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**Protective effect against neosporosis induced in
mice by mucosal immunisation with
Neospora caninum antigens**

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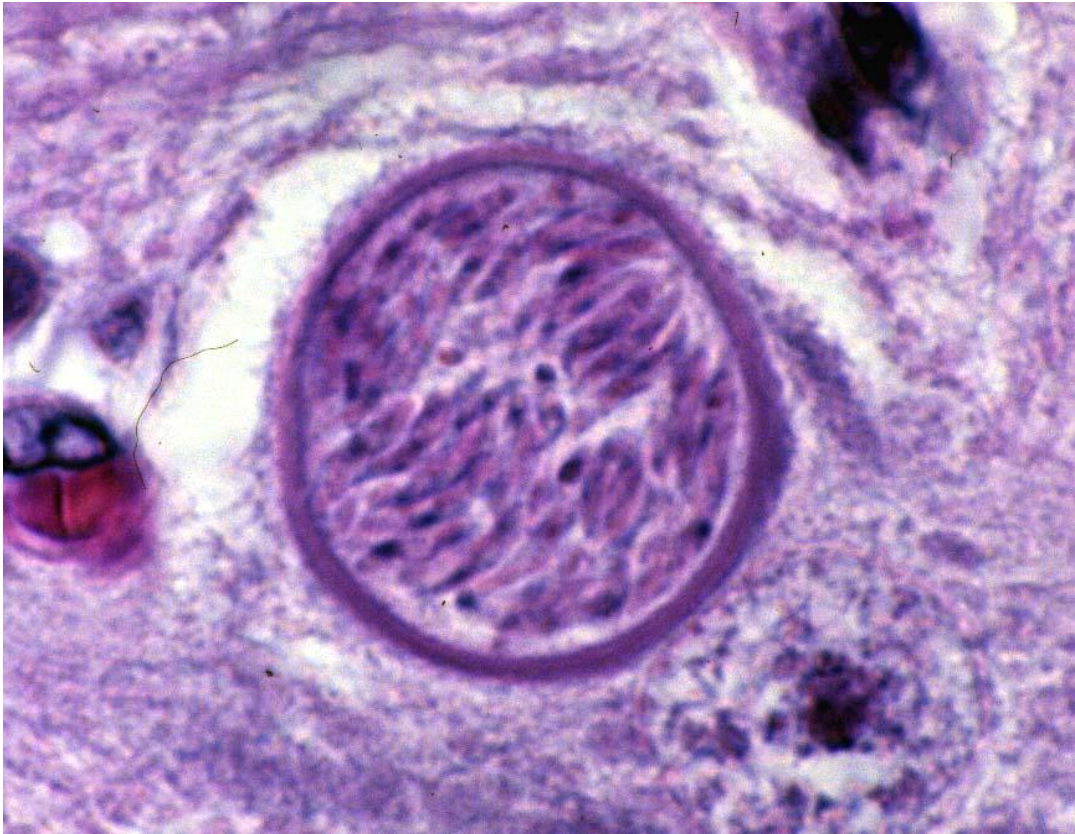


Image: *Neospora caninum* spinal cord cyst.

Credits: Steve J. Upton, Division of Biology, Kansas State University.

«Examining this water...I found floating therein divers earthy particles, and some green streaks, spirally wound serpent-wise...and I judge that some of these little creatures were above a thousand times smaller than the smallest ones I have ever yet seen, upon the rind of cheese, in wheaten flour, mould, and the like.»

Antonie van Leeuwenhoek, first recorded observation of protozoa.

In Letter to the Royal Society, London (7 September 1674)

Abstract

Neospora caninum is an obligate intracellular parasite of animals. Dogs, coyotes and dingoes are definitive hosts, while cattle are intermediate hosts and the most commonly affected animals in the field of neosporosis, the disease caused by *N. caninum*. This parasite is a major cause of abortion in cattle around the world leading to heavy economic losses to dairy and beef industry. The only vaccine commercially available against neosporosis has only a limited effect in preventing abortion in cattle herds. The parasite can enter cattle hosts through the gastrointestinal tract and this entry route is indispensable for sustaining infection within a herd. Thus, the present work aimed at 1) characterizing the immune response induced in mice by mucosal (intra-nasal) CpG-adjuvanted immunisation with membrane proteins from *N. caninum* tachyzoites (NcT) and 2) evaluating the protective effect conferred by this strategy against *N. caninum* infection induced by the intragastric route. Host protection is generally associated with a *N. caninum*-specific T_H1 immune response whereas parasite invasion and infection are favoured by a T_H2-biased immune response.

The immunisation with NcT membrane proteins plus CpG was performed by two intra-nasal administrations three weeks apart. It was shown here to be effective at promoting a *N. caninum*-specific immune response in the intestinal mucosa, as indicated by the significantly higher anti-NcT IgA ELISA titres detected in mice that received this treatment. The detection, by ELISA, of anti-NcT IgG in the serum suggested that the immunisation strategy used also induced a systemic immune response. The IgG1/IgG2a ratio, lower than 1.00, indicated that the *N. caninum*-specific immune response was predominantly of the T_H1-type, the prototypic protective response against *N. caninum*. Protection against this parasite was confirmed by the absence of parasites in the liver and brain of the immunised mice, seven days after infection with NcT, as evaluated by qPCR. At last, serum IgG antibodies produced upon the immunisation were shown, by flow cytometry, to bind the NcT surface *in vitro*. Western blot analysis indicated that IgG1 and IgG2a bound, respectively, NcT membrane proteins of 35 and 39 kDa and antibodies of both the IgG subclasses also bound a 17 kDa protein.

Altogether, this study suggests that intra-nasal CpG-adjuvanted immunisation with NcT membrane proteins is a promising strategy to prevent neosporosis. If successfully applied in cattle, this strategy can aid combating the horizontal transmission route of *N. caninum* in this group of animals and prevent, at least in part, the economic losses associated with neosporosis.

Keywords: *Neospora caninum*; neosporosis; gastrointestinal tract; mucosal immunity; immunisation; adjuvant; CpG

Resumo

Neospora caninum é um parasita intracelular obrigatório de animais. Os cães, coiotes e dingos são os hospedeiros definitivos, enquanto que os bovinos são hospedeiros intermediários e o grupo de animais mais afetado no que diz respeito à neosporose, doença causada por *N. caninum*. Este parasita é a principal causa mundial de aborto em bovinos e conduz a prejuízos económicos elevados na indústria de laticínios e da carne. Uma vacina contra a neosporose atualmente disponível no mercado demonstrou prevenir apenas parcialmente a ocorrência de abortos em manadas. Em bovinos, o parasita pode entrar pelo trato gastrointestinal, sendo esta via de entrada indispensável para manter a infeção numa manada. Deste modo, o presente estudo teve como objetivo 1) caracterizar a resposta imunitária induzida em ratinhos por imunização na mucosa (intranasal) com proteínas de membrana provenientes dos taquizoítos de *N. caninum* (NcT) e com o adjuvante CpG, e 2) avaliar o efeito protetor conferido por esta estratégia na infeção por *N. caninum* induzida pela via intragástrica. A proteção do hospedeiro está geralmente associada a uma resposta imunitária específica para *N. caninum* do tipo T_H1, enquanto que a invasão e infeção pelo parasita são favorecidas por uma resposta enviesada para o tipo T_H2.

A imunização com proteínas de membrana de NcT juntamente com CpG foi efetuada por duas administrações intranasais, com um intervalo de três semanas entre elas. Esta mostrou ser eficaz na indução de uma resposta específica para *N. caninum* na mucosa intestinal, como demonstrado pelos níveis significativamente mais elevados de IgA específica para NcT, detetados por ELISA, em ratinhos que receberam este tratamento. A deteção, também por ELISA, de IgG específica para NcT no soro sugeriu que a imunização em estudo era também capaz de induzir uma resposta imunitária sistémica. O facto de o rácio IgG1/IgG2a ser menor que 1.00 nestes ratinhos indicou que a resposta imunitária específica para *N. caninum* induzida pela imunização era predominantemente do tipo T_H1, portanto presumivelmente protetora contra *N. caninum*. A proteção contra este parasita foi confirmada, usando qPCR, pela ausência de parasita no fígado e cérebro dos ratinhos imunizados, sete dias após a infeção com NcT. Por fim, demonstrou-se, por citometria de fluxo, que IgGs do soro produzidas em resposta à imunização em estudo se ligavam à superfície de NcT. A análise efetuada por Western blot demonstrou que a IgG1 e IgG2a se ligavam, respetivamente, a proteína de membrana de NcT com 35 e 39 kDa e os anticorpos de ambas as subclasses também se ligavam à proteína de 17 kDa.

Em resumo, este estudo sugere que a imunização intranasal com proteínas de membrana de NcT e com o adjuvante CpG é promissora. Se aplicada com sucesso semelhante em bovinos, no futuro, esta estratégia poderá ajudar a combater a via horizontal de transmissão de *N. caninum*

neste grupo de animais e evitar, pelo menos em parte, os prejuízos económicos associados à neosporose.

Palavras-chave: *Neospora caninum*; neosporose; trato gastrointestinal; imunidade na mucosa; imunização; adjuvante; CpG.

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List of Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AP	Alkaline phosphatase
APC	Antigen-presenting cell
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BDL	Below detection limit
BSA	Bovine serum albumin
CALT	Conjunctiva-associated lymphoid tissue
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CMI	Cell-mediated immunity
CMIS	Common mucosal immune system
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte
Cy	Cychrome
DC	Dendritic cell
DG	Dense granule
DTH	Delayed-type hypersensitivity
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent-activated cell sorter
FAE	Follicle-associated epithelium
FBS	Foetal bovine serum
FCA	Freund's complete adjuvant
FcRn	Neonatal Fc receptor
FIA	Freund's incomplete adjuvant
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
GPI	Glycophosphatidylinositol
HBSS	Hank's Buffered Salt Solution
HEL	Hen egg lysozyme
HEV	High endothelial venule

i.g.	Intragastric
i.n.	Intra-nasal
IEL	Intraepithelial lymphocyte
IFAT	Indirect fluorescent antibody test
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
Iscom	Immune stimulating complex
J-chain	Joining chain
LN	Lymph node
LP	Lamina propria
MALT	Mucosa-associated lymphoid tissue
MFI	Mean fluorescence intensity
MLN	Mesenteric LN
<i>N. caninum</i>	<i>Neospora caninum</i>
NALT	Nasopharynx-associated lymphoid tissue
NAT	<i>Neospora</i> agglutination test
NBT	Nitro blue tetrazolium
NcT	<i>N. caninum</i> tachyzoites
NK	Natural killer
NTPase	Nucleotide triphosphate hydrolase
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PE	Phycoerythrin
PerCP	Peridin-chlorophyll protein
pIgR	Polymeric Ig receptor
PLG	Poly(D,L-lactide-co-glycolide)
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocyte
PP	Peyer's patch
PRR	Pattern recognition receptor
qPCR	Real-time quantitative PCR

RNS Reactive nitrogen species
s.c. Subcutaneous
SAG Surface antigen
SDS Sodium dodecyl sulfate
SED Subepithelial dome
sIgA Secretory IgA
SRS SAG-related sequence
T_C T cytotoxic
TCR T cell receptor
T_H T helper
TLR Toll-like receptor
TNF- α Tumour necrosis factor-alpha

1. Introduction

1.1 Project background

Neospora caninum is an obligate intracellular parasite that causes disease, neosporosis, in a wide range of veterinary species including dogs, sheep, goats and cattle. The latter are the most commonly affected animals and the economically relevant hosts. Cattle can be infected transplacentally, a very frequent and effective way of parasite transmission, or post-natally via the gastrointestinal (GI) tract. Although less common, this infection route is indispensable to sustain infection within a herd. Neosporosis is a major cause of abortion in cattle worldwide and, to date, no completely effective control measure against the parasite has been developed. Moreover, although a vaccine is commercially available, it has proven to be only partially effective in preventing abortions within herds.

1.2 Project objectives

The present work aimed at characterizing the immune response elicited in mice by mucosal (intra-nasal) (i.n.) CpG-adjuvanted immunisation with membrane proteins of *N. caninum* tachyzoites (NcT) and at evaluating the protective effect conferred by this immunisation procedure against neosporosis established by the GI tract. The ultimate purpose would be to establish an effective immunisation protocol that could be applied in cattle to prevent post-natal *N. caninum* transmission. Since horizontally transmitted neosporosis can also result in foetal infection and possibly abortion, the development of a novel vaccine that could confer successful immunisation would significantly contribute to improve the management and decrease the worldwide impact caused by *N. caninum* infections.

1.3 Thesis organisation

This document is organised into six chapters: *Introduction, State of the Art, Materials and Methods, Results, Discussion and Conclusions and future work*. The first four chapters are divided into several subheadings so that the different subjects and ideas can be more easily accessed.

Chapter 2, *State of the Art*, entails a comprehensive literature review in the field and provides the theoretical framework where the project is included.

Chapter 3, *Materials and Methods*, describes the reagents and data collection instruments used, as well as the methods employed to carry out the research study. A section of this chapter named *Experiment set-up* explains how the research was conducted by describing the organisation in time of the several tasks that the project comprised.

Chapter 4, *Results*, includes a detailed presentation of the data obtained which are then critically appreciated in the context of the stated objectives and other reported data in chapter 5, *Discussion*.

Conclusions drawn from the study as well as recommendations for future studies are documented in chapter 6, *Conclusions and future work*.

2. State of the Art

2.1 *Neospora caninum*

N. caninum is an obligate intracellular protozoan parasite that was first recognised in Norway in 1984 as the causative agent of encephalomyelitis and myositis in dogs, which had no antibodies to *Toxoplasma gondii*¹. It was classified later on, in 1988, by Dubey *et al.*², who described the new genus *Neospora* and the type species *Neospora caninum* belonging to the Phylum Apicomplexa and the family Sarcocystidae. In the same year, the parasite was isolated from paralyzed dogs³ and that strain was then designated NC-1⁴.

Unlike *T. gondii*, viable *N. caninum* is difficult to isolate⁵ and it has been obtained from only a few hosts, namely cattle⁶, sheep⁷, water buffaloes⁸, white-tailed deers⁹ and bison¹⁰, besides dogs. Prevalence of *N. caninum*-specific antibodies has been demonstrated in cattle, sheep, goats, dogs, pigs, coyotes and other domestic and wild animals⁵. *N. caninum* specific DNA in turn was found in chickens, rats and rabbits, among others, but its finding should not be regarded as equivalent to finding viable *N. caninum*⁵. Altogether, these reports stress out that a wide range of domestic and wild animals have been exposed to *N. caninum*.

At present, the zoonotic potential of *N. caninum* is still questionable. Neither the parasite itself nor its DNA were so far detected in human tissues¹¹. Moreover, in one study using serum samples from women with a history of idiopathic repeated abortions, no *N. caninum*-specific antibodies were detected by any of the serological tests performed¹². Nevertheless, *N. caninum* infection was successfully established in macaque fetuses that were infected with the parasite, either directly or transplacentally¹³, and *N. caninum*-specific immunoglobulin G (IgG) were detected in HIV-infected patients and in patients with neurological disorders¹⁴.

2.1.1 Life cycle

N. caninum is a single-celled coccidian parasite with a heteroxenous life cycle, which means that more than one host is needed to complete it (Figure 1)¹⁵. To date, the only identified definitive hosts of *N. caninum*, i.e., the animals in which sexual reproduction of the parasite takes place¹⁶, are the domestic dogs (*Canis lupus familiaris*)^{17,18}, Australian dingoes (*Canis lupus dingo*)¹⁶ and coyotes (*Canis latrans*)¹⁹. Intermediate hosts, i.e., animals in which parasite multiplication occurs by asexual reproduction¹⁶, include a wide range of warm-blooded animals like sheep, goats and felids²⁰, being however cattle the most common and economically important host animals²¹.

Three parasite infectious stages have been identified during *N. caninum* life cycle, namely oocysts, tachyzoites (short-lived, rapidly dividing stage) and bradyzoites (longer-lived, slowly dividing stage)²⁰. Oocysts, environmentally resistant and measuring approximately $10 \times 12 \mu\text{m}$ ^{22,23}, constitute the sexual stage of the parasite and are excreted in an unsporulated phase in the feces of acutely infected dogs, after ingestion of *N. caninum*-infected tissues^{24,25}. Low numbers of oocysts are usually excreted⁵. Shedding can start between 5 and 13 days after consumption of bradyzoite-containing tissue cysts and last for up to 27 days²⁶ or even continue for several months in some dogs²⁷. Oocysts sporulate in the environment within 24 to 72 hours after having been excreted, giving rise to two sporocysts, each containing four sporozoites^{24,26} that measure $6.5 \times 2 \mu\text{m}$ ²². Once cattle or other intermediate hosts ingest water or food contaminated with sporulated oocysts they enable the sporozoites to be released in the intestinal tract, where they invade cells and differentiate into tachyzoites²⁴ which are lunate-shaped and measure approximately $6 \times 2 \mu\text{m}$ ¹⁵. Tachyzoites divide within host cells by endodyogeny (process of asexual reproduction in which two daughter cells develop within the parent cell), forming numerous organisms that are released in the blood^{15,28}. The resulting parasitaemia provides a means for dissemination of the parasite throughout the body¹⁵. Okeoma *et al.*²⁹ detected *N. caninum* DNA in the leukocyte fraction of blood from naturally infected pregnant heifers. Tachyzoites were reported to infect many different cell types, including myocytes, hepatocytes, neural cells, vascular endothelial cells, alveolar macrophages, renal cells and placental trophoblasts, the last leading to transmission of the parasite to the foetus¹⁵. At a cellular level, tachyzoites are located within a parasitophorous vacuole in the host cell cytoplasm⁴. If the host is immunocompetent, its immune system will be activated in response to the invasion and the multiplication of tachyzoites in order to contain the spread of the parasite³⁰. To be protected from the attack mounted by the host immune system, tachyzoites differentiate into bradyzoites, which are slender, measuring approximately $6.5 \times 1.5 \mu\text{m}$ ³¹, and are usually located in the central nervous system surrounded by a cyst wall, thus forming intracellular tissue cysts^{24,32}. However, they have also been identified in the retina of a naturally infected dog³³ and in skeletal muscles of naturally infected dogs and cattle³⁴. Tissue cysts are often round or oval in shape and vary in size depending on the number of bradyzoites enclosed^{15,34}. Their diameter and cyst wall thickness are, respectively, up to $107 \mu\text{m}$ and up to $4 \mu\text{m}$ in dogs and approximately $50 \mu\text{m}$ and less than $2.5 \mu\text{m}$ in cattle^{4,35,36}. Bradyzoites represent a quiescent stage of the parasite that remain latent until the host's immune response is suppressed, which allow them to recrudesce²⁴, and may act as a source of foetal infection in subsequent pregnancies²⁸. Dogs can then ingest tachyzoites in placental tissue or bradyzoites in tissue cysts, which allow them to implant in the GI tract and undergo maturation. Oocysts are then excreted in the feces of these animals and the *N. caninum* life cycle starts all over again^{24,26,37} (Figure 1).

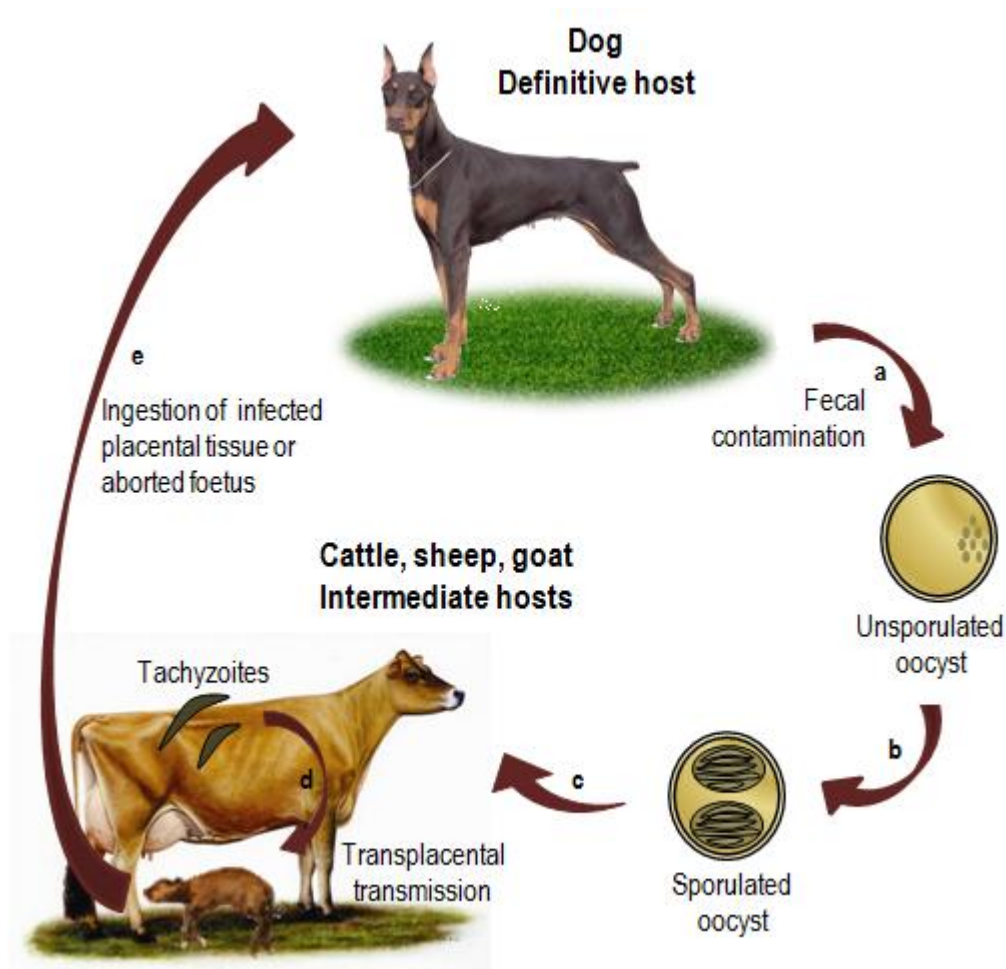


Figure 1. Life cycle of *N. caninum*. After ingestion of *N. caninum*-infected tissues, dogs excrete unsporulated oocysts in the feces (a). Oocysts sporulate in the environment (b) and are then ingested by intermediate hosts (c). Tachyzoites thus formed can be transmitted to the foetus by crossing the placenta, leading to foetal infection and eventually abortion (d). Infected tissues of intermediate hosts are a source of food for dogs, allowing the cycle to start all over again (e).

2.1.2 Modes of transmission in cattle

Two distinct transmission routes of *N. caninum* have been reported, the horizontal (post-natal or lateral) and the vertical (congenital or transplacental) transmission route¹¹. In cattle, the first occurs when sporulated oocysts are ingested, whereas the last refers to transmission of the parasite from an infected dam to her offspring during pregnancy¹⁵. Vertical transmission can be further divided into two distinct mechanisms, namely the exogenous and endogenous transplacental transmission. Exogenous transplacental transmission occurs when a naïve dam ingests sporulated oocysts that will differentiate into tachyzoites which will cross the placenta and infect the foetus. Endogenous transplacental transmission in turn occurs when a persistent *N. caninum* infection within a dam, previously acquired either vertically or horizontally, is reactivated during pregnancy^{15,21}. In this case, bradyzoites are thought to differentiate into tachyzoites that

spread to distinct host tissues including the uteri, where they cross the placenta and infect the foetus²¹.

In cattle, vertical transmission is a very efficient way of spreading *N. caninum* to a new host, with transmission rates ranging from approximately 80% to 95%^{38,39}, and can occur over several and consecutive pregnancies^{40,41} or intermittently⁴¹. The endogenous route of vertical transmission is thought to account for the majority of infections occurring in these animals⁴², being, in contrast, less frequent the occurrence of horizontal transmission (between 1% and 2% per cow per year)^{39,43}. However, it was shown by theoretical models that even low levels of horizontal transmission are needed to sustain *N. caninum* infection within a herd⁴⁴.

Other natural post-natal modes of transmission were tested for the possibility of also being responsible for *N. caninum* infection in cattle. Cow to cow transmission does not seem to occur⁴⁵ and was not observed so far⁵ and venereal transmission is unlikely to exist. Dams naturally bred with experimentally infected bulls did not seroconvert⁴⁶ and even though *N. caninum* DNA has been detected in the semen of seropositive bulls, only a few parasites were present there⁴⁷. Furthermore, although neonatal calves may become experimentally infected after ingestion of tachyzoite-contaminated milk, lactogenic transmission does not appear to be an important natural route of *N. caninum* transmission in cattle^{48,49}. Therefore, the only demonstrated natural mode of infection in cattle is through the ingestion of sporulated oocysts from the environment⁵.

2.1.3 Surface and secretory proteins of NcT

Two major immunodominant glycosylphosphatidylinositol (GPI)- anchored proteins populate the NcT surface and contribute to the initial low-affinity host-parasite contact. These are homologues of *T. gondii* surface antigen 1 (SAG1) and SAG-related sequence 2 (SRS2) and are commonly designated NcSAG1 and NcSRS2 with 29 and 35 kDa, respectively^{32,50}. Once the initial contact has been established, NcT discharge secretory organelles, namely micronemes, dense granules (DGs) and rhoptries. Microneme proteins, released at the onset of host-parasite adhesion and therefore mediators of the physical interaction between the parasite and the host cell surface⁵¹, include soluble molecules, such as NcMIC1, NcMIC2 and NcMIC4 as well as membrane-bound molecules, such as NcMIC3³². NcSUB1, a serine protease produced by the parasite, also displays microneme localization⁵². As suggested by *in vitro* studies, rhoptry proteins, in particular NcROP2, may be involved in host cell invasion, consistent with the fact that these organelles are exocytosed upon invasion of the host cell^{53,54}. Also, rhoptry components, as well as proteins from DGs which are secreted during the invasion process, are important for the formation and functioning of the parasitophorous vacuole in which the parasite develops^{53,54}. Proteins from DGs

include NCDG1, NCDG2, nucleotide triphosphate hydrolase (NTPase), NcGRA2 and NcGRA7⁵³. Another protein, existent on the surface of NcT and in the micronemes but absent in rhoptries and DGs, is protein disulfide isomerase (PDI)⁵⁵. NcPDI is involved in parasite-host cell interactions since it catalyses the reduction, oxidation and isomerisation of disulfide bonds and many components of both the adhesion and invasion machinery of *N. caninum* are cysteine-rich and depend on correct folding through the formation of disulfide bonds⁵⁵.

2.2 Neosporosis in cattle

Since its discovery, neosporosis, the disease caused by *N. caninum*, has emerged as a major cause of disease in dogs²⁶ and, in particular, abortions in cattle around the world¹¹. Before recognition of *N. caninum* as a new species and the characterization of the disease itself, neosporosis was often misdiagnosed as toxoplasmosis due to close similarities between either the causative parasites or the respective diseases both clinically and pathologically^{2,56}. *N. caninum* and *T. gondii* are, in fact, morphologically, genetically and antigenically closely related parasites^{4,50,57} and have similar life cycles¹¹. However, they are biologically different as neosporosis is mainly a disease of cattle and dogs and is not considered zoonotic, whereas toxoplasmosis affects primarily humans, sheep and cats, the last being definitive hosts of that parasite^{5,20}.

2.2.1 Clinical signs

In cattle, neosporosis manifests in the placenta and developing foetus but rarely in adult animals³⁰. Foetuses may either be aborted from the third month of gestation until the end, with most abortions occurring between the fifth and sixth month, or born weak with neurological symptoms or born clinically normal but persistently infected^{5,57}. The outcome depends on the timing in gestation at which the infection occurs³⁰, with infections occurring early in pregnancy being more harmful to the foetus than infections occurring later on⁵⁸. Cows infected with *N. caninum* are three to seven times more likely to abort than uninfected cows and the risk is highest in the first pregnancy^{41,59,60}. Other clinical signs of neosporosis have only been reported in cattle with less than 2 months of age and include underweight and inability to rise. The hind limbs or forelimbs may be flexed or hiperextended and neurological problems such as ataxia, decreased patella reflexes and loss of conscious proprioception may develop. Calves may also have exophthalmia or an asymmetrical appearance in the eyes and, occasionally, birth defects such as hydrocephalus and narrowing of the spinal cord may occur⁵.

2.2.2 Diagnostic techniques

A variety of serological tests can be performed to diagnose *N. caninum* infection in cattle, including the Indirect Fluorescent Antibody Test (IFAT), several Enzyme-Linked Immunosorbent Assays (ELISAs), the *Neospora* Agglutination Test (NAT) and Western Blot⁶¹. Even though IFAT is very specific for *N. caninum*, it is an expensive and time-consuming test, not being commonly performed to screen cattle populations for that infection^{24,62}. Avidity-ELISA is a useful test to distinguish between acute and chronic infections, based, respectively, on the lower or higher binding strengths (avidity) of IgG antibodies to a *N. caninum* antigen⁶³. Another way of doing that is by performing ELISAs using the recombinant proteins NcGRA7 and NcSAG4 (a bradyzoite stage-specific protein) to detect antibodies of the acute phase and chronic phase, respectively⁶⁴. However, it is worth mentioning that antibody levels can fluctuate considerably and may even drop below the cut-off value of the test used, giving rise to a negative result when *N. caninum*-specific antibodies actually exist^{65,66}. Therefore, negative serological results are not informative⁶¹.

Antibody fluctuations also occur in bovine fetuses as factors such as the stage of gestation and the time between infection and abortion influence antibody production⁶¹. In addition, serological results in the foetus only provide information about exposure to the parasite and histologic examination should therefore be carried out for a definitive diagnosis of neosporosis abortion⁶¹. However, few *N. caninum* parasites are generally present in autolysed tissues and these are hardly visible in histological sections. Therefore, immunohistochemistry is usually performed. The foetal brain is the tissue of choice either for histology or immunohistochemistry, as it is the most consistently affected organ^{24,61}. In severely autolysed fetuses, however, immunohistochemistry is insensitive and may underestimate the existence of infection⁶¹. In such cases, PCR can thus be used to detect *N. caninum* in the brain tissues of the aborted calves⁶¹. Diagnosis of bovine abortion is often difficult since finding *N. caninum*-specific DNA, antibodies or even the parasite itself in foetal tissues may not necessarily mean that *N. caninum* was the cause of the abortion, as asymptomatic and congenitally infected cows, many of them being serologically positive, are commonly found^{5,11}. Thus, a comprehensive diagnostic approach is usually conducted in which the aforementioned serologic, histologic and molecular methods are integrated to investigate the existence of a cause-effect relationship between *N. caninum* and abortion¹⁵.

Although *N. caninum* is closely related to *T. gondii* and *Sarcocystis* species, cattle serum cross-reactivity has not been a major issue⁶¹. *N. caninum* can also be distinguished from those parasites by immunohistochemistry and, at the DNA level, by PCR⁶¹. In addition, infection of bovine fetuses with *T. gondii* is rare and *Sarcocystis cruzi* is rarely (< 0.1%) found in aborted foetal

brains⁶¹. However, there is the possibility of serum cross-reaction with *Neospora hughesi*, a parasite closely related to *N. caninum* about which little is known⁶⁷.

2.2.3 Economic impact

N. caninum is a major cause of epidemic and endemic abortions in both dairy and beef cattle around the world^{5,15} and economic losses due to *Neospora*-associated abortions have been estimated to exceed hundreds of millions of dollars every year⁵³. In addition to that direct cost, indirect costs include veterinary assistance, diagnostic procedures, rebreeding, replacement costs when aborted cows have to be culled and possible loss of milk production⁵. Post-natal losses caused by *N. caninum* are difficult to assess since clinical disease has not been reported in calves older than 2 months of age⁵.

2.2.4 Prevention and control

In cattle, control measures that may reduce neosporosis include embryo transfer, test-and-cull strategies, chemotherapeutic treatment, vaccination and increased biosecurity in herds. Transfer of embryos from *N. caninum*-infected dams to only uninfected and seronegative recipient cows is a way of recovering uninfected calves and preventing endogenous transplacental transmission of the parasite^{5,68}. The test-and-cull approach includes testing and culling seropositive dams or seropositive aborting dams or testing and either excluding the progeny of seropositive dams from breeding or inseminating the progeny with beef bull semen only⁵ which has been demonstrated to reduce the risk of abortion in *Neospora*-seropositive heifers and cows^{69,70}. Despite having been successfully implemented in the field⁷¹ and considered one of the most efficacious solutions to control neosporosis, the test-and-cull approach is very expensive and likely uneconomical, especially in herds with a large proportion of infected cattle^{53,72}. Until now, no chemotherapeutic treatment has proven to be safe and effective enough to treat neosporosis in either dairy or beef cattle. Moreover, safety concerns arising from the possibility that drug administration could produce undesirable residues in milk or meat, may render these products unacceptable for consumption⁵. In addition, and similarly to the test-and-cull strategy, chemotherapy does not seem to provide an economically viable control option, as suggested by previous analysis⁷². In contrast, efficacious vaccination of all susceptible animals (both seropositive and seronegative cattle in a herd) does seem to be economically viable⁷² (further discussion on this topic in 2.6 *N. caninum* and vaccination). Farmers can also adopt biosecurity measures to prevent horizontal transmission of *N. caninum*, such as the avoidance of contact between dogs and infected

bovine tissues to hinder oocysts shedding and the coverage of feed, silage and bedding materials to minimize contamination with dog feces²⁸. No control measures directed to public health are recommended since, at present, neosporosis is not considered a zoonotic disease¹¹.

2.3 Mucosal immunity

The intestine is the largest area of the organism in contact with the external environment⁷³. In there, permanent antigenic challenge occurs from birth to death, in the form of food antigens, antigens of the microbiota and pathogens acquired by the oral route. This organ is thus considered a particularly vulnerable source of infection and a constant threat of disease^{74,75}. However, it has the ability to maintain the number of resident bacteria and simultaneously protect against pathogens by inducing mucosal innate and adaptive immune responses⁷⁶.

2.3.1 Innate immunity

In the intestinal mucosa, the innate immune response is carried out by strongly interconnected physical, chemical and cellular components of the epithelial barrier. A single epithelial cell layer, with tight junctions between the cells, accounts for the physical barrier but yet allows selective entry of essential nutrients. Chemical obstacles include the mucus overlying the epithelium as well as the bile, pancreatic enzymes and antimicrobial peptides. The cellular barrier in turn is composed of immune cells that trigger nonspecific defences against pathogens successful in breaching the epithelial barrier⁷⁶⁻⁷⁸. Epithelial cells lining the intestinal mucosal surface are able to recognize pathogen-associated molecular patterns (PAMPs) which are usually highly conserved and essential for pathogens survival but absent in the host through pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs)^{76,77}. That recognition induces the production and secretion of cytokines and chemokines that attract and activate polymorphonuclear leukocytes (PMNs), macrophages and dendritic cells (DCs), ultimately resulting in a nonspecific immune response aimed at clearing the pathogen^{76,77}. Interleukin-8 (IL-8), produced by epithelial cells, attracts and activates PMNs, which are usually the first cells to arrive at the site of infection⁷⁹. Interferon-gamma (IFN- γ) can be secreted by natural killer (NK) cells stimulated by cytokines secreted by infected epithelial cells such as IL-15, and is known to activate DCs and macrophages whose microbicidal activity is largely dependent on production of reactive nitrogen species (RNS)^{76,79}. Both macrophages and DCs are also a source of cytokines, such as IL-12, that are able to trigger adaptive immune responses^{76,77}.

2.3.2 Adaptive immunity

The production and presence of secretory IgA (sIgA) at luminal mucosal surfaces is considered the hallmark of mucosal adaptive immunity⁸⁰. However, more complex antibody and cellular interactions can occur, depending on the degree of pathogen invasion and pathogenicity⁸⁰. Whatever the mechanism of adaptive immunity could be, it requires the existence of inductive and effector sites, as well as acquisition of antigens by antigen-presenting cells (APCs).

2.3.2.1 Inductive and effector sites

In the inductive sites, antigens are taken up and B- and T-cell responses are induced with the help of APCs, whereas in the effector sites, the previously stimulated effector cells accomplish their function upon extravasation, retention and differentiation. An illustrative example may be their contribution to the formation of sIgA⁸¹.

The inductive sites are composed of mucosa-associated lymphoid tissue (MALT) and mucosa-draining lymph nodes (LNs) (Figure 2A-B)⁸¹. MALT is an organised lymphoid tissue in close contact to mucosal surfaces and, according to the anatomical localization, it can be subdivided into several components, such as the conjunctiva-associated lymphoid tissue (CALT), nasopharynx-associated lymphoid tissue (NALT) and gut-associated lymphoid tissue (GALT)⁸², with highly variable composition and distribution among different species⁸¹. All MALT subtypes are structurally organised into several compartments, namely the lymphoid follicles, containing predominantly B cells, the interfollicular areas, rich in T cells and high endothelial venules (HEVs), the follicle-associated epithelium (FAE), containing M-cells, and the subepithelial dome (SED), which separates the FAE from the follicle^{81,83}. These MALT subtypes also contain a variety of APCs, such as DCs and macrophages, and in contrast to LNs, lack afferent lymphatics, being the antigens directly sampled from mucosal surfaces through M-cells⁸¹. These specialized epithelial cells, so called because they display a thin *membranous* apical membrane with microfolds or microvilli⁸⁴, have the ability to endocytose and transport microorganisms and macro- and soluble molecules from the gut lumen directly to the SED region, thereby allowing the lymphoid tissue to access luminal antigens^{81,83}. Due to their reduced mucus layer and microvilli, these cells are more susceptible to pathogen invasion⁸⁰. It should be mentioned that since mucosa-draining LNs do not sample antigens directly from the lumen but have instead afferent lymphatic drainage, they are not considered MALT structures, yet they constitute mucosal inductive sites (Figure 2A-B)⁸¹. For antigens derived from the intestinal lumen, Peyer's patches (PPs), isolated lymphoid follicles and the appendix, all of them

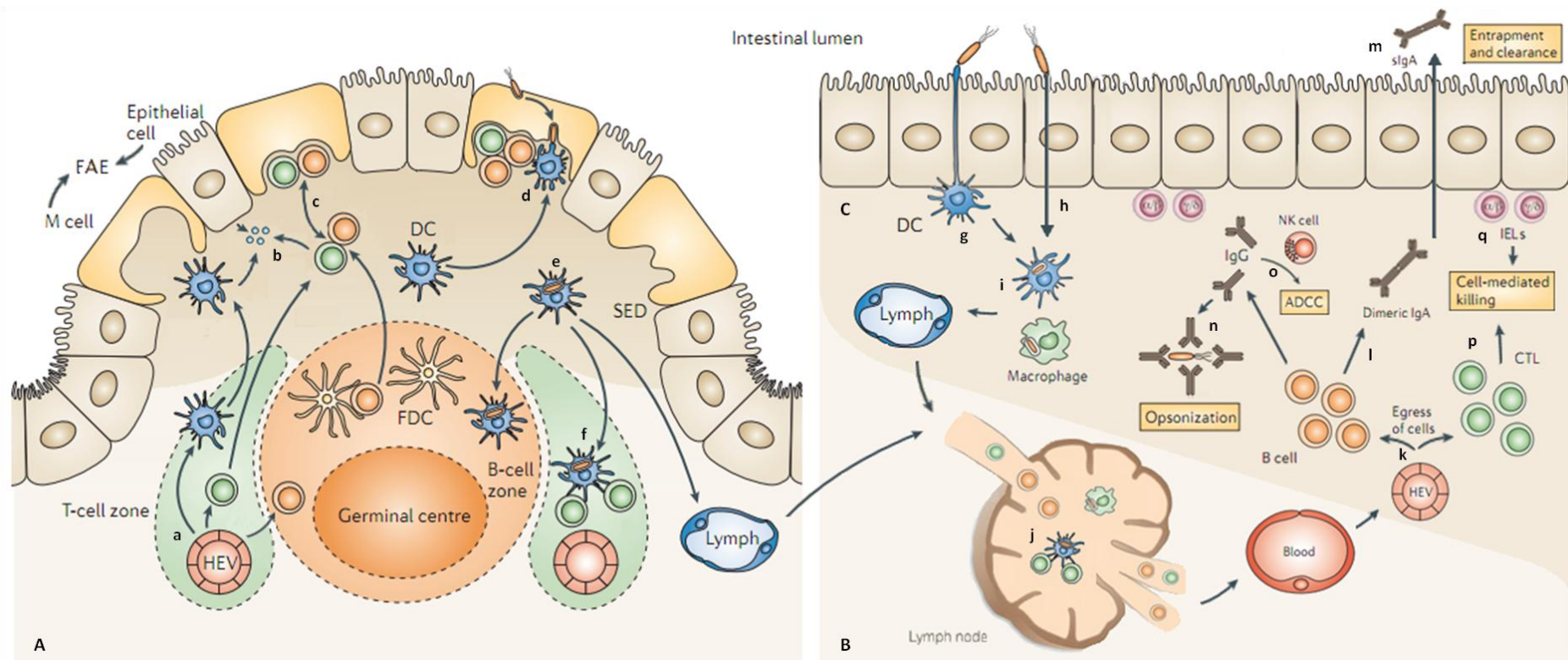


Figure 2. Composition and cellular traffic in inductive and effector sites. Inductive sites include MALT (A), composed of lymphoid follicles (B-cell zone), interfollicular areas (T-cell zone), FAE and SED, and mucosa-draining LNs (B) which unlike MALT contain afferent lymphatic drainage. B and T cells as well as DCs and macrophages populate these sites. Effector sites (C) contain mostly T cells, IgA plasma cells and IELs and fewer DCs and macrophages. In PP (inductive site), B and T cells and immature DCs enter the mucosa through HEVs (a) and some of them are attracted to the SED region in response to chemokines released by the FAE (b). Some B and T cells migrate to the intraepithelial pockets of M-cells (c) which transport luminal pathogens that are captured by DCs (d). Those DCs migrate to the lymphoid follicles and interfollicular areas and maybe to the draining LNs (e). LP (effector site) DCs can capture pathogens by extending their dendrites across the epithelium (g) and pathogens that are able to actively penetrate the epithelium can also be internalized by LP DCs (h), which then migrate to the nearest draining LN (i). Both in the interfollicular areas of MALT (f) and in the LNs (j), DCs present antigens to T cells. After activation, both T and B cells migrate to the peripheral blood and extravasate preferentially at mucosal tissues (k). IgA plasma cells secrete dimeric IgA (l) that is exported to the lumen forming sIgA, which is essential to prevent mucosal invasion (m). IgG promotes opsonization of pathogens (n) and collaborates with NK cells to kill infected cells (ADCC) (o). Infected cells can also be killed by CTLs (p) and IELs (q). ADCC, antibody-dependent cell-mediated cytotoxicity; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FAE, follicle-associated epithelium; FDC, follicular DC; HEV, high endothelial venule; IEL, intraepithelial lymphocyte; LN, lymph node; LP, lamina propria; MALT, mucosa-associated lymphoid tissue; NK, natural killer; PP, Peyer's patch; SED, subepithelial dome. Adapted from Neutra, M. R. & Kozlowski, P. A. (2006)⁷⁷.

GALT structures, are considered inductive sites for mucosal B and T cells, together with the mesenteric LNs (MLNs)^{85,86}.

The effector sites include different compartments, such as the lamina propria (LP) of various mucosal tissues and the stroma of exocrine glands⁸¹. At a cellular level, they are composed of T-cells (mostly CD4⁺), IgA plasma cells, fewer IgM and IgG plasma cells and few B cells, DCs and macrophages (Figure 2C)⁸³. Another effector site is the intraepithelial lymphocyte (IEL) compartment which generally refers to the epithelium of the small intestine where most IELs occur⁸¹ (Figure 2C). IELs consist mostly of T cells belonging to the T cell receptor- $\gamma\delta$ (TCR $\gamma\delta$)⁺ and TCR $\alpha\beta$ ⁺ lineages^{87,88}. They are in direct contact with epithelial cells and very close to antigens in the gut lumen and thereby contribute to the physical barrier against invading pathogens⁸⁷. Unlike conventional T cells, they are antigen-experienced T cells that upon encountering antigens mediate killing of the infected target cells or release cytokines right away^{87,88}. IELs are therefore able to mount and regulate both innate and adaptive immune responses^{87,88} (Figure 2C).

2.3.2.2 Cellular traffic in the mucosa and antigen sampling

At intestinal mucosal tissues with organised MALT, specifically at PPs, B and T lymphocytes as well as immature DCs, essential APCs in initiating mucosal T cell responses, enter the mucosa through HEVs^{77,89} and some of these cells can be then attracted to the SED region due to chemokines released by FAE (Figure 2). CC-chemokine ligand 20 (CCL20) and CCL9, produced by FAE of mouse small-intestinal PPs, are known to attract lymphocytes and DCs expressing CC-chemokine receptor 6 (CCR6) or CCR1, respectively⁹⁰⁻⁹². Some B and T lymphocytes migrate into the intraepithelial pockets formed by M-cells, where they express maturation or memory markers, while most of the immature DCs remain in the SED region⁷⁷. DCs capture antigens and microorganisms that are transported across the FAE by M-cells⁹³ and then migrate from the SED region to underlying B-cell follicles and interfollicular T-cell areas, where they upregulate the expression of maturation markers and MHC molecules^{93,94}. In the interfollicular areas, they process the antigens and present them to naïve T cells⁷⁷. It is postulated that some DCs can also carry the antigens to draining LNs where they are presented to T cells⁹⁵ (Figure 2).

Other strategies for antigen sampling have been reported, like the one that is exploited by LP DCs (Figure 2). These cells use exclusive proteins to separate the epithelial junctions, which allow them to extend their dendrites across the epithelium and directly sample the pathogens within the gut lumen, yet preserving the monolayer integrity^{96,97}. This strategy can also occur in tissues with a stratified, pseudostratified or simple epithelium that lack organised lymphoid follicles and M-cells, such as the female genital tract and, for that reason, it may also be more

immunologically important there⁷⁷. After antigen capture, those DCs can migrate to the nearest draining LN and present antigens to T cells⁷⁷. Alternatively, pathogens able to damage or actively penetrate the mucosal epithelium may also be captured by LP DCs that will be innately activated through recognition of PAMPs by their PRRs and will then carry the internalised antigens to draining LNs^{76,80} (Figure 2).

After being primed, naïve T and B cells become memory or effector cells and migrate from MALT to LNs and from LNs to the peripheral blood, and then extravasate at mucosal effector sites⁹⁸ (Figure 2). Therefore, mucosal infection at one site can provide protection at a distant mucosal site, which created the concept of *common mucosal immune system* (CMIS)⁹⁹. This term has been then refined to *compartmentalised mucosal immune system* since not all the mucosal tissues seem to be interconnected and cells may preferentially migrate to certain mucosal effector tissues than to all mucosal tissues non-selectively¹⁰⁰. Whatever the exact tissue is, the guiding of those cells to the mucosa again is dictated by the upregulation of tissue-specific adhesion molecules and chemokine receptors that bind complementary ligands in the endothelial cells of the mucosal vasculature^{101,102}.

2.3.2.3 Humoral immune response

Production and secretion of sIgA is the most important defence factor against mucosa-invading pathogens⁷³. It starts with class switching from IgM to IgA by activated B cells in the inductive sites followed by their migration through the bloodstream to various mucosal tissues^{80,103}. Within the LP (effector site), those B cells differentiate into plasma cells, which results in secretion of dimeric IgA whose monomers are linked by a joining chain (J-chain)⁸⁰. The interaction of the IgA dimer with the polymeric Ig receptor (pIgR) on the basolateral surface of epithelial cells induces their transcytosis to the apical surface¹⁰⁴. There, pIgR is proteolytically cleaved giving rise to a polypeptide named *secretory component* that remains attached to the IgA dimer, thus forming sIgA^{80,104}.

sIgA displays unique properties when compared with other classes of antibodies, namely, it is resistant to protease degradation, for which its dimerization, high degree of glycosylation and association with the secretory component account for, and can interact with mucus and effector leukocytes^{77,80}. However, sIgA is unable to activate complement or stimulate the release of inflammatory mediators by innate immune cells^{80,103}. Through a mechanism known as *immune exclusion*, sIgA promotes the entrapment of pathogens in the mucus, thus preventing their direct contact with mucosal surfaces⁷⁷. Also, sIgA molecules of appropriate specificity might sterically hinder pathogen surface molecules involved in epithelial attachment¹⁰⁵ or might intercept, during its pIgR-mediated transport, incoming pathogens retained in vesicles of epithelial cells¹⁰⁶.

Moreover, it was shown that association of sIgA with pathogens might facilitate the antigen sampling by DCs¹⁰⁷. Dimeric IgA molecules present in the fluid underlying the epithelial barrier and produced by local IgA plasma cells can also prevent mucosal infection by carrying pathogens that have breached the epithelium back to the lumen through the pIgR-mediated transport (Figure 2)^{77,108}.

IgG also contributes to adaptive immune responses at mucosal surfaces by promoting *antibody-dependent cell-mediated cytotoxicity* (ADCC), a mechanism through which antibody-coated infected cells are lysed by NK cells (Figure 2)⁷⁷. In addition, and through the interaction with an IgG-specific Fc receptor, the neonatal Fc Receptor (FcRn), IgG can be exported across the intestinal epithelial barrier to the lumen, where it binds antigens, and then be transported back into the LP, where the antigens are taken up by DCs for further processing and presentation to CD4⁺ T lymphocytes¹⁰⁹. IgG is the predominant antibody class in bovine secretions, specifically in the colostrum and milk, in contrast to IgA which is present there at much reduced concentrations and is the major Ig present in the human milk¹¹⁰.

2.3.2.4 Cellular immune response

When it comes to cell-mediated immunity (CMI), both cytotoxic T lymphocytes (CTLs, i.e., effector T cytotoxic (T_C) CD8⁺ cells), and T helper (T_H, CD4⁺) cells have been found to be important for mucosal immune defence against pathogens (Figure 2)¹¹¹. Upon antigen presentation by APCs, naïve antigen-specific T cells are activated and become effector cells⁷⁹. Induction of IgA production against mucosal pathogens is dependent on effector T_H cells¹¹¹. Depending on the cytokine environment in which T_H cells differentiate, distinct cell subsets, such as T_H1 or T_H2, may arise. T_H1 cells secrete IFN- γ and promote secretion of IL-12, a key cytokine for differentiation of T_H1 cells, by macrophages and DCs⁷⁹. T_H2 cells in turn secrete IL-4, which induces the differentiation of naive T cells into T_H2 cells, and also IL-10, which along with IL-4 suppresses the expansion of T_H1 populations⁷⁹. In the same way, IFN- γ inhibits the development and activity of the T_H2 subset⁷⁹. T_H1 immune responses, usually set up against intracellular pathogens, are further characterized by secretion of tumour necrosis factor-alpha (TNF- α) and opsonizing antibodies, such as those of the IgG2a isotype, and strong CTL responses that mediate killing of target cells^{79,112}. T_H2 immune responses are generally activated to control helminthic pathogens and allergic diseases, with production of IgG1 and IgE antibodies but weak CTL responses^{79,112}. Another type of T_H cells exists, namely the T_H17 cells. They are activated by IL-23 and produce IL-17, a highly pro-inflammatory cytokine responsible for tissue damage in *delayed-type hypersensitivity* (DTH) reactions, as it increases chemokine production that leads to recruitment of monocytes and neutrophils to the site

of inflammation. DTH reactions are in turn considered a crucial component of the host immune response against bacteria and intracellular parasites^{79,113}. Moreover, IL-17, as well as IL-22, both produced by T_H17 cells, are important effectors of mucosal immunity, as they have been shown to regulate the expression of antimicrobial peptides¹¹⁴.

2.4 Mucosal immunisation

As mucosal surfaces are the main sites of entry and colonization for many pathogens, there is an increasing interest in effectively protecting them through activation of the mucosal immune system¹¹⁵. This can be accomplished by mucosal immunisation, as the administration of vaccines specifically onto mucosal surfaces results in the stimulation of mucosal and also systemic immune responses. Systemic immunisation in turn can effectively elicit systemic responses but is generally a poor inducer of mucosal immunity^{99,116}.

Compared with systemic immunisations, mucosal immunisations are safer since direct contact between potentially toxic components of the vaccine and systemic circulation can be avoided and adverse effects can thus be minimized. Also, mucosal immunisation reduces the need for trained personnel to administer the vaccine and it can also enhance its efficacy due to the ability of eliciting both mucosal and systemic immune responses¹¹⁵.

An ideal mucosal vaccine would adhere to mucosal surfaces, effectively induce innate responses and provide both humoral and cellular adaptive immunity at the relevant mucosal site of administration and also throughout the body⁷⁷. However, it comes out that vaccines administered in the mucosa face the same bottlenecks as do pathogens, namely, they are attacked by proteases and nucleases, diluted in mucosal secretions, captured in the mucus layer, excluded by epithelial barriers and, in the case of vaccine antigens, their uptake generally occurs at low levels^{77,115}. As a result, multiple administrations of vaccine (prime-boost combinations) and in large doses are usually required to induce a suitable protective immune response^{77,115}.

Various routes of mucosal immunisation, namely the i.n., oral, rectal or vaginal, are being continuously tested for their efficacy and the choice of the optimal route may depend on the species to be immunised, the expected site of challenge and the type of vaccine used (live, killed or subunit, among others)⁷⁷.

2.4.1 Types of vaccines

Live vaccines consist of attenuated pathogens, i.e., pathogens that have lost the ability to cause disease but are still capable of growing within an inoculated host⁷⁹. This kind of vaccine is

more likely to stimulate appropriate CMI against intracellular pathogens as it more closely mimics what happens during natural infection^{57,79}. Its effectiveness as mucosal vaccine is in part due to the adaptation mechanisms of the component pathogens to survive in the luminal environment and effectively invade mucosal tissues⁷⁷. However, these vaccines have a limited shelf-life and safety concerns arise from the possibility of reversal to virulence^{30,79}. Killed vaccines in turn comprise inactivated pathogens that are not able to replicate in the host, yet they can raise an immune response⁷⁹. They are generally regarded as safe but are unable to induce CMI and can lack some of the immune-relevant antigens due to the inactivation process or because they are only secreted by the pathogens alive^{30,79}. Moreover, killed vaccines can include immunosuppressive antigens or antigens inducing undesired responses and subunit vaccines, which consist of specific and purified pathogen-derived macromolecules, thereby constitute an advantageous alternative^{30,79}. Several systems can be used to deliver the antigen at mucosal surfaces, such as lipid particles (e.g., liposomes and immune stimulating complexes (iscoms), the last being cage-like structures formed by phospholipids, cholesterol and saponins) and polymeric particles (made up of, e.g., poly(D,L-lactide-co-glycolide) (PLG) or chitosan)^{115,117}. DNA vaccines are in turn composed of plasmid DNA that encodes antigenic proteins, allowing these molecules to be expressed in the host in their natural conformation, and induce both humoral and CMI⁷⁹. Finally, recombinant vector vaccines make use of attenuated organisms, such as bacteria or viruses, to introduce antigen-coding genes from infectious pathogens into the host and can therefore maximize CMI to those antigens⁷⁹.

As with systemic immunisation, the administration at mucosal surfaces of antigens that have no inherent immunostimulatory properties generates a weak or undetectable immune response and co-delivery of substances known as *adjuvants* is therefore required^{80,118}.

2.4.2 Adjuvants

Adjuvants are substances incorporated into vaccine formulations in order to enhance, accelerate and prolong the immune response elicited towards vaccine antigens¹¹⁹. Most of them are chemicals, microbial components or mammalian proteins and can be grouped according to their mechanism of action¹¹⁷, which includes enhancement of antigen presentation, improvement of antigen stability or immunomodulation (in this case, adjuvants alter the cytokine network, increasing the concentration of some cytokines while decreasing the concentration of others, in that way, shifting the type of immune response)¹¹⁸. A single adjuvant can also combine more than one mechanism of action¹²⁰.

By enhancing antigen immunogenicity, adjuvants allow the number of boost immunisations and the amount of antigen needed to provide a successful immune response to be reduced,

making the vaccine more cost-effective^{117,119}. If they display immunomodulatory properties, they may also change the type of immune response to the most effective one against a given pathogen^{117,119}. However, as immune potentiators, adjuvants may also induce adverse reactions. Systemic adverse effects observed in laboratory animals include fever, arthritis, uveitis and malaise, and adjuvants that act locally can cause inflammation and more rarely granulomas or sterile abscesses¹¹⁸. In most instances, these effects are mild and the benefits coming out of their use outweigh the potential drawbacks¹¹⁷.

A wide variety of substances with adjuvant activity exists nowadays with different mechanisms of action and host range for application. These include alum compounds, the first adjuvants licensed to be used in humans, oil emulsions such as the Freund's complete adjuvant (FCA), which consists of a water-in-oil emulsion with mycobacteria and was abandoned due to high toxicity, and the Freund's incomplete adjuvant (FIA), that contains no mycobacteria and is still used when the inflammation is not a major issue and when a strong adjuvant is needed^{77,117}. Also, cytokines, microparticles and nanoparticles have adjuvant properties as well as diverse bacterial products including lipid A, cholera toxin (CT) and CpG, all of them being candidates for mucosal adjuvants^{77,117}.

CpG oligonucleotides are adjuvants that contain a central unmethylated CpG dinucleotide preferentially flanked by two 5' purines and two 3' pyrimidines¹²¹. They are underrepresented in vertebrates and mimic a DNA motif from bacteria which, contrarily to vertebrates, lack a cytosine methylase^{117,122}. CpG oligonucleotides stimulate macrophages¹²³, DCs¹²⁴ and NK cells¹²⁵ as well as B-cell proliferation¹²⁶ and one of their mechanisms of action in cells has been reported to be dependent on TLR-9¹²⁷. They are immunomodulators¹¹⁷, particularly effective in inducing a T_H1 antigen-specific immune response¹²¹ and induce production of cytokines such as IL-6¹²⁸, IL-12¹²⁵, IFN- γ ¹²⁵ and TNF- α ¹²³. When delivered systemically with diverse antigens, CpG oligonucleotides are strong adjuvants¹¹⁵ and when used as mucosal adjuvants, both systemic and mucosal immune responses were shown to be induced^{77,122}.

2.5 Immune response to *N. caninum*

In vivo studies on the immunology of *N. caninum* infection are commonly performed in mice. The availability of murine models with selective immunological defects as well as the fact that vertical transmission of the parasite has been demonstrated in these animals is of great advantage^{129,130}. Exposure of mice to *N. caninum* has been associated with encephalitis, myositis, acute primary pneumonia and pancreatitis¹³¹. However, particular mouse strains appear to be

more susceptible to brain infection than others. BD10.2 mice do not develop clinical neosporosis, whereas BALB/c and C57BL/6 mice are highly susceptible to *N. caninum*-induced encephalitis¹³².

Several components of the innate immune system seem to be activated upon *N. caninum* infection. It was shown that NK cells are able to produce IFN- γ and kill *N. caninum*-infected cells¹³³ and that activation of macrophages with IFN- γ resulted in increased production of RNS and killing activity against *N. caninum*¹³⁴.

As *N. caninum* is an obligate intracellular parasite, CMI is likely to play a major role in protective immunity. Evidences for the major importance of a T_H1 immune response came from *in vitro* studies, showing that IFN- γ and TNF- α significantly inhibited intracellular multiplication of the parasite^{135,136}, as well as from *in vivo* studies, where mice whose IFN- γ or IL-12 were depleted by means of specific monoclonal antibodies^{137,138} and IFN- γ -knockout mice¹³⁹ exhibited a significantly increased vulnerability to *N. caninum* infection. The importance of CD4⁺ T cells in protective immunity was demonstrated *in vivo* when most of the mice in which CD4⁺ T cells had been depleted succumbed to fatal neosporosis within 30 days upon challenged with *N. caninum*, contrarily to mice with no CD8⁺ T cells or those of the control group¹⁴⁰. Also, CD4⁺ T cells from infected cattle were shown to directly lyse parasite-infected autologous target cells *in vitro*¹⁴¹. Humoral immunity was also shown to be important to control *N. caninum* infection, since B-cell (and consequently antibody)-deficient μ MT mice experimentally infected with NcT were found to be more susceptible to infection than the wild-type mice¹⁴². It is also known that the humoral component of the protective immunity against neosporosis is characterized by a major predominance of serum IgG2a antibodies over the IgG1 subclass^{132,138}, which further stresses out the importance of T_H1 immunity in protection against the parasite.

Antigen-specific IL-4 responses as well as a predominance of IgG1 antibodies are associated with susceptibility to neosporosis, which shows that a T_H2-biased immune response, in detriment of the aforementioned T_H1 protective immune response, favours invasion and infection by *N. caninum*^{30,132,143}.

2.6 *N. caninum* and vaccination

When attention has been paid at the economic impact of neosporosis in both beef and dairy industry as well as at the lack of successful and cost-effective measures to control the disease, a urgent need for the development of a safe and efficacious vaccine has come out in order to reduce the overall losses caused by *N. caninum*. The assumption that vaccination may be a feasible goal is supported by observations showing that *N. caninum*-infected cows may develop protective immunity against abortion and transmission of the parasite¹¹. Therefore, many attempts

have been made along the last years to develop an effective immunisation protocol aimed at preventing *N. caninum* infection, transmission, disease or abortion¹²⁹. The efficacy of those attempts can be firstly evaluated in mouse models but it ultimately needs to be carried out in cattle, for these are the target hosts⁵³.

Several live vaccines have been tested for their efficacy against *N. caninum*. In particular, temperature-sensitive strains have been reported to induce significant protection against *N. caninum* in mice¹⁴⁴. Also, natural *N. caninum* isolates attenuated in their ability to cause disease in mice have emerged and include NC-Nowra and Nc-Spain-1H. Administration of NC-Nowra tachyzoites in mice¹⁴⁵ and cattle¹⁴⁶ before pregnancy resulted, respectively, in a dramatic reduction in transplacental transmission and total prevention of foetal death after challenging during pregnancy. Nc-Spain-1H in turn has been reported to prevent abortion in a pregnant mouse model¹⁴⁷. Prevention of foetal death has therefore been experimentally demonstrated in exogenously, but not in endogenously, *N. caninum*-infected cows⁵.

When it comes to killed vaccines, irradiated NcT protected mice from an otherwise lethal challenge¹⁴⁸ and a crude *N. caninum* extract prevented vertical transmission of the parasite in mice¹⁴⁹. On the other hand, cattle immunised with a NcT preparation together with the adjuvant POLYGEN induced IFN- γ production but failed to prevent vertical transmission in pregnant cattle after experimental challenge with NcT¹⁵⁰. The only vaccine commercially available at present, NeoGuard[®] marketed by Intervet Inc. (Merriam, KS, USA), is an Havlogen-adjuvanted vaccine containing inactivated NcT^{30,53}. However, it has proven to only partially protect herds in New Zealand¹⁵¹ and to reduce only in some extent the incidence of abortions in Costa Rican dairy cattle¹⁵², with a calculated efficacy of 46%¹¹.

Among the large number of proteins existing in *N. caninum*¹⁵³, the choice of candidate molecules to subunit vaccines has been focused on those located on the parasite surface, as well as on those involved in parasite survival (host cell invasion and intracellular development)⁵³. In the latter case, proteins present and excreted by secretory organelles constitute a common choice⁵³. Immunisation of mice with recombinant NcSAG1 and NcSRS2 together with the corresponding DNA vaccine significantly protected mice from cerebral infection¹⁵⁴. Other studies reported that BALB/c mice subcutaneously (s.c.) immunised with iscoms containing recombinant NcSRS2 displayed lower cerebral *N. caninum* burden when compared to the control group¹⁵⁵ and that s.c. inoculation of BALB/c mice with native NcSRS2 prevented transplacental transmission of the parasite¹⁵⁶. In cattle, Freund's adjuvanted-injection of NcSRS2 coupled to palmitic acid elicited T-cell activation and IFN- γ production, similarly to what happens upon a live parasite infection¹⁵⁷. In turn, immunisation of C57BL/6 mice with recombinant NcMIC1¹⁵⁸ and NcMIC3¹⁵⁹ significantly protected mice from cerebral infection, and FIA or saponin-adjuvanted administration of recombinant NcROP2

protected C57BL/6 mice from clinical signs of neosporosis and also significantly reduced the cerebral parasite burden in vaccinated mice⁵⁴. Both NCDG1 and NCDG2 are known to be immunogenic in cattle and they were used in immunological assays to demonstrate antibody responses in these animals¹⁶⁰. I.n. immunisation of recombinant NcPDI was recently reported to provide highly protective immunity against *N. caninum*. It was also shown that immunisation of BALB/c mice with plasmid DNA coding for NcGRA7 protected against vertical transmission of *N. caninum*¹⁶¹ and the protective effect was increased when the plasmid DNA was administered together with the CpG adjuvant¹⁶².

Intraperitoneal vaccination of BALB/c mice with a recombinant vaccinia virus carrying either NcSAG1 or NcSRS2 gene protected mice against cerebral infection¹⁶³ and vertical transmission of the parasite¹⁶⁴, being the best protection achieved with the vaccinia virus expressing the NcSRS2 antigen. *Brucella abortus* strain RB51 was also used to deliver several *N. caninum* antigens, namely NcMIC1, NcMIC3, NcGRA2, NcGRA6 and NcSRS2, and has shown to protect C57BL/6 mice from lethal infection¹⁶⁵ and vertical transmission of *N. caninum*¹⁶⁶. However, that approach might not be acceptable in cattle populations of countries that also want to prove to be free of bovine brucellosis⁵³.

The aforementioned studies of subunit, DNA and vector vaccines reflect a preferential use of purified proteins or recombinant DNA as vaccine agents in most of the approaches aimed at developing an effective immunisation protocol against *N. caninum*. However, native proteins also show promise as vaccine agents, as suggested by the already mentioned study with native NcSRS2¹⁵⁶, and they should therefore also be considered in future studies⁵³.

3. Materials and Methods

3.1 Experiment set-up

The experiment, generally consisting of two i.n. immunisations followed by a NcT intragastric (i.g.) infection, comprised several time-specific tasks that are schematized in Figure 3. At day 0, which corresponds to the starting day of the experiment, mice were immunised i.n. (prime immunisation) with NcT membrane proteins together with CpG (*Protein + CpG* group) or with only PBS, only CpG or only NcT membrane proteins (control groups named *PBS*, *CpG* and *Protein*, respectively) (Table 1). At day 14, vaginal lavage fluids were collected from all mice for NcT-specific IgA measurement and at day 21 all mice received a boost immunisation in the same conditions as in the prime immunisation. At day 28, vaginal lavage fluids were collected again and blood was also collected to measure NcT-specific IgG1 and IgG2a levels. At day 42, all mice were infected i.g. with NcT and at day 49 blood was collected and mice were euthanized. Intestinal lavage fluids were collected to measure NcT-specific IgA levels and several organs were also collected for subsequent histopathological and immunohistochemical analysis and DNA extraction. Spleen and MLNs cells were collected to evaluate cytokine production by flow cytometry (Figure 3).

Until day 28, data were obtained by two independent experiments, from now on named E1 and E2, and from day 28 until the end, only by the first experiment (E1) (Figure 3).

In E1, the *Protein + CpG* group and the control groups were composed, respectively, of 6 and 4 mice, while in E2, the *PBS* and *CpG* groups contained 10 animals and the *Protein* and *Protein + CpG* groups contained 11 mice (Table 1).

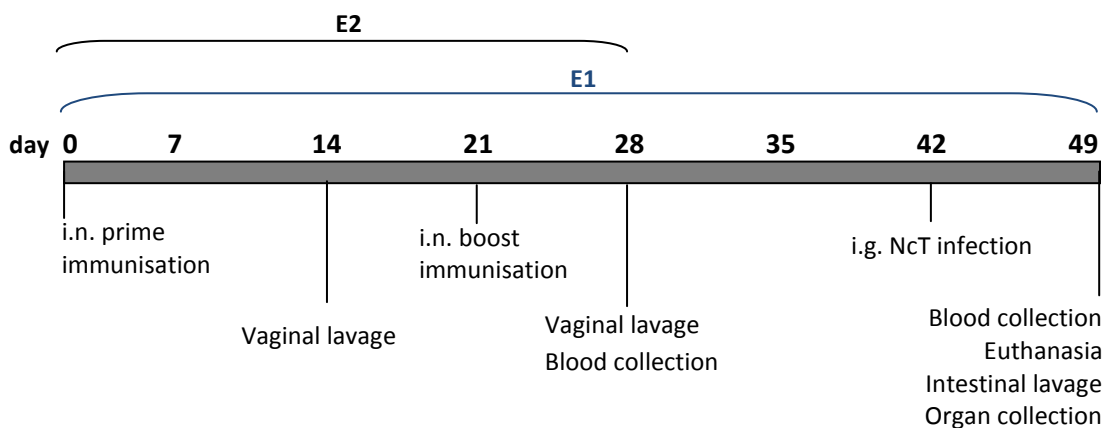


Figure 3. Timeline of tasks performed during the experiment. The protocol of prime-boost immunisation, in which *N. caninum* membrane proteins and the adjuvant CpG were administered i.n., was followed by i.g. infection with the parasite. E1, first experiment; E2, second experiment; i.n., intra-nasal; i.g., intragastric; NcT, *N. caninum* tachyzoites.

Table 1. Description of the groups of mice used in the experiments. E1, first experiment; E2, second experiment; NcT, *N. caninum* tachyzoites

Groups		Preparation administered (in 20 µL)		Number of mice	
		Substance(s)	Amount	E1	E2
Control	<i>PBS</i>	PBS	20 µL	4	10
	<i>CpG</i>	CpG	10 µg	4	10
	<i>Protein</i>	NcT membrane proteins	30 µg	4	11
<i>Protein + CpG</i>		NcT membrane proteins and CpG	30 µg 10 µg	6	11

3.2 Mice

Female C57BL/6 mice (8 weeks old) were purchased from Charles River (Barcelona, Spain). Animals were kept at the animal facilities of the Institute Abel Salazar during the experiments. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and the 86/609/EEC Directive and Portuguese rules (DL 129/92).

3.3 *N. caninum*

NcT of the NC-1 isolate were cultured and serially passaged in VERO cells maintained at 37 °C in minimum essential medium containing Earle's salts (Sigma, St. Louis, USA) supplemented with 10% foetal bovine serum (FBS) (PAA Laboratories GmbH, Pasching, Austria), L-glutamine (2mM) (Sigma), penicillin (200 IU/mL) and streptomycin (200 µg/mL) (Sigma) in a humidified atmosphere of 5% CO₂ in air.

To obtain the free parasitic forms of *N. caninum*, infected VERO cells were cultured until the host cell monolayer was 70% destroyed. Culture supernatants and adherent cells harvested using a cell-scraper were centrifuged at 1500 *g* for 20 min. The pellet was passed through a 25G needle and then washed with phosphate-buffered saline (PBS) by three serial centrifugations at 1500 *g* for 20 min each. The pellet thus obtained was resuspended in 3 mL of PBS, passed through a 25G needle and then through a PD-10 column filled with Sephadex™ G-25M (Amersham Biosciences Europe GmbH, Freiburg, Germany). Parasite concentration was determined with a haemocytometer.

3.4 Immunisations

Mice were immunised i.n., at day 0 and 21 (twice at 3-week intervals), with 20 µL of the preparation containing 30 µg of NcT membrane proteins and 10 µg of CpG (Invitrogen, Carlsbad, CA, USA) in PBS (*Protein + CpG* group). Control animals received 20 µL of PBS alone (*PBS* group), 20 µL of PBS with 10 µg of CpG (*CpG* group) or 20 µL of PBS with 30 µg of NcT membrane proteins (*Protein* group) (Table 1). Mice were briefly anaesthetized by isoflurane (IsoFlo®, Veterinaria Esteve, Bologna, Italy) inhalation before the immunisations.

3.5 Challenge infections

At day 42, *N. caninum* infection was performed in all mice by the i.g. route. Briefly, 4 hr before infection, mice were deprived of food. At the time of infection, animals were anaesthetized by intramuscular injection of 20 µL of a 4:5 mixture containing xylazine (Rompum®, Bayer Portugal, S.A., Carnaxide) and ketamine (Imalgene® 1000, Merial, Rio de Mouro, Portugal). In order to neutralize stomach acidity, 50 µL of a 10% sodium hydrogenocarbonate solution in water were then directly administered into the stomach, through a gavage probe. Ten minutes later, the same procedure was followed to inoculate 200 µL PBS containing 5×10^7 NcT.

3.6 Extraction of membrane proteins of NcT

NcT membrane proteins were obtained by using a protocol previously described by Zintl *et al.*¹⁶⁷. Namely, parasites were resuspended in PBS containing 0.75% Triton-X114 ($5 \times 10^7 - 5 \times 10^8$ NcT per 3 mL of solution), gently vortexed and incubated on ice for 10 min. The preparation was centrifuged at 10000 *g* for 30 min (4 °C) to remove cytoskeletal proteins and the supernatant was incubated at 30 °C for 3 min, cooled on ice for 10 min and then centrifuged at 10000 *g* for 30 min (4 °C) to remove possible aggregates. The resulting supernatant was incubated at 30 °C for 3 min and then centrifuged at 1000 *g* for 4 min (room temperature). The upper hydrophilic phase was discarded and absolute ethanol was added to the lower detergent phase (volume of ethanol equivalent to four times the volume of the detergent phase). This final fraction was gently vortexed and then incubated on ice for 1 hr with vortex-mixing each 15 min. Following incubation, a centrifugation step was performed at 12000 *g* for 20 min (4 °C) and the pellet was resuspended in PBS. Protein concentration was determined by the method of Lowry and using the spectrophotometer 6300 (Jenway, Essex, UK).

3.7 Collection of biological samples

At day 14 and 28, vaginal lavages were performed with three times 50 μ L PBS. Samples of vaginal lavage fluids were then centrifuged at 3500 g for 8 min (4 $^{\circ}$ C) and stored at -20 $^{\circ}$ C for NcT-specific IgA measurement.

At day 28, blood was collected from the submandibular vein and allowed to clot overnight. Serum was obtained by centrifugation at 16000 g for 15 min (4 $^{\circ}$ C) and was then stored at -20 $^{\circ}$ C for NcT-specific IgG1 and IgG2a measurement.

At day 49, mice were briefly anaesthetized by isoflurane inhalation and blood was collected by retro-orbital bleeding for NcT-specific IgG1 and IgG2a measurement. The animals were then euthanized by cervical dislocation and brain, liver, pancreas, stomach, heart, lungs and kidneys were collected and sectioned longitudinally. Large intestine was also collected and the colon and cecum were transversally divided in two parts each. Small intestine was used to collect intestinal secretions for NcT-specific IgA measurement and it was then transversally cut in three parts. Half of each organ/part was preserved in 10% buffered formalin for histopathological and immunohistochemical analysis and the other half was stored at -80 $^{\circ}$ C for DNA extraction. To collect intestinal secretions, small intestines were washed with 2 mL of a solution of protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Mannheim, Germany) in PBS. The intestinal lavage fluids were centrifuged, first at 4000 g for 15 min (4 $^{\circ}$ C) and then at 8500 g for 60 min (4 $^{\circ}$ C), and were then stored at -20 $^{\circ}$ C. Spleen and MLNs were also removed and placed in Hank's Buffered Salt Solution (HBSS, Gibco, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 2% FBS (PAA Laboratories GmbH) and penicillin (100 IU/mL) and streptomycin (100 μ g/mL) (Sigma) for *in vitro* cell culture.

3.8 *In vitro* cell culture

Spleen and MLN cells were obtained by gently teasing the organs in HBSS (Gibco) supplemented with 2% FBS (PAA Laboratories GmbH) and penicillin (100 IU/mL) and streptomycin (100 μ g/mL) (Sigma). Cell suspensions were centrifuged at 300 g for 10 min (4 $^{\circ}$ C) and the pellets of MLN cells were resuspended in RPMI-1640 medium (Sigma) supplemented with 10% FBS (PAA Laboratories GmbH), HEPES (10 mM) (Sigma), β -mercaptoethanol (7 mM) (Merck, Darmstadt, Germany) and penicillin (100 IU/mL) and streptomycin (100 μ g/mL) (Sigma). The pellets of spleen cells were resuspended in ACK lysing solution, consisting of NH_4Cl (150 mM), KHCO_3 (10 mM) and ethylenediamine tetraacetic acid (EDTA, 0.1 mM) in water, and incubated for 4 min at room temperature. Spleen cell suspensions were then centrifuged at 300 g for 10 min and the pellets

were resuspended in the RPMI medium just described above. Cells were distributed in 96-well plates (1×10^6 cells/well) and cultured for 5 hr at 37 °C in a humidified atmosphere of 5% CO₂ in air with 10 µg/mL of Brefeldin A (Epicentre Biotechnologies, Madison, WI, USA) and with the 1X phorbol myristate acetate (PMA) (Sigma) and ionomycin (Merck) preparation, for cytokine intracellular staining and flow cytometric analysis.

3.9 Cytokine intracellular staining

Spleen and MLN cells, incubated at 37 °C in RPMI-1640 medium (Sigma) supplemented with 10% FBS (PAA Laboratories GmbH), HEPES (10 mM) (Sigma), penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Sigma) and 7 mM β-mercaptoethanol (Merck) with 10 µg/mL of Brefeldin A (Epicentre Biotechnologies) and the 1X PMA (Sigma) and ionomycin (Merck) preparation, were subsequently pre-incubated with anti-FcγR mAb and then surface stained with phycoerythrin-cyochrome 5 (PE-Cy5) anti-mouse CD4 (clone RM4-5) or peridinin-chlorophyll-protein-Cy5.5 (PerCP-Cy5.5) anti-mouse CD8 (clone 53-6.7) (both from BD Biosciences Pharmingen, San Diego, CA, USA). Cells were then fixed with 2% formaldehyde, washed, permeabilized with 0.5% saponin (Sigma) and pre-incubated with anti-FcγR mAb before intracellular staining with the following antibodies: fluorescein isothiocyanate (FITC) anti-mouse IFN-γ (clone XMG1.2) (BD Biosciences) or the FITC isotype-matched control (FITC Rat IgG1, κ, clone R3-34) (BD Biosciences), PE anti-mouse IL-10 (clone JES5-16E3) or PE anti-mouse IL-4 (clone BVD4-1D11) (BD Biosciences).

3.10 Antibody measurement

Antibodies specific for NcT in vaginal and intestinal lavage fluids as well as in sera were quantified by ELISA. Briefly, polystyrene microtitre plates (Nunc, Roskilde, Denmark) were coated with 5 µg/mL of NcT membrane proteins and incubated overnight at 4 °C. Wells were then saturated with TST buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 20) with 2% bovine serum albumin (BSA), for 1 hr at room temperature. Serial dilutions of the samples in TST buffer with 1% BSA were subsequently plated and incubated for 1 hr at room temperature. After washing, bound antibodies were detected by addition of alkaline phosphatase (AP)-coupled monoclonal goat anti-mouse-IgA, diluted 1:500 in TST buffer with 1% BSA, goat anti-mouse IgG1 or goat anti-mouse IgG2a, both diluted 1:1000 in TST buffer with 1% BSA, for 1 hr at room temperature (all antibodies from Southern Biotechnology Associates, Birmingham, AL, USA). Substrate solution containing *p*-nitrophenyl phosphate (Sigma) diluted in AP buffer (1 mM MgCl₂, 50 mM NaCO₃ [pH 9.8]) was added after washing and the reaction was stopped by addition of 0.1 M EDTA pH 8.0. Absorbance

values were measured at 405 and 570 nm using the plate reader Multiskan EX (Thermo Electron Corporation, Vantaa, Finland) and the Ascent software version 2.6 (Thermo Electron Corporation). To determine the antibody titres, the absorbance values at 570 nm were first subtracted to the absorbance values measured at 405 nm in order to compensate for imperfections of the plate, as described by the manufacturer. The ELISA antibody titres were expressed as the reciprocal of the highest dilution that gives an absorbance equal to that of the control (no sample added).

3.11 DNA extraction

DNA from the brain and liver was extracted by digesting each organ overnight at 55 °C in SE buffer (75 mM NaCl, 25 mM EDTA, pH 8.0) with 1% sodium dodecyl sulfate (SDS) solution and 0.5 mg/mL proteinase K (Sigma). DNA was then purified by the phenol (Sigma)-chloroform (Merck) method and ethanol precipitated. DNA concentration was determined using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the NanoDrop ND-1000 software version 3.6.0 (Thermo Scientific) and was adjusted to 200 ng/μL for real-time quantitative polymerase chain reaction (qPCR) analysis.

3.12 qPCR

N. caninum DNA amplification was performed with 1 μL of template DNA in a final volume of 10 μL containing 1X EXPRESS SYBR® GreenER™ qPCR Supermix (Invitrogen) and 0.5 μM of each primer (forward primer Np21plus: 5' – CCC AGT GCG TCC AAT CCT GTA AC, reverse primer Np6 plus: 5' – CTC GCC AGT CAA CCT ACG TCT TCT) (TIB-Molbiol, Berlin, Germany). The PCR programme was run as follows: (1) denaturation at 95 °C, 10 min; (2) amplification in 45 cycles (including denaturation [95 °C, 30s], annealing [63 °C, 15s] and extension [72 °C, 45s]); (3) cooling at 4 °C. A melting temperature curve was made from 65 °C to 95 °C with increments of 0.02 °C/s to confirm the specificity of the qPCR product through the presence of a single melting-temperature peak. Amplifications were made in a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) and the quantitative evaluation of fluorescent signals from the PCR products was performed using the Rotor-Gene 6000 software version 1.7 (Corbett Life Science). Standards consisting of known amounts of *N. caninum* DNA, from 10⁻³ ng to 10 ng, were included in each run and a standard curve was plotted with the absorbance values thus obtained. Quantification of the fluorescent signals from the samples was performed by interpolation of the standard curve. In each run, DNA of uninfected organs and NcT DNA were used, respectively, as negative and positive controls.

3.13 SDS-PAGE and Western blot

NcT membrane proteins were denaturated by boiling for 5 min in loading buffer before electrophoresis, which was then carried out at 45 mA constant current on a 4% polyacrylamide stacking gel (pH 6.8) followed by a 10% polyacrylamide resolving gel (pH 8.8). Gels were either stained by the silver nitrate staining protocol or electrophoretically transferred overnight at 60 mA constant current to a nitrocellulose membrane (Sigma) for Western blot. After transferring, the membrane was saturated with TST buffer with 1% BSA for 1 hr at room temperature and blotted proteins were subsequently incubated with vaginal lavage fluids or sera, diluted 1:500 and 1:1000 in TST buffer with 0.1% BSA, respectively, for 2 hr at room temperature with shaking. After washing, AP-coupled monoclonal goat anti-mouse-IgA, diluted 1:500 in TST buffer with 0.1 % BSA, goat anti-mouse IgG1 or goat anti-mouse IgG2a, both diluted 1:1000 in TST buffer with 0.1 % BSA (all antibodies from Southern Biotechnology Associates), were added and the membrane was incubated for 1 hr at room temperature with shaking. Bound secondary antibodies were detected by adding the substrate solution containing nitro blue tetrazolium / 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Roche) diluted in AP buffer (5 mM MgCl₂, 0.1 M NaCl, 0.1 M Tris [pH 9.5]) and the reaction was stopped by transferring the membrane to water.

3.14 Antibody binding assay to NcT

NcT were incubated at 37 °C for 30 min with mice serum (containing the antibodies), previously decomplexed at 56 °C for 30 min and diluted 1:10, 1:100, 1:500 and 1:1000 in fluorescent-activated cell sorter (FACS) buffer (PBS with 1% BSA and 10 mM NaN₃) (5×10^5 NcT per antibody dilution). After a centrifugation step at 6000 *g* for 15 min (4 °C), parasite suspensions were incubated on ice for 30 min with FITC anti-mouse IgG (BD Biosciences), diluted 1:100 in FACS buffer, in order to detect parasite-bound serum IgG. Unbound secondary antibody was then removed by centrifuging the preparations at 6000 *g* for 15 min (4 °C).

3.15 Flow cytometric analysis

All immunofluorescence cytometric analyses were performed on an EPICS XL Flow Cytometer (Beckman Coulter, Miami, FL, USA) using the CellQuest Software (Becton Dickinson, San Jose, CA, USA).

3.16 Statistical analysis

Statistical difference of the results between the pairs of groups to be compared, i.e., the *PBS* with the *CpG* group and the *Protein* with *Protein + CpG* group, was determined by Student *t*-test, using the GraphPad Software, Inc. version 5.00. $p < 0.05$ was considered to be statistically significant. In the following bar charts, the bars represent the mean plus 1 SEM of the data obtained for each group. Groups contained a variable number of mice (see 3.1 *Experiment set-up*). The absence of SEM in some bars in the following charts means that the parameter in study was detected in only one mouse among all that constitute that group. When this happens in the *Protein* group, no statistical analysis could be made in relation to the *Protein + CpG* group, yet the difference in the parameter in study was considered significant.

4. Results

4.1 Extraction of NcT membrane proteins

NcT membrane proteins were extracted using the non-ionic detergent Triton X-114 in a protocol previously described by Zintl *et al.*¹⁶⁷. Every time proteins were extracted, the quality of the procedure was assessed by running a SDS-PAGE and comparing the migration pattern of the bands with the one shown by Zintl *et al.*¹⁶⁷. Figure 4A depicts the pattern commonly obtained and Figure 4B the one used for comparison.

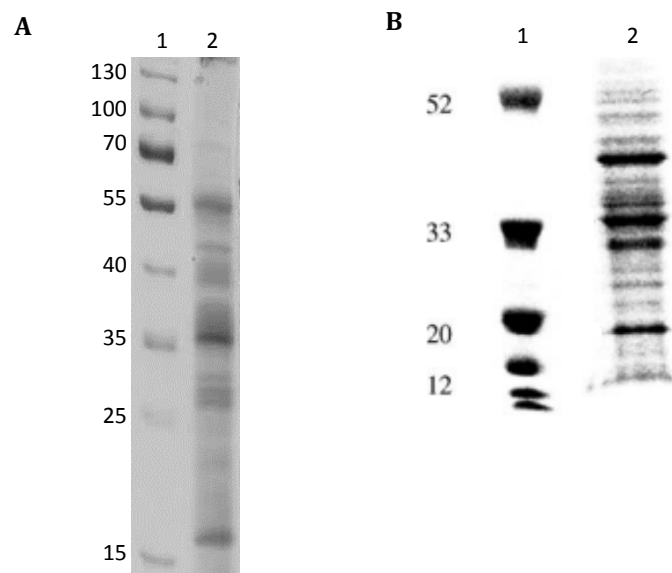


Figure 4. SDS-PAGE gels of NcT membrane protein extracts obtained by the Triton X-114 extraction procedure. Silver nitrate stained 4-10% SDS-PAGE gel commonly obtained (A). Coomassie Brilliant Blue stained 4-12% SDS-PAGE gel obtained by Zintl *et al.*¹⁶⁷ (B). Lane 1, molecular weight marker; lane 2, protein extract. Numbers on the left of each gel correspond to molecular weights, in kDa.

The extracts comprised proteins of approximately 47, 35, 27 and 17 kDa (Figure 4A) that were similar in size to the four main proteins obtained by Zintl *et al.*¹⁶⁷, specifically of 44, 34, 29.5 and 17 kDa (Figure 4B), which suggests that the protocol employed to extract membrane proteins of NcT has been successfully performed. In addition, proteins of 55 and 39 kDa were extracted by that protocol, yet they have not been obtained by Zintl *et al.*¹⁶⁷ (Figure 4).

4.2 Antibody production in mice upon CpG-adjuvanted immunisation with NcT membrane proteins and following NcT infection

Since the GI tract is one of the major entry routes of *N. caninum* in the host, eliciting a specific immune response against the parasite specifically in the intestinal mucosa can be of great advantage to counteract *N. caninum* infection by the horizontal route. Yet the concept of CMIS has been renamed to *compartmentalised mucosal immune system*¹⁰⁰, it still supports the idea that immunisation at one mucosal site can provide protection at a distant mucosal site⁹⁹. Recently, Ciabattini *et al.*¹⁶⁸ have shown that following i.n. CpG-adjuvanted immunisation with OVA, T cells are primed in the draining LNs and then disseminate towards non-draining LNs, including the MLNs, and the spleen. Therefore, immunisation of mice was performed i.n. at day 0 and 21 (3 weeks apart). The boost immunisation aimed at augmenting the immune response induced by the first immunisation as well as at extending it to a systemic level.

4.2.1 NcT-specific IgA production

While the experiments were in progress, the induction of a *N. caninum*-specific immune response in the mucosa was evaluated by ELISA quantification of NcT-specific IgA in the vaginal lavage fluids, for those being the most closely associated mucosal fluids in relation to the intestinal mucosa that can be non-invasively sampled. Vaginal lavage fluids were collected either after the prime immunisation, at day 14 (Figure 5), or after the boost immunisation, at day 28 (Figure 6).

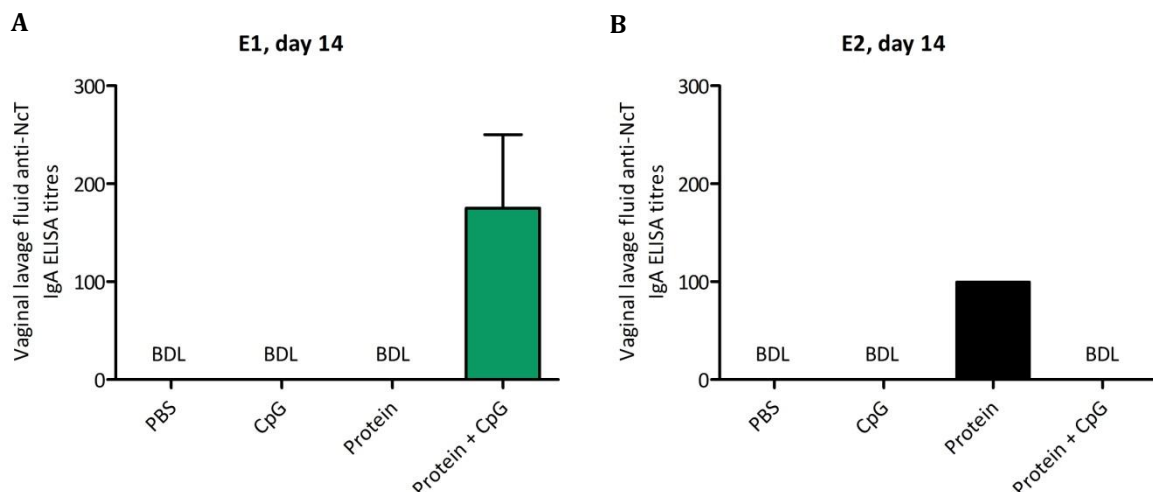


Figure 5. Anti-NcT IgA ELISA titres in mice vaginal lavage fluids after the prime immunisation. ELISA titres of NcT-specific IgA in mice vaginal lavage fluids at day 14 of E1 (A) or E2 (B), i.e., 14 days after the prime immunisation. Mice were immunised i.n. with 20 μ L of: only PBS (*PBS* group), PBS with 10 μ g of CpG (*CpG* group), PBS with 30 μ g of NcT membrane proteins (*Protein* group) and PBS with 30 μ g of NcT membrane proteins and 10 μ g of CpG (*Protein + CpG* group). BDL, below detection limit; E1, first experiment; E2, second experiment; i.n., intra-nasal, NcT, *N. caninum* tachyzoites.

After the prime immunisation, NcT-specific IgA was detected in the vaginal lavage fluids of mice from the *Protein + CpG* group in E1 (Figure 5A) and of just one mouse of the *Protein* group in E2 (Figure 5B). Although performed in exactly the same conditions, the experiments did not lead to similar data, being therefore inconclusive the results regarding the IgA titres in these groups and at this time point. However, both experiments were in agreement for the *PBS* and *CpG* groups, where, as expected, no mice produced NcT-specific IgA (Figure 5).

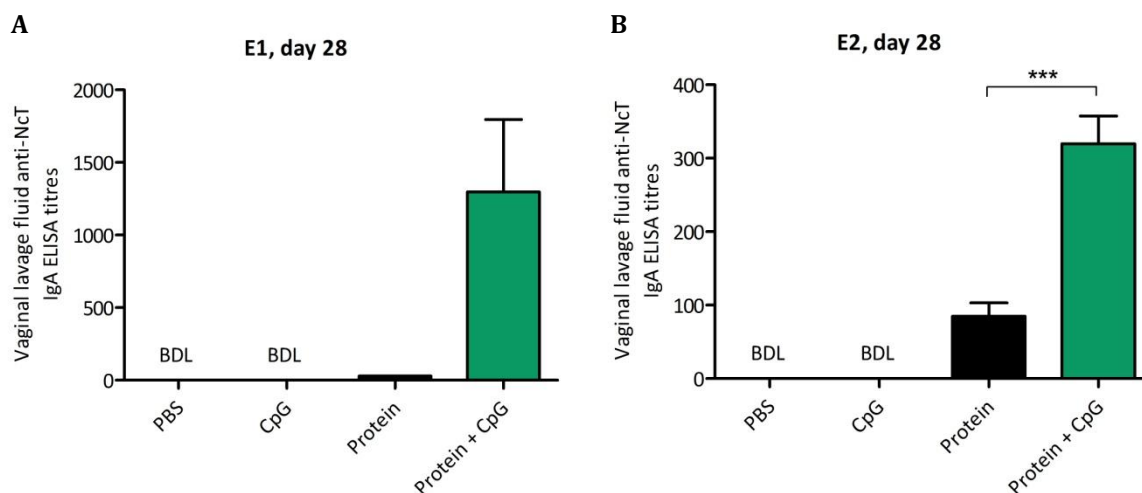


Figure 6. Anti-NcT IgA ELISA titres in mice vaginal lavage fluids after the boost immunisation. ELISA titres of NcT-specific IgA in mice vaginal lavage fluids at day 28 of E1 (A) or E2 (B), i.e., 7 days after the boost immunisation. Mice were boosted i.n. with 20 μ L of: only PBS (*PBS* group), PBS with 10 μ g of CpG (*CpG* group), PBS with 30 μ g of NcT membrane proteins (*Protein* group) and PBS with 30 μ g of NcT membrane proteins and 10 μ g of CpG (*Protein + CpG* group). BDL, below detection limit; E1, first experiment; E2, second experiment; i.n., intra-nasal, NcT, *N. caninum* tachyzoites (***) $p < 0.001$.

At day 28 and in E1, anti-NcT IgA was mostly detected in the vaginal lavage fluids of mice of the *Protein + CpG* group, with higher levels when compared with the only one detected in the *Protein* group (Figure 6A). In E2, mice of both the *Protein* and *Protein + CpG* groups produced NcT-specific IgA, with significantly higher titres in the *Protein + CpG* group (Figure 6B). As expected again, neither in E1 nor in E2 did mice of the *PBS* or *CpG* groups produce NcT-specific IgA (Figure 6). Altogether, these results suggest that the immunisations with NcT membrane proteins and CpG as adjuvant were able to induce a specific immune response against *N. caninum* at the mucosal level.

By looking at the levels of anti-NcT IgA in E1, it is noteworthy that they are much higher at day 28 (Figure 6A) than at day 14 (Figure 5A), which suggests that, as expected, the boost strengthened the mucosal immune response elicited by the prime immunisation.

In order to directly evaluate the anti-NcT IgA titres in the intestinal mucosa, IgA was quantified in the intestinal secretions obtained from mice at the day of euthanasia, i.e., at day 49 (Figure 7).

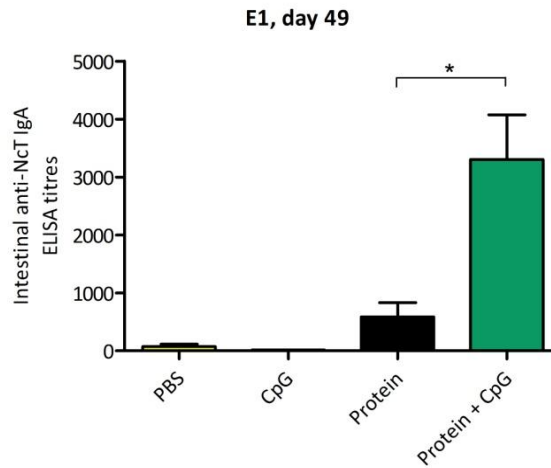


Figure 7. Anti-NcT IgA ELISA titres in mice intestinal lavage fluids after the immunisations and the NcT challenge infection. ELISA titres of NcT-specific IgA in mice intestinal lavage fluids at day 49 of E1, i.e., 7 days after the NcT infection. Mice were previously immunised i.n. twice and 3 weeks apart with 20 μ L of: only PBS (*PBS* group), PBS with 10 μ g of CpG (*CpG* group), PBS with 30 μ g of NcT membrane proteins (*Protein* group) and PBS with 30 μ g of NcT membrane proteins and 10 μ g of CpG (*Protein + CpG* group). At day 42, all mice were i.g. infected with 200 μ L PBS containing 5×10^7 NcT. BDL, below detection limit; E1, first experiment; i.g., intragastric; i.n., intra-nasal, NcT, *N. caninum* tachyzoites (* $p < 0.05$).

At the day of euthanasia, i.e., after both i.n. immunisations and NcT infection, anti-NcT IgA was detected in the intestinal lavage fluids of mice of all groups, being however the levels significantly higher in the *Protein + CpG* group, when compared with the *Protein* group (Figure 7). This result indicates that the protocol of immunisation under study was effective at inducing a *N. caninum*-specific immune response in the intestinal mucosa of mice.

4.2.2 NcT-specific IgG1 and IgG2a production

As mentioned before, the boost was performed with the aim of reinforcing the immune response promoted by the prime immunisation, but also of extending the *N. caninum*-specific immune response to a systemic level. In order to evaluate the ability of the i.n. boost to promote a systemic immune response, IgG1 and IgG2a levels were measured by ELISA in mice serum collected at day 28 (Figure 8). This measurement would also allow, through the IgG1/IgG2a ratios, to characterize the immune response induced by the protocol of immunisation in study, namely T_H1 -vs. T_H2 immunity, and consequently, to infer about the protective effect of that strategy against neosporosis.

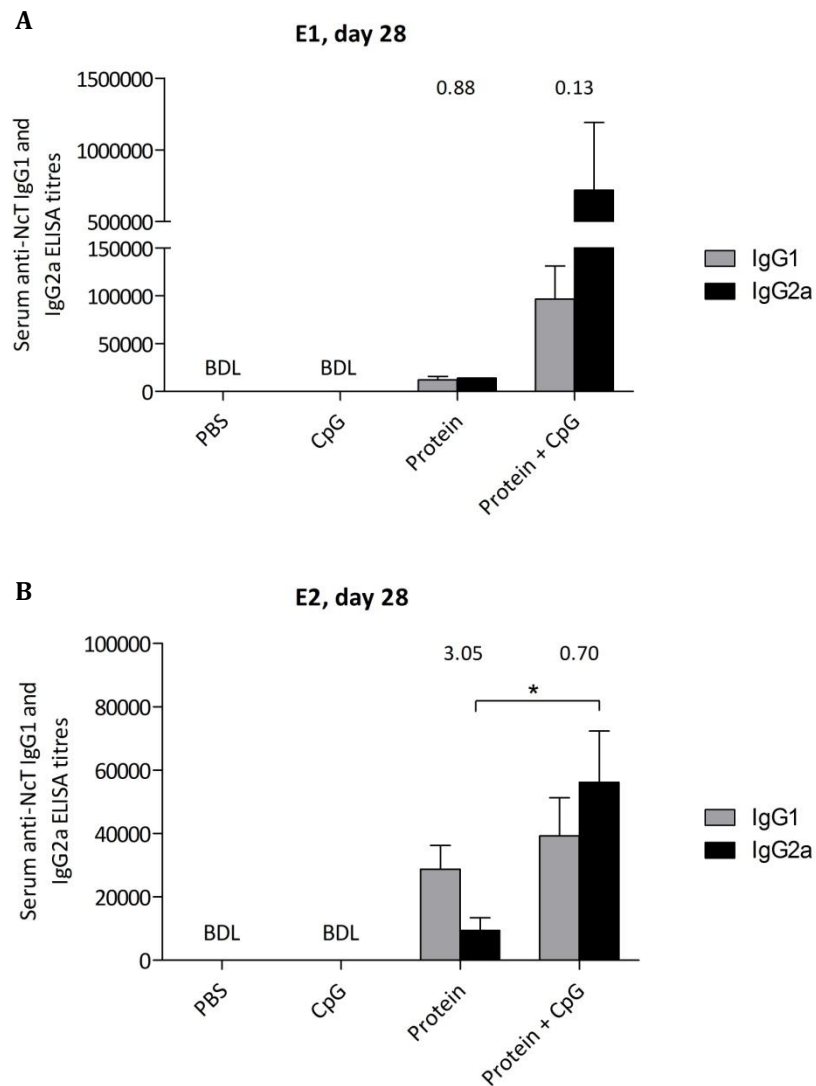


Figure 8. Anti-NcT IgG1 and IgG2a ELISA titres in mice serum after the boost immunisation. ELISA titres of NcT-specific IgG1 and IgG2a in mice serum at day 28 of E1 (A) or E2 (B), i.e., 7 days after the boost immunisation. Mice were boosted i.n. with 20 μ L of: only PBS (*PBS* group), PBS with 10 μ g of CpG (*CpG* group), PBS with 30 μ g of NcT membrane proteins (*Protein* group) and PBS with 30 μ g of NcT membrane proteins and 10 μ g of CpG (*Protein + CpG* group). Numbers above the bars correspond to the IgG1/IgG2a ratios for each group. BDL, below detection limit; E1, first experiment; E2, second experiment; i.g., intragastric; i.n., intra-nasal, NcT, *N. caninum* tachyzoites (* $p < 0.05$).

At day 28, IgG1 and IgG2a were detected in the serum of mice of both the *Protein* and *Protein + CpG* groups (Figure 8) which demonstrates that the boost immunisation induced a systemic immune response against *N. caninum*. No significant differences in the levels of serum NcT-specific IgG1 were detected between the *Protein* and *Protein + CpG* group in any of the experiments (Figure 8). However, IgG2a was mostly produced and in higher amounts by mice of the *Protein + CpG* group in E1 (Figure 8A) and its levels were significantly higher in mice of the *Protein + CpG* group in E2 (Figure 8B). As production of IgG1 and IgG2a is induced, respectively, by T_H2 -type and T_H1 -type cytokines, this suggests that in mice of the *Protein + CpG* group a bias towards the

production of T_H1-type cytokines was induced, in contrast to what happened in mice of the *Protein* group. When it comes to the IgG1/IgG2a ratios, although they were below 1.00 for both the *Protein* and *Protein + CpG* group in E1, a lower value was obtained for the *Protein + CpG* group (Figure 8A), which suggests that the immune response induced by immunisation was more biased to the T_H1-type in this group. In E2, the ratios above and below 1.00 in the *Protein* and *Protein + CpG* groups (Figure 8B) clearly demonstrate a predominant T_H2 and T_H1 immune response, respectively.

IgG1 and IgG2a levels were also measured in the serum collected at the day of euthanasia, i.e., at day 49 (Figure 9).

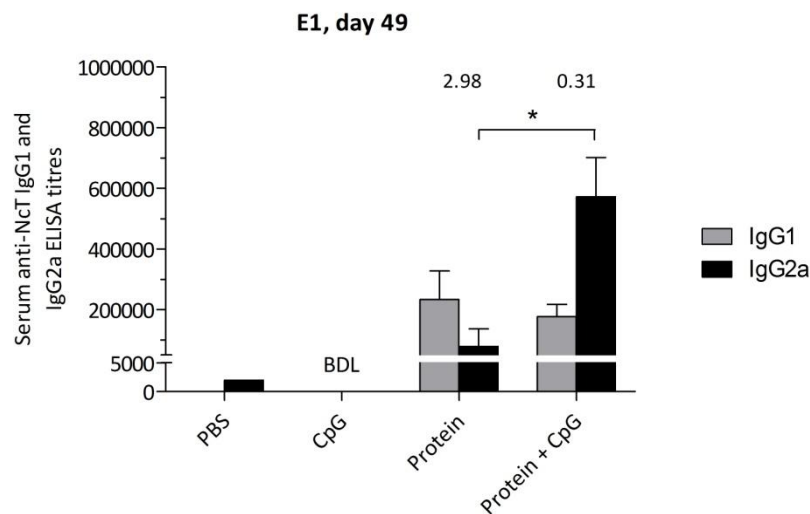


Figure 9. Anti-NcT IgG1 and IgG2a ELISA titres in mice serum after the immunisations and the NcT challenge infection. ELISA titres of NcT-specific IgG1 and IgG2a in mice serum at day 49 of E1, i.e., 7 days after the NcT infection. Mice were previously immunised i.n. twice and 3 weeks apart with 20 µL of: only PBS (*PBS* group), PBS with 10 µg of CpG (*CpG* group), PBS with 30 µg of NcT membrane proteins (*Protein* group) and PBS with 30 µg of NcT membrane proteins and 10 µg of CpG (*Protein + CpG* group). At day 42, all mice were i.g. infected with 200 µL PBS containing 5×10^7 NcT. Numbers above the bars correspond to the IgG1/IgG2a ratios for each group. BDL, below detection limit; E1, first experiment; i.g., intragastric; i.n., intra-nasal, NcT, *N. caninum* tachyzoites (* $p < 0.05$).

Similarly to the results obtained after the boost, following both the immunisations and the NcT challenge infection no differences in the IgG1 levels were detected between the *Protein* and the *Protein + CpG* group, whereas the IgG2a levels were significantly higher in mice of the *Protein + CpG* group when compared with the *Protein* group (Figure 9). The IgG1/IgG2a ratios were well above and below 1.00, in the *Protein* and *Protein + CpG* group (Figure 9), suggesting again that the immune response is predominantly T_H2 and T_H1, respectively.

In summary, results in Figure 8 and Figure 9 suggest that the immunisation strategy in study, consisting on the administration of NcT membrane proteins together with CpG, was able to induce a systemic *N. caninum*-specific immune response of the T_H1 type, which is known to be protective against neosporosis.

4.3 Cytokine production by MLN and spleen cells following CpG-adjuvanted prime-boost immunisation with NcT membrane proteins and NcT infection

In order to further characterize the immune response induced in mice after the prime-boost CpG-adjuvanted i.n. immunisation strategy with NcT membrane proteins and to infer about its protective effect against neosporosis, cells from MLNs and spleen, collected at day 49, were intracellularly stained and the number of IFN- γ producing CD8⁺ cells, as well as of IFN- γ -, IL-10- and IL-4- producing CD4⁺ cells was evaluated by flow cytometry (Figure 10).

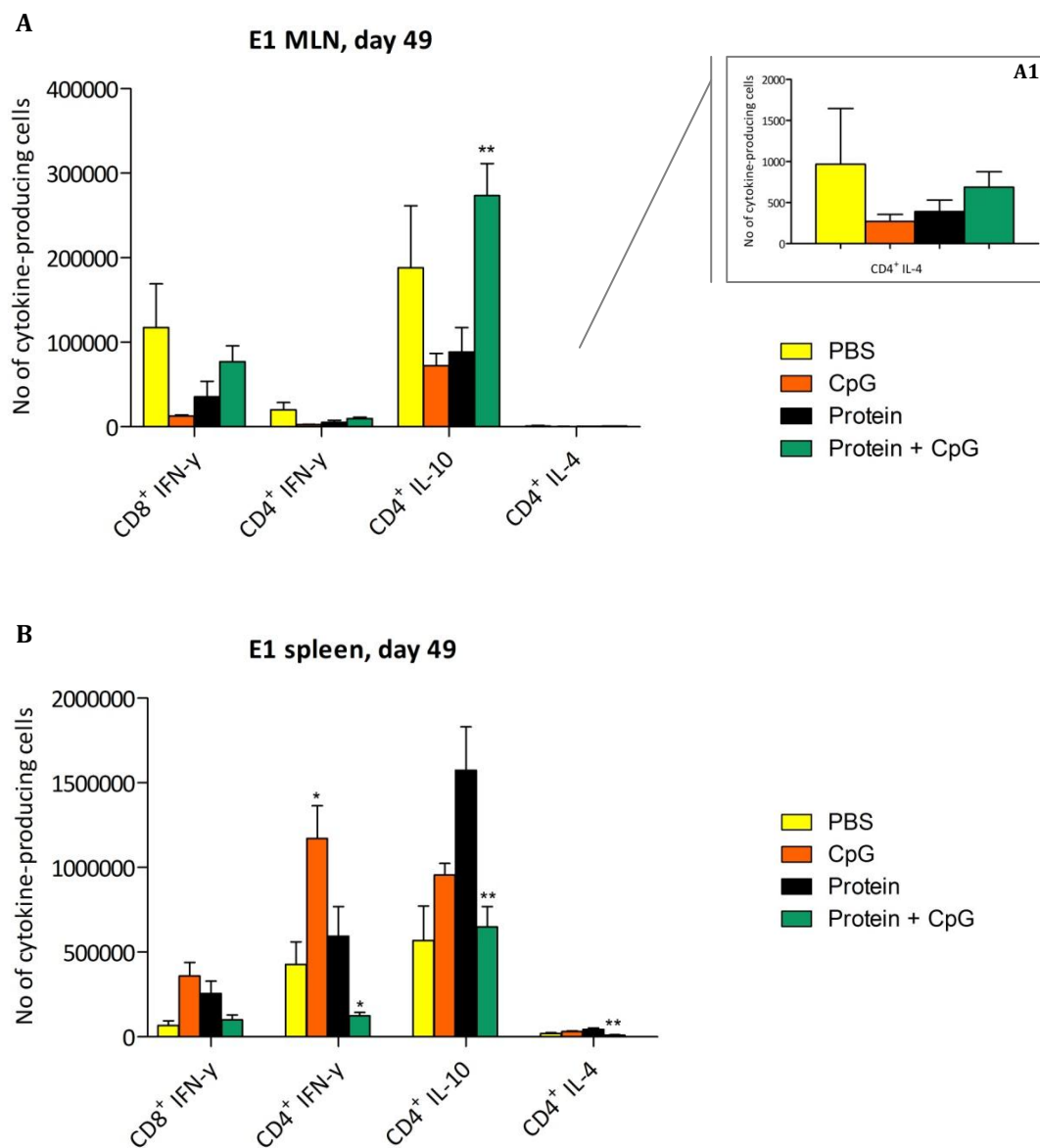


Figure 10. Cytokine production by CD4⁺ and CD8⁺ cells from MLNs and spleen after the immunisations and the NcT challenge infection. MLN (A) and spleen (B) cells were collected at day 49 of E1, i.e., 7 days after the NcT infection and the production of IFN- γ by CD4⁺ cells and IFN- γ , IL-10 and IL-4 by CD8⁺ cells was assessed by flow cytometry. The graph representing the number of MLN CD4⁺ IL-4-producing cells is amplified in (A1). Mice were previously immunised i.n. twice

and 3 weeks apart with 20 μL of: only PBS (*PBS* group), PBS with 10 μg of CpG (*CpG* group), PBS with 30 μg of NcT membrane proteins (*Protein* group) and PBS with 30 μg of NcT membrane proteins and 10 μg of CpG (*Protein + CpG* group). At day 42, all mice were i.g. infected with 200 μL PBS containing 5×10^7 NcT. BDL, below detection limit; E1, first experiment; i.g., intragastric; i.n., intra-nasal, NcT, *N. caninum* tachyzoites. Statistical significance between the compared groups, i.e., *PBS* with *CpG* group and *Protein* with *Protein + CpG* group, * $p < 0.05$; ** $p < 0.01$).

Significant differences were detected only in the number of MLN CD4^+ cells producing IL-10, which was higher in mice of the *Protein + CpG* group when compared with that of the *Protein* group (Figure 10A), and in the number of spleen CD4^+ cells producing the same cytokine, as well as IFN- γ and IL-4, which was lower in the *Protein + CpG* group than in the *Protein* group (Figure 10B). No significant differences were detected between the compared groups in the number of IFN- γ -producing CD8^+ cells, from either the MLNs or the spleen (Figure 10). It is also noteworthy that, independently of the group and the organ, the number of CD4^+ cells producing IL-4 was the lowest when compared with the other cells, either CD4^+ or CD8^+ , producing the other cytokines in study (Figure 10).

4.4 Parasite load in liver and brain of mice following prime-boost CpG- adjuvanted immunisation with NcT membrane proteins and NcT infection

In order to evaluate whether or to what extent the strategy of immunisation under study protected mice against neosporosis, the parasite load was evaluated in the mice liver and brain after both i.n. CpG-adjuvanted immunisations with NcT membrane proteins and i.g. infection with NcT. This was made by qPCR measurement of *N. caninum*-specific DNA in those organs, collected at day 49 (Figure 11).

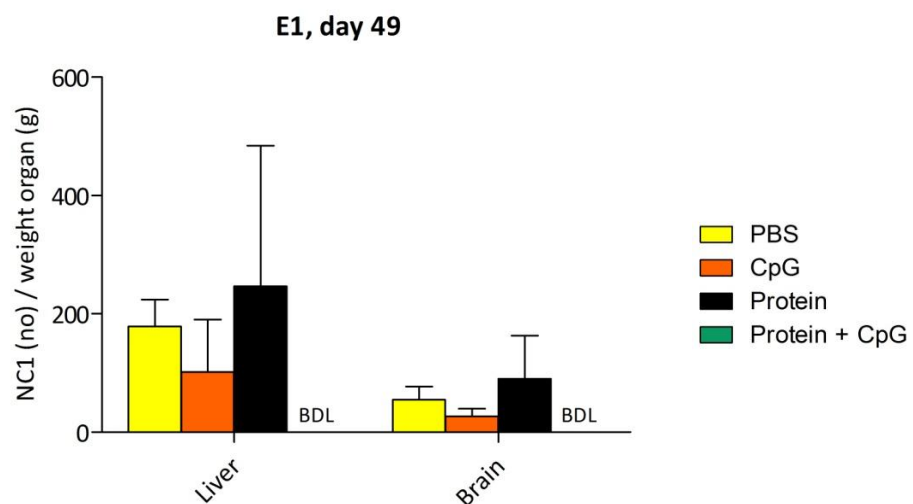


Figure 11. *N. caninum* load in liver and brain of mice after the immunisations and the NcT challenge infection. Number of parasites per gram of liver or brain at day 49 of E1, i.e., 7 days after the NcT infection. Mice were immunised i.n. twice

and 3 weeks apart with 20 μL of: only PBS (*PBS* group), PBS with 10 μg of CpG (*CpG* group), PBS with 30 μg of NcT membrane proteins (*Protein* group) and PBS with 30 μg of NcT membrane proteins and 10 μg of CpG (*Protein + CpG* group). At day 42, all mice were i.g. infected with 200 μL PBS containing 5×10^7 NcT. BDL, below detection limit; E1, first experiment; i.g., intragastric; i.n., intra-nasal, NcT, *N. caninum* tachyzoites.

Seven days after the NcT challenge infection, the parasite was detected in the liver and brain of mice of all groups except for the *Protein + CpG* group (Figure 11). These results indicate that the immunisation strategy assessed in this study effectively protected mice from parasite colonization in those organs. Taking into account the number of parasites in the control groups, one can also infer that brains presented lower parasite loads than livers (Figure 11).

4.5 Serum IgG binding to the surface of NcT

IgG antibodies present in the serum collected at day 49 from mice of the *Protein + CpG* group of E1, that have proven to be specific to NcT membrane proteins, as assessed by ELISA, were investigated *in vitro* for the ability to bind the NcT surface. NcT were incubated with that serum, from now on named *immune serum*, followed by the FITC-conjugated anti-IgG secondary antibody, and the mean fluorescence intensity (MFI) of the labeled parasites was then determined by flow cytometry. Figure 12A depicts the FITC MFI values obtained using the different serum dilutions and Figure 12B is a representative example of the flow cytometry histograms thus obtained.

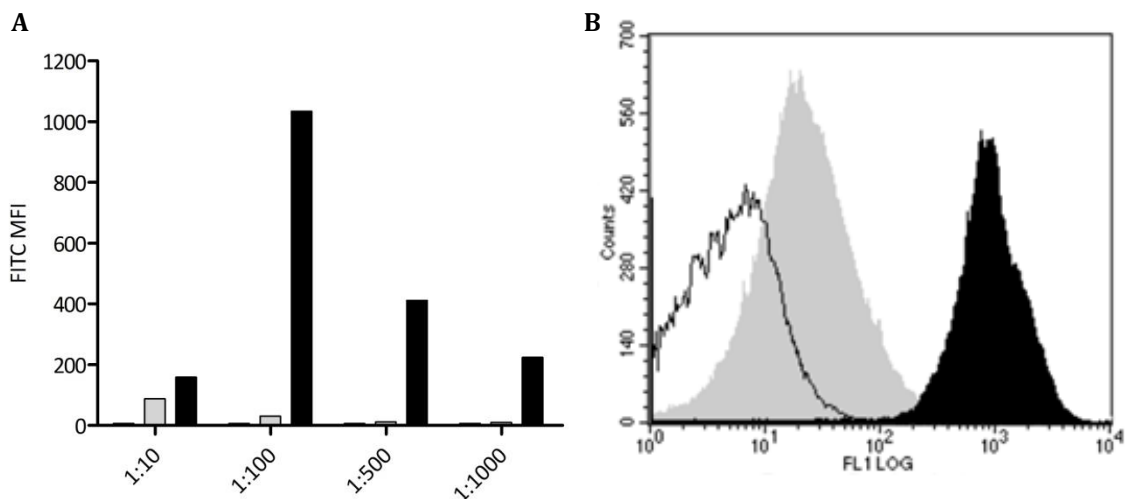


Figure 12. *In vitro* binding of mice serum IgGs to the NcT surface. NcT were incubated with serum for 30 min at 37 °C. Sera in which IgGs were demonstrated by ELISA to bind or not to bind NcT membrane proteins were named, respectively, *immune serum* and *non-immune serum* and were used at the following dilutions: 1:10, 1:100, 1:500 and 1:1000. The immune and non-immune serum came, respectively, from mice of the *Protein + CpG* group and from mice of the *PBS* and *CpG* group, all from E1. After a further incubation step with the FITC-conjugated anti-IgG secondary antibody, MFI was measured by flow cytometry. Open bars, NcT only; grey solid bars, NcT incubated with non-immune serum; black solid bars, NcT incubated with immune serum (A). Flow cytometry histogram with serum diluted 1:500. Open histogram, NcT only; grey solid histogram, NcT incubated with non-immune serum; black solid histogram, NcT with immune serum (B).

Data shown are representative results of three independent experiments. FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; NcT, *N. caninum* tachyzoites.

Figure 12 shows that, at any of the serum dilutions used, FITC MFI values were higher when NcT were incubated with immune serum than with non-immune serum, which indicates that IgGs induced by the immunisation are specific for NcT and able to bind the surface of the parasite. When comparing the MFI values of NcT incubated with immune serum at the different dilutions, the MFI value was the lowest for the ten-fold dilution, the highest for the one hundred-fold dilution and then decreased progressively when the serum was diluted 1:500 and 1:1000.

In order to further identify the NcT membrane proteins to which IgGs of the immune serum were bound, a Western blot was carried out using as capture antigen NcT membrane proteins obtained by the same procedure as the proteins used for the immunisations (Figure 13).

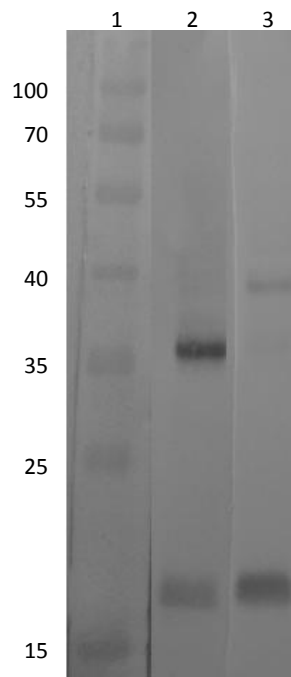


Figure 13. Western blot analysis of the NcT membrane proteins to which IgG1 and IgG2a from the immune serum were bound. The NcT membrane proteins used as capture antigens were obtained using Triton X-114 in the extraction procedure. Immune serum contains antibodies that were demonstrated by ELISA to bind NcT membrane proteins. Lane 1, molecular weight marker; lane 2, IgG1 ligands; lane 3, IgG2a ligands. Numbers on the left are molecular weights, in kDa.

As depicted in Figure 13, IgG1 and IgG2a were shown to bind the same membrane protein of approximately 17 kDa. In addition, IgG1 and IgG2a bound, respectively, the membrane protein of approximately 35 kDa and 39 kDa (Figure 13).

5. Discussion

In cattle, *N. caninum* infection can be acquired either post-natally or transplacentally, being, respectively, the GI tract and the placenta the parasite entry routes into the host¹¹. Since most cattle are infected transplacentally, a successful vaccine targeting that mode of infection could counteract neosporosis. However, it would be only partially effective since some form of post-natal transmission is known to be required in order for the infection to be sustained in a herd⁴⁴. Therefore, the development of an immunisation strategy targeting this last route of infection is essential to cope with the disease. Taking into account that *N. caninum* post-natal infection originates in the GI tract, stimulation of an immune response directly in the intestinal mucosa could be a way of counteracting infection and dissemination of the parasite soon after it contacts with the host. The existence, at the very beginning, of antibodies specific to membrane proteins essential for adhesion and/or invasion by the parasite could impede it of penetrating host cells and thus cross the intestinal mucosal barriers, by antibody-mediated neutralization. Therefore, this project aimed at characterizing the immune response promoted in mice by mucosal immunisation with NcT membrane proteins as well as at evaluating the protective effect of that strategy against i.g. established murine neosporosis. This infection route was chosen in order to more closely simulate the horizontal route of *N. caninum* transmission that naturally happens in herds.

The NcT membrane proteins used for immunisation were obtained by employing a protocol that uses Triton-X114. Extraction protocols using this non-ionic detergent have been employed in previous studies to isolate cell-surface membrane-associated proteins from *N. caninum*¹⁶⁹ and other parasites, such as *T. gondii*¹⁷⁰ and *Leishmania major*¹⁷¹. The specific protocol followed in this project has been previously described and compared by Zintl *et al.*¹⁶⁷ with others aimed at obtaining *N. caninum* antigens, namely using sonication of parasites or solubilisation of NcT with SDS or detergent and non-detergent sulfobetaines. Compared with those methodologies, the Triton-X114 extraction is highly selective and has been considered a useful method to concentrate a subset of *N. caninum* membrane proteins¹⁶⁷. By employing that procedure, a protein extract comprising six proteins with approximately 55, 47, 39, 35, 27 and 17 kDa, was obtained and resulted in a SDS-PAGE migration pattern similar to the one obtained by Zintl *et al.*¹⁶⁷ (Figure 4). Therefore, the extraction protocol was considered successful and used every time NcT membrane proteins were needed, either for the immunisations or to set ELISA and Western blot tests (as capture antigens). The observation that two proteins of 55 and 39 kDa were commonly obtained in

this work, but not by Zintl *et al.*, could be explained by a slight different composition of the NcT membrane. Although the strains of *N. caninum* were the same in both protocols (NC-1), the fact that the isolates could have been passed different times and/or in different strains of mice could have accounted for the existence of different antigens in the membrane and, consequently, slight differences in the virulence between them (the strain used in this study has been previously passed once in C57BL/6 IL-12KO mice). Regarding the components of the extract, the proteins of approximately 35 and 27 kDa may most likely correspond to NcSRS2 and NcSAG1, respectively, which are the two immunodominant surface proteins of NcT⁵⁰. Nonetheless, the exact identification of the proteins composing the extract would require the performance of peptide mass fingerprint.

Since a protective immune response at one mucosal site can be induced by immunisation at a distant mucosal site (concept of CMIS, then renamed to *compartmentalised mucosal immune system*)^{99,100}, and supported by a recently published study demonstrating that T cells primed following i.n. CpG-adjuvanted immunisation with OVA migrate to the spleen and to non-draining LNs, including the MLNs¹⁶⁸, the i.n. route was chosen to immunise the mice. This choice was further encouraged by the fact that, once its efficacy has been proven in mice, i.n. immunisation, contrarily to other routes of mucosal immunisation such as the rectal or vaginal ones, could be easily applied in cattle, the ultimate target species of this protocol. In addition to the first immunisation, a boost was administered to all mice three weeks later in order to strengthen the immune response promoted by the prime immunisation and enlarge the scope of the *N. caninum*-specific immunity to a systemic level.

To investigate the efficacy of the immunisation strategy under study, the evaluation of a *N. caninum*-specific immune response at the mucosa of the GI tract, a site of entry of the pathogen into the host, seemed essential. For that purpose, IgA, the most characteristic mediator of mucosal immunity⁸⁰, would ideally be measured in the intestinal mucosal secretions. However, their sampling requires invasive methods that are not feasible for routine diagnostics¹⁷². Therefore, the IgA measurement was made, either after the prime or the boost immunisation, in the vaginal lavage fluids, as those are the closest mucosal fluids in relation to the intestinal secretions that could be collected by a non-invasive procedure. At the day of euthanasia, intestinal secretions were nevertheless collected to quantify NcT-specific IgA directly at the intestinal mucosal site. The protocol of immunisation studied here, namely the i.n. prime-boost immunisation with NcT membrane proteins plus CpG, was demonstrated to induce an immune response against the parasite at the mucosal level (Figure 6) and specifically at the intestinal mucosal site (Figure 7). This was indicated by the significantly higher ELISA titres of anti-NcT IgA, respectively detected in the vaginal lavage fluids after the boost (Figure 6) and in the intestinal lavage fluids at the day of

euthanasia (Figure 7), in the *Protein + CpG* group when compared with the *Protein* group. The results concerning the anti-NcT IgA titres detected after the prime immunisation were inconclusive since different data were obtained in the two independent experiments performed in the same conditions (Figure 5). Further replicate experiments would therefore be necessary to better ascertain the anti-NcT IgA titres in mice of the *Protein* and *Protein + CpG* groups, at this time point. Nevertheless, they were useful, by suggesting that the boost induced an augmentation in the magnitude of the immune response promoted by the prime immunisation, as assessed by the increased anti-NcT IgA titres in the vaginal lavage fluids of the *Protein + CpG* group at day 28, when compared with those at day 14 (Figure 5A and 6A). The fact that mice of the control *PBS* and *CpG* groups did not produce IgA specific to *N. caninum* neither after the prime nor after the boost immunisation (Figure 5 and 6) was already expected since until these time points those animals were not administered with *N. caninum* antigens, contrarily to those of the *Protein* and *Protein + CpG* groups. However, it is understandable that NcT-specific IgA had been detected in the intestinal lavage fluids of those mice (Figure 7) since they had been infected with NcT seven days before sampling these secretions. The lower IgA titres detected when compared with those of the *Protein* and *Protein + CpG* groups (Figure 7) further stresses out the role of the encounter with the antigen before the infection, and consequently the importance of the immunisation, to mount a significant mucosal immune response against the pathogen once the infection has occurred.

Besides the aim of strengthening the immune response promoted by the prime immunisation, the boost was also performed to extend it to a systemic level and thus to make the immune response even more protective against the parasite. This second aim was achieved since IgG1 and IgG2a specific for *N. caninum* were detected after the boost in the serum of mice of the *Protein* and *Protein + CpG* groups (Figure 8) and also after the NcT challenge infection (Figure 9). As already expected, and similarly to what has been reported in respect to the IgA titres, mice of the *PBS* and *CpG* groups did not produce parasite-specific antibodies of any of the IgG isotypes after the boost immunisation (Figure 8). One mouse of the *PBS* group did however produce IgG2a in residual amounts after the NcT challenge infection (Figure 9), which is consistent with only having encountered *N. caninum* antigens from the infection on.

It is well established that the production of IgG2a antibodies is induced by T_H1 cytokines, such as IL-12 and IFN- γ , whereas the production of IgG1 antibodies is favoured by T_H2 cytokines, such as IL-4³². In this way, the levels of antibody subclasses may reflect the *in vivo* production of cytokines, as well as the type of immune response mounted against the pathogen, namely when attention is paid to the ratio IgG1/IgG2a³². In this case, the significantly higher levels of serum IgG2a in the *Protein +CpG* group when compared with the *Protein* group, either after the boost (Figure 8) or after both immunisations and the NcT infection (Figure 9), suggest that these mice

were producing more T_H1-type cytokines than the ones of the *Protein* group. In contrast, the levels of T_H2-type cytokines did not appear to significantly differ between the compared groups, as no statistically significant differences in the serum IgG1 titres were reported (Figure 8 and 9). By looking at the IgG1/IgG2a ratios, it was clearly established that the immune responses promoted in mice of the *Protein* and *Protein + CpG* group were, respectively, of the T_H2-type (ratios above 1.00) and T_H1-type (ratios below 1.00), either after the boost immunisation (Figure 8B) or after both immunisations and the NcT challenge infection (Figure 9). Only for the first experiment and after the boost were the ratios for both groups below 1.00 (Figure 8A). Nevertheless, since the ratio for the *Protein + CpG* group was much lower than the ratio for the *Protein* group (0.13 vs. 0.88, respectively), one can also infer that the immune responses were, respectively, more biased to the T_H1- and T_H2-type.

From the antibody analyses just presented, it can be inferred that the administration of NcT membrane proteins together with CpG (*Protein + CpG* group) has been able to induce in mice, both at the mucosal and systemic level, a protective immune status effective against *N. caninum*. This status was characterized by significantly higher antigen-specific IgA and IgG2a titres, when compared with mice of the *Protein* group, and biased to the T_H1-type of immunity. The immune protection conferred by the immunisation was supported by data on liver and brain parasite load, measured seven days after the i.g. NcT challenge infection (Figure 11). In fact, no mouse of this group was colonized by *N. caninum*, neither in the liver nor in the brain, contrarily to mice of the *Protein* group. This observation is consistent with the previously inferred T_H2 immune response for these mice that is known to be associated with susceptibility to *N. caninum* infection. Also, and as expected, mice of the *PBS* and *CpG* groups presented *N. caninum* colonization in both organs (Figure 11), in agreement with the fact that none of these animals had been previously immunised with *N. caninum* antigens in order to be protected at the time of infection. Although not statistically significant, mice of the *CpG* group were less colonized than those of the *PBS* group and this could be explained by CpG being an immunomodulator effective at inducing T_H1-type immune responses¹²¹. This type of immune response is known to be protective against neosporosis and may have been responsible for the lower *N. caninum* colonization observed, when compared with that in animals treated with PBS alone. Comparing the number of parasites in the livers and brains of mice of the control groups, it is noteworthy that the first appeared to be more colonized than the last (Figure 11). A possible explanation could be that at the time point at which the *N. caninum* load was assessed (seven days after the NcT challenge infection), the parasite had still not evolved into the bradyzoite stage, which is preferentially located in the central nervous system^{24,32}, and was still disseminating throughout the organism in the tachyzoite form.

After the prime-boost immunisation and the NcT challenge, the cytokine production by MLN and spleen cells was evaluated with the aim of further characterizing the induced immune response. A study conducted by Velge-Roussel *et al.*¹⁷³ supported the analysis of the cytokine production in cells coming from the MLNs to evaluate the mucosal immune response, as they demonstrated that i.n. CT-adjuvanted immunisation with *T. gondii* SAG1 induced a specific cellular response in the NALT, the cervical LN and MLNs. On the other hand, systemic immunity could be evaluated using cells obtained from the spleen¹⁷³. If the type of immune response in the *Protein* and *Protein + CpG* groups, either T_H1 or T_H2, has been deduced from the serum IgG1/IgG2a ratios, the same did not happen when the production of cytokines was analysed (Figure 10). In fact, the only significant difference in the MLNs was noticed in the total number of CD4⁺ IL-10-producing cells that was significantly higher in mice of the *Protein + CpG* groups than in animals of the *Protein* group (Figure 10A), which contrasts with the T_H1 immune response for the first group, as could be deduced from the IgG1/IgG2a ratios. On the other hand, the number of spleen CD4⁺ cells producing IL-4 and IL-10 was significantly lower in the *Protein + CpG* group than in the *Protein* group, which could be in agreement with the T_H1 immune response previously inferred if the number of IFN- γ producing cells was not also significantly lower than in the *Protein* group, as it has actually been demonstrated (Figure 10B). Low levels of IFN- γ in mice that contain higher IgG2a than IgG1 titres could be explained by IgG2a production independent of IFN- γ as has been demonstrated to occur upon parasitic infections¹⁷⁴. In this study, Markine-Goriaynoff *et al.*¹⁷⁴ have shown that either IFN- γ receptor-knockout mice or wild-type mice treated with anti-IFN- γ did produce IgG2a after infection with *T. gondii* and *Trypanosoma cruzi*, respectively, clearly indicating that at least some of the *in vivo* production of IgG2a is independent of IFN- γ . One can also hypothesize that the low levels of IFN- γ in mice of the *Protein + CpG* group were due to a decrease in the number of parasites able to enter the host, due to NcT-specific IgA-mediated neutralization in the intestinal mucosa. Alternatively, if they could have actually entered, it can be hypothesized that the NcT-specific IgG1 and IgG2a generated upon immunisation have been successful in preventing its dissemination. If one of those hypotheses did happen, the parasite could have not been able to stimulate the immune system and therefore to induce the production of IFN- γ , typically associated with protection against neosporosis. Also, it could be that, as mice were shown to be protected against *N. caninum*, by assessing the parasite load in the liver and brain, the threshold level of IFN- γ for protection had been achieved with the low amounts detected, which means that more IFN- γ would not lead to more protection. At last, it should be noted that it is possible that the cytokine analysis was made too late after the infection and the peak of IFN- γ production already subsided by the time the analysis was made. Therefore, the cytokine analysis must be done in another experiment where the animals would be analysed sooner than seven days after the NcT challenge.

When it comes to the significant differences between the *Protein* and the *Protein + CpG* group besides the cytokine analysis, it is reasonable to postulate that they are generally assigned to the effect caused by the administration of CpG. In fact, CpG has been reported to be a potent mucosal adjuvant when delivered i.n. together with bacterial antigens¹²² and its administration with NcT membrane proteins in the present study has likely been responsible for the significantly higher IgA titres detected in the *Protein + CpG group*. In the same study, CpG has demonstrated to induce not only a mucosal but also a systemic immune response when delivered i.n.¹²². In this case, however, the systemic immune response promoted by the boost immunisation can not be entirely attributed to CpG, since mice that only received NcT membrane proteins also produced detectable amounts of antibodies of the IgG isotypes. One can infer that the NcT membrane proteins used in the immunisation are slightly immunogenic by themselves and capable of stimulating an immune response, both at the systemic level and at the mucosal level, which in this last case would also explain the fact that mice of the *Protein* group produced some NcT-specific IgA. Also, it is reasonable to hypothesize that the route of administration (i.n.) was itself responsible for promoting not only a mucosal but also a systemic immune response in both groups, as discussed below. Nonetheless, CpG is most likely responsible for the significantly higher IgG2a titres detected in the *Protein + CpG* group when compared with the *Protein* group and for the T_H1 immune response inferred in these mice based on the IgG1/IgG2a ratios. A study carried out by Teixeira *et al.*¹⁷⁵ showed that intradermal immunisation of BALB/c mice with *N. caninum* sonicates in the alum or Freund's adjuvant or with irradiated NcT without adjuvant led to an increased susceptibility to the parasite, with predominant production of IgG1 comparatively to IgG2a and a decrease in the IFN- γ mRNA expression upon NcT challenge. Also, in a clear explanatory study, Chu *et al.*¹²¹ showed that co-administration of CpG with hen egg lysozyme (HEL), in either T_H1- or T_H2-biased mice, induced a T_H1 immune response characterized by high levels of IFN- γ production and low levels of IL-5 secretion. Moreover, a T_H1 immune response with high production of IgG2a was switched on when FIA, that was shown to induce T_H2 immune responses when co-administered as the sole adjuvant with HEL, was administered together with CpG and HEL. The potent T_H1-immunomodulatory ability of CpG has also been demonstrated in an experimental study conducted by Weeratna *et al.*¹¹² who showed that the combination of CpG with FIA and alum compounds, both of them strongly T_H2-biased, induced even stronger T_H1 immune responses than when CpG was administered alone. In the same study, CpG has also shown to incur only minimal tissue damage at the injection site upon intramuscular administration¹¹². Also, and according to its T_H1-immunomodulatory property, CpG is known to be a potent inducer of T_H1 cytokines, such as IL-12 and IFN- γ ¹²⁵, and Rosa *et al.*¹⁷⁶ highlighted the critical role of IFN- γ in the adjuvant activity of CpG when co-administered i.n. with recombinant proteins. Namely, significantly lower IgG titres were

detected in IFN- γ -knockout mice , an effect that was specific for the CpG adjuvant¹⁷⁶. In the same study, the importance of CD4⁺ and CD8⁺ cells as IFN- γ sources *in vivo* following CpG-adjuvanted immunisation has been demonstrated¹⁷⁶. According to this, the fact that neither a significantly higher number of CD4⁺ nor of CD8⁺ cells produced IFN- γ in the present study, further supports the idea that the cytokine analysis should be repeated.

In order to further understand the functional properties of IgG antibodies that have proven, by ELISA, to bind NcT membrane proteins, an assay was conducted in which these antibodies were investigated for the ability to bind the surface of NcT *in vitro*. Independently of the dilution at which the serum containing those IgGs (*immune serum*) was incubated with NcT, they have been shown to bind the NcT surface (Figure 12), which is in agreement with the fact that they were generated upon immunisation with NcT membrane proteins, the same to which they have then proven to bind, by ELISA, as previously referred. The IgG binding was proven by the higher MFI values of the opsonized parasites when compared with the ones obtained when the NcT were incubated with the *non-immune serum*. Nonetheless, it is of note that the ten-fold dilution of the serum was the one that led to the least binding, probably due to an excess of antibodies that competed for the same ligand on the membrane of the parasite and thus sterically hindered the binding of more IgG. The one hundred-fold dilution led, in turn, to the highest binding, among all the four dilutions tested. The five hundred- and one thousand-fold dilution resulted in minimal background, i.e., the differences in the MFI values between NcT incubated with non-immune serum and with no serum at all were minimal, but the MFI values progressively decreased from the one hundred- until the one thousand-fold dilution. One can hypothesize that this was due to a progressive lack of IgGs to bind all the surface of the parasite. In summary, the one hundred-fold dilution of the serum seems to lead to an ideal ratio antibody: parasite and is therefore recommended for future *in vitro* assays of this type. The specificity of these antibodies for the NcT membrane proteins was further investigated by Western blot where it was demonstrated that IgG1 and IgG2a bind, respectively, the membrane proteins of 35 and 39 kDa and both of them also bind the one of 17 kDa.

Several studies have used the i.n. route to immunise animals against a variety of parasites, including *T. gondii*. I.n. immunisation of CBA/J mice with *T. gondii* SAG1 protein and using non-toxic mutant heat-labile enterotoxins as adjuvants induced both mucosal and systemic immune response and protected mice against oral infection with *T. gondii*¹⁷⁷. More recently, i.n. CT-adjuvanted immunisation of BALB/c mice with the recombinant proteins TgROP2, TgGRA5 and TgGRA7 has been shown to induce partial protection against tissue cyst formation after oral challenge infection with tissue cysts of *T. gondii*¹⁷⁸. Besides mice, i.n. immunisation has also been applied in sheep using *T. gondii* tachyzoite antigens encapsulated into PLG microspheres, which

resulted in both systemic and mucosal, humoral and cell-mediated immune responses¹⁷⁹. When it comes to *N. caninum*, a pertinent study and the first on i.n. immunisation of mice against this parasite, has been recently published by Debache *et al.*¹⁸⁰, who demonstrated that the outcome of protection depends not only on the nature of the antigen but also on the route of delivery¹⁸⁰. Three recombinant *N. caninum* proteins, namely NcPDI, NcROP2 and NcMAG1, were used for saponin-adjuvanted i.p. immunisation or CT-adjuvanted i.n. immunisation of C57BL/6 mice which were then challenged by i.p. infection with NcT. In the case of recombinant NcPDI, the i.n. route provided 90% protection rate against neosporosis but only 20% when the protein was delivered i.p.¹⁸⁰.

The interest in developing vaccines of defined composition is progressively increasing. Although there is one commercially available vaccine against *N. caninum* which consists of a crude extract of inactivated NcT and the Havlogen adjuvant and has proven to be partially effective in reducing the incidence of abortions in herds, the composition of this kind of vaccines made of crude extracts may vary from one batch to another and thus can not be accurately controlled. Moreover, these vaccines may include non-protective or immunosuppressive factors³². Therefore, the possibility of combining purified *N. caninum* antigens (instead of the whole parasite) and the strong T_H1-immunomodulator CpG with the i.n. route of delivery, that has recurrently proven to induce both mucosal and systemic immunity, seemed promising in order to develop an efficient immunisation protocol against *N. caninum*. At least so far, this challenge seems to be on the good way for success.

6. Conclusions and future work

The present study was carried out with the aim of characterizing the immune response induced in mice by mucosal CpG-adjuvanted immunisation with NcT membrane proteins as well as of evaluating the protective effect of that protocol of immunisation against neosporosis. The strategy of immunisation under study was efficient at inducing an immune response against *N. caninum*, both at the intestinal mucosa and the systemic level, as indicated by the ELISA NcT-specific IgA and IgG titres, respectively. Taking into account that serum IgG1/IgG2a ratios were lower than 1.00, the immune response thus induced was predominantly of the T_H1 type, known to be protective against neosporosis. In accordance with this, seven days after i.g. infection with NcT, no parasites were detected in the liver and brain of mice subject to that protocol of immunisation, as evaluated by qPCR. At last, serum IgG1 and IgG2a produced by the immunisation were shown by flow cytometry to bind the NcT surface *in vitro* and were specific for three of the NcT membrane proteins used to immunise the animals as determined by Western blot. IgG1 was specific to the protein of 35 kDa, IgG2a to the protein of 39 kDa and antibodies of both IgG isotypes to the protein of 17 kDa.

In the near future, peptide mass fingerprint could be performed in order to precisely identify the proteins present in the extract that is used for immunisation. The knowledge of the NcT proteins to which intestinal IgA binds would be useful to infer about whether IgA binding to the parasite at the intestinal mucosa is important to prevent its adhesion to the intestinal cells and/or invasion of the host. The ability of the intestinal IgA to prevent parasite entry into cells could be experimentally investigated by incubating them with the parasite and then with the VERO cells. The role of intestinal IgA could be further studied by an *in vivo* experiment of the course of *N. caninum* infection as soon as it contacts with the host by the i.g. route. For this, immunised mice could be challenged with NcT at different time points and sections of the intestine could be used for histological examination and immunohistochemical analysis to investigate the damage caused by the parasite in the intestine as well as its localization along time. If the parasite is shown to invade the host after contacting with the intestinal mucosa, the consequences of serum IgG1 and IgG2a binding to the NcT membrane, namely which parasite functions are neutralized upon binding (adhesion, invasion), could be inferred if the proteins to which they bind have been identified by peptide mass fingerprint. The effect of serum IgG1 and IgG2a binding in parasite entry into cells could be also investigated by the aforementioned *in vitro* study. If IgG1 and IgG2a did not prove to prevent parasite invasion of host cells, their ability to activate the complement system, that would

then destroy the *N. caninum*-infected host cells, could be evaluated as a possible reason to explain why the parasite has not been detected in the liver and brain of the immunised mice.

With regard to the experiment set-up followed in this project, the possibility of decreasing the time between the NcT challenge infection and the euthanasia of the animals should be considered in order to probably get a better profile of the cytokine production by MLN and spleen cells after the infection. Also, it would be interesting to carry out the same type of experiment but with a longer time gap between the last immunisation and the NcT challenge infection. This would be useful to investigate if the immunisation could provide long-term protection against the parasite. Furthermore, the protective effect of the immunisation strategy in study, either the present or the long-term one, could be evaluated by carrying out survival studies.

The possibility of combining another adjuvant with CpG could be considered in order to possibly increase the efficiency of the immunisation. Weeratna *et al.*¹¹² reported that the combination of CpG with alum compounds induced the strongest immune responses to the hepatitis B surface antigen when delivered intramuscularly and with minimal damage in the injected muscle, among all the adjuvants alone and combinations of adjuvants tested.

The ultimate task of this project would be to apply in cattle the immunisation protocol that has proven to be successful in mice and evaluate the efficacy of the strategy in that group of animals. When proven to be successful, a way of preventing the post-natal route of *N. caninum* transmission will have been established and the overall negative impact caused by *N. caninum* in cattle will have, at least in part, been contradicted.

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