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Neospora caninum glycosylphosphatidylinositols used as adjuvants modulate cellular immune responses induced *in vitro* by a nanoparticle-based vaccine.

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Running title: *Adjuvant effects of Neospora GPIs in vitro*

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ABSTRACT

Neospora caninum causes abortion in ruminants, leading to important economic losses and no efficient treatment or vaccine against neosporosis is available. Considering the complexity of the strategies developed by intracellular apicomplexan parasites to escape immune system, future vaccine formulations should associate the largest panel of antigens and adjuvants able to better stimulate immune responses than natural infection. A mucosal vaccine, constituted of di-palmitoyl phosphatidyl glycerol-loaded nanoparticles (DGNP) and total extract (TE) of soluble antigens of *Toxoplasma gondii*, has demonstrated its efficacy, decreasing drastically the parasite burden. Here, DGNP were loaded with *N. caninum* TE and glycosylphosphatidylinositol (GPI) of *N. caninum* as Toll-like receptor (TLR) adjuvant able to induce specific cellular and humoral immune responses. Activation of TLR2 and TLR4 signalling pathway in HEK reporter cells induced by GPI was abrogated after its incorporation into DGNP. However, in murine bone marrow-derived dendritic cells, an adjuvant effect of GPI was observed with higher levels of interleukin (IL)-1β, reduced levels of IL-6, IL-12p40 and IL-10, and decreased expression of major histocompatibility complex (MHC) molecules. GPI also modulated the responses of bovine peripheral blood mononuclear cells, by increasing the production of IFN-γ and by decreasing the expression of MHC molecules. Altogether, these results suggest that GPI delivered by the DGNP might modulate cell responses through the activation of an intracellular pathway of signalisation in a TLR-independent manner. *In vivo* experiments are needed to confirm the potent adjuvant properties of *N. caninum* GPI in a vaccine strategy against neosporosis.

Keywords: Neospora caninum, vaccine, adjuvant, nanoparticle, glycosylphosphatidylinositol, antigen presenting cells, TLR, cytokine.

Abbreviations

BMDCs, bone marrow-derived dendritic cells; BSA, bovine serum albumin; DGNP, Di-palmitoyl phosphatidyl glycerol-loaded nanoparticles; 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 fibroblast; IFN, interferon; Ig, immunoglobulin; IL, interleukin; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RPMI, Roswell Park Memorial Institute; SEM, standard error of the mean; SEAP, secreted embryonic alkaline phosphatase; TE, total extract; TLC, thin layer chromatography; TLR, Toll-like receptor, TNF, tumour necrosis factor.

1. Introduction

Neosporosis, due to *Neospora caninum*, is an infectious disease responsible for abortion and infertility in cattle worldwide [1]. Cattle get naturally infected by ingestion of *N. caninum* oocysts released within dog faeces and congenital transmission occurs from an infected heifer to the foetus when parasites cross the placental barrier [2]. As *N. caninum* is widely distributed and largely sub-diagnosed, the financial losses due to neosporosis are certainly under-estimated [3]. There is no efficient treatment or vaccine available to fight this infectious disease. There is accumulating evidence that *N. caninum*-infected animals can develop a degree of protective immunity against abortion and/or congenital transmission, indicating that immunoprophylaxis is a feasible goal [4]. Furthermore, a recent predictive study suggested that vaccination is the most economical option compared to medication, selective breeding and test-and-cull in farms with prevalence ≥ 30% [5]. Many vaccines have been tested in mouse and ruminant models of chronic or congenital neosporosis [4,6]. Vaccines based on live attenuated parasites are the most protective, but they are expensive and difficult to manufacture, require a cold storage, have a short shelf life and are associated with safety concerns related to reversion to pathogenic form or recombination with wild strains. Subunit vaccines constituted of recombinant proteins of *N. caninum* should be safe, but they were partially protective in terms of parasite burden reduction or vertical transmission [4,6]. Optimal progress towards novel vaccines against intracellular apicomplexan parasites will depend on the selection of appropriate parasite antigens and their optimal presentation to the immune system. We demonstrated earlier that a lysate of soluble antigens of *T. gondii* loaded into nanoparticles made of maltodextrin and phospholipids (di-palmitoyl phosphatidyl glycerol-loaded nanoparticles, DGNP) induced protective immunity after nasal administration. This formulation led to 100% survival of mice challenged with a lethal dose of *T. gondii* and to almost 90% reduction in parasite load in offspring born to immunized mice infected during pregnancy [7,8]. Both, Th1 and Th17 immune responses developed in splenocytes of immunized mice. This vaccine has been also administered to sheep, in which toxoplasmosis causes abortion. No cerebral cyst could be microscopically detected 2 months after infection with *T. gondii* of ewes immunized by intranasal route [9]. Furthermore, cerebral cysts were present in only 12% (1/8) of the lambs born to immunized ewes infected 3 weeks before delivery versus 55% (5/9) of the lambs born to non-vaccinated infected ewes [9].

In cattle, repeated vertical transmission of *N. caninum* to foetus can occur due to the reactivation of tissue cysts. For this reason, a vaccine against neosporosis should induce protective cellular immune responses leading to the complete elimination of the parasite in case of infection of heifers. To this end, the use of a specific adjuvant might be decisive. Toll-like receptors (TLRs) are expressed on numerous cells of the immune system and TLR agonists are promising adjuvants in farm animals as demonstrated with TLR4 and TLR7/8 ligands in a vaccine against *Mycobacterium bovis* infection in cattle [10]. TLR2 activation has been demonstrated to be promising as a strategy to induce balanced immune responses important for pathogens as *N. caninum* [11]. Indeed, even Th1 inflammatory cytokines like interferon(IFN)-γ and interleukin(IL)-12 are key mediators in a protective immune response against *N. caninum* [12,13], the regulatory cytokine IL-10 is required to down-regulate inflammatory and avoid damages [14]. Importance of Th2 anti-inflammatory cytokines has been also raised. In sheep naturally infected with *N. caninum*, increased expression of both Th1 (TNF-α, IL-2 and IL-18) and Th2 (IL-4, IL-10) cytokines was detected in the placenta, but the occurrence of foetal death was associated with a positive IFN-γ/IL-4 ratio [15]. On the contrary, a lower IFN-γ/IL-4 ratio was found in sheep carrying viable foetuses, in which parasites were vertically transmitted [15]. In heifers experimentally infected at 110 days of gestation, the low-

virulence isolate Nc-Spain1H did not modify the balance of IFN-γ/IL-4 transcript expression compared to non-infected animals, whereas the high-virulence Nc-Spain7 isolate induced a predominant Th2 response in the caruncle (maternal side of the placenta) at 10 days post-infection, allowing parasite multiplication, and a Th1 bias in caruncle and cotyledon (foetal side of the placenta) at 20 days post-infection, contributing to the placental damage [16].

We have previously shown that glycosylphosphatidylinositols (GPIs) of *N. caninum* triggered the signalisation pathway of TLR2 and TLR4, and induced the production of IL-1β, IL-12 and TNF-α by mouse macrophages or dendritic cells and of IFN-γ and IL-10 by bovine splenocytes or peripheral blood mononuclear cells (PBMCs) *in vitro* [17]. Furthermore, mice infected with *N. caninum* produced anti-GPI antibodies that could participate in the resistance to the pathogen [17]. Thus, GPIs induce both cellular and humoral immune responses and represent good candidates as adjuvant specific for *N. caninum*. In this work, we evaluated whether *N. caninum* GPIs could modulate cellular responses when incorporated together with all soluble antigens of *N. caninum* into DGNP.

2. Materials and Methods

2.1. Preparation of the vaccine formulations

2.1.1. Preparation of total extract (TE) of soluble antigens of *N. caninum*

Tachyzoites of the *N. caninum* strain NC-1 (ATCC® 50843™) were cultivated in human foreskin fibroblasts (HFF, ATCC® SCRC-1041™) grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% foetal calf serum (FCS). After intracellular multiplication, cells were collected and tachyzoites were purified from cell debris by glass wool filtration as described [17]. Extract of total soluble antigens was obtained by disruption of tachyzoites in ultra-pure water by sonication (ultrasound bath 46 KHz, Bioblock Scientific). After centrifugation at 2000 x *g* for 20 min at 4°C, protein concentration was measured by a protein assay reagent kit (Pierce). TE was stored at -20 °C until use.

2.1.2. Purification of *N. caninum* GPIs

GPIs of *N. caninum* were extracted as previously described [17]. In brief, tachyzoite GPIs were extracted with chloroform-methanol (10:10, v:v) and chloroform-methanol-water (10:10:3, v:v:v) 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 separated by TLC on silica plates using a chloroform-methanol-water (10:10:3, v:v:v) solvent system, with spots of GPIs previously labelled with D-[6-^{3}H]-glucosamine hydrochloride used as tracers. 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 was checked with the Pierce® *Limulus* Amebocyte Lysate Chromogenic Endotoxin Quantitation kit according to the manufacturer's instructions (Thermo Fisher Scientific).

2.1.3. DGNP loading with *N. caninum* antigens

The formulations DGNP/TE, DGNP/GPI and DGNP/TE/GPI were obtained by mixing DGNP (10 or 30 µg/mL [18]) with TE (10 µg/mL), GPI (purified from 10^{8} tachyzoites in 1 µL *n*-butyl alcohol) or TE plus GPI, respectively. As negative control, the formulation DGNP/But was obtained by mixing DGNP with *n*-butyl alcohol (1 µL), the solvent of GPI.

2.2. Cell culture and stimulation with the vaccine formulations

2.2.1. Murine cells

Macrophages of the RAW 264.7 cell line (ATCC® TIB-71™) were stimulated in 96-well plate at $2x10^5$ cells in a final volume of 200 µL DMEM under different conditions of stimulation (2.2.4. section). After 24-hour incubation at 37 °C in 5% CO_2 atmosphere, supernatants were stored at -20 °C for quantification of cytokines (2.3.1. section).

Population enriched in dendritic cells were obtained by *in vitro* differentiation of bone marrow cells from femurs of 10-week old female OF1 mice (directive 2010/63/EU not applied) in the presence of granulocyte/macrophage-colony stimulating factor (GM-CSF) at 20 ng/mL for 8 days as described previously [17]. Bone marrow-derived dendritic cells (BMDCs) were stimulated for 24 h at 37 °C in 5% CO_2 atmosphere in 24-well plate at $5x10^5$ cells in a final volume of 600 µL Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2% FCS under different conditions of stimulation (2.2.4. section). After centrifugation of floating cells, supernatants were stored at -20 °C for quantification of cytokines (2.3.1. section) and cells were pooled with adherent cells detached by using accutase to study expression of major histocompatibility complex molecules of class II (MHC II) by cytometry (2.3.3. section).

2.2.2. Ruminant cells

All 6-18 month-old Holstein cows (agricultural school of Fondettes, France) and 18 month-old Charmoise ewes (INRAE Val-de-Loire, France) were free of the main communicable diseases (infectious bovine rhinotracheitis, bovine viral diarrhoea, bovine leucosis, brucellosis and tuberculosis). Absence of antibodies specific for *N. caninum* and *T. gondii* was verified by ELISA as described [17].

Bovine blood was collected in 7-mL Vacuette tubes containing 0.017 g EDTA (Greiner Bio-one). PBMCs were obtained from buffy coat after centrifugation onto Ficoll-Paque solution (Sigma-Aldrich) and lysis of erythrocytes as described [17].

Ovine 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.

PBMCs and splenocytes were seeded in 24-well plate at 10^6 cells in a final volume of 600 µL RPMI 1640 supplemented with 2% FCS under different conditions of stimulation (2.2.4. section). The cells were stimulated at 37 °C in 5% CO_2 atmosphere for 24 h and centrifuged, and supernatants were stored at -20°C for quantification of cytokines (2.3.1. section). Expression of MHC II was studied on the cells (2.3.3. section).

2.2.3. Human embryonic kidney (HEK) cells

HEK-Blue™ hTLR2 and hTLR4 cells (InvivoGen) are HEK293 cells co-expressing human TLR2 or TLR4 with secreted embryonic alkaline phosphatase (SEAP) reporter gene. The cells were maintained in DMEM supplemented with 10 % FCS and HEK-Blue™ Selection (InvivoGen). The cells were stimulated at 37 °C in a 5 % CO_2 atmosphere for 24 h and centrifuged. Supernatants were stored at -20°C for quantification of SEAP activity (2.3.2. section).

2.2.4. Conditions of stimulation

Viability of murine, ruminant, and HEK-Blue™ cells (2.2.1.-2.2.3. sections) was checked with trypan blue. The cells were stimulated with medium alone or with DGNP, But, DGNP/But, TE, DGNP/TE, GPI, DGNP/GPI and DGNP/TE/GPI (with 10 µg/mL of DGNP, 10 µg/mL of TE, 1 µL of *n*-butyl alcohol and GPI7 extracted from 10^8 tachyzoites for $2x10^5$ tested cells). Medium specific for each cell type supplemented with 1 % FCS was used for the stimulation. HEK-Blue™ cells were stimulated in absence of HEK-Blue™ Selection.

2.3. Analysis of cellular responses

2.3.1. Cytokine quantification

Cells were stimulated as described in 2.2. section. Levels of mouse cytokines were quantified in the culture supernatants by using TNF-α, IL-1β, IL-6, IL-10, IL-12p40 and IL-12p70 specific sandwich enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Affymetrix eBioscience). Levels of bovine/ovine cytokines were quantified in the culture supernatants of PBMCs and splenocytes by sandwich ELISA using antibodies specific for bovine/ovine IL-10, IL-12p40 and IFN-γ (Bio-Rad Laboratories) and ExtrAvidin®-Peroxidase (Sigma-Aldrich) as previously described [17]. Known concentrations of recombinant IL-10, IL-12p40 and IFN-γ proteins (Kingfisher Biotech, Inc.) permitted to calculate cytokine concentration in samples.

2.3.2. SEAP activity

After stimulation of HEK-Blue™ hTLR2 and hTLR4 cells (section 2.2.4), SEAP reporter gene activity was measured at 630 nm after addition of QUANTI-Blue™ detection medium (InvivoGen) to supernatant following the manufacturer's instructions.

2.3.3. Measurement of MHC expression

After stimulation and recovery by using accutase as described in 2.2. section, BMDCs and PBMCs were centrifuged at 300 x *g* and saturated for 30 min on ice in PBS with 1% bovine serum albumin (PBS-BSA). After centrifugation, 3 x 10^5 BMDCs were incubated for 30 min on ice in the dark in PBS-BSA with 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. After final centrifugation, cells of both types were suspended in 300 µL PBS-2% paraformaldehyde and analysed by flow cytometry using Miltenyi MACSquant operated by FlowLogic 7.2.1 software.

2.3.4. Statistics

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

3. Results

3.1. Adjuvant effect of N. caninum GPI on murine cells

3.1.1. Production of TNF-α by RAW 264.7 macrophages

When stimulated with TE of *N. caninum*, RAW 264.7 macrophages produced significantly higher amounts of TNF-α than with medium alone (Fig. 1A) and TE incorporation into DGNP did not change TNF-α levels. Incorporation of GPI into DGNP almost completely inhibited the GPI-induced TNF-α production (Fig. 1A). Not surprisingly, the DGNP/TE/GPI formulation induced similar TNF-α levels than TE and DGNP/TE. All negative controls (the GPI solvent *n*-butyl alcohol, DGNP alone and *n*-butyl alcohol-loaded DGNP) did not stimulate cells (Fig. 1A). To understand the mechanism of action of antigens on the target cells, TLR signalling was studied in HEK-TLR-Blue reporter cell lines. TE and DGNP/TE slightly increased phosphatase alkaline activity related to TLR2 signalling, but did not induce TLR4 signalling (Fig. 1B and C). GPI of *N. caninum* increased phosphatase alkaline activity in both HEK TLR2 and HEK TLR4 cells, whereas GPI incorporated into DGNP was unable to increase activity, suggesting that GPI was in the core and not at the surface of DGNP. As for TNF-α production by RAW 264.7 macrophages, activity of HEK cells in response to the DGNP/TE/GPI formulation was similar to those of TE and DGNP/TE.

3.1.2. Production of inflammatory and regulatory cytokines by BMDCs

Although GPI had no adjuvant effect on TNF-α production induced by DGNP/TE in RAW 264.7 macrophages, we further studied the adjuvant effect of GPI on murine BMDCs. TE stimulated the cells to produce significant higher levels of IL-6 (Fig. 2C) and IL-12p40 (Fig. 2D). Levels of TNF-α (Fig. 2A), IL-12p40/p70 (Fig. 2D) and of the regulatory cytokine IL-10 were increased but not significantly, and IL-1β was slightly decreased. (Fig. 2B). Delivery of TE by DGNP did not significantly modify the levels of the tested cytokines, except for IL-10 production, which reached a concentration significantly higher compared to medium alone (Fig. 2E). In contrast to the results obtained with RAW 264.7 macrophages, GPI presented a strong adjuvant effect on the cytokine pattern produced by BMDCs. Indeed, DGNP/TE/GPI induced 7-fold higher levels of IL-1β (Fig. 2B) and respectively 6-fold, 1.5-fold and 10-fold lower levels of IL-6 (Fig. 3C), IL-12p40 (Fig. 2D) and IL-10 (Fig. 2E) compared to DGNP/TE.

3.1.3. Expression of MHC II by BMDCs

Expression of MHC II was measured on murine BMDCs. In our conditions, TE of *N. caninum* did not affect MHC II expression, neither the percentage of positive cells nor the intensity of expression measured by flow cytometry (Fig. 3). When TE was delivered by DGNP, it slightly enhanced the intensity of expression, but not the percentage of expressing cells. *N. caninum* GPI has the tendency to counteract this last effect (Fig. 3A) and drastically diminished the intensity of MHC II expression (Fig. 3B).

3.2. Adjuvant effect of N. caninum GPI on bovine/ovine cells

3.2.1. Production of inflammatory and regulatory cytokines by bovine/ovine cells

Although mouse is the animal model used to develop strategies against neosporosis, the target species of an anti-neosporosis vaccine are farm ruminants. It is thus important to test the potent adjuvant effect of *N. caninum* GPI on cells isolated from toxoplasmosis and neosporosis seronegative cows and ewes. Cellular responses were highly variable between individuals and PBMCs from only 3 out of 9 cows were able to produce cytokines after stimulation *in vitro*. TE increased the production of IFN-γ and IL-10 in both bovine PBMCs and ovine splenocytes (Fig. 4) and its incorporation into DGNP led to a slight increase in IL-1β production by bovine PBMCs (Fig. 4A) and in IFN-γ levels by both bovine PBMCs (Fig. 4B) and ovine splenocytes (Fig. 4D). *N. caninum* GPI increased the production of IL-1β (Fig. 4A) and IFN-γ (Fig. 4B) production by bovine PBMCs compared to the condition DGNP/TE, but the differences were not statistically different due to the high variability. GPI did not modulate the production of IL-10 (Fig. 4C) by bovine cells and of IFN-γ (Fig. 4D) and IL-10 (Fig. 4E) by ovine cells.

3.2.2. Expression of MHC II by bovine cells

Expression of MHC II was measured on bovine PBMCs. The percentage of MHC II positive cells decreased after stimulation with TE, DGNP/TE and DGNP/TE/GPI, but the differences were not significant (Fig. 5A). In contrast, TE decreased the intensity of expression of MHC II only when it was incorporated into DGNP (Fig. 5B). Contrary to the adjuvant effect of *N. caninum* GPI on the intensity of expression of MHC II by murine BMDCs (Fig. 5B), GPI did not affect MHC II intensity of expression compared to DGNP/TE (Fig. 5B).

Altogether, these results show that *N. caninum* GPI added to DGNP/TE has a marked adjuvant effect on cytokine production and MHC II expression by primary murine cells, but a more modest adjuvant effect on primary ruminant cells *in vitro*.

4. Discussion

Several studies on immunization with one or more recombinant proteins of *N. caninum* revealed a lack of protection of this strategy in mouse models as well as in cattle target species [4,6]. A vaccine containing a large panel of antigens, closest to the entire parasite, should be more efficient

against neosporosis. *N. caninum* has a tropism for mucosa, from the tachyzoite stage in the digestive system to the bradyzoite stage in the uterus. So, a vaccine delivery system adapted to mucosa would be appropriated to develop both local and systemic immune responses. In this context, a total extract of soluble antigens of *N. caninum* was incorporated into cationic maltodextrin-based porous nanoparticles with anionic lipid core (DGNP), demonstrated to have high affinity for mucosa [19]. A vaccine composed of DGNP loaded with *T. gondii* TE significantly reduced vertical transmission of the parasite in both mice and ewes [8,9]. However, recent comparative studies highlighted more important differences between *T. gondii* and *N. caninum* as previously admitted. These differences take place in their genomes [20], but also in the transcriptomic profiles of infected cells *in vitro* [21]. Furthermore, the use of inert or live vectors as delivery systems of *N. caninum* antigens did not lead to protection against neosporosis [3]. For example, subcutaneous immunization of heifers with a native antigen extract from *N. caninum* tachyzoites formulated with Immune Stimulating COMplexes (ISCOMs) did not protect against vertical transmission when challenge occurred at day 70 of gestation [22].

From the past studies arose the need of immune modulatory adjuvants to reach sterilizing protection. The TLR3 ligand CpG has shown an adjuvant effect to an intranasal vaccine based on membrane proteins extracted from *N. caninum* tachyzoites [23]. Higher titres of IgA were obtained in vaginal and intestinal lavage fluids and the number of mice without detectable parasites increased twice compared with vaccine without adjuvant. However, this vaccine was not tested in a model of congenital neosporosis [23]. In pregnant cattle, soy-lecithin/β-glucan were used as TLR adjuvant of an anti-neosporosis vaccine, but no abortion was recorded in the naive/infected groups of the study and the protection could not be evaluated [24]. It has been shown that TE of *T. gondii* triggered TLR2 and, in a lesser extent, TLR4 signalling in HEK cells, and its incorporation into DGNP did not modify the reporter gene activity [7]. TE of *N. caninum*, free or incorporated into DGNP, triggered only TLR2 signalling, confirming the specificity of antigens. We have previously shown that GPI4 to GPI10 of *N. caninum* stimulated RAW 264.7 murine macrophages to produce TNF-α, with the highest levels obtained with GPI7 and GPI8 [17]. When incorporated into nanoparticles, GPIs were no more able to induce TNF-α production by these cells. Free *N. caninum* GPIs are ligands of TLR2 and TLR4, but their incorporation into DGNP blocked their recognition by TLR2 and TLR4 of HEK cells, explaining the absence of signalling leading to the transcription of the TNF-α gene. So, GPI incorporated into DGNP cannot be considered as a TLR adjuvant. In a study on chicken macrophages, LPS incorporated into poly lactic-*co*-glycolic acid (PLGA) was suspected to activate NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in the cytosol, leading to expression of the *IL-1β* gene [25]. Furthermore, infection of murine BMDCs by *N. caninum* activated NLRP3 inflammasome [26]. Thus, GPI could be suspected to activate inflammasome, leading to the elevated production of IL-1β in BMDCs stimulated by the DGNP/TE/GPI formulation.

In order to develop an efficient vaccine, it is crucial to know which cytokine profile is required for protection against congenital neosporosis. After *N. caninum* infection of cattle, levels of abortifacient Th1 cytokines (IFN-γ, IL-2, IL-12p40 and TNF-α), Th2 cytokines (IL-4 and TGF-β1) and the regulatory cytokine IL-10 increased in the placenta and a much greater increase resulted in foetal death [27]. The majority of the cytokines detected were of maternal origin, but IFN-γ, TNF-α, IL-4 and IL-10 could be expressed either by the foetal trophoblast cells or by infiltrating maternal mononuclear cells [27]. *In vitro*, infection of bovine macrophages with either low- or high-virulence strains led to an increase in IL-12p40 and IL-10 transcript expression compared to non-infected macrophages [28]. In another study, expression of the Th1 cytokines TNF, IL-6 and IL-1β was also upregulated in bovine macrophages infected with both strains [29]. The levels of IL-8 and TNF-α cytokines were increased in the supernatants of both caruncular and trophoblast cell lines infected with *N. caninum*, while the expression of IL-6, IL-12p40 and TGF-β1 transcripts was downregulated. In addition, messengers for IFN-γ, IL-17, IL-4 and IL-10 were not detected [30]. So, the cellular response to infection depends on the cell type. In the present study, DGNP/TE of *N. caninum* induced IL-12p40, TNF-α and IL-10 production by murine dendritic cells and addition of

N. caninum GPI as adjuvant reduced the levels of these cytokines, leading to a profile that could benefit to the foetus survival in case of infection. In contrast, GPI has no or low immunomodulatory effect on bovine PBMCs and ovine splenocytes. Cells of only one third of the animals responded to the stimulation. This lack of reactivity is a frequent phenomenon as observed in a study on proliferation of PBMCs in response to LPS, in which cell count did not increase for 4 out of 9 cows [31]. The variability in cell populations constituting PBMCs is also well known in ruminants [32] and the GPI might have or not an adjuvant effect depending on the individual.

Different adjuvants have been tested in BALB/c mice subcutaneously immunized with recombinant NcSRS2 and the antigen-specific cytokine response has been analysed in splenocytes [33]. The association with water-in-oil, xanthan gum and alum hydroxide induced a significant increase in the transcription of TNF-α, of IL-4, IL-10, IL-12 and of IFN-γ, TNF-α, IL-12, IL-4, IL-10, respectively, while all formulations induced downregulation in IL-17A transcription compared to the group that was inoculated with the protein alone [33]. This study highlighted the variation in the cytokine profiles induced by the adjuvant according to its mechanisms of action. Intranasal vaccine containing *N. caninum* disulphide isomerase emulsified in cholera toxin as mucosal adjuvant protected mice against death due to intraperitoneal challenge with tachyzoites, but did not protect against vertical transmission [34]. The loss of protection in pregnant mice was associated with increased expression of splenic Th1 cytokine and IL-17A transcripts. $CD4^+$ T cells differentiate into $CD4^+$ Th17 cells under the influence of IL-6 and TGF-β [34]. Here, DGNP/TE of *N. caninum* induced IL-6 production by murine dendritic cells and addition of *N. caninum* GPI as adjuvant drastically reduced the production of this cytokine, suggesting that the DGNP/TE/GPI formulation could orientate the cytokine response in favour to protection of the foetus *in vivo*.

It has been established by immunohistochemistry that increased MHC II expression by cells of the placenta and of the central nervous system is related to the local inflammatory response, and particularly IFN-γ expression triggered by *N. caninum* tachyzoites [35,36]. MHC II is a marker of antigen presenting cell maturation, and its expression increased on peritoneal macrophages of *N. caninum*-infected Balb/c mice [37]. In a previous study, when peritoneal macrophages and dendritic cells were incubated *in vitro* with *N. caninum* tachyzoites, MHC II expression increased and decreased, respectively, while MHC II expression increased on both cell types stimulated with an antigen extract [38]. On splenic dendritic cells, an initial increase in MHC II expression at 12 h post-infection was followed by a downregulation 5 days after infection in comparison with dendritic cells from non-infected Balb/c mice [39]. *In vitro* infection of bovine macrophages with low- or high-virulence strains led to a significant reduction in the percentage of cells expressing MHC II [28]. So, the strong down-regulation of MHC II on murine BMDCs and the low down-regulation of MHC II on bovine PBMCs induced by *N. caninum* GPI when associated to DGNP/TE is not predictive of the cell response *in vivo*, but might be in favour of a protective response.

N. caninum GPI has proven an immunomodulatory adjuvant effect on cytokine production and MHC II expression on cells of both mouse and ruminant origin. Thus, it would be interesting to test the adjuvant effect of GPI in animals immunized with DGNP/TE.

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Legends to figures

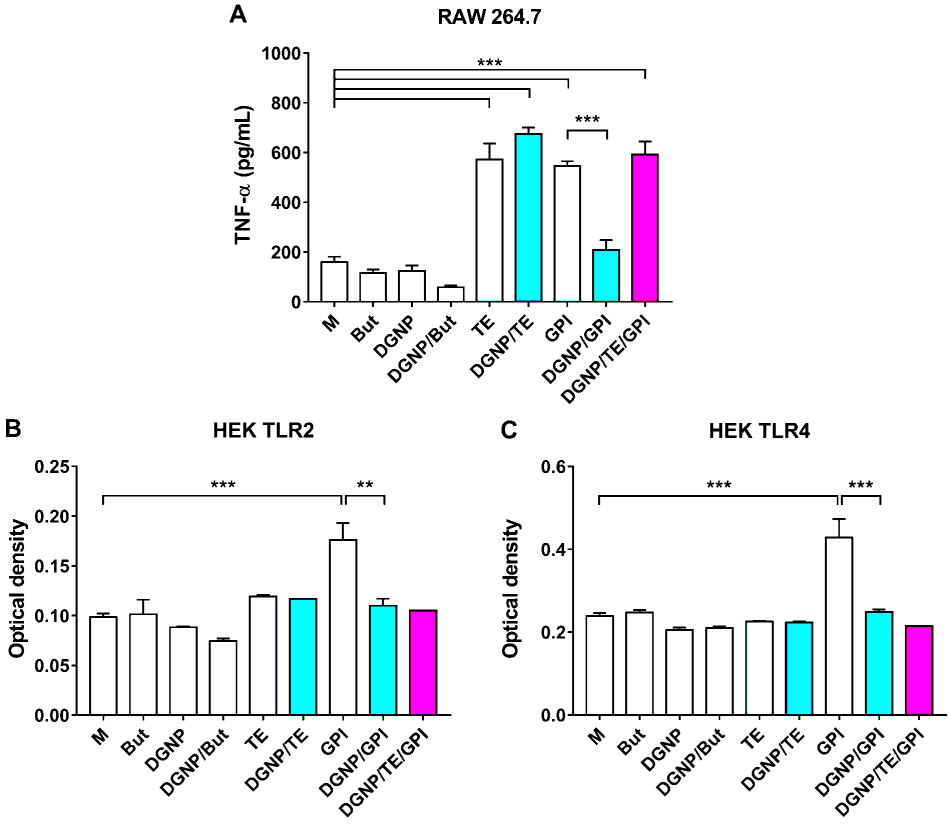
Fig. 1. TNF-α production by RAW 264.7 macrophages and TLR signalling in HEK cells. RAW 264.7 macrophages (A) and HEK-Blue^{TM} hTLR2 (B) or hTLR4 (C) cells were stimulated for 24 h with medium alone (M) or with DGNP (10 µg/mL), *n*-butyl-alcohol solvent (But, 1 µL), DGNP/But (10 µg/mL-1 µL), TE (10 µg/mL), DGNP/TE (10 µg/mL-10 µg/mL), GPI (from 10^8 tachyzoites in 1 µL But), DGNP/GPI (10 µg/mL-10^8 tachyzoites), and DGNP/TE/GPI (10 µg/mL-10 µg/mL-10^8 tachyzoites). (A) TNF-α was quantified by sandwich ELISA in supernatant of RAW 264.7 cell culture. Results are expressed in pg/mL as means + SEM with n = 3 and are representative of 2 independent experiments. (B, C) 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 + SEM with n = 2. ** $p < 0.01$, *** $p < 0.001$ (Sidak's multiple comparison test).

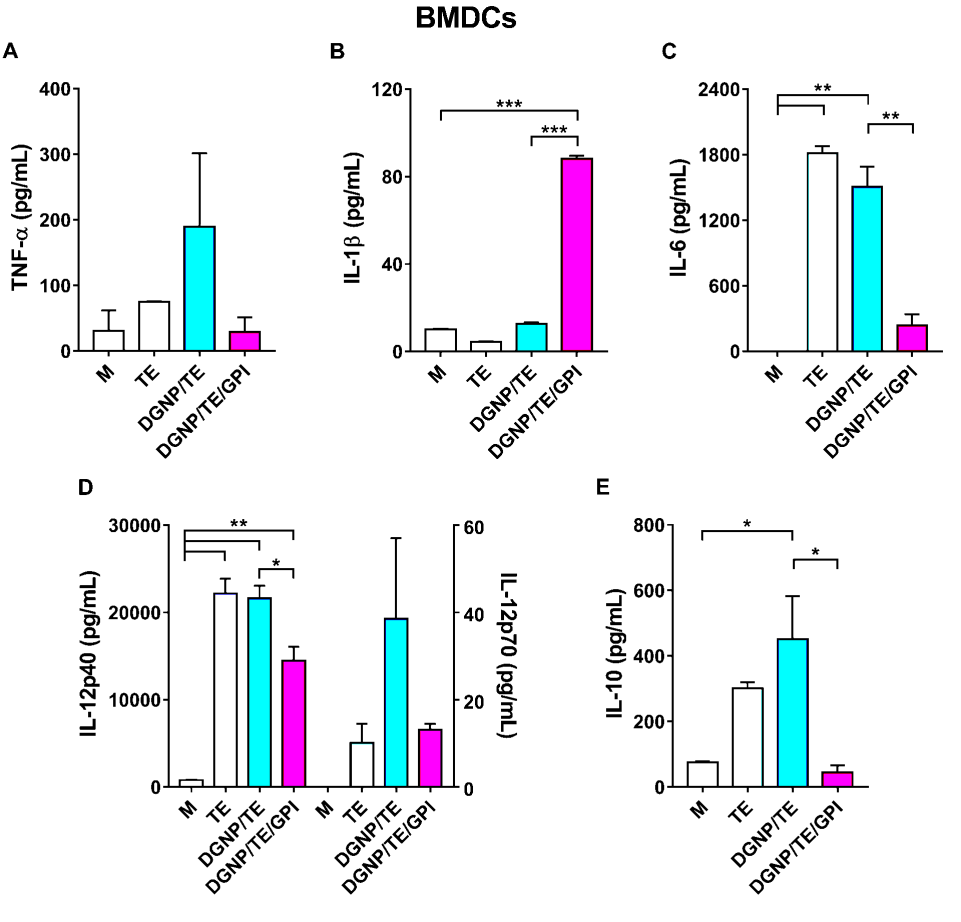
Fig. 2. Adjuvant effect of *N. caninum* GPI on cytokine production by BMDCs. BMDCs were stimulated for 24 h with medium alone (M), TE (10 µg/mL), DGNP/TE (10 µg/mL-10 µg/mL) or DGNP/TE/GPI (10 µg/mL-10 µg/mL-10^8 tachyzoites). TNF-α (A), IL-1β (B), IL-6 (C), IL-12p40 (D), and IL-10 (E) levels were quantified by sandwich ELISA in supernatants of BMDCs. Results are expressed in pg/mL as means + SEM with n = 2 independent mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Holm-Sidak's multiple comparison test).

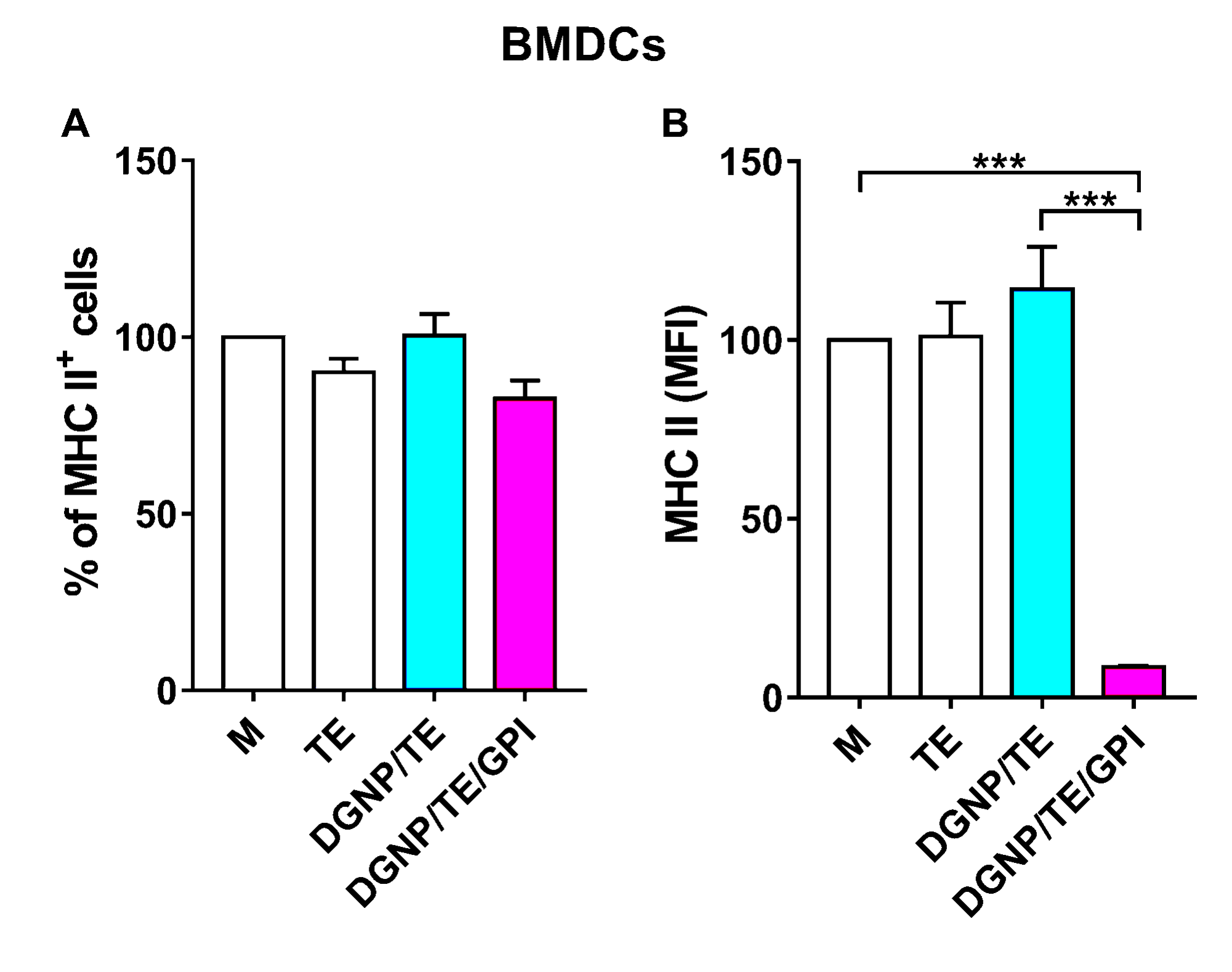
Fig. 3. Adjuvant effect of *N. caninum* GPI on MHC class II expression by BMDCs. BMDCs were stimulated for 24 h with medium alone (M), TE (10 µg/mL), DGNP/TE (10 µg/mL-10 µg/mL) or DGNP/TE/GPI (10 µg/mL-10 µg/mL-10^8 tachyzoites). Expression (A) and median of fluorescence intensity (B) of MHC molecules of class II were measured at the surface of BMDCs by flow cytometry after labelling with specific antibodies. Results are expressed as means + SEM relatively to the control condition (M = 100) with n = 3. *** $p < 0.001$ (Sidak's multiple comparison test).

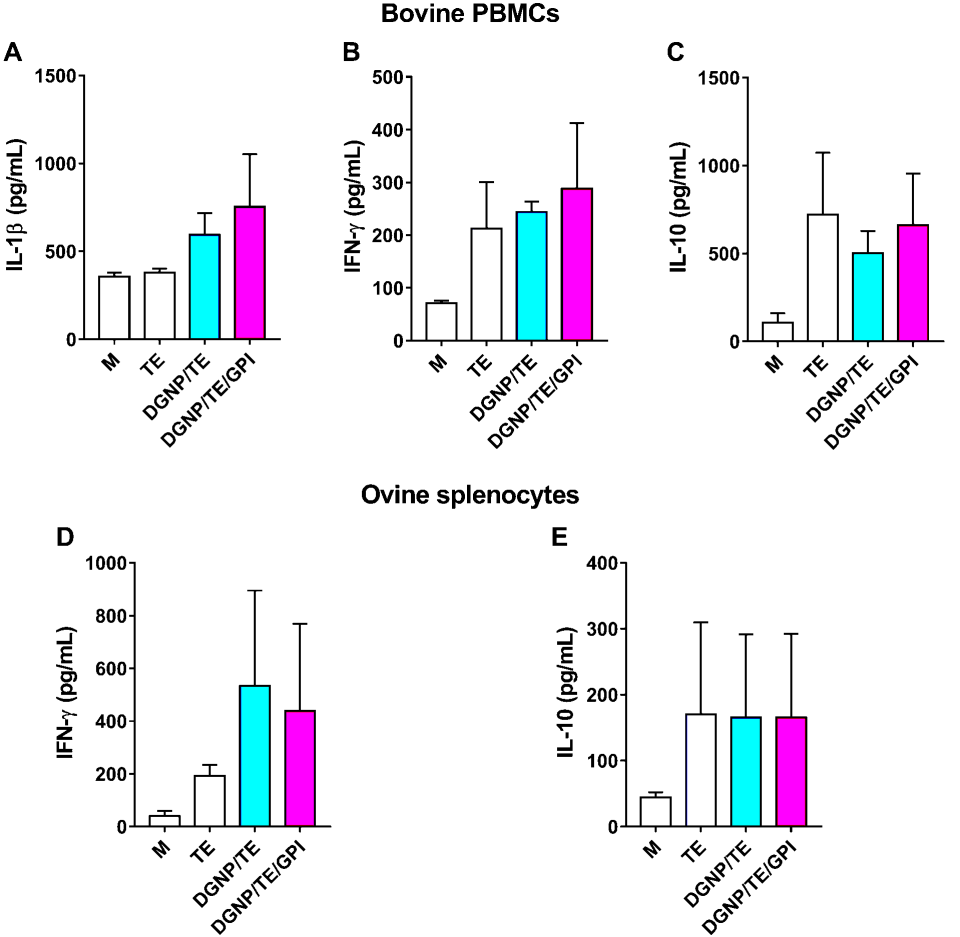
Fig. 4. Adjuvant effect of *N. caninum* GPI on cytokine production by cells isolated from ruminants. Bovine PBMCs (A-C) and ovine splenocytes (D-E) were stimulated for 24 h with medium alone (M) TE (10 µg/mL), DGNP/TE (10 µg/mL-10 µg/mL) or DGNP/TE/GPI (10 µg/mL-10 µg/mL-10^8 tachyzoites). IL-1β (A), IFN-γ (B, D) and IL-10 (C, E) levels were quantified by sandwich ELISA in cell culture supernatants. Results are expressed in pg/mL as means + SEM with n = 3 animals.

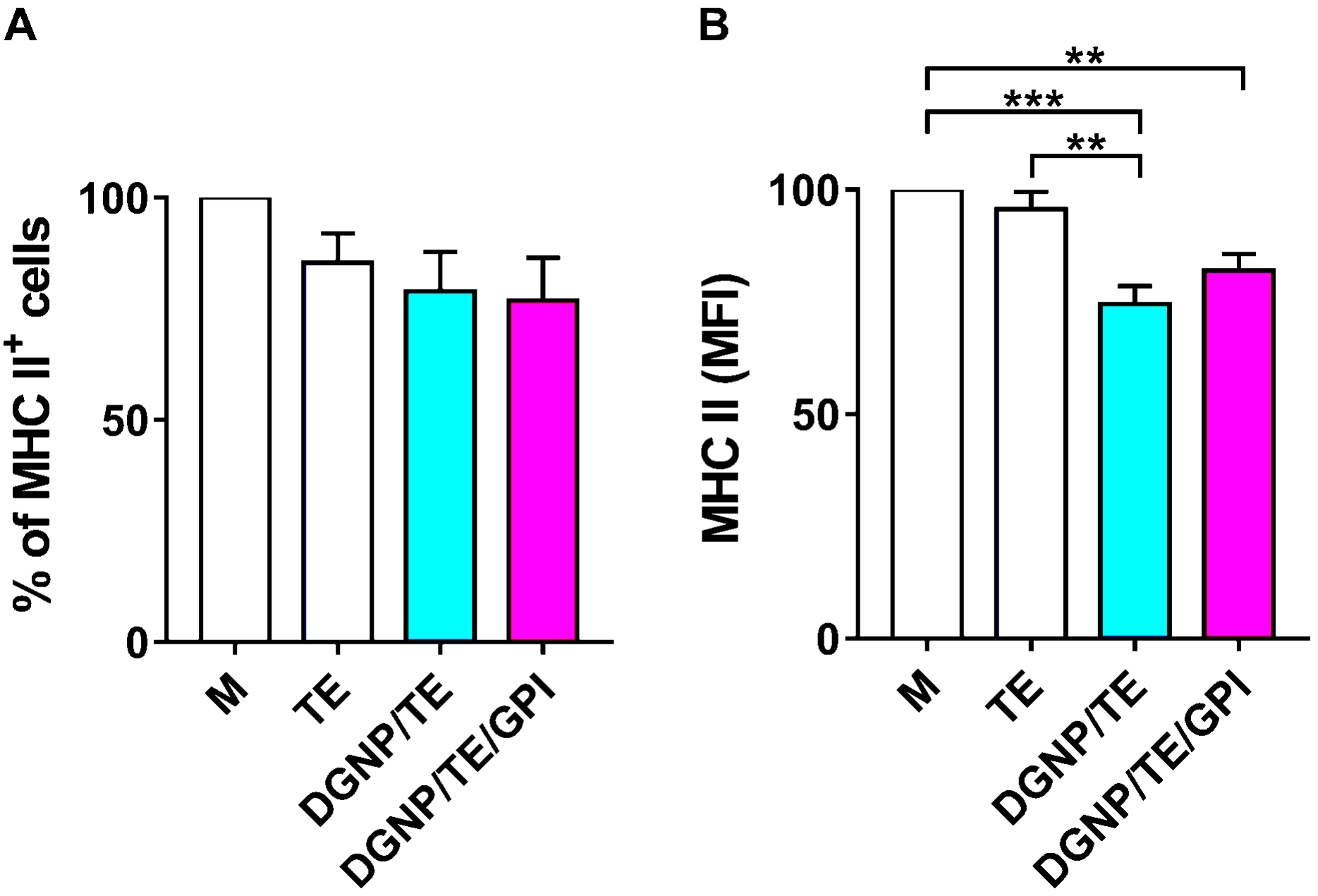
Fig. 5. Adjuvant effect of *N. caninum* GPI on MHC II expression by bovine PBMCs. Bovine PBMCs were stimulated for 24 h with medium alone (M), TE (10 µg/mL), DGNP/TE (10 µg/mL-10 µg/mL) or DGNP/TE/GPI (10 µg/mL-10 µg/mL-10^8 tachyzoites). Expression (A) and median of fluorescence intensity (B) of MHC II were measured at the surface of PBMCs by flow cytometry after labelling with specific antibodies. Results are expressed as means + SEM relatively to the control condition (M = 100) with n = 5 cows. ** $p < 0.01$, *** $p < 0.001$ (Sidak's multiple comparison test).


A
RAW 264.7
TNF-α (pg/mL)
1000
800
600
400
200
0
***
***
M
But
DGNP
DGNP/But
TE
DGNP/TE
GPI
DGNP/GPI
DGNP/TE/GPI
B
HEK TLR2
Optical density
0.25
0.20
0.15
0.10
0.05
0.00
***
**
M
But
DGNP
DGNP/But
TE
DGNP/TE
GPI
DGNP/GPI
DGNP/TE/GPI
C
HEK TLR4
Optical density
0.6
0.4
0.2
0.0
***
***
M
But
DGNP
DGNP/But
TE
DGNP/TE
GPI
DGNP/GPI
DGNP/TE/GPI

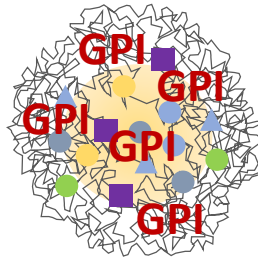

BMDCs
A
TNF-α (pg/mL)
400
300
200
100
0
M
TE
DGNP/TE
DGNP/TE/GPI
B
IL-1β (pg/mL)
120
80
40
0
***
***
C
IL-6 (pg/mL)
2400
1800
1200
600
0
**
**
D
IL-12p40 (pg/mL)
30000
20000
10000
0
IL-12p70 (pg/mL)
60
40
20
0
**
*
E
IL-10 (pg/mL)
800
600
400
200
0
*
*

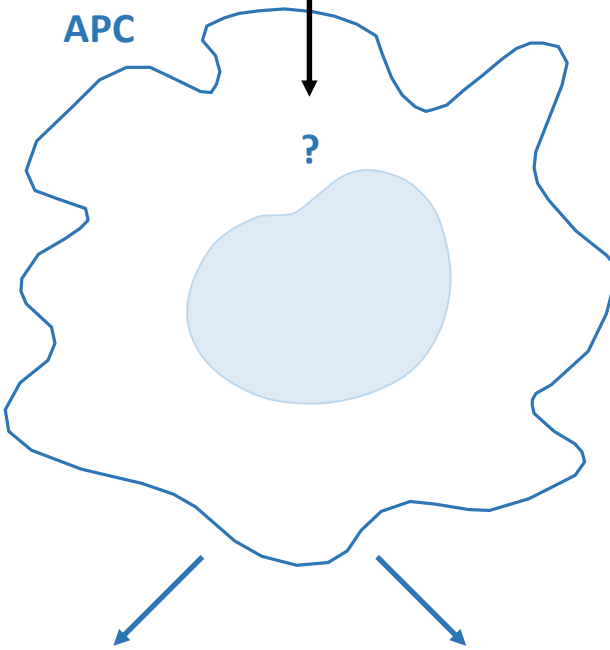

BMDCs
A
% of MHC II+ cells
150
100
50
0
M
TE
DGNP/TE
DGNP/TE/GPI
B
MHC II (MFI)
150
100
50
0
***
***
M
TE
DGNP/TE
DGNP/TE/GPI


Bovine PBMCs
A
IL-1β (pg/mL)
1500
1000
500
0
M
TE
DGNP/TE
DGNP/TE/GPI
B
IFN-γ (pg/mL)
500
400
300
200
100
0
M
TE
DGNP/TE
DGNP/TE/GPI
C
IL-10 (pg/mL)
1500
1000
500
0
M
TE
DGNP/TE
DGNP/TE/GPI
Ovine splenocytes
D
IFN-γ (pg/mL)
1000
800
600
400
200
0
M
TE
DGNP/TE
DGNP/TE/GPI
E
IL-10 (pg/mL)
400
300
200
100
0
M
TE
DGNP/TE
DGNP/TE/GPI


A
% of MHC II+ cells
100
50
0
M
TE
DGNP/TE
DGNP/TE/GPI
B
MHC II (MFI)
**
***
**
100
50
0
M
TE
DGNP/TE
DGNP/TE/GPI

Stimulation with DGNP/TE/GPI





Bovine

IL-1β ↗

IFN- γ ↗

Murine

IL-1β ↗↗

IL-6 ↘↘

TNF-α ↘

IL-12 ↘

IL-10 ↘

MHC ↘↘

compared to stimulation with DGNP/TE