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Version of Record: https://www.sciencedirect.com/science/article/pii/S1043466619300912
Manuscript_677261f4d3ca937d6fdf0a5a5cf09418

# *In vitro* cellular responses to *Neospora caninum* glycosylphosphatidylinositols depend on the host origin of antigen presenting cells

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Running title: *Cellular responses induced by Neospora GPIs*

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ABSTRACT

Neosporosis due to *Neospora caninum* causes abortions in farm animals such as cattle. No treatment and vaccine exist to fight this disease, responsible for considerable economic losses. It is thus important to better understand the immune responses occurring during the pathogenesis to control them in a global strategy against the parasite. In this context, we studied the roles of *N. caninum* glycosylphosphatidylinositols (GPIs), glycolipids defined as toxins in the related parasite *Plasmodium falciparum*. We demonstrated for the first time that GPIs could be excreted in the supernatant of *N. caninum* culture and trigger cell signalling through the Toll-like receptors 2 and 4. In addition, antibodies specific to *N. caninum* GPIs were detected in the serum of infected mice. As shown for other protozoan diseases, they could play a role in neutralizing GPIs. *N. caninum* GPIs were able to induce the production of tumour necrosis factor-α, interleukin(IL)-1β and IL-12 cytokines by murine macrophages and dendritic cells. Furthermore, GPIs significantly reduced expression of major histocompatibility complex (MHC) molecules of class I on murine dendritic cells. In contrast to murine cells, bovine blood mononuclear cells produced increased levels of IFN-γ and IL-10, but reduced levels of IL-12p40 in response to GPIs. On these bovine cells, GPI had the tendency to up-regulate MHC class I, but to down-regulate MHC class II. Altogether, these results suggest that *N. caninum* GPIs might differentially participate in the responses of antigen presenting cells induced by the whole parasite in mouse models of neosporosis and in the natural cattle host.

*Keywords*:
*Neospora caninum*; glycosylphosphatidylinositol; antigen presenting cell; major histocompatibility complex; TLR.

## Footnotes

*Abbreviations*: BMDCs, bone marrow-derived dendritic cells; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; GPI, glycosylphosphatidylinositol; FITC, fluorescein isothiocyanate;

HEK, human embryonic kidney; HFF, human foreskin fibroblasts; IFN, interferon; Ig, immunoglobulins; IL, interleukin; LPS, lipopolysaccharide; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PECs, peritoneal exudate cells; PI, propidium iodide; RPMI, Roswell Park Memorial Institute; SD, standard deviation; SEAP, secreted embryonic alkaline phosphatase; T, Tween-20; TLC, thin layer chromatography; TLR, Toll-like receptor, TNF, tumor necrosis factor.

## 1. Introduction

Neosporosis, an infectious disease due to *Neospora caninum*, a *Toxoplasma gondii*-related protozoan parasite, affects a variety of animal species, mainly cattle and dogs. After ingestion of oocysts (shed by canids) by these intermediate hosts, the parasite multiplies intracellularly as tachyzoite and differentiates into bradyzoite inside tissue cysts persisting in the brain and muscles throughout the whole life of the host [1]. Congenital transmission can also occur from an infected dam to the foetus when tachyzoites cross the placental barrier [2].

Neosporosis is emerging as a major cause of abortion and infertility in cattle, worldwide [3]. The estimate for the *N. caninum*-related losses exceeds US $1.3 billion annually [4]. There is no commercialized drug and no efficient vaccine that can be used to clear *N. caninum* infection and the practices established in farms consist solely in limiting the number of infected animals in the herd. The difficulty to develop an efficient vaccine against congenital neosporosis is due to the fact that immune responses specific to *N. caninum* in non-pregnant animals do not always protect against foetal infection during pregnancy [5]. It is thus important to decipher the mechanisms underlying natural resistance to infection to improve them and fight this pathogen.

Cytokine production is a parameter systematically studied in animals infected by *N. caninum* [6]. Immunological studies in mouse models of experimental neosporosis indicate that the cytokines IFN-γ and interleukin(IL)-12 are key mediators in a protective immune response against *N. caninum* [7,8]. Although IFN-γ is important to control the parasite, the regulatory cytokine IL-10 is required to down-regulate inflammatory response and avoid damages [9]. In non-pregnant infected cows, IFN-γ is also particularly important for the development of host protective immunity [10]. Furthermore, production of IFN-γ is associated with protection against abortion in cows naturally infected with *N. caninum* [11].

The production of cytokines in response to *N. caninum* has also been studied *in vitro*. Stimulation of splenocytes from infected mice with parasite antigens enhanced IFN-γ production [7]. IL-12 added to fibroblasts infected with *N. caninum* increased IFN-γ production by IL-2-activated bovine NK cells [12]. At the molecular level, little is known on parasite-host cell relationships. Recombinant *N. caninum* profilin increased IFN-γ production by murine splenocytes *in vitro*, as well as serum IFN-γ and IL-12 levels after intraperitoneal injection [13]. The antigen NcGRA7 is involved in up-regulation of cytokines *in vitro* (IL-6 and IL-12p40) and *in vivo* (IFN-γ), related to parasite virulence in mice [14]. Glycosylphosphatidylinositols (GPIs) of *Plasmodium falciparum* have been described as toxins playing a role in malaria disease [15,16]. GPIs are glycolipids ubiquitous in eukaryotic cells that have the primary function to anchor proteins at the cell membrane surface. Most of the surface proteins of *N. caninum* tachyzoites are anchored by GPIs, and the first identified, NcSAG1 (Surface Antigen Glycoprotein) and NcSRS2 (SAG-Related Sequence), have been suspected to be involved in the parasite-host cell interactions [17,18]. In *N. caninum* tachyzoites, more than 7 different GPIs and GPI-intermediates of biosynthesis have been identified [19]. The aim of the present work was to understand the role of *N. caninum* GPIs in the cellular responses, notably the cytokine production, of antigen presenting cells of model (mouse) and natural (cattle) host animals.

## 2. Materials and Methods

2.1. *Metabolic labelling of GPIs*

Human foreskin fibroblasts (HFF, ATCC® SCRC-1041™) were cultivated in Roswell Park Memorial Institute (RPMI, Gibco) 1640 medium supplemented with 10 % foetal calf serum (FCS) and Vero (ATCC® CCL-81TM) cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10 % FCS. HFF and Vero cells were infected with tachyzoites of the *N. caninum* strain NC-1 (ATCC® 50843™) at a ratio of 0.5 parasite per cell in flasks of 175 $cm^2$. After intracellular multiplication for 3 days at 37 °C in a 5 % $CO_2$ atmosphere, adherent infected cells were washed with phosphate buffered saline (PBS) to remove medium containing serum and glucose. Metabolic labelling of *N. caninum* was performed in 15 mL glucose-free RPMI 1640 medium (Sigma-Aldrich) or glucose-free DMEM (Sigma-Aldrich) supplemented with 2.44 mM sodium pyruvate and 0.5 mCi D-[6-$^3$H]-glucosamine hydrochloride (Hartmann Analytic GmbH) for 7 h at 37 °C in a 5 % $CO_2$ atmosphere. From this step, the protocol was the same for HFF and Vero cell cultures. Infected cells were scraped and centrifuged at 2000 x *g* for 10 min. Supernatant containing free GPIs was removed and the pellet containing infected cells was suspended in 1 mL DMEM. Tachyzoites aggregates were disrupted by using glass beads (diameter of 1-1.5 mm) and the Mixer Mill homogenizer (Retsch) at 20 shakes/sec. for 5 min. Subsequently, tachyzoites were purified by glass wool (Assistent®, Glaswarenfabrik Karl Hecht GmbH & Co) filtration. Briefly, a 10-ml syringe was filled with 0.75 g of glass wool in the first 5-ml volume and with 0.5 g of glass wool in the second 5-ml volume. This column was equilibrated with DMEM containing 5 % FCS prior to the passage of the suspension containing parasites and host cell debris. The column was eluted with 50 ml of the same medium to recover only tachyzoites. After centrifugation of the effluent at 3000 x *g* for 5 min, the supernatant was discarded and the parasite pellet was washed with PBS and centrifuged. Glycolipids of tachyzoites were extracted with chloroform-methanol (10:10, by volume) by sonication (ultrasound bath 47 MHz, Branson 3200). After centrifugation, a second extraction was performed on the pellet with chloroform-methanol-water (10:10:3, by volume) by sonication. After concentration by solvent evaporation under a stream of nitrogen, GPIs were partitioned between water-saturated *n*-butyl alcohol and water (1:1, by volume) and recovered in the *n*-butyl alcohol phase by centrifugation at 3000 x *g* without braking for 10 min. GPIs from 10000 cpm were separated by thin layer chromatography (TLC) on silica gel 60 plate (Merck) using a chloroform-methanol-water (10:10:3, by volume) solvent system. Free GPIs liberated in culture supernatant (sample volume of 800 µL) during metabolic labelling were recovered in the *n*-butyl alcohol phase after partitioning between supernatant and water-saturated *n*-butyl alcohol (1:1, by volume) by centrifugation at 3000 x *g* without braking for 10 min. GPIs were concentrated by evaporation of the solvent under a stream of nitrogen and separated by TLC. The silica plate was scanned for radioactivity in both supernatant and tachyzoite samples by using a Berthold LB 2842 linear analyser.

2.2. *Purification of individual GPIs*

Tachyzoites of *N. caninum* were cultivated in HFF in DMEM supplemented with 10 % FCS. Cells were scraped and parasites were purified as described in 2.1. section. GPIs were extracted with chloroform-methanol (10:10, by volume) and with chloroform-methanol-water (10:10:3, by volume) by sonication, dried under a nitrogen stream and recovered in the *n*-butyl alcohol phase by water-saturated *n*-butyl alcohol/water partition. GPIs were precipitated under a stream of nitrogen to remove contaminating phospholipids [20]. GPIs were then separated by TLC on 0.5 mm silica gel 60 plate (Merck, GPIs from 5 x $10^9$ parasites/plate) using a chloroform-

methanol-water (10:10:3, by volume) solvent system, with spots of metabolically labelled GPIs (section 2.1.) applied in parallel tracks as tracers (4000 cpm). GPIs were detected by using the Berthold LB 2842 linear analyser and areas corresponding to individual GPIs were scraped off the plate, re-extracted with chloroform-methanol and chloroform-methanol-water by sonication and residual silica was removed by water-saturated *n*-butyl alcohol/water partition. GPIs were stored at -20 °C in *n*-butyl alcohol until use. Absence of endotoxin in each GPI sample was checked with the Pierce® *Limulus* Amebocyte Lysate Chromogenic Endotoxin Quantitation kit according to the manufacturer's instructions (Thermo Fisher Scientific). For cell stimulation, the needed amount of individual GPIs was dried under a nitrogen stream to remove the solvent *n*-butyl alcohol. Each GPI was then suspended in the appropriated cell culture medium by sonication and added to the cells. As negative control, cells were incubated with the solvent *n*-butyl alcohol alone dried prior to addition of medium and treatment by sonication.

2.3. *Evaluation of TLR signalling*

HEK-Blue™ hTLR2 and hTLR4 cells (InvivoGen) were seeded at 2 x $10^5$ in 96-well plates in 100 µL DMEM supplemented with 1 % FCS without selection antibiotics. The cells were stimulated at 37 °C in a 5 % $CO_2$ atmosphere for 24 h with GPIs purified from $10^8$ tachyzoites of *N. caninum*. Secreted embryonic alkaline phosphatase (SEAP) reporter gene activity was measured at 630 nm after addition of QUANTI-Blue™ detection medium (InvivoGen) to supernatant following the manufacturer's instructions.

2.4. *Stimulation of mouse cell lines with N. caninum GPIs*

Macrophages of the RAW 264.7 cell line (ATCC® TIB-71™) were seeded at 2 x $10^5$ in 96-well plates in 100 µL DMEM. Dendritic cells of the SRDC cell line (Applied Biological Materials Inc.) were seeded at $10^6$ in 24-well plates in 300 µL RPMI 1640 medium. Individual GPIs purified from 3 x $10^8$ tachyzoites of *N. caninum* were dried, suspended in the same medium (100 µL/well for RAW 264.7, 300 µL/well for SRDC) by sonication and added to the cells. After 24-hour stimulation at 37 °C in a 5 % $CO_2$ atmosphere, non-adherent RAW 264.7 and SRDC were centrifuged and the supernatants were stored at -20 °C for quantification of cytokines (2.7. section). Pellets of SRDC were pooled with adherent SRDC detached by using accutase (Affymetrix eBioscience). Expression of MHC molecules was further studied on these cells (2.8. section).

2.5. *Stimulation of primary murine cells with N. caninum GPIs*

Population enriched in dendritic cells were obtained by *in vitro* differentiation of primary bone marrow cells. For this, femurs of 10-week old female OF1 mice were dissected using scissors after euthanasia (directive 2010/63/EU not applied [chapter 1, article 1.5.]). After removal of connected muscles, bones were disinfected in 70 % ethanol solution for 2 min and rinsed with RPMI 1640 medium. Femurs were placed with 70 µL RPMI 1640 medium into a 0.5-mL tube pierced at the bottom with a needle. This tube was placed in a 1.5-mL tube and centrifuged at 10000 x *g* for 2 spins of 30 sec., each to collect bone marrow cells. Erythrocytes were lysed by an osmotic shock with 1 mL ultra-pure water, stopped by addition of RPMI 1640 medium. After 10 min centrifugation at 600 x *g*, cells were cultivated in bacteriological Petri dishes at 2 to 4 x $10^6$ in 10 mL complete medium (RPMI 1640, 15 mM Hepes, 10 % FCS, 50 µM β-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL of streptomycin, 1 % non-essential amino acids, 1mM sodium pyruvate) supplemented with the supernatant of J558 cells as source of granulocyte/macrophage-colony stimulating factor (GM-CSF) at the final concentration of

20 ng/mL. After 3 days, 10 mL of complete medium with GM-CSF were added for additional 3-day culture. The supernatant was then replaced by fresh medium for additional 2-day culture. Floating and semi-adherent cells were collected and percentages of dendritic cells and macrophages were determined by flow cytometry (Miltenyi MACSquant Miltenyi and FlowLogic 7.2.1 software) after labelling with 0.25 µg/5 x $10^5$ cells of fluorescein isothiocyanate(FITC)-rat anti-mouse F4/80 antibody (clone BM8, eBioscience), with 0.25 µg/5 x $10^5$ cells allophycocyanin-Armenian hamster anti-mouse CD11c antibody (clone N418, eBioscience) or with corresponding isotype controls (rat immunoglobulins[Ig]G2a and Armenian hamster IgG). Total cells defined as bone marrow-derived dendritic cells (BMDCs) were seeded in 24-well plate at 5 x $10^5$ cells in 300 µL RPMI 1640 medium supplemented with 2 % FCS. The cells were stimulated at 37 °C in a 5 % $CO_2$ atmosphere for 24 h with individual GPIs purified from 3 x $10^8$ tachyzoites of *N. caninum* suspended in 300 µL RPMI 1640 medium. BMDCs were centrifuged and supernatants were stored at -20 °C for quantification of cytokines (2.7. section). Expression of MHC molecules was further studied on the cells (2.8. section).

Peritoneal exudates cells (PECs) rich in macrophages were harvested from Swiss OF1 mice by washing the peritoneum with 5 mL DMEM after euthanasia (directive 2010/63/EU not applied [chapter 1, article 1.5.]) and immediately centrifuged at 600 x *g* for 5 min. PECs were seeded at $10^6$/300 µL DMEM in 24-well plates. The cells were stimulated at 37 °C in a 5 % $CO_2$ atmosphere for 24 h with individual GPIs purified from 3 x $10^8$ tachyzoites of *N. caninum* suspended in 300 µL DMEM. Floating cells were centrifuged and pooled with adherent cells detached by using accutase. Apoptosis was further studied on the cells (2.9. section).

### 2.6. *Stimulation of primary bovine cells with N. caninum GPIs*

All animals were free of the main communicable diseases (infectious bovine rhinotracheitis, bovine viral diarrhea, bovine leucosis, brucellosis and tuberculosis). Blood of 6-18 and 24 month-old Holstein cows (agricultural school of Fondettes and INRA Val-de-Loire, France) was collected in 7-mL dry tubes. Absence of antibodies specific for *N. caninum* and *T. gondii* was verified by ELISA using 10 µg/mL of soluble antigens extracted from tachyzoites of *N. caninum* or *T. gondii* by sonication. Limited dilutions of serum samples in PBS-3% BSA were added and IgG were detected with alkaline phosphatase-conjugated goat anti-bovine IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted at 1/5000 in PBS-3% BSA. Complexes were detected by p-nitro-phenyl-phosphate at 405 nm. Optical density of the samples was compared to those of positive and negative controls for *Toxoplasma* and *Neospora* (IDEXX). For purification of peripheral blood mononuclear cells (PBMCs), blood was collected in 7-mL Vacuette tubes containing 0.017 g EDTA (Greiner Bio-one). Buffy coat rich in mononuclear cells was separated by centrifugation at 700 x *g* for 20 min with low braking. The buffy coat diluted with equal volume of PBS was over-layered onto Ficoll-Paque solution (d = 1.077, Sigma-Aldrich) and centrifuged at 700 x *g* for 30 min without braking. Peripheral blood mononuclear cells (PBMCs) were collected and washed with 10 mL RPMI 1640 medium. After centrifugation at 1300 x *g* for 10 min, erythrocytes were lysed by osmotic shock with water (see 2.5. section) and eliminated by centrifugation of PBMCs.

After slaughtering, part of the spleen was mechanically disrupted in RPMI 1640 medium and filtered on 100 µm Nylon membrane (Millipore). After centrifugation at 1300 x *g* for 10 min, erythrocytes were lysed by osmotic shock with water and eliminated by centrifugation of splenocytes.

Bovine PBMCs and splenocytes were seeded in 24-well plate at $10^6$ cells in 300 µL RPMI 1640 supplemented with 2 % FCS. The cells were stimulated at 37 °C in a 5 % $CO_2$ atmosphere for 24 h with individual GPIs purified from 3 x $10^8$ tachyzoites of *N. caninum* suspended in 300 µL of the same medium. PBMCs were centrifuged and supernatants were stored at -20 °C for

quantification of cytokines (2.7. section). Expression of MHC molecules on PBMCs was further studied on the cells (2.8. section).

### 2.7. *Cytokine quantification*

RAW 264.7, SRDC and BMDCs were stimulated as described in 2.4. and 2.5. sections. Levels of mouse cytokines were quantified in the culture supernatants by using IL-1β, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-23 and TNF-α specific sandwich enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Affymetrix eBioscience). Bovine PBMCs and splenocytes were stimulated as described in 2.6. section and levels of cytokines were quantified in the culture supernatants. For this, 50 µL of capture antibody specific for bovine IL-10, IL-12p40, or IFN-γ (clones CC318, CC301 and CC330, respectively, Bio-Rad Laboratories) diluted at 5 µg/mL in PBS were coated overnight at 4 °C onto Nunc™ MaxiSorp™ 96-well plate (Thermo Fisher Scientific). Wells were washed 3 times with 200 µL of PBS with 0.05 % Tween-20 (PBS-T). Saturation was performed with 200 µL of PBS-T supplemented with 1 % bovine serum albumin (PBS-T-BSA) and 5 % sucrose for 1 h. After 3 washes with 200 µL of PBS-T, 50 µL of cell culture supernatants or recombinant cytokines diluted in PBS-T-BSA (IL-10 from 0 to 32000 pg/mL, IL-12p40 from 0 to 50 pg/mL, Kingfisher Biotech, Inc.; IFN-γ from 0 to 4000 pg/mL, Thermo Fisher Scientific) were added for 1 h at room temperature. Wells were washed 4 times with PBS-T and 50 µL of specific detection antibodies coupled to biotin (clones CC320, CC326 and CC302, respectively, Bio-Rad Laboratories) were added at 2.5 µg/mL in PBS-T-BSA for 1 h. After 4 washes, 50 µL of ExtrAvidin®-Peroxidase (Sigma-Aldrich) were added at 1/2000 in PBS-T-BSA for 20 min in the dark. After 4 washes, 50 µL of tetramethylbenzidine (Sigma-Aldrich) substrate were added. Reaction was stopped with 50 µL of 1M phosphoric acid when the wells containing the highest concentration of recombinant cytokines turned to dark blue. Optical density was measured at 450 nm on a microtiter plate reader (BioTek Instruments, Inc.).

### 2.8. *Measurement of MHC expression*

SRDC and PBMCs were stimulated for 24 h with *N. caninum* GPIs as described in 2.4. and 2.6. sections. Adherent cells were detached by using accutase and pooled with floating cells. After centrifugation at 300 x g, cells were saturated for 30 min on ice in PBS-BSA. After centrifugation, 3 x $10^5$ SRDC were incubated for 30 min on ice in the dark in PBS-BSA with 0.5 µg FITC mouse anti-mouse H-2K[k] MHC class I antibody (clone 36-7-5, BD Pharmingen™), 0.5 µg FITC mouse anti-mouse I-E[k] MHC class II antibody (clone 14-4-4S, BD Pharmingen™) or 0.5 µg FITC mouse IgG2a, κ isotype control (clone G155-178, BD Pharmingen™), while 3 x $10^5$ PBMCs were incubated for 30 min on ice in the dark in PBS 5 % FCS with R. phycoerythrin (RPE) mouse anti-sheep MHC class II DQ DR polymorphic reacting with bovine antigens (clone 28:1, Bio-Rad Laboratories) or with RPE mouse IgG1 isotype control (Bio-Rad Laboratories) at 1/20, and with mouse anti-human MHC class I HLA-A/B/C (reacting with bovine antigens) from supernatant of W6/32 hybridoma [21] at 1/10 followed by PE goat anti-mouse IgG (Thermo Fisher Scientific) at 1/20 after centrifugation or with PE goat anti-mouse IgG alone as control. After final centrifugation, cells were suspended in 300 µL PBS-2 % paraformaldehyde and analysed by flow cytometry.

### 2.9. *Measurement of apoptosis*

PECs were stimulated for 24 h with *N. caninum* GPIs as described in 2.5. section. Adherent cells were detached with accutase and pooled with floating cells. After centrifugation at 300 x

*g*, PECs were incubated for 15 min at 4 °C in the dark in 100 µl binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) with 5 µl annexin V-FITC (BD Pharmingen™) and 5 µg/ml propidium iodide (PI, Sigma-Aldrich). After centrifugation at 300 x *g*, PECs were suspended in 300 µL binding buffer and analysed by flow cytometry. Non-apoptotic population is annexin V-FITC$^-$/PI$^-$, apoptotic population is annexin V-FITC$^+$/PI$^-$ due to phosphatidylserine externalization at the surface of intact cell membrane and necrotic population is annexin V-FITC$^+$/PI$^+$ due to the loss of cell membrane integrity in both primary and secondary necrotic cells.

### 2.10. *Detection of anti-GPI antibodies by dot blot*

Blood was collected from the sub-mandibular vein of female 3-week-old Swiss OF1 mice (purchased from JANVIER LABS and maintained in the University of Tours animal house) 3 weeks after intraperitoneal injection of 2 x $10^6$ tachyzoites of the *N. caninum* strain NC-1 (ATCC® 50843™). The experimental protocol, carried out in accordance with the European Union Directive (2010/63/EU), was approved by the Val-de-Loire Ethics Committee for Animal Experimentation and the French Ministry for Research (permit numbers APAFIS#3971-20160207102745.v3). Anti-*Neospora* IgG were detected by ELISA using soluble antigens extracted from tachyzoites of *N. caninum* by sonication at 10 µg/mL. Limited dilutions of serum samples in PBS-3% BSA were added and IgG were detected with alkaline phosphatase-conjugated anti-mouse IgG (Sigma-Aldrich) diluted at 1/5000 in PBS-3% BSA. Complexes were detected by p-nitro-phenyl-phosphate at 405 nm. A sample is considered positive when the optical density is at least 2.5 times higher than those of a sample from non-infected mice.

GPIs extracted from 3 x $10^8$ tachyzoites of *N. caninum* were spotted onto nitrocellulose membrane (Schleicher & Schuell) in *n*-butyl alcohol. Equal volume of *n*-butyl alcohol was spotted as negative control. Nitrocellulose strips were saturated for 1 h with 5 % defatted dry milk in Tris-buffered saline (15mM Tris–HCl pH 8, 140mM NaCl) with 0.05 % Tween-20 (TBS-T). Blots were incubated for 1 h at room temperature with serum of mice infected with *N. caninum* or with serum of non-infected mice diluted at 1/250 in TBS-T. After washing with TBS-T, blots were incubated for 1 h at room temperature with rabbit anti-mouse IgG conjugated to human alkaline phosphatase (Promega) at 1/2500. After washing with TBS-T, blots were reacted with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate substrate (Promega) in 100 mM Tris/HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$. The colour reaction was stopped by washing with water and blots were scanned for documentation.

### 2.11. *Statistics*

The parametric one-way ANOVA test followed by the Dunnett's or the Sidak's multiple comparisons test was used for statistical evaluation (GraphPad Prism 7).

## 3. Results

### 3.1. *Comparison of GPI profiles from N. caninum cultivated in two different cell lines*

Multiplication of *N. caninum* tachyzoites in HFF increased the yield of parasite production compared to Vero cells (personal observation). It has been shown that two of the six GPIs of *T. gondii* tachyzoites grown in Vero cells were no more detectable when cultured in HFF [22]. We thus compared GPI profiles of *N. caninum* tachyzoites after metabolic labelling in both cell lines. Ten peaks of GPI species were detected in parasites grown in Vero cells (Fig. 1A), but

with lower radioactivity for GPI1 (I), GPI2 (II) and GPI8 (VI) and higher radioactivity for GPI9 (VII) than in the study of Schares *et al*. [19]. All 10 peaks were present on the GPI profile obtained from *N. caninum* tachyzoites cultivated in HFF with reduced but still detectable GPI7 (V) and GPI8 (VI). These results indicate that contrary to *T. gondii* [22], similar profiles of GPIs were obtained when *N. caninum* divided in Vero cells and HFF. We also analysed the profile of GPIs secreted in the supernatants. All GPIs except GPI1 and GPI10 were detected in supernatant of both HFF and Vero cultures (Fig. 1B). Except for GPI7, more abundant in the supernatant of Vero cells, the two profiles were similar. In regard to these results, GPIs purified from parasites grown in HFF were further tested for their cell responses.

### 3.2. *TLR signalling and responses of murine cell lines stimulated with N. caninum GPIs*

It has been shown that GPIs of *Trypanosoma cruzi*, *T. gondii* and *P. falciparum* induce intracellular signalling through TLR2 and TLR4 [23–25]. HEK293 cells expressing a reporter gene under the control of transcription factors activated after binding of a ligand of TLR2 or TLR4 were used to determine whether GPIs of *N. caninum* also signal *via* these receptors. As shown on Fig. 2A, GPIs of *N. caninum* induced two-fold activation of the reporter gene in both HEK TLR2 and HEK TLR4 cells compared to the negative control (one-way ANOVA, $p = 0.0033$). However, this increase was only significant for HEK TLR4, suggesting that GPIs of *N. caninum* were better recognized by this receptor. Secretion of GPIs might explain how they could enter physically into contact with the receptors.

We have shown that all GPI species of *T. gondii* and *P. falciparum* induced TNF-α production by macrophages of the RAW 264.7 cell line [26,27]. Here, the production of TNF-α by these cells was significantly increased in response to GPIs of *N. caninum* (one-way ANOVA, $p < 0.0001$) (Fig. 2B). This increase was stimulated by GPI4 to GPI10, but not by GPI1 to GPI3. As in our previous studies [26,27], cells were not stimulated with defined concentrations of GPIs, but with GPIs extracted from the same number of parasites (here 3 x $10^8$ tachyzoites) to respect their proportions. The relative amounts of GPIs cannot only explain the cell response since the GPI7 peak was at radioactivity count as low as GPI1 to GPI3 (Fig. 1A). Furthermore, higher amounts of GPI1 to GPI3 did not lead to higher cell stimulation (data not shown). The production of other cytokines in response to *N. caninum* GPIs was investigated: almost no IL-1β and no IL-5 were secreted by the RAW 264.7 cells and GPIs did not increase this secretion, while IL-6, IL-10, IL-12p40, IL-12p70 and IL-23 were produced by the cells but GPIs did not significantly modify the basal levels (data not shown). We also studied the production of cytokines by SRDC, a murine dendritic cell line. The GPIs of *N. caninum* did not drastically modify the levels of IL-1β, IL-12p40 and TNF-α produced by these cells (data not shown).

Live *N. caninum* tachyzoites induced an increase in expression of MHC molecule of class II by DCs after 24-hour incubation [28]. The expression of MHC molecules was explored to study the effect of the GPIs on the maturation of antigen presenting cells. Expression of MHC molecules was rapidly and strongly regulated in SRDC stimulated with *T. gondii* antigens or with bacterial lipopolysaccharide (LPS) [29]. For this reason, these cells were chosen to study this parameter. The cells were stimulated for 24 h with the 10 different GPI species of *N. caninum* and expression of MHC molecules of classes I and II was measured on the total cells by flow cytometry. Our results show that all GPIs induced globally a significant decrease in the percentages of positive cells (Fig. 2C) and intensity of fluorescence (Fig. 2D) of MHC class I (one-way ANOVA, $p = 0.001$ and 0.024, respectively). Individually, GPI7 to GPI10 significantly decreased both percentage and mean, while GPI3 decreased mean. This decrease of MHC class I expression was detected as early as 12 h of incubation with GPIs on SRDC (data not shown). In contrast, percentages of positive cells and intensity of fluorescence of MHC

class II were not significantly modified by GPIs (one-way ANOVA, $p$ = 0.54 and 0.29, respectively) (Fig. 2E and F).

Altogether, experiments done on murine cell lines showed that *N. caninum* GPIs were able to induce TNF-α production by macrophages and to reduce the number of dendritic cells expressing MHC molecules, with GPI7 and GPI8 the most efficient molecules.

### 3.3. *Responses of primary murine cells stimulated with N. caninum GPIs*

Since cell lines not always reflect what happens *in vivo*, the effects of *N. caninum* GPIs on the innate immune responses of primary murine antigen presenting cells were studied. For this, bone marrow cells were differentiated in the presence of GM-CSF for 8 days. According to the literature [30], the differentiated cells designed as bone marrow-derived dendritic cells (BMDCs) were heterogeneous with more than 50 % of $CD11c^{+}F4/80^{high}$ macrophages and about 20 % of $CD11c^{+}F4/80^{low}$ dendritic cells. BMDCs were incubated for 24 h with the 10 different GPIs. Contrary to the murine cell lines tested, BMDCs did not produce TNF-α (data not shown), but were able to produce significantly increased levels of IL-1β and IL-12p40 (Fig. 3A and B, one-way ANOVA, $p < 0.0001$) in response to *N. caninum* GPIs. GPI1 to GPI5 and GPI7 increased only moderately the levels of both cytokines, whereas a marked increase was obtained with GPI6 and GPI8 to GPI10. Expression of MHC molecules was not modulated by GPIs on BMDCs (data not shown).

*In vivo*, an increase in apoptotic B-cell precursors has been observed in bone marrow of mice infected with *N. caninum* [31]. *In vitro*, *N. caninum* increased apoptosis of fibroblasts treated with IFN-γ, but inhibited apoptosis of mouse embryonic fibroblasts induced by TFN-α plus cycloheximide [32,33]. Thus, GPIs of *N. caninum* could be responsible for either inhibition or activation of apoptosis. Since cell death modulations are hardly measurable in immortalized cell lines, only primary cells were used in the present work. A very low percentage of apoptotic cells was obtained in BMDCs both in absence (less than 3 %) or in presence of GPIs (from 0.2 to 10 %). On the contrary, basal apoptosis of non-elicited PECs rich in macrophages reached a mean of 40 %. This percentage increased in the presence of GPIs of *N. caninum* (Fig. 3C), but the global difference with the control was not statistically significant (one-way ANOVA, $p$ = 0.8) due to the high variability between cells from individual mice. However, GPIs might participate in apoptosis observed in *N. caninum*-infected cells or animals. No cytokines were secreted by PECs in our culture conditions (data not shown) and this could be due a consequence of the elevated cell death.

These results show that *N. caninum* GPIs were able to induce the production of different inflammatory cytokines by primary murine cells than murine cell lines. On primary cells, GPI8 to GPI10 induced the highest cytokine levels.

### 3.4. *Responses of primary bovine cells stimulated with N. caninum GPIs*

Mouse is used as model to study neosporosis but it is not a major host of the parasite. Due to different immune responses, viable parasites could be indeed isolated from cattle [34] but not from wild mice in which only DNA has been detected [35]. It was thus important to study the effects of *N. caninum* GPIs on cells from animals naturally susceptible to neosporosis. For this, PBMCs and splenocytes were purified from cows seronegative for toxoplasmosis and neosporosis and stimulated *in vitro* with the different GPIs. The first observation was that cells from only one third of all tested animals (3/9) responded to the GPI stimulation. In bovine PBMCs, GPI4 to GPI10 strongly increased levels of IL-10 (Fig. 4A, one-way ANOVA, $p < 0.0001$), but all GPIs reduced the basal levels of IL-12p40 (Fig. 4B, one-way ANOVA, $p$ = 0.0444) and more markedly GPI2, GPI5, GPI6 and GPI10. Only GPI7 induced IFN-γ

production by PBMCs (297.2 ± 56.3 pg/mL *versus* 69.7 ± 3.1 for the control), whereas all GPIs, but not GPI3 and GPI8, induced IFN-γ by bovine splenocytes (Fig. 4C), even the differences with the control condition were not significant due to very low levels produced and high variability between the individuals (one-way ANOVA, $p = 0.061$).

The expression of MHC molecules was analysed on the whole population of PBMCs in response to stimulation with GPI10. The percentage of cells expressing MHC class I was not regulated by the GPI (Fig. 5A), but intensity of fluorescence of MHC class I slightly increased on cells isolated from 3 of the 6 cows studied (Fig. 5B, not significant). On the contrary, GPI decreased the percentage of MHC class II molecules on cells of 2 cows, but did not modulate the intensity of fluorescence (Fig. 5C and D, not significant).

These results show that, in contrast to murine cells, bovine PBMCs produced IL-10 in response to GPIs of *N. caninum*, and almost all GPIs induced similar cell responses. Although expression of MHC molecules class I decreased on murine cells, GPI had the tendency to up-regulate MHC class I and to down-regulate MHC class II on bovine PBMCs.

### 3.5. *Humoral responses to N. caninum GPIs*

Antibodies specific for *T. gondii* GPIs and for *P. falciparum* GPIs have been detected in patients with toxoplasmosis and malaria, respectively [36,37], suggesting that antibodies against *N. caninum* GPIs could be produced by infected animals. GPIs of *N. caninum* were submitted to dot blot as described in the 2.10. method section. By using this immunoblotting approach, antibodies specific for all 10 GPI species were detected in the serum of a mouse infected with tachyzoites of *N. caninum* (Fig. 6A), but not in the serum of a non-infected mouse (Fig. 6B). GPI6 showed a weak reaction, whereas GPI10 showed the strongest one. Since GPI10 is less expressed than GPI6 (Fig. 1A), its higher antigenicity could be related to specific structure.

## 4. Discussion

*N. caninum* is known to regulate TLR2 expression and host immune responses to infection are dependent on this TLR [28]. *N. caninum* soluble antigens enhanced the percentage of TLR2 positive peritoneal murine macrophages and BMDCs, while tachyzoites induced the upregulation of TLR11 expression of peritoneal murine macrophages [28,38]. TLR2 is involved in host resistance to *N. caninum*. Indeed, TLR2 deficiency did not lead to the death of infected mice but parasite loads were higher than in wild type mice [28]. Association of TLR variants with viral and bacterial diseases have been identified in cattle [39] and the risk of neosporosis was associated to *tlr4* genetic variants in dogs from Kenyan villages [40]. Our results argue for a TLR4 signalling triggered by *N. caninum* GPIs and TLR4 polymorphism could be one hypothesis to explain why some bovine cells did not respond to GPIs. About 70 % of IL-12p40 production by infected mouse macrophages were attributed to signalling through TLR11 and extracellular signal-regulated protein kinases 1/2 phosphorylation [38]. However, production of other cytokines could be attributed to TLR2 and TLR4. It would be interesting to study infection of TLR4-deficient mice to determine its role in the host responses to the pathogen. As for *T. gondii* [22], we hypothesize that some GPIs of *N. caninum* are expressed free at the parasite surface. This could permit the direct binding of their glycan moiety to TLR on host cells. The other possibility is the secretion of entire GPIs by tachyzoites, since we demonstrate for the first time the presence of GPIs in culture supernatants. Promastigotes of *Leishmania* sp. released GPI-related structures (lipophosphoglycan and hydrophilic phosphoglycan) into the culture medium possibly by release of micelles from the cell surface [41]. In spleen of mice

infected with *N. caninum*, intracellular IL-12 was detected in infected DCs but also in bystander cells [28], suggesting a paracrine effect of secreted molecules that could be GPIs.

In this report, we have shown that GPIs of *N. caninum* induce the production of different cytokines by murine macrophages and DCs. Similar observations were made with the entire parasite and antigen extracts for IL-12, TNF-α and IL-10 [28,42,43]. In general, whole *N. caninum* tachyzoites were more effective than antigen preparations in inducing IL-12, TNF-α and IFN-γ by murine DCs and splenocytes [44]. Despite the production of the p40 subunit common to IL-12 and IL-23, the production of this last cytokine by murine macrophages was not increased in response to GPIs. Mineo *et al*. demonstrated a protective role of IL-12, but could not detect IL-17 neither in the peritoneum nor in the serum of mice infected with *N. caninum*, and concluded that Th17 responses do not play a role in this infection [45]. However, IL-17A was produced directly by infected bovine fibroblasts or indirectly by bovine $CD4^+$ T cells co-cultured with *N. caninum*-infected macrophages [46,47].

In contrast to murine cells, IL-12p40 production by bovine PBMCs was reduced in the presence of *N. caninum* GPIs. This could be due to differences in the cell populations in peripheral blood of cows and in murine BMDCs. We could also hypothesise that IL-10 produced by bovine and not by murine cells is responsible for the down-regulation of IL-12. An important point is that cells from all cows did not respond to GPI stimulation. Similarly, PBMCs of only one to four calves have been able to produce IFN-γ in response to *N. caninum* antigens [48]. Bovine $CD4^+$ T cells co-cultured with macrophages primed with LPS produced greater levels of IFN-γ than T cells co-cultured with non-primed macrophages [46]. In our study, cows were not infected with *N. caninum* or *T. gondii*. However, asymptomatic infection with other pathogen(s) than those routinely diagnosed could be responsible for higher cell response to GPIs.

A marked upregulation of MHC molecules of class II was observed on the surface of spleen DCs 12 h and 2 days after intraperitoneal infection of mice with *N. caninum* tachyzoites [49]. *In vitro*, differences were once again observed depending on the cell type used. Viable parasites down-regulated the expression of MHC molecules of class II on murine DCs, but up-regulated this expression at the surface of murine macrophages, while antigens up-regulated MHC II expression on both cells [43]. Here we show that *N. caninum* GPIs decreased expression of MHC class I on murine DCs, but had no significant effect on MHC class II expression. Thus, it is unlikely that GPIs are responsible for the up-regulation observed with antigen extracts. Furthermore, GPIs are insoluble and absent from preparations of soluble antigens. Boysen *et al*. suggested that NK cells did not produce IFN-γ in response to *N. caninum* antigen preparation because of the absence of GPIs [12]. On bovine cells, GPIs acted differently than on murine cells with a tendency to up-regulate MHC class I, but to down-regulate MHC class II.

Discrepancy between all results obtained with murine and bovine cells might be due to co-evolution of the parasite with cattle, its natural intermediate host. For this reason, it is essential to work on cells of target species like cattle to study immune responses to *N. caninum*.

Intraperitoneal inoculation of *N. caninum* tachyzoites into mice induced an increase in the numbers of Ig-secreting splenic B cells and a rise in serum levels of *N. caninum*-specific IgG2a and IgM isotypes [31]. Optimal production of IgG specific for soluble antigens of *N. caninum* required the expression of TLR2 by mice [28], suggesting a role of TLR2 ligands in adaptive immune responses. It is supposed that antibodies play a role in neutralizing cell invasion by tachyzoites, hypothesis strengthened by parasite opsonisation observed with specific anti-*Neospora* IgG from serum of immunized mice [50–52]. In an epidemiological study on malaria, about 75 % of all individuals older than 7 years had long-lived IgG specific to *P. falciparum* GPIs [37]. In contrast, only 10 % of the children under 2 years exhibited a persistent anti-GPI antibody response, associated with lower febrile illness and higher haemoglobin levels. These results suggest that anti-GPI antibodies neutralize the toxic effects of GPIs. *Plasmodium*-

induced TNF-α production was neutralized by specific anti-GPI antibodies, supporting this hypothesize [53]. Antibodies directed against *N. caninum* GPIs found in the serum of infected mouse might be important in the global resistance mechanisms against neosporosis. Immunization with GPIs of *P. falciparum* and *T. brucei* did not provide sterilizing, but "anti-disease" vaccine by reducing symptoms of malaria and trypanosomiasis, respectively [53,54]. Thus, GPIs of *N. caninum* could be tested as specific adjuvant in a vaccine strategy against neosporosis.

It has been shown that the GPI anchoring NcSRS2 has the core glycan composition obtained from the peak IV, characterized by the conserved trimannosyl core glycan unmodified by additional hydrophilic substituents and an inositol modified by a fatty acid to give the proposed structure: ethanolamine – phosphate – mannose – mannose – mannose – glucosamine – acyl – phosphatidylinositol [19]. GPIs from peaks III to VII also possess a modified inositol structure and all GPIs carry only ester-linked fatty-acids as their hydrophobic components [19]. The present study shows that all GPIs did not induce same cellular and humoral responses. Thus, extensive analyses (chemical and exoglycosidase treatments in combination with high pH anion exchange chromatography, gel-filtration and lectin affinity chromatography, gas chromatography-mass spectroscopy and negative ion electrospray mass spectrometry) will be necessary to elucidate their carbohydrate and lipid composition and to establish structure-activity relationships.


## Acknowledgments

The W6/32 hybridoma was kindly provided by François Lemonnier from the Cochin Institute in Paris.

## Funding

This work was supported by the Deutsche Forschungsgemeinschaft (to RTS), the Institute for Virology of Marburg (to RTS), the University of Tours (to FDG and IDP), the INRA (to NM), the Region Centre Val de Loire (to HD) and the bilateral programme PHC PROCOPE 33324XA of EGIDE/DAAD (to FDG and RTS).


## Conflict of interest

The authors declared that they have no conflicts of interest.

**Author Contributions**: All authors have made substantial contributions to (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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## Legends to figures

Fig. 1. Comparison of GPI profiles from *N. caninum* tachyzoites in Vero cells and HFF. *N. caninum* GPIs were labelled with D-[6-$^{3}$H]-glucosamine during their biosynthesis by tachyzoites cultivated in Vero cells (white with black line profile) or in HFF (grey profile). Glycolipids were extracted from tachyzoites (A) by organic solvents or from culture supernatant (B) by partitioning as described in the 2.1. method section. GPIs were separated by TLC and chromatograms were scanned for radioactivity using a Berthold linear analyser (cpm: counts per minute). Roman peak numbers are from Schares *et al.*[ 20].

Fig. 2. TLR signalling and modulation of cellular responses of murine cell lines by *N. caninum* GPIs. (A) HEK-Blue$^{TM}$ hTLR2 or hTLR4 cells were stimulated for 24 h with GPIs purified from $10^8$ tachyzoites of *N. caninum* or with dried solvent *n*-butyl-alcohol (But) as negative control. Alkaline phosphatase activity of the reporter gene was quantified in supernatant by addition of QUANTI-Blue$^{TM}$ detection medium and measurement at 630 nm. Results are expressed in optical density as means + standard deviation (SD) with n = 2 (from 1 experiment). [* $p < 0.05$ (Sidak's multiple comparison test)]. RAW 264.7 macrophages (B) and SRDC dendritic cells (C-F) were stimulated for 24 h with the 10 individual GPIs (G1 to G10) purified from 3 x $10^8$ tachyzoites of *N. caninum* or with dried solvent *n*-butyl-alcohol (But) as negative control in triplicate. TNF-α was quantified by sandwich ELISA in supernatant of RAW 264.7 cell culture (B). Results are expressed in pg/mL as means + SD. Results are representative of 5 independent experiments. Expression and median of fluorescence intensity (MFI) of MHC molecules of classes I (C, D) and II (E, F) were measured at the surface of SRDC by flow cytometry after labelling with specific antibodies. Results are expressed as percentage relatively to the control condition (But = 100 %) with n = 3 pooled from 3 independent experiments. [* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Dunnett's multiple comparison test)].

Fig. 3. Modulation of cellular responses of murine primary cells by *N. caninum* GPIs. BMDCs (A, B) or PECs (C) were stimulated for 24 h with the ten individual GPIs (G1 to G10) purified from 3 x $10^8$ tachyzoites of *N. caninum* or with dried solvent *n*-butyl-alcohol (But) as negative control. IL-1β (A) and IL-12p40 (black columns)/IL-12p70 (grey columns) (B) levels were quantified by sandwich ELISA in BMDC culture supernatants. Results are expressed in pg/mL as means + SD with n = 2 combined from 2 different mice. Apoptosis was evaluated by flow cytometry and results are expressed in percentage of annexin V-FITC$^{+}$/PI$^{-}$ cells as means + SD with n = 4 combined from 4 different mice. [** $p < 0.005$, *** $p < 0.001$ (Dunnett's multiple comparison test)].

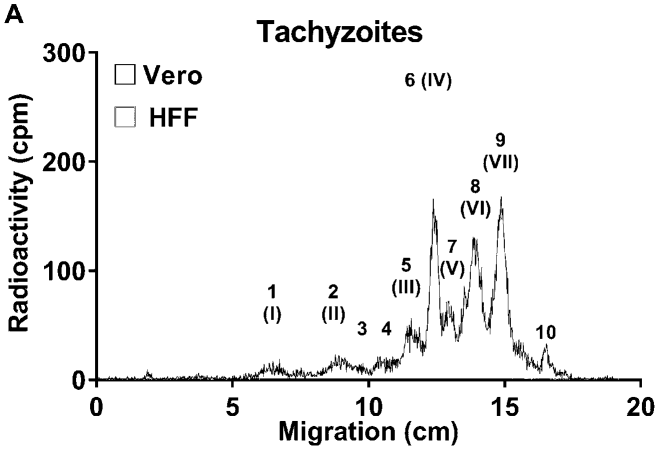
Fig. 4. Modulation of cytokine production by bovine primary cells stimulated by *N. caninum* GPIs. Bovine PBMCs (A, B) or splenocytes (C) were stimulated for 24 h with the 10 individual GPIs (G1 to G10) purified from 3 x $10^8$ tachyzoites of *N. caninum* or with dried solvent *n*-butyl-

alcohol (But) as negative control in duplicate. IL-10 (A), IL-12p40 (B) and IFN-γ (C) levels were quantified by sandwich ELISA in cell culture supernatants. Results are expressed in pg/mL as means + SD with n = 2 to 4 combined from 2 to 4 different cows. [* $p < 0.05$, *** $p < 0.001$ (Dunnett's multiple comparison test)].

Fig. 5. Modulation of MHC expression of bovine primary cells by *N. caninum* GPIs. Bovine PBMCs were stimulated for 24 h with GPI10 purified from 3 x $10^8$ tachyzoites of *N. caninum* or with dried solvent *n*-butyl-alcohol (But) as negative control. Expression and mean of fluorescence intensity (MFI) of MHC molecules of classes I (A, B) and II (C, D) were measured at the cell surface by flow cytometry after labelling with specific antibodies. Results are expressed as percentage relatively to the control condition (But = 100 %) with n = 6 combined from 6 different cows.

Fig. 6. Humoral responses to *N. caninum* GPIs. The 10 individual GPIs (G1 to G10) purified from 3 x $10^8$ tachyzoites of *N. caninum* or the corresponding volume of *n*-butyl-alcohol (But) as negative control were spotted onto nitrocellulose membranes and incubated with serum of a mouse infected with *N. caninum* tachyzoites (A) or with serum of a non-infected mouse (B). The presence of specific anti-GPI antibodies was detected by using anti-mouse IgG conjugated to alkaline phosphatase followed by incubation with a precipitating substrate of the enzyme giving coloured spots.

Fig. 1 (single-column)



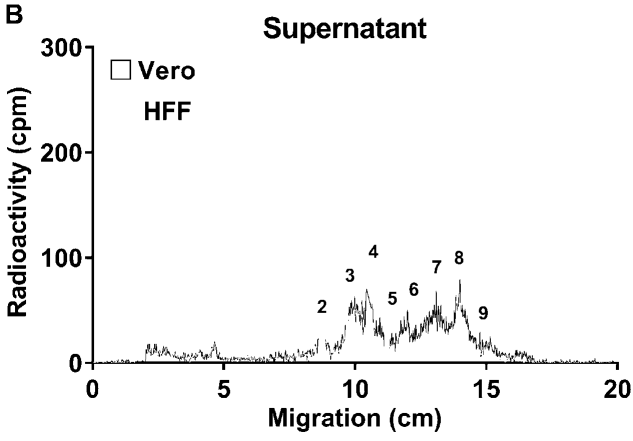
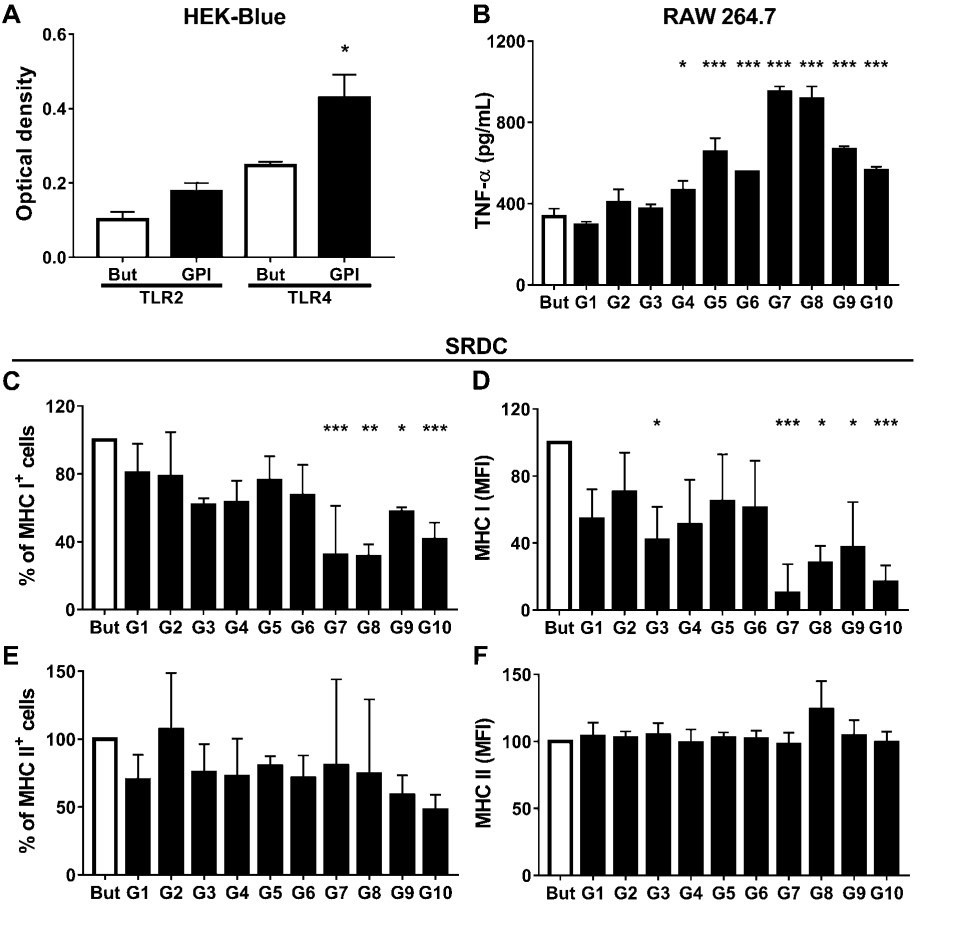



Fig. 2 (two-column)



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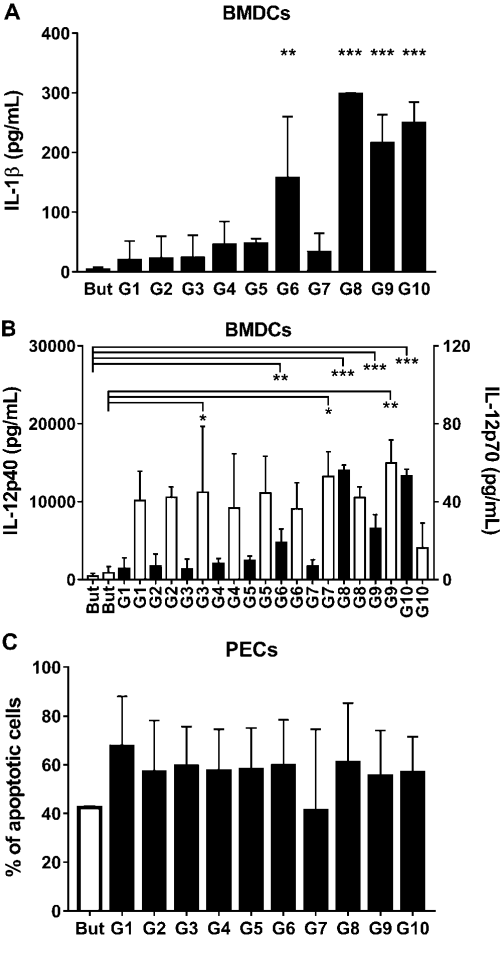

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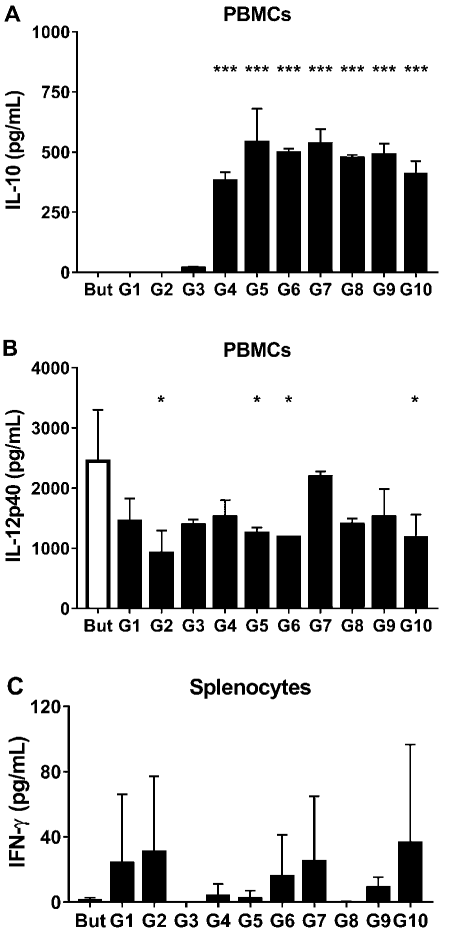


Fig. 5 (1.5-column)

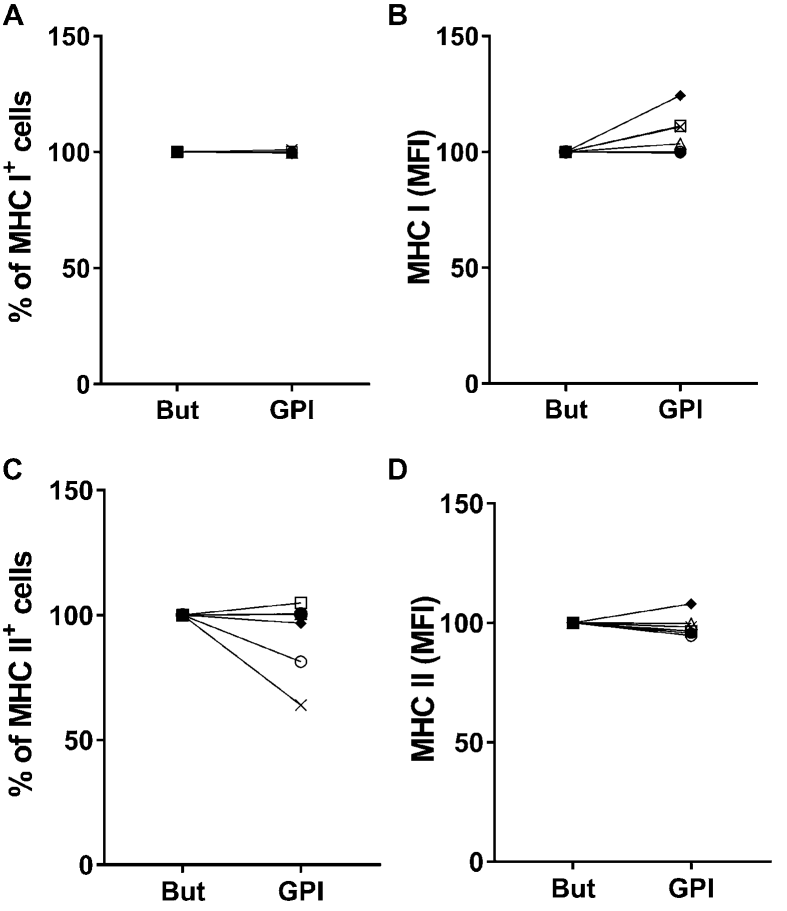
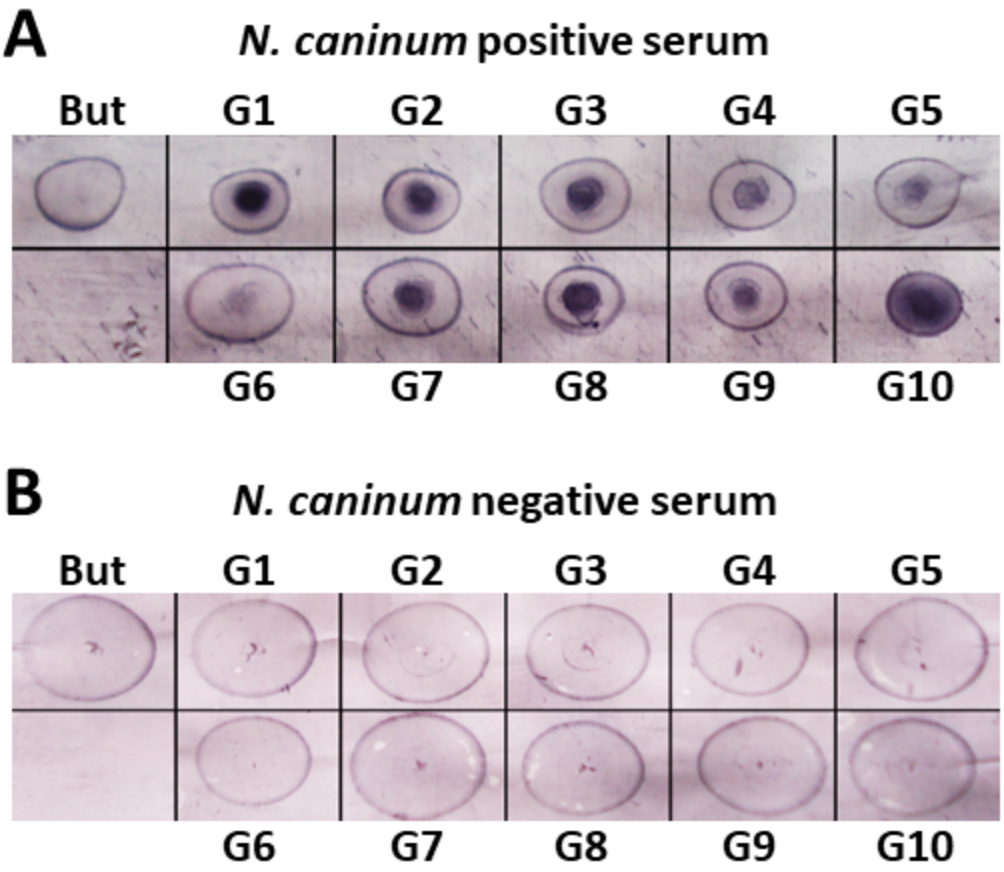
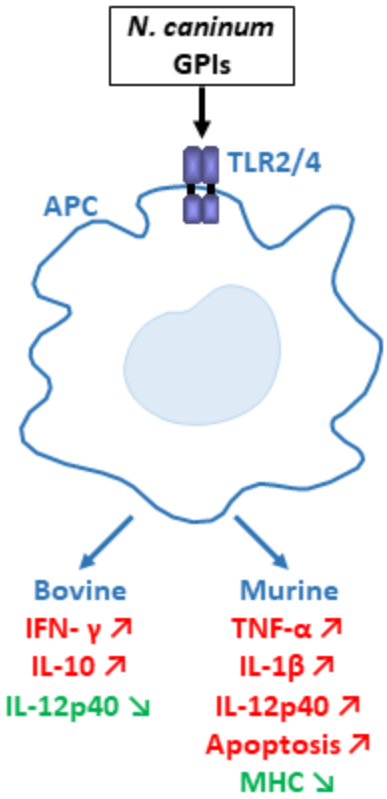


Fig. 6 single-column




N. caninum
GPIs
TLR2/4
APC
Bovine
IFN- γ ↗
IL-10 ↗
IL-12p40 ↘
Murine
TNF-α ↗
IL-1β ↗
IL-12p40 ↗
Apoptosis ↗
MHC ↘