5 . Detection by ELISA of cytokine levels in the supernatants of cultured lung mononuclear cells unstimulated or stimulated with NcMP or NcS for 3 days. Cells were collected 3 weeks upon the last of two i.n immunizations (non-infected) with NcMP and Carbigen™ (NcMP+Carbigen) or sham-immunization with Carbigen™ (Carbigen) or 7 days upon the infectious challenge (infected). Bars represent means plus SD. Number of mice per group in each experiment non-infected/infected: Carbigen (n=4/6), NcMP+Carbigen (n=4/7). Each condition was set in duplicate. DL- Detection limit (4 pg/mL) is indicated by a horizontal line. Statistical significances between groups were calculated using one-way ANOVA and Sidak *post-hoc* test (* $p < 0.05$; ** $p < 0.01$; ns – not significant).

Carbigen™ adjuvant effect on NcMP antigens, as shown here, boosted the IFN-γ

secretion in splenocytes of unchallenged mice as detected 3 weeks upon the last of two

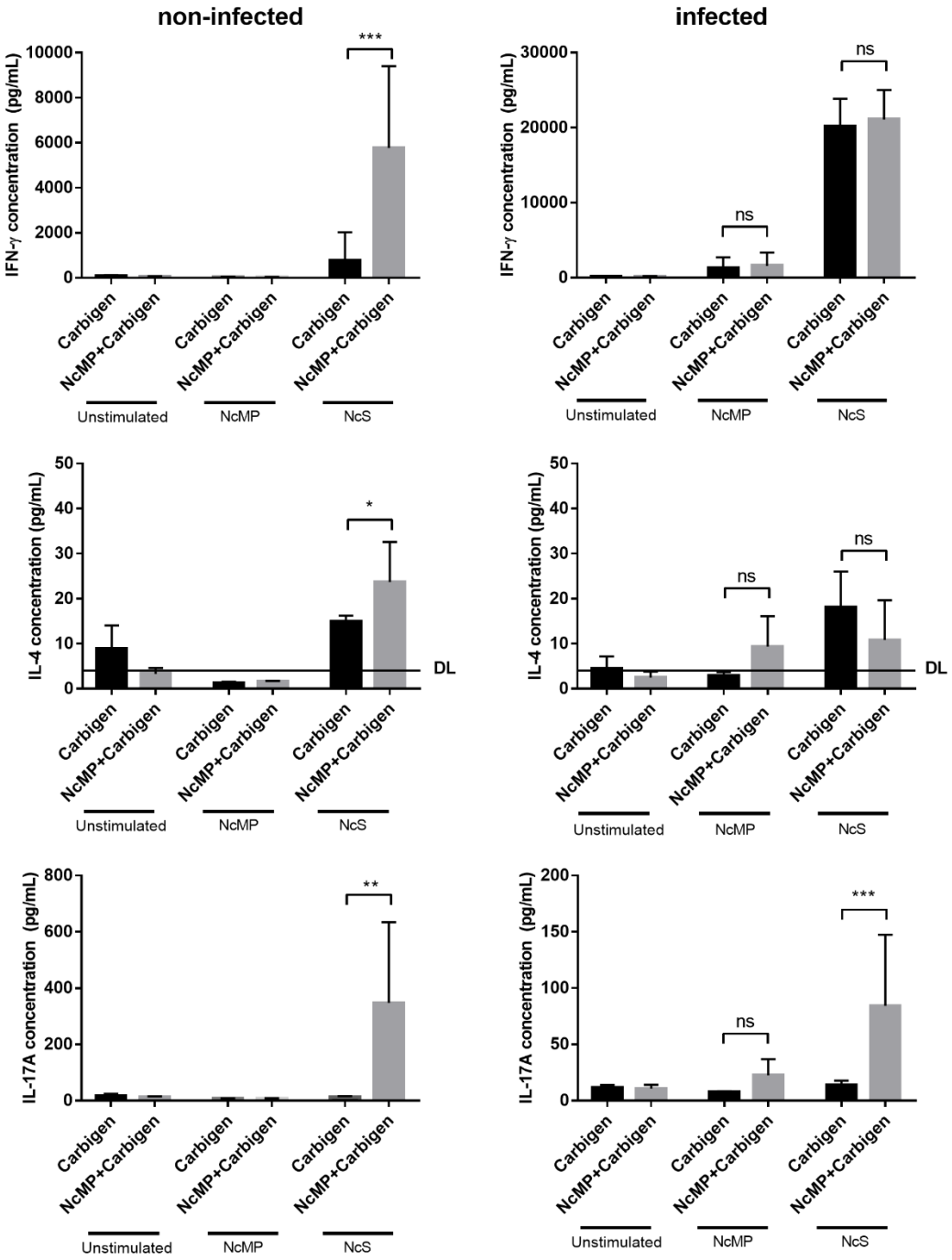

Figure 6. Detection by ELISA of cytokine levels in the supernatants of cultured liver mononuclear cells unstimulated or stimulated with NcMP or NcS for 3 days. Cells were collected 3 weeks upon the last of two i.n immunizations (non-infected) with NcMP and Carbigen™ (NcMP+Carbigen) or sham-immunization with Carbigen™ (Carbigen) or 7 days upon the infectious challenge (infected). Bars represent means plus SD. Number of mice per group in each experiment non-infected/infected: Carbigen (n=4/6), NcMP+Carbigen (n=4/7). Each condition was set in duplicate. DL - Detection limit (4 pg/mL) is indicated by a horizontal line. Statistical significances between groups were calculated using one-way ANOVA and Sidak *post-hoc* test (** $p < 0.01$; *** $p < 0.001$; ns – not significant).

immunizations. Using CpG as adjuvant, Ferreirinha *et al.* reported long-term memory cells with the ability to produce of IFN-γ that prevailed as far as 19 weeks following the last immunization. (Ferreirinha *et al.*, 2016). Also, the authors refer that differences in IFN-γ production could only be detected in the supernatants of non-infected mice. Indeed, 7 days after infection, the levels of IFN-γ in the supernatants of immunized mice were even lower than the ones found in sham-immunized mice. One reason would be a very quick response by immunization-driven memory cells that would already be diminished 7 days after infection. Using different time-points, particularly very soon after infection, to assess cytokine production in mice immunized with NcMP and Carbigen™ would be useful to fully understand the potential of Carbigen™ in boosting a protective immune response.

The role of IL-17A in innate immunity comprises neutrophil activation and recruitment through induction of CXC chemokine expression, as well as inducing the production of antimicrobial peptides in epithelial cells (Murphy *et al.*, 2016). Higher IL-17A levels were detected in culture supernatants of spleen (Figure 4), lungs (Figure 5) and liver (Figure 6) cells collected from the non-infected immunized mice, upon stimulation with NcS. The levels of IL-17A were also found slightly elevated in the lungs and liver cell culture supernatants in the NcS-stimulated groups using cells obtained from infected mice, comparatively to groups corresponding to sham-immunized mice (Figures 5 and 6). However, the levels of IL-17A were lower in the cultures of cells obtained from immunized infected animals than in counterpart groups from non-infected mice (Figure 5 and 6). This effect was less pronounced in the liver of immunized mice, since the levels of this cytokine were mainly unchanged by infection. Within this organ, IFN-γ secretion was the highest detected among the three organs of immunized mice upon infection. Therefore, this may indicate that the liver is inflicted with exacerbated inflammation at 7 days post-infection. The function of IL-17A in host immunity is not entirely elucidated. Antibody-mediated neutralization of IL-17A in mice infected with *Staphylococcus aureus* led to elevated bacterial colonization in skin lesions along with decreased detection of murine β-defensin and psoriasin peptides near injured tissues (Chan *et al.* 2015). However, as reviewed by Jin & Dong (Jin & Dong, 2013), IL-17A has been consistently associated to inflammatory conditions, such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and psoriasis. Also, chronic infection with *Pseudomonas aeruginosa* in the lungs of cystic fibrosis correlates with higher levels of IL-17A in the respiratory sputum of affected patients (McAllister *et al.*, 2005). The pathology of *N. caninum* infection has been previously characterized in the liver, lungs, brain and heart of BALB/C mice (Collantes-Fernández *et al.*, 2004). Of all organs, lesions were frequently detected in the liver and with considerable severity.

During *N. caninum* infection, IFN-γ acts as a negative regulator of IL-17A production (Peckham *et al.*, 2014), so it might be that control of inflammation is delayed in the liver. Th17 cells are the most common source of this cytokine and are also associated with mucosal immunity, providing a link with the presence of IgAs in the gut mucosa (Hirota *et al.*, 2013). Interestingly, mice deficient in IL-17RA are highly susceptible to *T. gondii* infection (Kelly *et al.*, 2005). Despite that neutrophil function in IL-17RA-deficient mice remains normal, reduced chemokine expression and consequent neutrophil recruitment may account for the increased susceptibility to infection. As *N. caninum* resembles *T. gondii* mechanism of infection, it might be expected that IL-17A could also have an important role in the early immune response mounted against the parasite. Moreover, other than neutrophils, IL-17RA is expressed in fibroblasts, epithelial cells and macrophages. In response to IL-17A stimulation, fibroblasts produce chemokine (C–X–C motif) ligand 1 (CXCL1), chemokine (C–C motif) ligand 2 (CCL2), CCL7, CCL20 and matrix metalloproteinases (MMPs) 3 and 13 (Jin and Dong, 2013), which may facilitate leukocyte recruitment and migration through tissue extracellular matrix.

4.3 Flow cytometry analysis of activated/effector memory T cell populations

Cells were collected prior to infection and 7 days upon the i.p. parasitic challenge from the spleens, lungs and liver of mice immunized twice i.n with NcMP+Carbigen™ or sham-

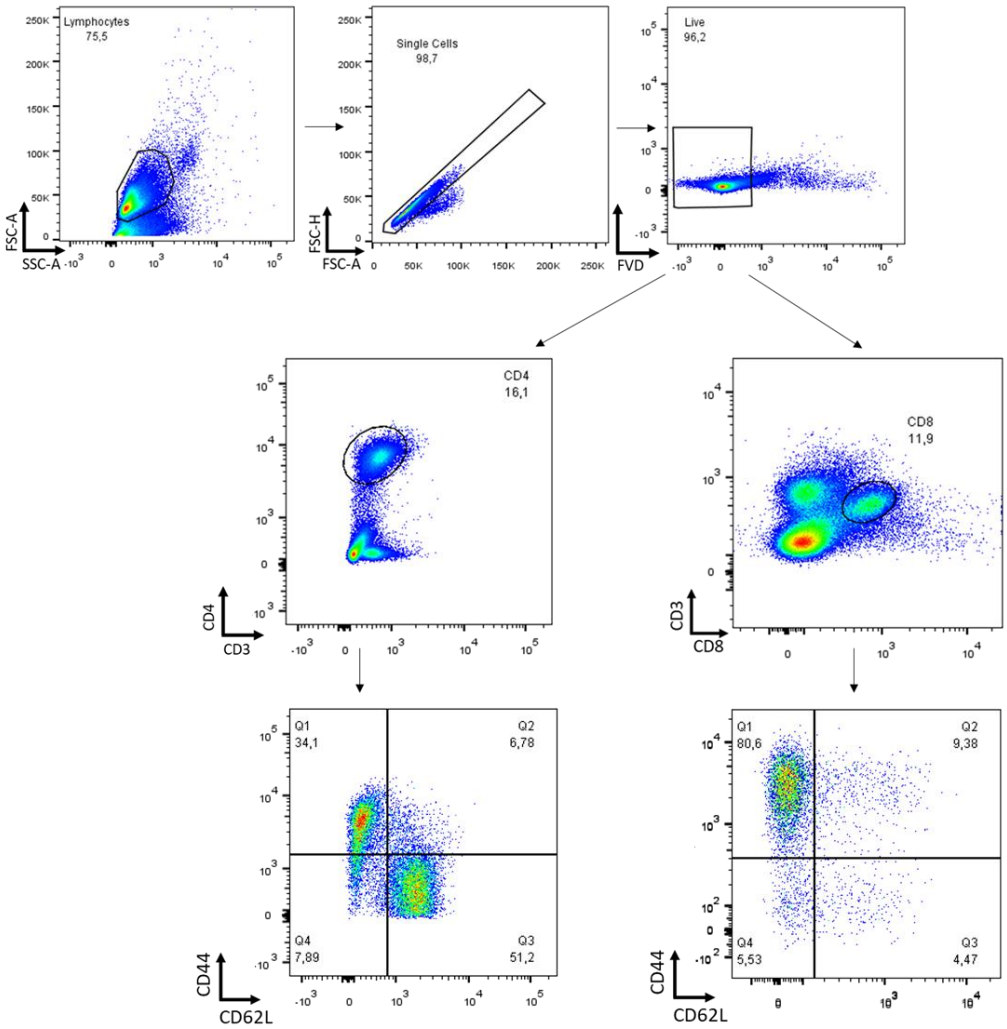

Figure 7. Gating strategy used to identify activated/effector memory CD4⁺ and CD8⁺ T cells. $CD4^+$ or $CD8^+$ activated/memory Effector T cells were further defined as $CD44^+CD62L^-$. Gating was aided by FMO controls for each used antibody.

immunized with Carbigen™ alone. To assess the presence of activated/effector memory T cells which display a $CD44^+CD62L^-$ phenotype, the expression of CD44 and CD62L in $CD3^+CD4^+$ and $CD3^+CD8^+$ cells was quantified using flow cytometry analysis. In Figure 7, the gating strategy used to define these populations is shown. Before infection, significant differences between immunized and sham-immunized mice were detected for both total splenic $CD4^+$ T cells and splenic $CD44^+CD62L^-$ $CD4^+$ T cells (Figure 8A). Immunization seems to significantly increase $CD44^+CD62L^-$ $CD4^+$ T cell numbers, that persist after infection in the lungs, but not in the liver (Figure 9A and 10A). In addition, the dynamics of $CD8^+$ T cell activation was also assessed due to

their relevance in the immune response against intracellular parasites (Jordan *et al.*, 2010) and of *N. caninum* in particular (Correia *et al.*, 2015).

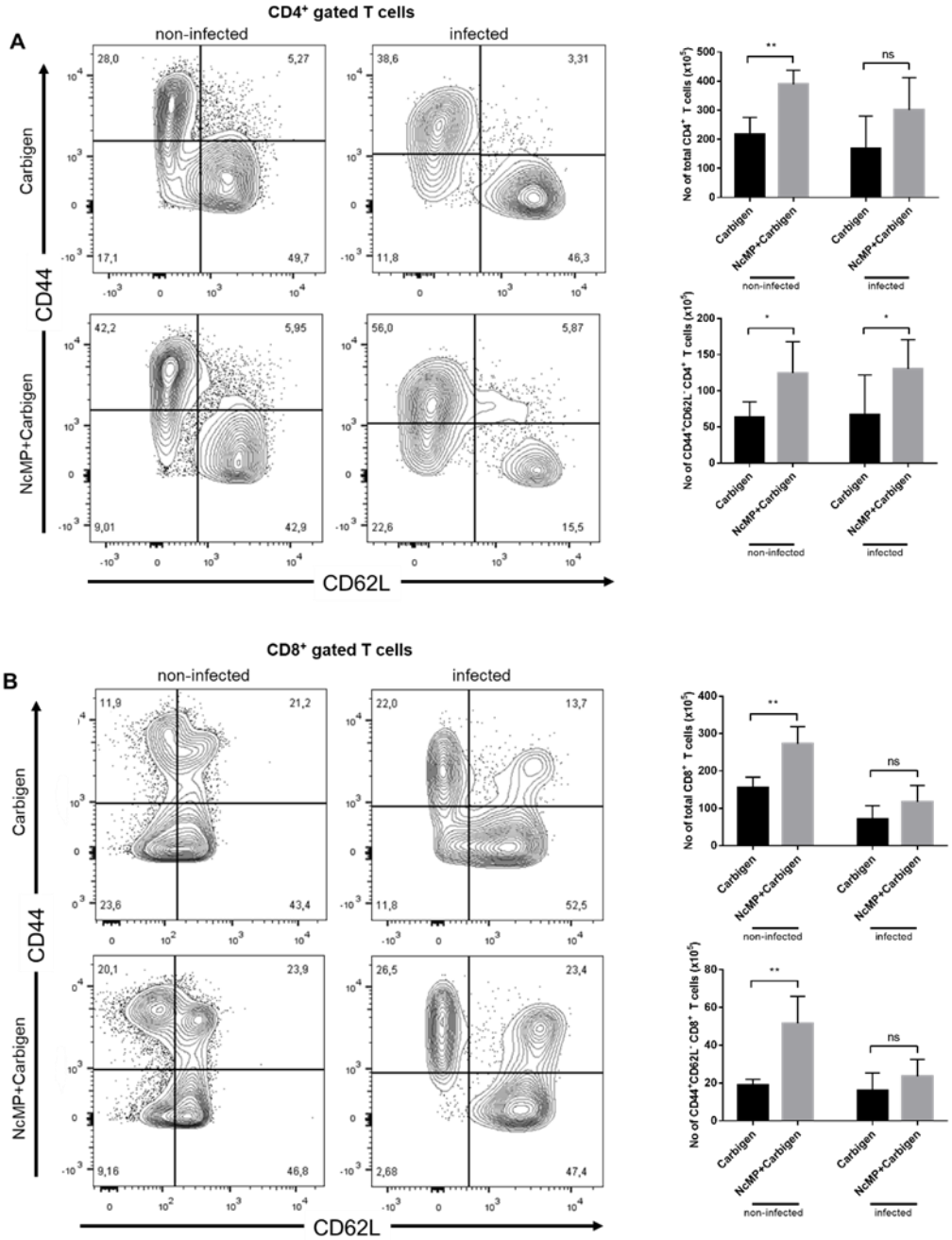

Figure 8. Splenic CD4$^+$ T cells and CD8$^+$ T cells expansion and differentiation into CD44$^+$CD62L$^-$ Activated or Effector Memory T cells. (A) CD4$^+$ T cells. (B) CD8$^+$ T cells. Values expressed in number of cells (x10^5). Cells were collected from the spleens of mice immunized i.n twice (non-infected) and of mice identically immunized upon 7 days of challenge (infected). Bars represent means plus SD. Number of mice per group in non-infected/infected independent experiments: Carbigen (n=4/6), NcMP+Carbigen (n=4/7). Unpaired two-tailed t-test was used to compare sham-immunized with immunized mouse groups. Contour plots are illustrative examples of gated cells. Quadrants were set according to Fluorescence Minus One (FMO) controls. Numbers within quadrants correspond to the percentage of cells in that quadrant (** $p < 0.01$; * $p < 0.05$; ns – not significant).

Significant differences between immunized and sham-immunized mice in total CD8$^+$ T cells and

$CD8^+$ activated/effector memory T cells were only detected in the spleen of unchallenged mice (Figure 8B). Upon infection, total and activated/effector memory $CD8^+$ cell numbers decreased in the spleen and increased in the lungs and liver (Figure 9B and 10B).

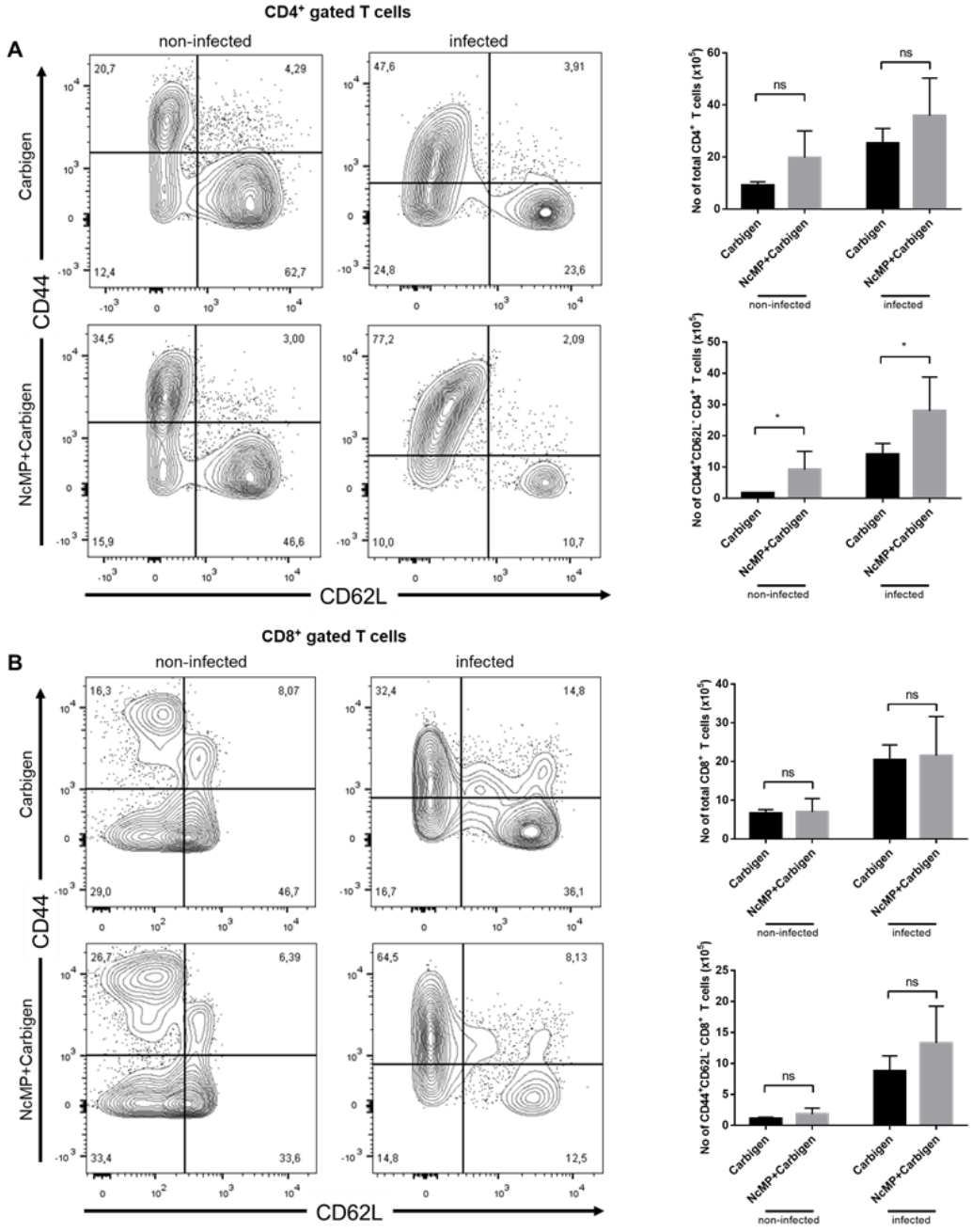

Figure 9. Lung $CD4^+$ T cells and $CD8^+$ T cells expansion and differentiation into $CD44^+CD62L^-$ Activated or Effector Memory T cells. (A) $CD4^+$ T cells. (B) $CD8^+$ T cells. Values expressed in number of cells ($x10^5$). Cells were collected from the lungs of mice immunized i.n twice (non-infected) and of mice identically immunized upon 7 days of challenge (infected). Bars represent means plus SD. Number of mice per group in non-infected/infected independent experiments: Carbigen (n=4/6), NcMP+Carbigen (n=4/7). Unpaired two-tailed t-test was used to compare sham-immunized with immunized mouse groups. Contour plots are illustrative examples of gated cells. Quadrants were set according to Fluorescence Minus One (FMO) controls. Numbers within quadrants correspond to the percentage of cells in that quadrant (* $p < 0.05$; ns – not significant).

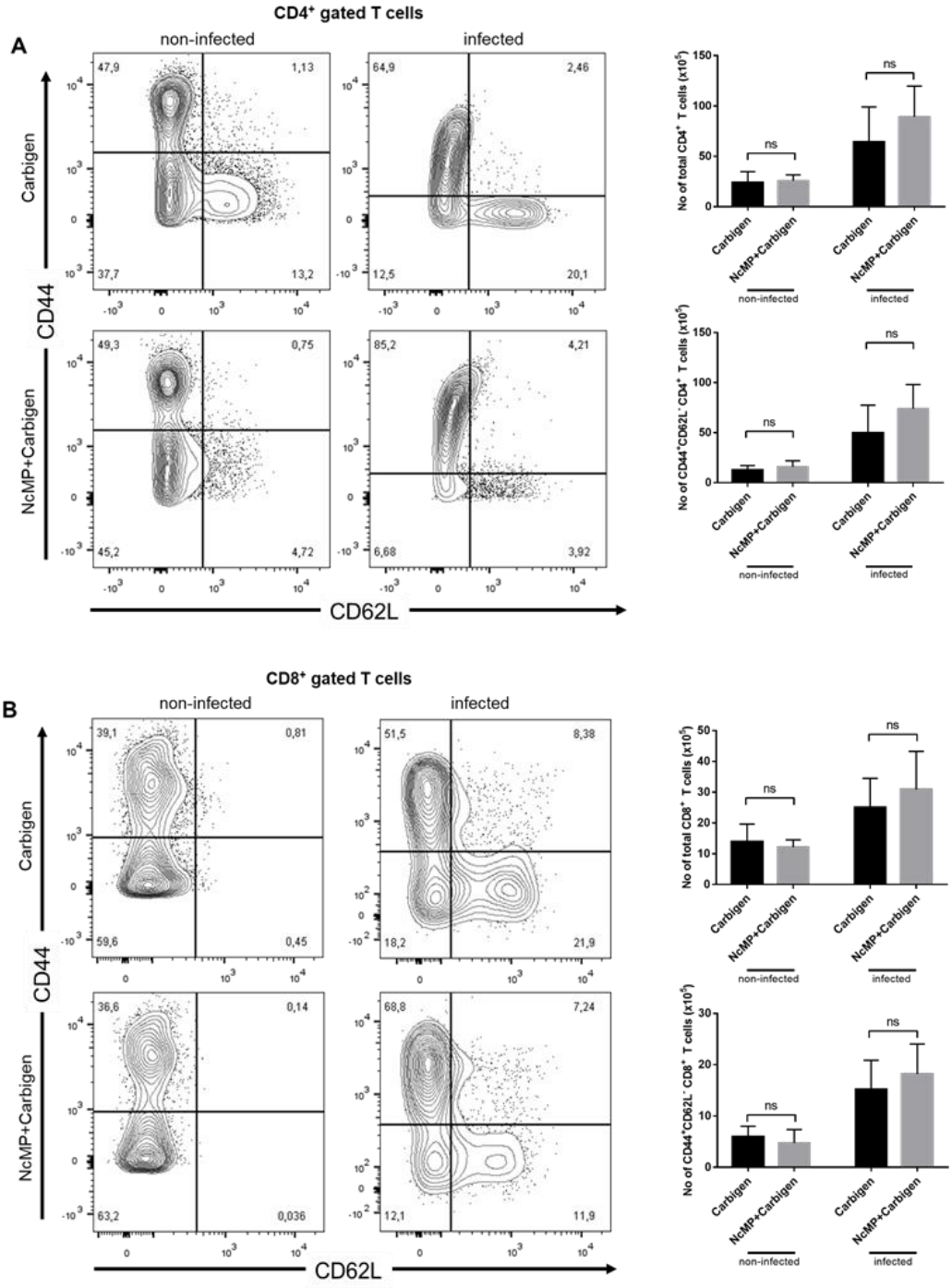

Figure 10. Liver CD4$^+$ T cells and CD8$^+$ T cells expansion and differentiation into CD44$^+$CD62L$^-$ Activated or Effector Memory T cells. (A) CD4$^+$ T cells. (B) CD8$^+$ T cells. Values expressed in number of cells (x10^5). Cells were collected from the lungs of mice immunized i.n twice (non-infected) and of mice identically immunized upon 7 days of challenge (infected). Bars represent means plus SD. Number of mice per group in non-infected/infected independent experiments: Carbigen (n=4/6), NcMP+Carbigen (n=4/7) Unpaired two-tailed t-test was used to compare sham-immunized with immunized mouse groups. Contour plots are illustrative examples of gated cells. Quadrants were set according to Fluorescence Minus One (FMO) controls. Numbers within quadrants correspond to the percentage of cells in that quadrant (ns – not significant).

In general, immunization with NcMP+Carbigen™ induced the differentiation of CD4$^+$ and CD8

activated/effector memory T cells in the spleens. The subset of effector memory $CD4^+$ T cells, depending on their polarization, produces large amounts of IFN-γ, IL-4 and IL-17A in response to stimulus, as compared with $CD44^+CD62L^+$ central memory T cells (Sallusto *et al.*, 2004). Thus, polarization of activated or activated/effector memory T cells to Th1, Th2 and Th17 phenotypes was further assessed by flow cytometry analysis of Tbet, Gata3 and RORγt transcription factors (Zhu *et al.*, 2010), respectively, seven days after infection (Figure 11).

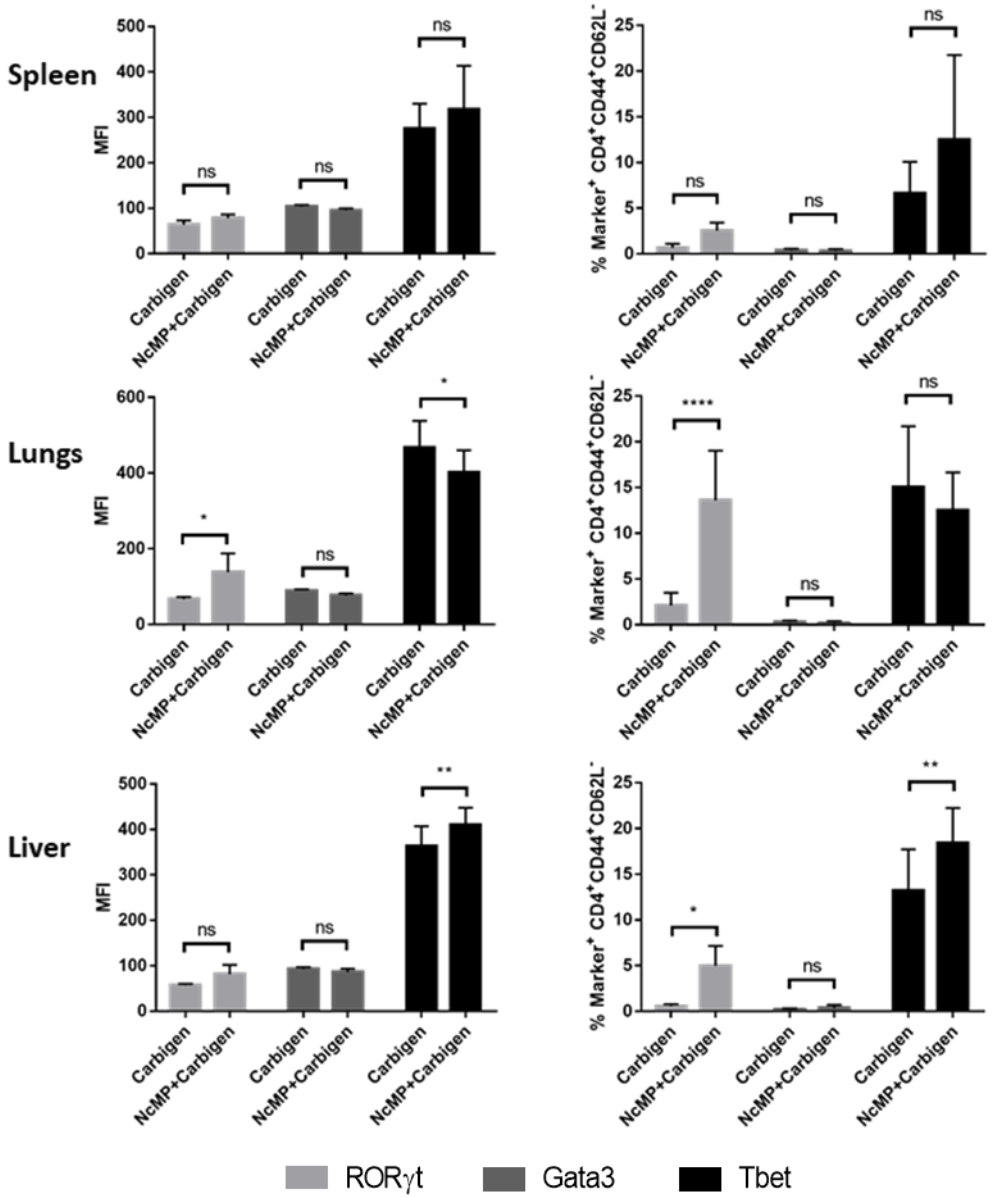

Figure 11. Mean fluorescence intensity (MFI) and percentage of RORγt, Gata3 and Tbet in $CD4^+$ $CD44^+CD62L^-$ T cells, obtained by flow cytometry. Cells were collected from the spleen, lungs and liver of mice immunized i.n twice, and 7 days after challenge. Bars represent means plus SD. Number of mice per group in: Carbigen (n=6), NcMP+Carbigen (n=7) Unpaired two-tailed t-test was used to compare sham-immunized with immunized mouse groups (**** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$; ns – not significant).

Detection of significant IL-17A in cultured lung and liver mononuclear cells (Figure 5 and 6)

correlates with increased expression of RORγt and recruitment of activated CD4$^+$ Th17 cells into these organs (Figure 11). Immunization did only induce a significant effect on Tbet expression on activated/effector memory CD4$^+$ T cells in the liver, whereas populations of activated/effector memory CD4$^+$ T cells expressing Gata3 in all tested organs remained unchanged between sham-immunized and immunized mice upon 7 days of infection (Figure 11).

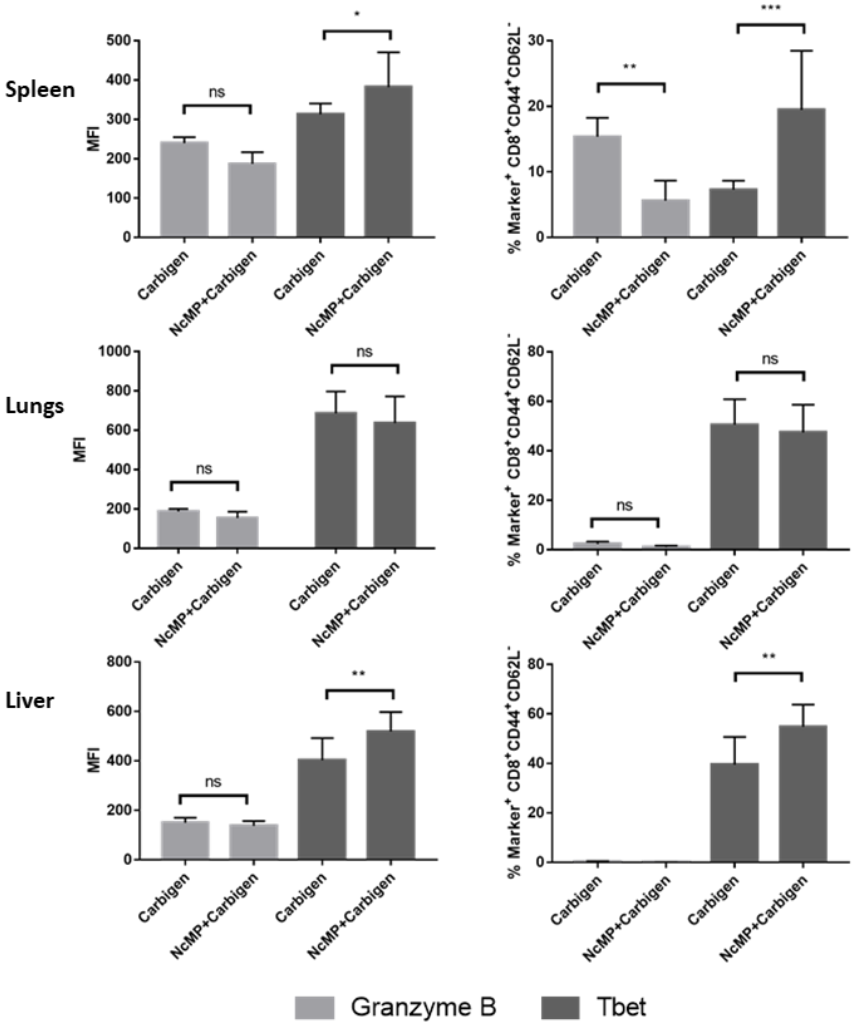

Figure 12. Mean fluorescence intensity (MFI) and percentage of Granzyme B or Tbet expression in CD8$^+$CD44$^+$CD62L$^-$, obtained by flow cytometry. Cells were collected from the spleen, lungs and liver of mice immunized i.n, 7 days of challenge. Bars represent means plus SD. Number of mice per group: Carbigen (n=6), NcMP+Carbigen (n=7). Unpaired two-tailed t-test was used to compare sham-immunized with immunized mouse groups (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns – not significant).

Total and CD8$^+$ activated/effector memory T cells numbers decreased in the spleen upon infection (Figure 8), while their numbers were found increased in the liver and lungs, irrespectively of immunization (Figure 9 and 10). A possible explanation may reside in the homing of CD8$^+$ T cells to peripheral tissues. In the spleen and liver, immunization led to an increase of Tbet expression in activated/effector memory CD8$^+$ T cells (Figure 12). Unexpectedly,

in the spleen, a larger population of activated/memory $CD8^+$ T cells expressing Granzyme B was detected in sham-immunized mice concomitantly with the observation of preferential Tbet expression in the same subset of T cells but in immunized mice (Figure 12). However, no differences were observed regarding IFN-γ production by leukocytes isolated from mice immunized and infected comparatively to sham-immunized counterparts (Figure 6).

Apoptosis mediated by perforin and granzyme is a mechanism used by $CD8^+$ T cells to destroy parasite-infected cells and may help restrain parasite dissemination. In *T. gondii* infection, dissemination through the host tissues has been accounted to infected APCs inability to destroy the PV, therefore acting as a Trojan horse (Lambert *et al*., 2006).

Interestingly, the reported importance of $CD8^+$ T cells in response to *N. caninum* infection did not correlate with perforin function, as perforin-deficient ($Prf1^{-/-}$) mice did not have increased parasite burden in the brain and lungs, compared with WT mice, 7 days after i.p challenged with 1×10^7 tachyzoites (Correia *et al*., 2015). Nevertheless, from these results it can be inferred that NcMP+Carbigen™ i.n. immunization did not induce the differentiation of $CD8^+$ T cells with a cytotoxic phenotype. The number of $CD8^+$ T cells expressing the transcription factor Tbet is nevertheless significantly increased in the spleen and liver of immunized mice (Figure 12). These cells could be contributing to the elimination of the parasite by producing IFN-γ, a key cytokine in the control of *N. caninum* infection.

4.4 Humoral response to immunization and infection

The production of NcMP-specific IgAs in intestinal lavage fluids from the different mice was assessed using ELISA. The assessment of the IgA-mediated response to immunization before and after infection comprised Carbigen™ sham-immunized and NcMP+Carbigen™ immunized mouse groups. A significant increase in the levels of NcMP-specific IgA titres was detected in

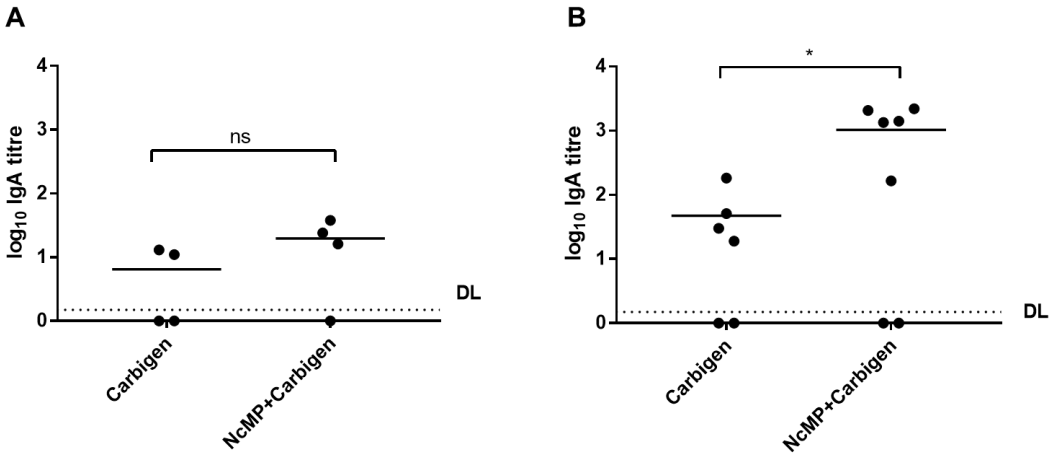

Figure 13. Intestinal N. caninum-specific IgA in non-infected and infected mice. (A) intestinal lavage fluids collected 3 weeks upon the last of two i.n immunizations with NcMP and Carbigen™ (NcMP+Carbigen) or sham-immunizations with Carbigen™ (Carbigen). (B) intestinal lavage fluids were collected from mice 7 days upon challenge. Mice were previously immunized twice i.n with NcMP and Carbigen™ (NcMP+Carbigen) or sham-immunized with Carbigen™ (Carbigen). Data is presented as log_{10} of the antibody titres. Each circle represents an individual mouse. Horizontal bars represent mean values in each group. Number of mice in (A): Carbigen (n=4), NcMP+ Carbigen (n=4). Number of mice in (B): Carbigen (n=6), NcMP+Carbigen (n=7). Detection limit (DL) is indicated by a horizontal line. One-way ANOVA was used to compare both groups (* $p < 0.05$, ns – not significant).

mice immunized with NcMP+Carbigen™ following infection, as compared to sham-immunized controls (Figure 13). This indicates that IgA-producing memory B-cells remained until infection and IgA production was boosted by infection, even though the infection was done by the intraperitoneal route. Intestinal IgA levels detected in sham-immunized mice 7 days following infection may indicate that mice develop antibodies against the parasite in the absence of previous immunizations. As previously mentioned, the importance of mucosally-produced IgA in limiting *N. caninum* invasion through the gut epithelium was hypothesized (Ferreirinha *et al.*, 2016). The present work used an intraperitoneal route instead of the intragastric route of infection previously used to assess protection in NcMP+CpG immunized mice (Ferreirinha *et al.*, 2014). By using the latter, infection with tachyzoites would be conditioned by the intestinal mucosa and IgA-mediated immune exclusion would be expected. In accordance, IFN-γ levels in cultures from splenocytes obtained from immunized mice 7 days upon the parasitic challenge was lower than what is here reported with NcMP+Carbigen™ immunized mice and could be attributed to the reduced parasite burden reaching systemic circulation. Complementary to the

IFN-γ and IL-4 secretion, serum IgG2a and IgG1 are also commonly used as markers of Th1- and Th2-type responses, respectively.

The isotype IgG1 was always prevalent, leading to IgG1/IgG2a ratios above one. (Figure 14). In this context, it seems that Carbigen™ induces a Th1/Th2 mixed response with high IFN-γ secretion. The titers of IgGs increased in mice sham-immunized with Carbigen™ upon infection and the IgG1/IgG2a ratio was lower than the one observed in immunized mice. Then, within 7 days after challenge, non-immunized mice develop antibodies against *N. caninum* antigens, though the overall titer is considerably lower than the one measured in immunized mouse serum. Comparison between groups was not statistically significant and may be explained by the low size of the sample tested.

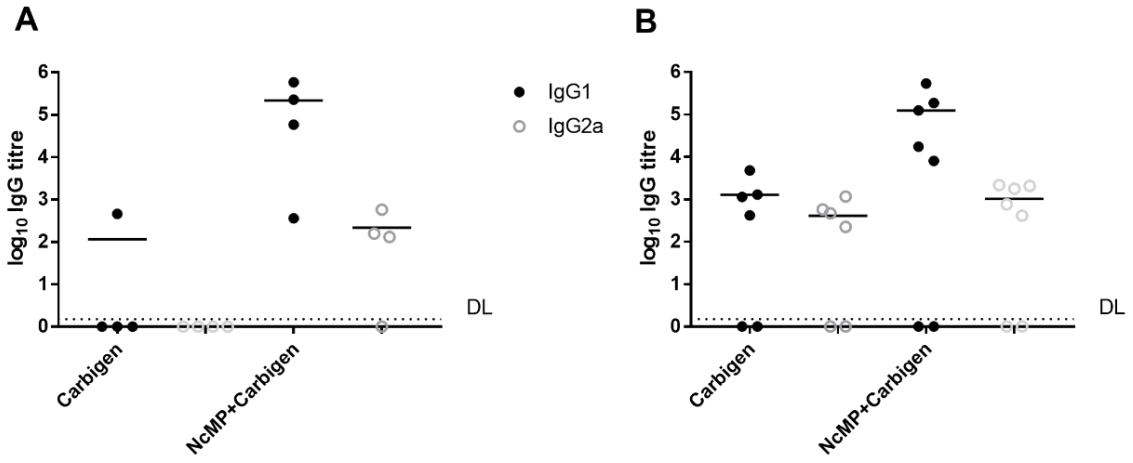

Figure 14. Serum anti-N. caninum IgG1 (black circles) and IgG2a (white circles), detected by ELISA, in immunized mice. (A) Sera were collected 3 weeks upon the last of two i.n immunizations with NcMP and Carbigen™ (NcMP+Carbigen) or sham-immunizations with Carbigen™ (Carbigen). (B) Sera were collected 7 days upon infection of previously immunized mice. Data is presented as $\log_{10}$ of the antibody titres. Each circle represents an individual mouse. Horizontal bars represent mean values in each group. One-way ANOVA was used to evaluate statistically significant differences between groups. All comparisons between groups were not statistically significant. Number of mice in: Carbigen (n=4); NcMP+ Carbigen (n=4). Detection limit (DL) is indicated by a horizontal line.

During the host immune response, antibodies act towards the neutralization of pathogens and as effectors through the interaction of their constant Fc region with Fc receptors and complement proteins (Huber *et al.*, 2006). IgG1 interacts preferentially with inhibitor FcγRIIB receptor while IgG2a interacts with activator FcγRIV. In addition, the administration of recombinant IgG1 and IgG2a antibodies in mice either lacking FcγRIIB or FcγRIV receptor, respectively, corresponded with enhanced and diminished ADCC activity on platelet depletion and lung metastasis (Nimmerjahn & Ravetch, 2005). In its turn, the latter may be mostly involved in the neutralization of pathogens. Taken together, it makes sense that high levels of

IgG2a are related with increased immunity to intracellular parasites such as *N. caninum*, whose infection is counteracted by cellular immune responses to which IgG2a may contribute at a larger extent than IgG1. Host resistance to neosporosis is associated with a high ratio of IFN-γ/IL-4 cytokine production. Considering the spleen as a central player on the immunological surveillance of blood-circulating pathogens, systemic infections would soon reach the spleen and prime lymphoid cells before homing to other tissues. Thus, different routes of infection may account for the divergence on pro-inflammatory cytokine levels. It would be worthwhile testing if a different effect on mice may occur when infected through the intragastric route. IL-17A was also consistently detected in cultures of antigen stimulated splenocytes, liver and lung mononuclear cells collected before infection (Figure 4, 5 and 6). In addition, this cytokine levels correlated with the expression of RORγt in activated/effector memory $CD4^+$ T cells. Notwithstanding the immunopathologic potential of this cytokine, its transverse role on innate and adaptive immunity through IgA secretion may indeed confer protection, especially in the gut mucosa where infection is established. Based on this result, the detected effect of Carbigen™ as an adjuvant may be providing a depot effect for the antigen rather than the suitable immunomodulating effect needed to a Th1-type immune response shift. However, further optimizations on the immunization formulation would be necessary to clearly evaluate Carbigen™ effect.

4.5 Parasite burden in the brain and lungs of challenged mice

Brains and lungs collected from 7-day-infected sham-immunized and immunized mice were homogenized and DNA was extracted using the phenol-clorophorm method. The lungs are

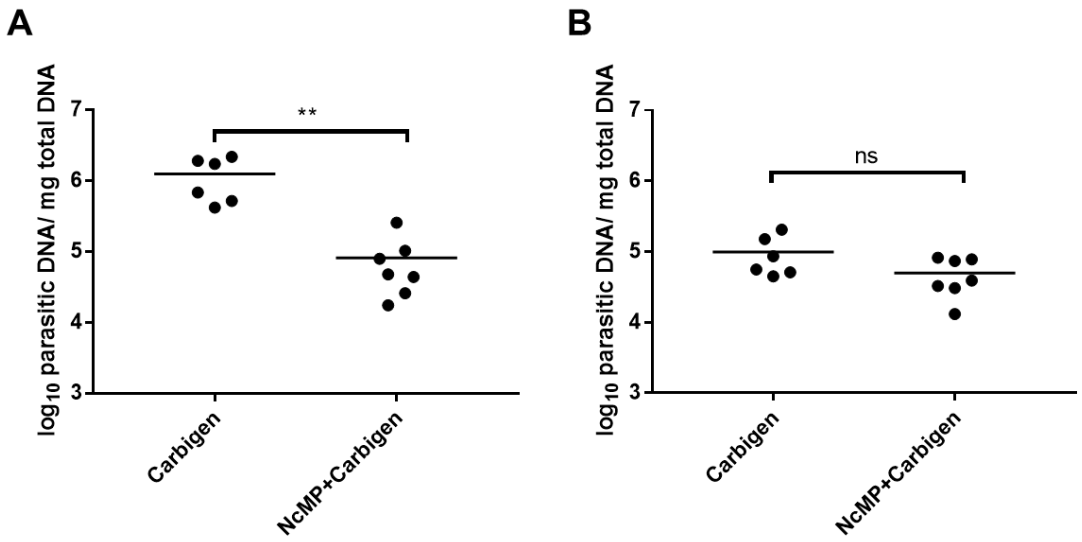

Figure 15. Parasite burden in the brain (A) and lungs (B) of mice 7 days upon i.p challenge with 1x10^7 tachyzoites. Mice were previously immunized twice i.n with NcMP and Carbigen™ (NcMP+Carbigen) or sham-immunized with Carbigen™ (Carbigen). Parasite burden was assessed by qPCR for *N. caninum* Nc5 DNA sequence. Data is presented as $\log_{10}$ of detected parasitic DNA per mg of total template DNA. Each circle represents an individual mouse. Horizontal bars represent mean values to each group. Number of mice per group: Carbigen (n=6), NcMP+Carbigen (n=7). Statistical significances between groups were calculated using unpaired two-tailed t-test. (** $p < 0.01$; * $p < 0.05$).

a target organ in the acute phase of neosporosis. As previously reported (Collantes-Fernandez *et al.*, 2006), parasites were detected in the lungs of BALB/c mice infected i.p with 1 x 10^6 *N. caninum* Nc-1 strain tachyzoites as soon as 24 hours post-infection and remained detectable therein until 11 days post-infection. On the other hand, the authors reported that parasite DNA was detected in the brain of the infected animals by 7 days post-infection and remained detectable until at least 63 days post-infection.

Here, the reported protective effect achieved with NcMP and CpG i.n. immunization (Ferreirinha *et al.*, 2014) was not detected when Carbigen™ was used as an adjuvant. Indeed, brain parasite burden between sham-immunized and immunized mice did not reach statistical significance (Figure 15A). Nevertheless, a partial protection was conferred by immunization in the lungs (Figure 15B) showing its potential in boosting mucosal immunity, likely through a Th17-type immune response. Previous work had showed that NcMP alone conferred immunized mice a partial protection, yet insufficient, against the establishment of a chronic infection (Ferreirinha *et al.*, 2014). As the secretion of IFN-γ was preferentially induced in splenocytes of

NcMP+Carbigen™ mice, it would be worth testing, in a new experiment, how its level of secretion differed from mice immunized with NcMP+CpG upon 7 days of i.p infection and how significantly different would be the parasitic colonization in the brain and lungs.

4.6 Comparison of cytokine production and humoral response: NcMP and Carbigen™ *vs* NcMP and CpG

Splenocytes were collected from immunized and sham-immunized mice and stimulated *in vitro* with NcS along with unstimulated splenocytes as controls. Following 3 days of culture, culture supernatants were collected and used to detect IFN-γ, IL-4 and IL-17A levels using ELISA. As shown in Figure 16, splenocytes from mice immunized with NcMP plus CpG or Carbigen™ adjuvant showed a tendency for increased IFN-γ and IL-17A production in response to NcS antigen stimulation, as compared with splenocytes from mice treated with PBS or

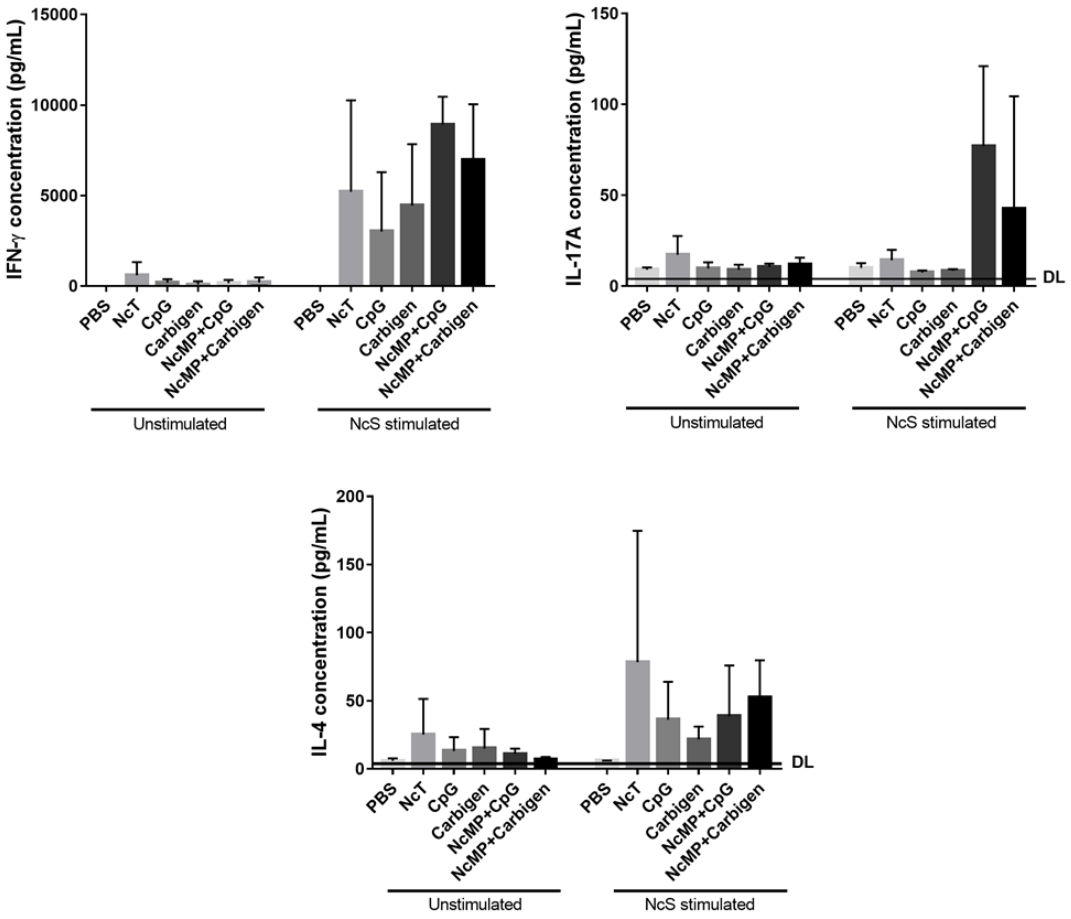

Figure 16. Detection of cytokine levels by ELISA in supernatants of cultured splenocytes unstimulated or stimulated with NcS for 3 days. Cells were isolated from the spleens of mice 7 days upon challenge. Mice were previously immunized twice i.n with NcMP and Carbigen™ (NcMP+Carbigen), NcMP and CpG (NcMP+CpG), sham-immunized with Carbigen™ (Carbigen), CpG (CpG) or PBS alone (PBS). PBS mice were not challenged. NcT mice were challenged without any previous immunization. Bars represent means plus SD. Number of mice per group: PBS (n=3); NcT (n=3); CpG (n=4); Carbigen (n=5); NcMP+CpG (n=5); NcMP+Carbigen (n=5). DL- Detection limit (4 pg/mL) is indicated by a horizontal line. Statistical significances between groups were calculated using one-way ANOVA and Tukey's *post-hoc* test. All comparisons were non-significant.

adjuvants alone. These results indicate that NcMP antigens may induce Th1 and Th17-type responses when combined with either CpG or Carbigen™ (Figure 16). The adjuvant effect of CpG

is known to encompass a shift in the immune response towards a Th1-type, hence mediated by IFN-γ production (Chu *et al.*, 1997). Carbopol-974p, another carbomer-based adjuvant combined with viral gp140 antigen induced lower levels of IFN-γ than when CpG adjuvant was used, in a similar antigen recall assay using splenocytes (Gartlan *et al.*, 2016). As we did not observe significant differences in IFN-γ production when either adjuvant was used, this might indicate that the used antigen *per se* can play an important role in favoring the production of this cytokine. Also, IL-17A detection was mainly restricted to mice immunized with NcMP irrespective of the adjuvant used and it might be that Carbigen™ can also enhance Th17 priming effect. Thus, the development of a Th17 immune response upon immunization with NcMP+Carbigen™ is significant and may hold a protective effect against an intragastric infection

Although not being statistically significant, it seems that NcMP+Carbigen™ vaccine formulation does not boost higher IgA titres in comparison with NcMP+CpG (Figure 17). Detectable IgA titres in sham-immunized mice 7 days following infection may indicate that mice develop antibodies against the parasite in the absence of previous immunizations. Carbigen™ did not show the effectiveness of CpG as adjuvant in a similar immunization procedure (Ferreirinha *et al.*, 2014).

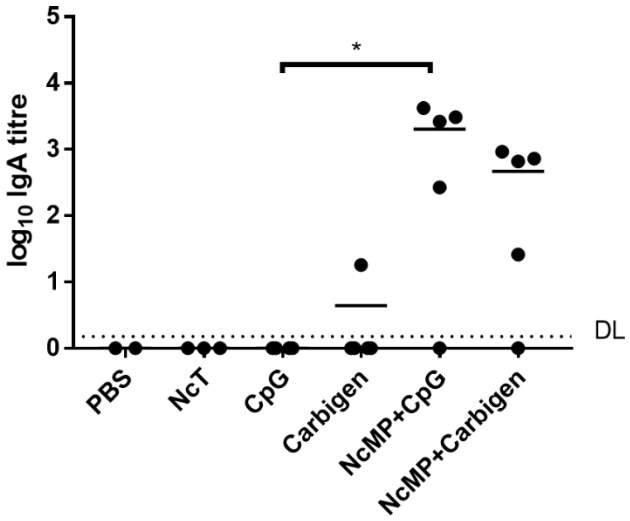

Figure 17. Intestinal N. caninum-specific IgA in infected mice. Mice were previously immunized twice i.n with NcMP and Carbigen™ (NcMP+Carbigen), NcMP and CpG (NcMP+CpG) sham-immunized with Carbigen™ (Carbigen), CpG (CpG) or PBS alone (PBS). PBS mice were not challenged. NcT mice were challenged without previous immunization. Data is presented as log_{10} of the antibody titres. Each circle represents an individual mouse. Horizontal bars represent mean values in each group. Number of mice in (A): – Carbigen (n=4), NcMP+ Carbigen (n=4). Number of mice in (B): PBS (n=3), NcT (n=3), CpG (n=4), Carbigen (n=5), NcMP+CpG (n=5), NcMP+Carbigen (n=5). Detection limit (DL) is indicated by a horizontal line. One-way ANOVA was used to evaluate statistically significant differences between groups. All comparisons but CpG vs NcMP+CpG were non-significant. (* $p < 0.05$).

Upon 7 days of challenge, CpG adjuvant effect led a IgG1/IgG2a ratio below one whereas Carbigen™ led to extremely high IgG1/IgG2a ratios (Figure 18). The low size of the tested sample may account for lack of statistical significance in any comparison between groups. Preferential IgG2a secretion with NcMP+CpG immunization has been consistently reported (Ferreirinha *et al*., 2014 and 2016). Based on these results, only NcMP+CpG immunized mice developed an immune response characterized by preferential IFN-γ secretion as suggested by the IgG1/IgG2a ratio below 1. Previous unpublished work of the group had shown that mice immunized with NcMP+CpG were also protected against intraperitoneally established neosporosis. Although the parasite burden was not assessed here in NcMP+CpG immunized mice, the higher levels of IgG2a than IgG1 could indicate that these mice developed a strongly polarized Th1-type immune response that could be protective against an i.p challenge.

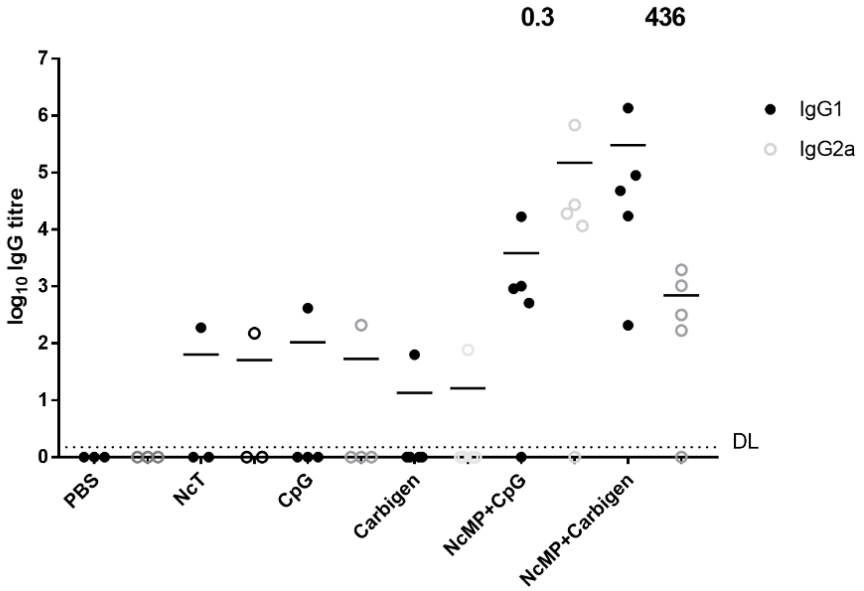

Figure 18. Serum anti-N. caninum IgG1 (black circles) and IgG2 (white circles), detected by ELISA, in immunized mice. Sera were collected from mice 7 days upon challenge. Mice were previously immunized twice i.n with NcMP and Carbigen™ (NcMP+Carbigen), NcMP and CpG (NcMP+CpG), sham-immunized with Carbigen™ (Carbigen), CpG (CpG) or PBS alone (PBS). PBS mice were not challenged. NcT mice were challenged without previous immunization. Data is presented as $\log_{10}$ of the antibody titres. Numbers above relate to the ratio of IgG1/IgG2a, calculated using the mean $\log_{10}$ titres of each IgG subclass. Each circle represents an individual mouse. Horizontal bars represent mean values in each group. One-way ANOVA was used to evaluate statistically significant differences between groups. All comparisons were non-significant. Number of mice in: PBS (n=3); NcT (n=3); CpG (n=4); Carbigen (n=5); NcMP+CpG (n=5); NcMP+ Carbigen (n=5). Detection limit (DL) is indicated by a horizontal line.

The levels of IFN-γ and IL-17A measured in the supernatants of cultures from the spleens of mice immunized with CpG plus NcMP were not considerably different from the ones found in the Carbigen™ plus NcMP immunized counterparts. However, the IgG1/IgG2a ratio below one in the sera of CpG plus NcMP immunized mice points out to a much stronger Th1-type response in these mice than in Carbigen™ plus NcMP immunized animals. It would be interesting to

evaluate the expression of the transcription factor Tbet by $CD4^+$ and $CD8^+$ T cells in these animals and also evaluate earlier time points upon infection to clarify if the production of this cytokine was indeed distinctly produced and if that difference could account for protection.

4.7 Western Blot analysis

Pooled serum antibodies from immunized and non-immunized mice were used in Western Blot analysis to assess the presence of one or more immunodominant proteins (Figure 19). Proteins recognized by both immunized and sham-immunized mouse sera are non-specific and may account for reactivities of the set of natural antibodies developed in C57BL/6 mice that react with conserved antigens present in this parasite. Nevertheless, both NcMP+Carbigen™ and NcMP+CpG vaccinated mice developed antibodies specific to two membrane proteins of approximately 55 kDa and 45 kDa (black arrows). NcMP+Carbigen™ immunized mice also developed antibodies against a 35 kDa of molecular weight (blue arrows), while antibodies in the serum of mice immunized with NcMP+CpG also recognized a different protein of 100 kDa. However, the intensity of these unique bands was very weak and might not account for the differences in parasitic burden.

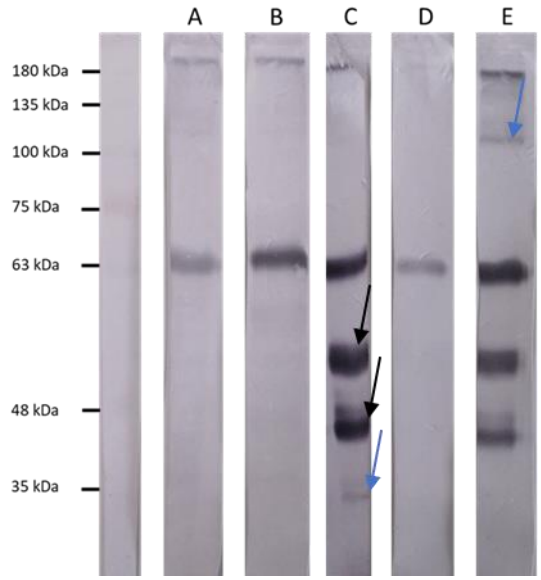

Figure 19. Western Blot of total serum IgG against NcMP antigens. The protein profile of the marker (NZYColour Protein Marker II) is identified in the first lane with the corresponding molecular weight of each band. Lanes A-E were incubated with serum from challenged mice previously immunized twice i.n with PBS (A), Carbigen™ (B), NcMP+Carbigen™ (C), CpG (D), NcMP+CpG (E).

5. Conclusions and further perspectives

In the context of Th1/Th2-type polarization, an immune response with high IFN-γ/IL-4 ratio combined with a IgG1/IgG2a ratio below 1 is thought to be protective against neosporosis. Herein, preferential IFN-γ and IgG1 secretion seems to confer partial protection to parasitic colonization in the lungs but not in the brain, indicating IgG2a relevance in parasite clearance. In addition, immunization induced the expansion of memory T cell populations both in $CD4^+$ and $CD8^+$ T cell subsets, whereas $CD4^+$ T cell lineage and $CD8^+$ effector phenotype remained mainly unchanged in comparison with sham-immunized mice. It would be interesting to evaluate if Carbigen™ could confer suitable protection when infection was performed by the intragastric route. Also, combination of immunization routes, such as intranasal plus subcutaneous, could also improve vaccine efficacy. Future testing using the bovine experimental model and eventual commercialization will depend on the combination of NcMP antigens with an adjuvant that is both economically viable in the context of scaled up testing in bovines and able to induce a protective immune response in the host. If Carbigen™ fails to induce considerable protection after the referred refinement of the immunization protocol, one class of adjuvants worth testing would be Immune stimulating complexes (ISCOMs). These are based on saponins that are combined with lipids to create a matrix in which antigens are encapsulated. In addition to enhanced bioavailability, ISCOMs display an immunomodulatory effect that both stimulates T-cell mediated cytotoxic responses and intestinal IgA secretion. From an economical perspective, it would also be a lot more affordable than CpG. Altogether, ISCOMs have the needed features to further refine the mucosal immunization against neosporosis here employed.

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