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Vaccination of squirrel monkeys (*Saimiri* spp.) with nanoparticle-based *Toxoplasma gondii* antigens: new hope for captive susceptible species

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ABSTRACT

Squirrel monkeys (*Saimiri* spp.), new world primates from South America, are very susceptible to toxoplasmosis. Numerous outbreaks of fatal toxoplasmosis in zoos have been identified around the world, resulting in acute respiratory distress and sudden death. To date, preventive hygiene measures or available treatments are not able to significantly reduce this mortality in zoos. Therefore, vaccination seems to be the best long-term solution to control acute toxoplasmosis. Recently, we developed a nasal vaccine composed of total extract of soluble proteins of *Toxoplasma gondii* associated with muco-adhesive maltodextrin-nanoparticles. The vaccine, which generated specific cellular immune responses, demonstrated efficacy against toxoplasmosis in murine and ovine experimental models. In collaboration with six French zoos, our vaccine was used as a last resort in 48 squirrel monkeys to prevent toxoplasmosis. The full protocol of vaccination includes two intranasal sprays followed by combined intranasal and s.c. administration. No local or systemic side-effects were observed irrespective of the route of administration. Blood samples were collected to study systemic humoral and cellular immune responses up to 1 year after the last vaccination. Vaccination induced a strong and lasting systemic cellular immune response mediated by specific IFN-γ secretion by peripheral blood mononuclear cells. Since the introduction of vaccination, no deaths of squirrel monkeys due to *T. gondii* has been observed for more than 4 years suggesting the promising usage of our vaccine. Moreover, to explain the high susceptibility of naive squirrel monkeys to toxoplasmosis, their innate immune sensors were investigated. It was observed that Toll-like and Nod-like receptors appear to be functional following *T. gondii* recognition suggesting that the extreme susceptibility to toxoplasmosis may not be linked to innate detection of the parasite.

1. Introduction

Toxoplasma gondii is an intracellular protozoan well known in veterinary medicine and human health. The felids are definitive hosts responsible for oocysts dissemination in the environment leading to infection of intermediate hosts after ingestion of

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contaminated water or vegetables. An increasing number of studies have documented wildlife toxoplasmosis especially in captive species. New World Primates (NWP, Platyrrhini) and more specifically squirrel monkeys (*Saimiri* spp.) exhibit an unusual susceptibility. Since the late 1960s (McKissick et al., 1968), several outbreaks in captive squirrel monkeys due to *T. gondii* infection have been reported worldwide (Inoue, 1997; Cedillo-Peláez et al., 2011; Pardini et al., 2015; Nishimura et al., 2019). Acute toxoplasmosis was responsible for 30% mortality in the squirrel monkey colony of a London zoo (Cunningham et al., 1992). More recently in South Korea, 54% of the colony died in less than a month (Oh et al., 2018). A retrospective study of clinical signs and lesions of toxoplasmosis (Epiphanio et al., 2003) described, a few hours before death, nonspecific symptoms like discomfort, dyspnea, lethargy, hypothermia and serosanguinous or foamy nasal discharge. Autopsy revealed pulmonary congestion or oedema, splenomegaly and mesenteric lymphadenitis associated with multi visceral lesions. All these outbreaks highlight the high susceptibility of captive squirrel monkeys to acute toxoplasmosis associated with severe respiratory distress and presence of tachyzoites in broncho-alveolar lavages suggesting a possible contamination by aerosol (Furuta et al., 2001). Contamination should more likely occur by oocysts carried by vector-like cockroaches (Espinosa-Avilés, 2007) or humans (Oh et al., 2018) or by cysts contained in small preys. Despite drastic sanitary measures introduced in zoos to limit the source of infection (Lindsay and Dubey, 2014), enzootic toxoplasmosis resulting in squirrel monkey death is still observed without usual effective treatments (pyrimethamine/sulfadiazine or spiramycin) (*Fowler's Zoo and Wild Animal Medicine, Volume 8 - 1st Edition*, n.d.). As type I, II or III *T. gondii* strains are responsible for saimiris death (Carme et al., 2009; Salant et al., 2009; Cedillo-Peláez et al., 2011), the main hypothesis to explain squirrel monkey's susceptibility remains the absence of co-evolution with the parasite. NWPs evolved more than 20 million years before felids, and therefore prior to *T. gondii* arrival in South America (Cunningham et al., 1992; Sibley et al., 2009; Perelman et al., 2011; Fleagle, 2013). Moreover, squirrel monkeys, due to their exclusively arboreal way of life, had very little contact with the parasite, which may explain the lack of adaptation of their immune system to *T. gondii* (Cunningham et al., 1992; Innes, 1997).

Detection of *T. gondii* by innate immune sensors is the first step that drives the development of protective adaptative immunity. Interestingly, humans and saimiris Toll-like and Nod-like receptors (TLRs, NLRs) are closely related (Gazzinelli et al., 2014; Chavarría-Smith et al., 2016). In mice and humans, TLR11/12 and TLR7/8/9 respectively are involved in *T. gondii* sensing and IL-12 secretion. Moreover, glycosylphosphoinositols (GPIs), MIC1 and MIC4 proteins are known to bind to TLR2 and TLR4 (Debierre-Grockiego et al., 2007; Sardinha-Silva et al., 2019). Functionalities of TLR4 and TLR9 have already been assessed in response to their agonists in squirrel monkeys (Tougan et al., 2013; Nehete et al., 2013, 2020). *Toxoplasma gondii* interaction with NLRP1 and NLRP3 leads to the activation of inflammasome that process and induce release of mature IL-1β and IL-18 essential for parasite control in mice in vivo. In vitro, IL-1β secretion by human peripheral blood mononuclear cells (PBMCs) infected with *T. gondii* is suppressed with NLRP3 inhibitor (Gov et al., 2017). *Toxoplasma gondii* sensing by neutrophils, macrophages and dendritic cells induces secretion of TNF-α and IL-12 that triggers IFN-γ secretion by NK cells. This early IFN-γ secretion plays a major role in controlling acute infection (Tato et al., 2003; Denkers et al., 2004; Debierre-Grockiego et al., 2010; Debierre-Grockiego and Schwarz, 2010) *via* reactive oxygen species (ROS), nitrogen intermediates (NO) (Halonen and Weiss, 2013) and indoleamine 2–3 dioxygenase (IDO) (Fujigaki et al., 2002).

Scarce data is available about the immune responses induced by *T. gondii* in squirrel monkeys. Investigations highlighted serum antibodies in surviving animals using modified agglutination test without distinguishing antibody classes (Minervino et al., 2017). Currently, no known data is available in the literature regarding the cellular immune response induced by *T. gondii* in squirrel monkeys compared with humans and mice. For the latter, it has been shown that both humoral and cellular immune responses are essential to control parasite dissemination during acute toxoplasmosis.

In humans, *Toxoplasma* infection induces specific humoral immune response characterised by serum IgA, IgM and IgG production that may play a role in neutralisation, activation of classical pathway of complement and inflammation (Correa et al., 2007) and intestinal secretory IgA involved in early protection (McLeod et al., 1988; Chardès et al., 1990; Mineo et al., 1993). However, cellular response, especially *via* IFN-γ (Suzuki et al., 1988; Gazzinelli et al., 1993), is the main mechanism of protection (Innes, 1997).

Effective adaptive immune response against *T. gondii*, initiated by IL-12 (Hou et al., 2011) is determined by the cooperation between CD4+ and CD8+ T cells (Gazzinelli et al., 1992). NK, CD8 + and CD4+ T cells constitute an essential source of IFN-γ, the major mediator of protection (Tait and Hunter, 2009). CD8+ T cells use several mechanisms to control *T. gondii* infection: inflammatory cytokines production, CD40/CD40L interactions resulting in autophagy or infected cell lysis by perforin (Gazzinelli et al., 1992; Denkers et al., 1997; Subauste, 2009). Squirrel monkey's PBMCs are well described in terms of cell populations and secreted cytokines, especially IFN-γ (Contamin et al., 2005; Nehete et al., 2013, 2018). This suggests that vaccination inducing high levels of IFN-γ would be sufficient to control *T. gondii* multiplication in saimiris.

To date, only one vaccine is available to prevent ovine toxoplasmosis. Ovilis Toxovax® (MSD Animal Health, USA) is a live attenuated vaccine from the S48 strain which partially protects sheep from abortion and can revert to a pathogenic form (Buxton et al., 1991; Buxton and Innes, 1995; Kur et al., 2009). Live vaccines, even attenuated, are not suitable for immunisation of highly susceptible species due to potential uncontrolled parasite multiplication. Other vaccination strategies using subunit vaccines with adjuvants or vehicles have been explored however they all failed to protect against infection irrespective of the route of administration (Buxton et al., 1989; Stanley, 2004). However, inert vaccine by mucosal route remains a valuable alternative as mucosal immunisation leads to local and systemic immune response establishment (Jenkins, 2001).

For more than a decade, a promising vaccine strategy against *T. gondii* has been developed which is composed of maltodextrin nanoparticles (NP) charged with phospholipids (DGNP). These porous DGNP, with a high capacity to transport a large quantity of proteins (Paillard et al., 2009), can be loaded with total extract of *T. gondii* proteins (TE). DGNP displays several characteristics essential to the vaccine strategy (Bernocchi et al., 2016; Lê et al., 2018, 2019), including a high capacity to deliver proteins to mucosal cells through nasal mucus penetration (Fasquelle et al., 2020). Therefore, DGNP/TE vaccination can be performed by intranasal (i.n.) route in order to deliver all immunogenic proteins to mucosal cells, particularly to resident immune cells. This vaccine has already been validated to protect against murine and ovine toxoplasmosis. Indeed, use of DGNP/TE allowed 100% survival against a lethal challenge in mice and significative decrease of brain cyst load (>70%) associated with a Th1/Th17 immune response (Dimier-Poisson et al., 2015). Similarly, two i.n. vaccinations of mice and sheep protected their fetuses from tachyzoites transplacental passage during pregnancy (Ducournau et al., 2017, 2020). These findings have pointed to the first potential prevention of fatal toxoplasmosis in saimiris and several French zoos facing heavy

losses (up to 86% of saimiri's population) due to toxoplasmosis have volunteered to test this new vaccine.

Due to the absence of efficient treatment or health measures, the DGNP/TE vaccine usage was authorised under the French veterinarian cascade regulation (The European directive n°2001/82/CE, The European directive n°2001/82/CE and rural French code, Article L2431). The present work aims to decipher the immune responses induced by the vaccination in squirrel monkeys, in field conditions. In addition, studies were performed on experimental naive animals to explore mechanisms of parasite recognition.

2. Materials and methods

2.1. Ethics statements

Forty-eight squirrel monkeys (*Saimiri boliviensis;* 28 females and 20 males aged 8 ± 6 years) included in the vaccination protocol were housed across six French zoos (Bioparc de Doué-la Fontaine, Doué en Anjou; Zoo d'Amnéville, Amnéville; Zoo de Mulhouse, Mulhouse; Zoo de Besançon, Besançon; Zoo de Branféré, Le Guerno; Zoo de Calviac, Calviac-en-Périgord). Each zoo has the responsibility for the saimiri well-being, health and medical decision and are not submitted to European animals' experimentation rules (Directive 1999/22/CE). All saimiris involved in the study were born and parent-reared in zoos and were daily monitored by zookeepers. Their enclosures differed depending on the institutions: aviaries (Amnéville, Besançon, Calviac, Mulhouse), opened-top enclosures with smooth walls (Doué la Fontaine) or islands (Branféré). Most animals were housed in single-species exhibits except in one institution (Bioparc de Doué-la Fontaine, Doué en Anjou) where they were mixed with spectacled bears (*Tremarctos ornatus*). The environment always contained rocks, trees, branches or wooden poles and ropes. Saimiris were fed various diets from two to four times a day, depending on the institution. However, all were given fruits, vegetables and monkey pellets, with some extra protein source (crickets, eggs, fish). Water was available *ad libitum*. All animals were provided with different types of environmental enrichment (hidden food pieces, olfactive stimuli in the environment). Some also received cognitive stimulation (manipulative devices either containing food or not). The saimiris were not used to directly interact with humans except during training sessions with goals that varied between institutions (reading a transponder, weighing, hand food distribution for individual drug delivery).

Sixteen naive squirrel monkeys (*Saimiri sciureus),* including 13 females and three males, aged 13.5 ± 3.3 years from the Mediterranean Primatology Center (MPRC) (13790 Le Rousset, FRANCE) were housed in a level I and II containment enclosure and declared free from any infectious disease. Blood sampling was performed under experimental authorization in accordance with the guidelines for animal experimentation (EU Directive 2010/63/EU by the French Ministry of Education and Research).

2.2. Vaccine preparation

DGNP was prepared as described previously (Paillard et al., 2009). Briefly, maltodextrin was dissolved in 2 N sodium hydroxide with magnetic stirring. Epichlorhydrin and glycidyltrimethylammonium chloride (GTMA) were added to obtain a cationic polysaccharide gel crushed with a high-pressure homogenizer (Emulsiflex C3, France). After ultrafiltration, NPs were mixed with 1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol (DPPG) to produce the 70 nm lipid nanoparticles (DGNP).

Toxoplasma gondii antigen extract (TE) was prepared from the RH strain tachyzoites (ATCC® PRA-310™) cultured on human foreskin fibroblasts (HFF; ATCC® number CRL-1634™) in DMEM medium (Dutscher, France) with 5% fetal bovine serum (FBS, Dutscher), 2 mM HEPES (Dutscher) as previously described (Dimier-Poisson et al., 2015). Tachyzoites were harvested, washed in endotoxin-free PBS (Sigma Aldrich, USA), and dry frozen at −20 °C. After three frozen/thawed cycles, the tachyzoites were lysed in endotoxin-free sterile water then sonicated for three 10 min periods at 60 W/s. Soluble extract were obtained by centrifugation at 700*g* during 10 min. The protein concentration was determined by the Micro BCA protein assay (Pierce, Rockford, Ill, USA) using BSA as the standard.

The TE of *T. gondii* proteins was mixed with DGNP at a 1:3 weight ratio (50 µg:150 µg) in water for 1 h at room temperature in 50 µL or 100 µL for i.n. or s.c. route of vaccination respectively. As already described in previous works (Dimier-Poisson et al., 2015), the complete antigen encapsulation was confirmed by loading the mixture onto native PAGE, followed by silver nitrate staining. DGNP and DGNP/TE formulations were characterized in terms of size and surface charge by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) respectively, in water, with a Nanosizer Nano ZS (Malvern Instrument, France) Before each vaccination, DGNP/TE formulations were characterised (Supplementary Fig. S1).

2.3. Vaccination schedule

Before the first vaccination, all squirrel monkeys were tested for the presence of serum *T. gondii* antibodies by High Sensitivity Direct Agglutination (HSDA) test performed by Parasitology-Mycology laboratory of the National Reference Center Toxoplasmosis, CHU Reims, France (Supplementary Table S1). Among the tested squirrel monkeys, only five were seropositive to *T. gondii*. These five saimiris were classified as infected subjects (at 5 months to more than 4 years earlier), were not vaccinated and were used as positive control of protection.

All seronegative subjects (n = 48) were vaccinated in the respect of the veterinary cascade (Lecu and Petit, 2012). The European directive n°2001/82/CE and rural French code, Article L2431, allow to prescribe medicines under the cascade. Extemporaneous preparations (also known as "veterinary specials") are products that do not hold a Marketing Authorisation but can legally be prescribed, supplied and used under the last step of the cascade.

The schedule was adapted from a previously used protocol in a sheep experimental model (Ducournau et al., 2020) and a custom spray consisting of a conical flacon and a nosepiece size-suitable for squirrel monkeys was used for nasal instillation. Two i.n. administrations (50 µg TE/150 µg NP in a 50 µL /dose) were performed 1 month apart. Due to the extreme susceptibility of squirrel monkeys to *Toxoplasma* infection, a 3rd dose was added 6 months later as a combined i.n. (50 µg TE/150 µg NP in a 50 µL/dose) and s.c. administration (50 µg TE/150 µg NP in a 100 µL/dose) and an annual and 2-year boost by combined i. n. and s.c. routes (Fig. 1). All four immunisations are subsequently called "full protocol". For vaccination, squirrel monkeys were anesthetized by Isoflurane® inhalation at 3–5%, with 2 L/min Oxygen supply. All sampling was performed during this medical procedure according to the field conditions of this study.

Although all squirrel monkeys were vaccinated (n = 48), only 21 adults from four zoos (Bioparc de Doué-la Fontaine; Zoo d'Amnéville; Zoo de Mulhouse; Zoo de Besançon) were included in the immunological study. This group was formed according to age and physiological status in order to be as homogeneous as possible. Indeed, young (<2 years) and old (>15 years) monkeys and pregnant females were not included in the immune response study. Table 1 provides details about these squirrel monkeys regarding

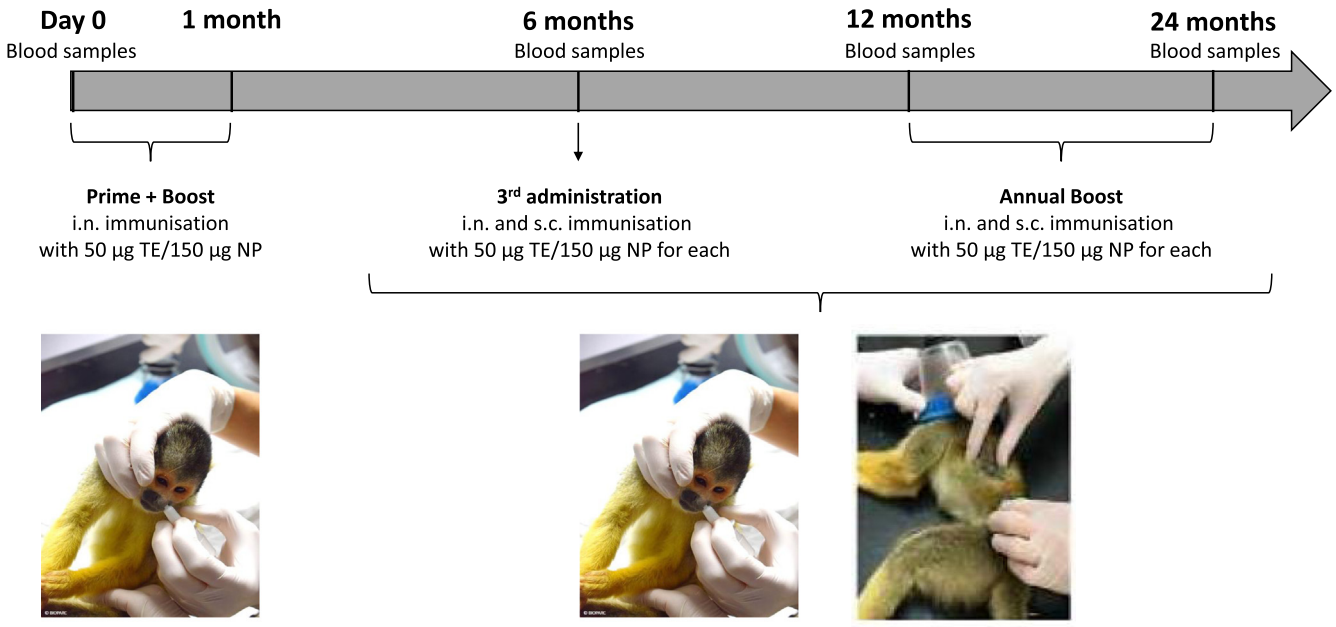

Fig. 1. Vaccination protocol and timeline for squirrel monkey sampling in the participating French zoos. Squirrel monkeys were vaccinated twice, 1 month apart by intranasal (i.n.) route with 50 µg of total extract of *Toxoplasma gondii* proteins (TE) and 150 µg of nanoparticles (NP) in 50 µL by spray. Two administrations at 6 and 12 months were performed with combined routes (i.n. and s.c) with 50 µg of TE and 150 µg of NP in 50 µL for i.n. and 50 µg of TE and 150 µg of NP in 100 µL for s.c. administration. Blood sampling was proceeded before vaccination and at 0, 6, 12 and 24 months.

Table 1
Details about vaccinated squirrel monkeys included in immune monitoring in the zoos.

Squirrel monkeys	Time point			
	Day 0	6 months	12 months	24 months
Number	5	21	21	14
Sex	4 Females	13 Females	13 Females	7 Females
	1 Males	8 Males	8 Males	7 Males
Age (year)				
mean ± S.D.	9.6 ± 3.1	8.57 ± 3.5	8.6 ± 3.5	8.3 ± 3.8
Herpes Simplex Virus(HSV2)	3/5 positive	14/21 positive	14/21 positive	9/14 positive
serological status	2/5 equivocal	4/21 equivocal	4/21 equivocal	2/14 equivocal
	0/5 negative	3/21 negative	3/21 negative	3/14 negative
Cytomegalovirus(CMV)	ND	13 ND	13 ND	8 ND
serological status		0/8 positive	0/8 positive	0/6 positive
		2/8 equivocal	2/8 equivocal	1/6 equivocal
		6/8 negative	6/8 negative	5/6 negative

ND, not done.

their age, their sex, and their Herpes simplex virus (HSV) and Cytomegalovirus (CMV) status.

2.4. Follow-up after vaccination

Squirrel monkeys were examined before and after each vaccination. The following physiological parameters were followed, and vaccine safety was checked until 10 days after the i.n. vaccination to notice any congestion, nasal bleeding, nasal irritation, dyspnea, cough, abnormal breath sounds. Subcutaneous administrations were also monitored to ensure minimal pain or to assess the presence of abnormalities like pruritus, edema, alopecia, abscesses, granuloma formation, or tumors at the injection site. The general condition of the vaccinated squirrel monkeys was carefully monitored for anaphylactic reaction, hyperthermia, digestive issues, respiratory distress, lethargy, or any disorder potentially induced by vaccination.

2.5. Faecal samples

Faecal samples were collected in 1x PBS supplemented with 1 mM PhenylMethylSulfonylFluoride (PMSF, Sigma Aldrich) and 100U/0,1mg/mL Penicillin-Streptomycin (Dutscher). Samples were centrifuged at 1,000*g* for 5 min to remove fragments. Supernatants were centrifuged at 13,000*g* for 5 min to collect the soluble fraction and immediately analysed.

2.6. Blood samples

Blood samples (approximately 4 mL) were collected in anticoagulant (heparin or EDTA) from the femoral vein of anesthetised captive animals before each vaccination at day 0, 6 months, 12 months and 24 months or on request for naive saimiris at the MPRC. All samples were treated as soon as possible within 24 h. Plasma was obtained after centrifugation at 13,000*g* for 5 min and stored at −20 °C until analysis.

2.7. Determination of anti T. gondii antibody titre by ELISA

One hundred µL/well of 10 µg/ml TE in carbonate buffer pH 9.6 was coated to 96-well flat-bottom plates (Maxisorp, Nunc, Denmark) overnight at 4 °C. Three washes using 200 µL 1x PBS/0.05% Tween-20 (PBS-T) were performed between each step. PBS-4% BSA (200 µL) was added for 90 min at 37 °C. The sera were incubated as 100 µL volumes with serial two-fold dilutions from 1/50 to 1/102400 in PBS-T for 1 h at 37 °C whereas the faeces samples were added without any dilution. Antibody anti-human IgG (Sigma Aldrich), and human IgA (Sigma Aldrich) coupled to alkaline phosphatase diluted in PBS-T were added in 100 µL/wells for 90 min at 37 °C. Antibody presence was revealed by the addition of a solution of p-Nitrophenylphosphate (pNPP) at 1 mg/ml (Interchim, France) in diethylamine (DEA)-HCl pH 9.6. OD was measured at 405 nm using a microtiter plate reader (Biotek Instruments, USA). The positive threshold was determined, independently for each assay, using the mean OD of negative samples plus 2.5 times the S.D..

2.8. In vitro sera neutralisation of T. gondii

As previously described by Moine et al. (2018), $2x10^4$ HFF cells were seeded in 96-well flat bottom plates in 100 µL in DMEM without red phenol (Dutscher), with 1% FBS, 100 U/0.1 mg/mL penicillin–streptomycin at 37 °C in 5% CO_2 atmosphere until confluence. Serum heat-treated for 30 min at 56 °C or not, was incubated (v:v 1:4) for 1 h at 37 °C with 1,000 *T. gondii* tachyzoites modified for β-galactosidase expression (RH β-gal) (Seeber and Boothroyd, 1996). After incubation, the mix (50 µl) was added to HFF cells (100 µL) and incubated 5 days at 37 °C under 5% CO_2. Proliferation controls were 1,000 RH β-gal tachyzoites alone or sera from naive animals. Inhibition controls were RH β-gal tachyzoites with sera from naturally infected animals. After 5 days, 100 µL of supernatants were discarded. Cells and tachyzoites were lysed by addition of 50 µL of Triton X-100 (Sigma) at 0.1% (v/v) in water, followed by the addition of chlorophenol red- β-D-galactopyranoside (CPRG, Sigma) solution diluted in 100 mM HEPES pH 8 at the final concentration of 1 mM. OD was measured after brown coloration development for 1 h at 565 nm.

Percentage of neutralising activity was determined as follows: 100 – (sample OD /proliferation positive control OD)*100.

2.9. Isolation of PBMCs

PBMCs were obtained from blood diluted up to 6 mL with sterile 1x PBS, gently laid down on equal volume of Histopaque®-1077 (Sigma Aldrich, USA) and centrifuged at 700g for 30 min at room temperature. The cellular ring containing the PBMCs was collected and washed in 1x PBS. After a 10 min centrifugation at 700g, erythrocyte pellets were lysed by adding 1 ml of sterile water. After a 700g centrifugation for 10 min, PBMCs were resuspended in RPMI (Dutscher) containing 5% FBS, 20 mM HEPES, 100 U/0.1 mg/mL penicillin–streptomycin, 1 mM sodium pyruvate (Dutscher) and 25 µM β-Mercaptoethanol and cultured as described below.

An enriched antigen-presenting cell (APC) population (adherent fraction) was obtained after 2 h of plastic adherence of $4x10^5$ PBMCs /mL at 37 °C in 5% CO_2 in RPMI containing 5% FBS, 20 mM HEPES, 100U/0.1 mg/mL penicillin–streptomycin, 1 mM sodium pyruvate and 25 µM β-Mercaptoethanol. Non-adherent cells containing lymphocytes were discarded (Delles et al., 2002).

2.10. Cell culture conditions

PBMCs ($4x10^5$/well) from vaccinated animals were seeded in 96-well flat bottom culture plates in triplicate and stimulated with 20 µg/mL TE or 50 ng/mL and 250 ng/mL phorbol 12-myristate 13-acetate (PMA)-ionomycin as positive control in a final volume of 200 µL. Culture was performed for 72 h at 37 °C in a 5% CO_2 atmosphere, then supernatants were harvested and frozen at −20 °C until cytokines analysis.

Both PBMCs ($4x10^5$) and enriched APCs cells ($8x10^4$) from naive animals, were cultured in 200 µL with agonists and/or antagonists of TLRs 1/2, 4, 8 and 9 (Supplementary Table S2). Ten µM of specific antagonists was added overnight before the addition of 20 µg/mL TE or RH *T. gondii* tachyzoites at a multiplicity of infection (MOI) of 1, for 3 days. Specific agonist controls (Supplementary Table S2) were used according to the recommended concentration.

PBMCs ($4x10^5$) and enriched APCs ($8x10^4$), from naive animals, were also cultured for 3 h with LPS at 10 ng/mL. Medium was changed before the addition of specific NLR antagonists for 2 h. Two NLRs antagonists were used: MCC950 (antagonist of NLRP3; InvivoGen, USA) at the concentration 4 µg/mL, and Ac-YVAD-cmk (antagonist of caspase-1; InvivoGen) at 30 µg/mL. Live tachyzoites of *T. gondii* at MOI = 1 were then added for 4 and 18 h as well as alum as a control agonist at 100 µg/mL. The supernatants were collected and stored at −20 °C until ELISA analysis.

Cell viability was checked using Propidium Iodide (PI) according to the manufacturing guidelines (R&D systems, USA). Two washes with 1X PBS were performed, then cells were suspended into 100 µL of flow cytometry buffer. Five µL of PI at 10 µg/mL were added to each well and cell acquisition was undertaken with Macsquant® cytometer (Miltenyi Biotec, Germany) and analyzed using FlowLogic® software (version 7.3). Controls conditions without PI were performed.

2.11. Cytokines analysis by ELISA

Interleukin-1β; 2; 4; 5; 6; 8; 10; 12p70; 13; 17; 23; GM-CSF; TNF-α; IFN- γ (Supplementary Table S3) were quantified using ELISA kits according to the manufacturing guidelines. The cross-reactivity has been demonstrated using supernatant of PBMCs activated with PMA and ionomycin or concanavalin A. Briefly, capture antibody solution in 1x PBS was coated overnight at 4 °C into a 96-well flat-bottom plate (Maxisorp, Nunc). Two washes with 200 µL/well were performed with 1x PBS. Saturation was performed by adding 1x PBS - 0.1% BSA for 1 h at room temperature. Five washes with 200 µL/well were then performed with PBS-T (repeated after each step). Culture supernatants diluted to 1:2 in PBS-T or recombinant human standards (supplied in the kits) (in 50 µL) were added for 2 h at room temperature. Detection antibody (50 µL) was used according to guidelines for 1 h at room temperature. Streptavidin-horseradish peroxidase (HRP) diluted to 1:1000 was added (in 50 µL) for 1 h followed by 3,3′,5,5′ tetramethylbenzidine (TMB) substrate (Sigma Aldrich) in 50 µL. Reactions were stopped with 50 µL of 1 M H_2SO_4. OD was measured at 450 nm/630 nm on a microtiter plate reader.

2.12. Electron microscopy

For scanning and transmission electron microscopy, PBMCs were cultured in 6-well plates and collected 24 h after infection with *T. gondii* tachyzoites at MOI = 1. Adherent cells were collected using a scraper, washed in 1x PBS and fixed in 0.1 M phosphate buffer (pH 7.2) containing 4% paraformaldehyde and 1% glutaraldehyde. Cells were fully dehydrated in a graded series of ethanol baths (50° – 70° – 90° – 100°). For the scanning electron microscopy, a polymerization step preceded the sectioning of samples with a microtome. Observation and data were obtained thanks to the assistance of the IBiSA Electron Microscopy Facility (University of Tours, France).

2.13. Statistical analysis

GraphPad Prism® software (version 7.05) was used for data analysis. Based on distribution, tested using Shapiro-Wilk test, nonparametric tests (Wilcoxon, Mann-Whitney or Kruskal-Wallis followed by a Dunn's multiple comparisons test) or parametric paired t-test were performed. All statistical tests were two-sided and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. DGNP/TE vaccine induces mild humoral immune response

Squirrel monkeys were first vaccinated by the nasal route to induce mucosal and systemic immune responses.

To assess the mucosal humoral immune response, presence of IgA and IgG was explored by endpoint ELISA in faeces of squirrel monkeys before and after vaccination at day 0 and 6, 12, and 24 months after vaccination. Irrespective of time or status (before vaccination, vaccinated or infected), no IgA or IgG was detected in the faeces of saimiris (data not shown).

Systemic humoral immune responses were explored in sera of squirrel monkeys. Serum IgA and IgG were analysed by endpoint ELISA before and 6, 12, and 24 months after vaccination compared with infected subjects (Fig. 2). Among the few squirrel monkeys showing a weak IgA titre (>day 0 titre) after vaccination, only three subjects had an IgA titre of 400 at 1 year after the full protocol. Most of vaccinated subjects never showed an increase of serum IgA (median = 0; interquartile [0–175]). No difference was observed depending on time ($P = 0.8727$) or status ($P = 0.5476$) (Fig. 2A). Among all samples, only five subjects could be tested from start to finish and never exceed a titre of 100 after vaccination. Regarding serum IgG (Fig. 2B), a slight increase was observed over time until it became significantly different 1 year after the full immunisation protocol (median titre = 200 [75–400]). At this point, the IgG titre was significantly lower than for infected subjects (102400; $P < 0.0001$). Again, the five squirrel monkeys followed from day 0 never exceed a titre of 100 after vaccination.

3.2. Neutralising activity of sera

The neutralising activity of sera from vaccinated squirrel monkeys was evaluated using a *T. gondii* multiplication assay with HFF cells. As shown in Fig. 3A, the percentage of inhibition of *T. gondii* multiplication by sera of vaccinated animals appeared to increase over time after the beginning of vaccination. Twelve months following the first vaccination, the inhibition of *T. gondii* multiplication reached 38%. Up to 80% inhibition at 12 and 24 months was observed for some sera although for half of the sera, no neutralising activity was noted (median = 38 [0–78.8] and median = 0 [0–79.2] respectively). The five samples tested from start to finish showed a non-significant increase of neutralising activity (20–80%) of sera after the vaccination. As shown in Fig. 3C, D, the neutralising activity observed for sera from vaccinated subjects was not correlated to the titre of serum IgA and IgG. The neutralising activity of sera from vaccinated subjects was greatly repealed when sera were heat inactivated (Fig. 3 E-G). On the contrary, sera from naturally infected subjects, with a high antibody titre, showed a decrease from 68.8% ± 3.4 to 47% ± 6.2 of neutralising activity after heat inactivation (Fig. 3B).

These results suggest that neutralising activity in vaccinated subjects with low antibody titres may be linked to vaccination and thermolabile serum components.

3.3. DGNP/TE vaccine induces specific IFN-γ and Il-17 cytokine secretion

Induction of cellular immune responses, especially IFN-γ mediated Th1, is crucial for the vaccine efficacy against *T. gondii*. To explore possible mechanisms of protection following vaccination, cytokines from the supernatants of TE-stimulated PBMCs of vaccinated squirrel monkeys were compared with those from naturally infected subjects. Interleukin-1β; 2; 4; 5; 8; 10; 12p70; 13; 23; GM-CSF were not detected irrespective of the status of animals or the time after vaccination (data not shown). As shown in Fig. 4A, IFN-γ secretion by PBMCs from vaccinated subjects increased over time and became significantly higher after full vaccination (median = 2,346 pg/mL [1,844–3,522]; *P*-value = 0.0114) and tended towards the level of naturally *T. gondii*-infected squirrel monkeys (median = 2,980 pg/mL [2,267–3,513]; *P*-value = 0.4998). Regarding IL-17 secretion by PBMCs, a significant increase was noted 1 year after full vaccination (median = 77.50 pg/mL [5.558–181.5]) compared with 6 months (median = 1.610 pg/mL [0–17.95]) and 12 months (median = 0.48 pg/mL [0–13.74]) after the beginning of vaccination (Fig. 4B). Moreover, this secretion was also significantly higher than that observed for long-time naturally infected subjects (median = 0 [0–6.845]; *P*-value = 0.0332). Finally, for TNF-α (Fig. 4C), no difference in secretion was observed, except at 12 months where an unexpected decrease was noted. No statistical difference was either noted, irrespective of the time or the status of subjects for IL-6 secretion by PBMCs (Fig. 4D).

To conclude, DGNP/TE vaccine induces a specific secretion of IFN-γ and IL-17 cytokines that reflects the establishment of an adaptive immune response against *T. gondii* in the squirrel monkey.

3.4. Pattern recognition receptors (PRRs) do not appear to be involved in a defective immune response

Given the efficacy of vaccination to induce a specific immune response, questions remain as to the high susceptibility of squirrel monkeys to toxoplasmosis. *Toxoplasma gondii* tachyzoites appear to interact with monkey host cells (Supplementary Fig. S2) similar to that described in humans and mice. PRR involvement in the defective immune response was explored through the recognition of *T. gondii* by TLRs and NLRs on squirrel monkeys' PBMCs and related enriched APCs. Assessment of TLRs and NLRs functionality was tested in two independent experiments. PBMCs were cultured with either *Toxoplasma* TE, live parasites (RH) and TLR or NLR antagonists, and the secretion of cytokines was measured. Absence of cytotoxicity was verified for each antagonist using PI staining of cells. A similar cell mortality was observed in each group: 15–20% with TLRs antagonists and 9–19% with NLRs antagonists (data not shown).

Regarding *T. gondii* recognition by TLRs, their functionality was assessed by secretion of TNF-α, IL-1β and IL-6. Fig. 5 shows the IL-6 secreted by squirrel monkeys' PBMCs in response to *T. gondii* stimulation, both with TE (median = 120.3 pg/mL [95.24–159]) and live RH tachyzoites (median = 272.5 pg/mL [166.5–327.8]). A significant decrease in IL-6 was observed when specific TLRs antagonists (see Supplementary Table S2) were added (Fig. 5B-H), except for TE-stimulated PBMCs in the presence of TLR1/2 antagonist (Fig. 5A). IL-6 secretion decreased when PBMCs were stimulated by TE and live RH parasites respectively to 90.17 and 243 pg/mL in the presence of antagonist for TLR4 (Fig. 5C, D); to 105.1 and 187.9 pg/mL for TLR8 antagonist (Fig. 5E, F) and to 94.57 and 187.8 pg/mL for TLR9 antagonist (Fig. 5G, H). The same tendency was observed with related enriched APCs but this was not significant (data not shown). For TNF-α and IL-1β measurement, no difference was observed (50 pg/mL and 20 pg/mL respectively)

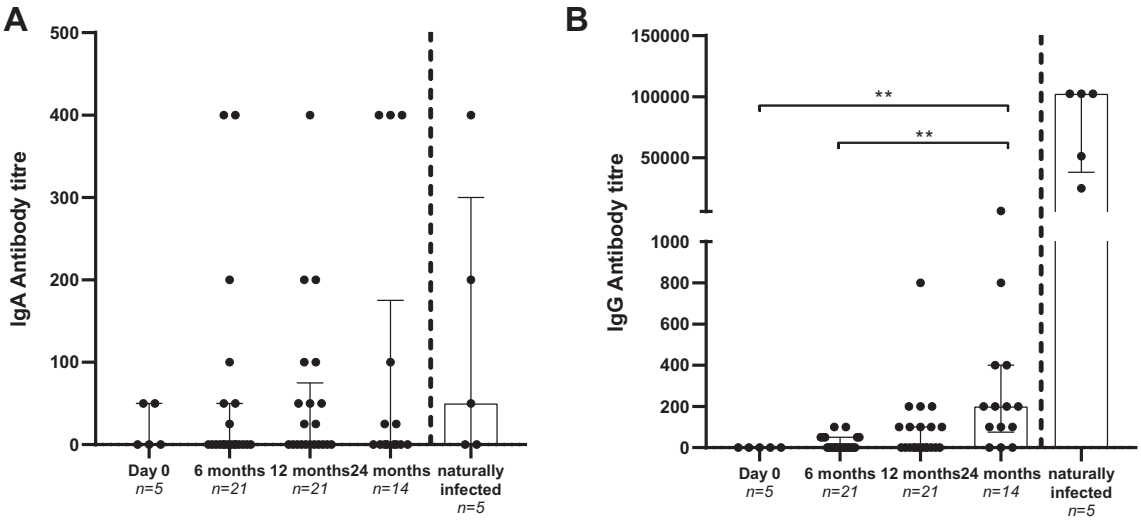

Fig. 2. Humoral immune responses of squirrel monkeys before and following vaccination (6, 12 and 24 months) compared with *Toxoplasma gondii* naturally infected subjects. Serologic analysis was done by endpoint ELISA for (A) *T. gondii*-specific IgA antibodies and (B) *T. gondii*-specific IgG antibodies. Results are represented as median with interquartile range and individual data point titres. Statistical analysis was performed using Kruskall-Wallis and Dunn's multiple comparisons post hoc, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

irrespective of the cell stimulation conditions i.e. no stimulation *versus* TE or RH +/- antagonists (data not shown).

Toxoplasma gondii recognition by NLRs was assessed by measuring IL-1β secreted by PBMCs or related enriched APCs in response to live RH *T. gondii* in presence of specific antagonists of NLRP3 and caspase-1. As shown in Fig. 6A, a high but not significative increase of IL-1β was noted 82.66 pg/mL [39.18–117.8] 4 h p.i. compared with cells alone (44.27 pg/mL [12.59–70.71]). In the presence of specific antagonists, we observed a significant decrease of IL-1β secretion compared with PBMCs stimulated by live RH *T. gondii* at 4 h p.i. (Fig. 6A), 41.94 pg/mL and 42.63 pg/mL for NLRP3 and caspase-1 antagonists respectively. As shown in Fig. 6B, no difference was noted between cells 18 h p.i. (65.88 pg/mL) or not (53.6 pg/mL). However, a significant decrease in IL-1β production was observed in the presence of RH and NLRP3 antagonist (23.76 pg/mL [18.5–28.65]), and RH and caspase-1 antagonist (31.06 pg/mL [23.45–43.24]). The same tendency was observed with related APC-enriched cells but not significantly (data not shown).

In conclusion, both TLRs and NLRs innate sensors of squirrel monkeys may be implicated in *T. gondii* recognition similar to humans and mice.

4. Discussion

Toxoplasmosis is an often-fatal disease of NWPs, and squirrel monkeys are highly susceptible hosts. Currently, there is only one commercial live vaccine available that cannot be administered to highly susceptible animals. We have developed a mucosal anti-*Toxoplasma* inert vaccine (DGNP/TE) for animal and human application. Intranasal instillation has shown efficacy against chronic and congenital murine and ovine toxoplasmosis decreasing mortality and parasite load (Dimier-Poisson et al., 2015; Ducournau et al., 2017, 2020).

The present work aimed to follow the immune response after immunisation of captive squirrel monkeys with the DGNP/TE vaccine under field conditions. Immunisations were performed by both i.n. and s.c. routes, the first two immunisations being only by i.n. route. In the literature, vaccines delivered *via* the i.n. route are known to enhance mucosal and systemic, cellular, and humoral immune responses (Zheng et al., 2018). Mucosal immunity is the first line of defense against *T. gondii*, which naturally invades the intestine of its host. Moreover, this route of immunisation is relevant for squirrel monkeys since contamination by aerosol has been hypothesised (Furuta et al., 2001). Subcutaneous route is widely used for vaccination and induces strong systemic humoral immunity but fails to induce efficient and long-term cellular protection (Levin et al., 2015). Uddback et al., (2016) observed that adenoviral vectors expressing influenza A nucleoprotein given by s.c. route induced short-term protection in mice. Intranasal immunisation elicited more lasting protection but the most robust protection was induced by simultaneous, combined vaccination.

Humoral and cellular immune responses induced by the vaccination were followed throughout the immunisation process. Although data recording was performed on animals of different sex and from different zoos, no difference in immune responses was observed also suggesting there was no impact of diet, enrichment and/or husbandry which allowed to subjects to be considered as one group. The immune response was recorded only for adults (8+/- 6 years) since Nehete et al., (2013) demonstrated that specific differences exist in immune function of lymphocytes between young (3–4 years), adult (8–10 years) and old (16–19 years) squirrel monkeys. Due to field conditions, samples of few animals could not be collected each time and the blood samples could only be performed at times of medical procedures. Additionally, invasive procedures like bronchoalveolar lavage for example, could not be performed to collect samples.

We used faecal samples to detect mucosal humoral responses after immunisations, but no antibody could be detected like in our previous experiments in mice (Dimier-Poisson et al., 2015; Ducournau et al., 2017). Absence of mucosal response may come from the lack of sensitivity of the technique or the sampling kinetics. After i.n. vaccination, EL-Malky et al. (2014) succeeded in detecting faecal *T. gondii* IgA 10 days after the third boost but at a low level. In accordance with most publications, Rashid et al., (2017) detected a weak mucosal humoral response after concentration of intestinal washes and only in some mice i.n. immunised three times with *T. gondii* ROP18 protein plus cholera toxin.

In the present work, antigen-specific serum IgG or IgA were not measured after two i.n. immunisations and significant IgG titers were observed only 1 year after the full immunisation protocol. Serum humoral response was very similar to that already observed in our previous studies in mice and sheep (Dimier-Poisson et al.,

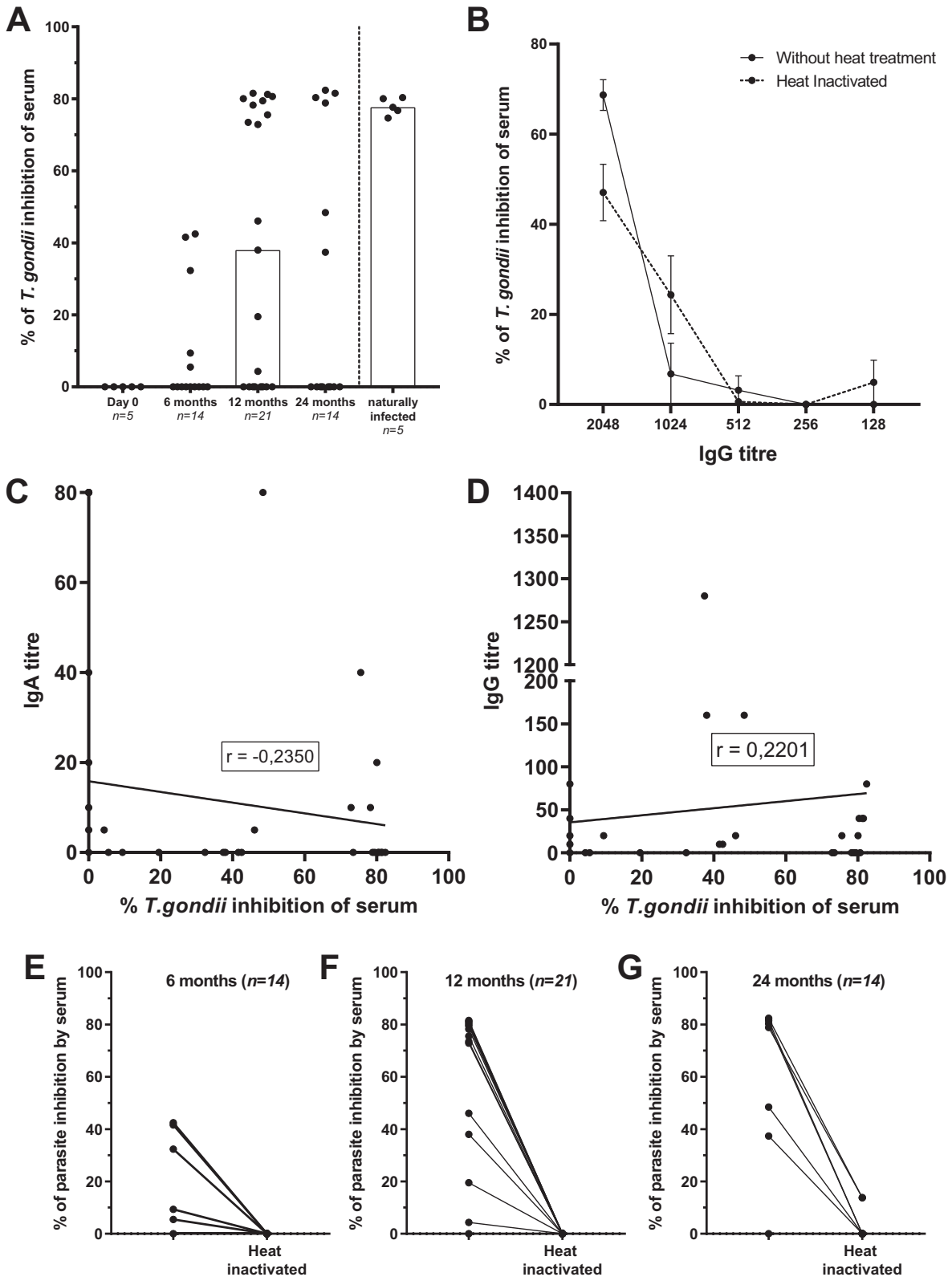

Fig. 3. Neutralising activity of squirrel monkey sera against *Toxoplasma gondii* tachyzoites. (A) Neutralising activity without heat inactivation of sera (1:5 dilution), before and following vaccination (6, 12 and 24 months) compared with *T. gondii* naturally infected subjects. Results are represented as median with interquartile range and individual data points. Statistical analysis was performed using Kruskall-Wallis and Dunn's multiple comparisons post hoc. (B) Neutralising activity with and without heat inactivation of sera (1:50 for the 1st dilution) from naturally infected squirrel monkeys (n = 5). Results are represented as mean +/- S.E.M. (C, D) Correlation between % of *T. gondii* inhibition and (C) IgA or (D) IgG titre (including data at 6, 12 and 24 months: n = 49). The box contains Spearman's correlation coefficient r. (E-G) Effect of heat inactivation of sera on the neutralising activity against *T. gondii* tachyzoites at (E) 6, (F) 12 and (G) 24 months. Each serum without or with heat treatment is connected by a line.

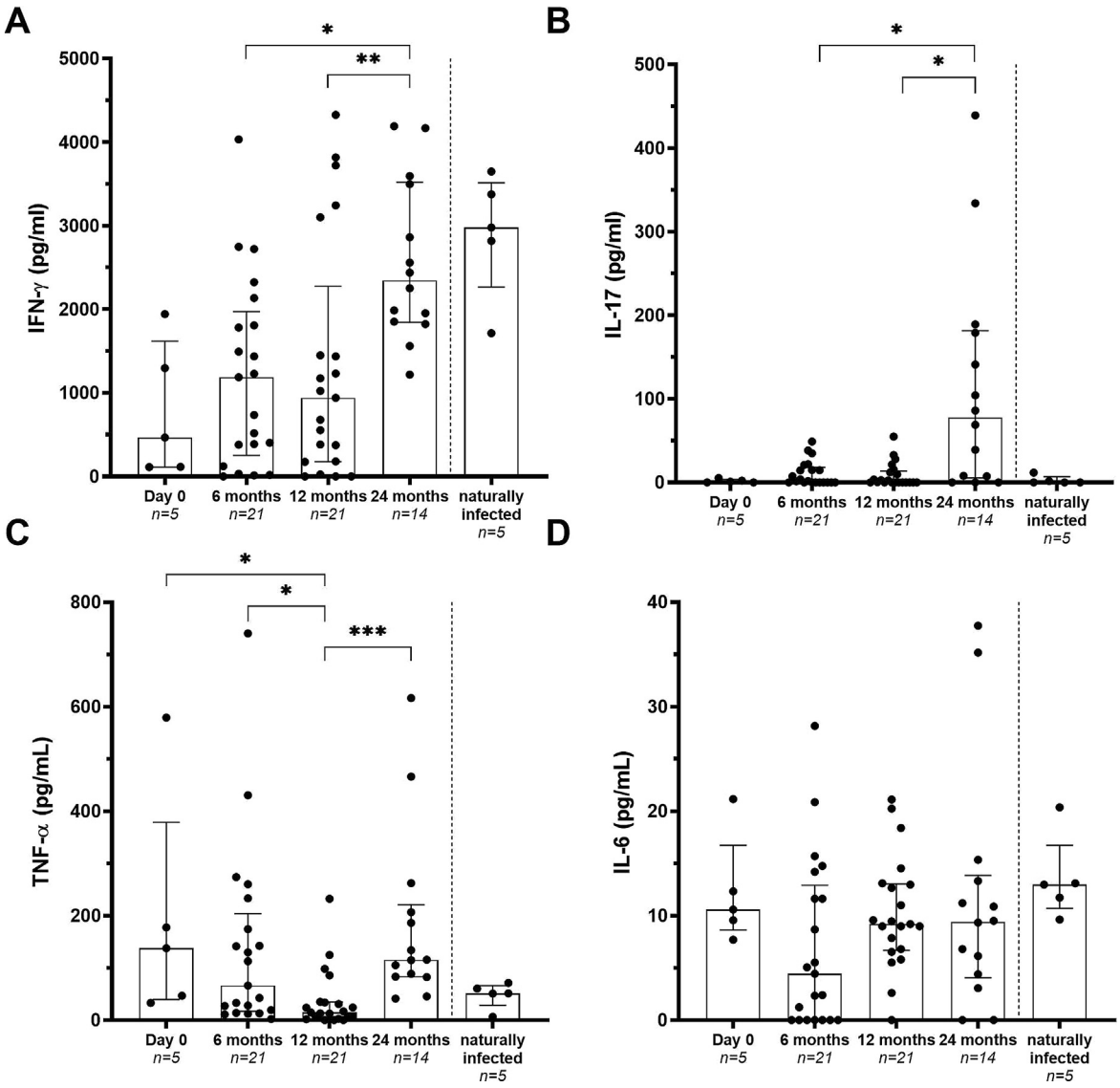

Fig. 4. Cellular immune responses induced by vaccination. ELISA was used to determine the concentration of cytokines in 72 h supernatants from peripheral blood mononuclear cells (PBMCs) stimulated with total extract of *Toxoplasma gondii* proteins (TE) before and following vaccination (6, 12 and 24 months) and compared with those from *T. gondii* naturally infected subjects. Cytokines are shown in (A) IFN-γ specific secretion; (B) IL-17A specific secretion; (C) TNF-α specific secretion and (D) IL-6 specific secretion. Results are represented as median with interquartile range and individual data point titres. Statistical analysis was performed using Kruskall-Wallis and Dunn's multiple comparisons post hoc, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. Cytokine detection threshold: IFN-γ = 4 pg/mL; IL-17 = 5 pg/mL; TNF-α = 4 pg/mL; IL-6 = 10 pg/mL.

2015; Ducournau et al., 2020). Rashid et al., (2017) detected specific humoral response after the third i.n. immunisation with recombinant ROP18 plus cholera toxin. However, the titres were lower than in mice vaccinated by s.c. route. Lakhrif et al. (2018) showed that three immunisations of mice by the s.c or combined routes induced high level of specific IgG titres in contrast to mice immunised i.n..

These collective results show that depending on the conditions, weak serum specific IgG can be induced after three i.n. immunisations,. The serum humoral response that we observed may be either induced by repeated i.n. immunisation or be dependent on the s.c immunisation. In our previous work (Ducournau et al., 2020), ewes vaccinated i.n. (despite no serum antibodies detected) were better protected against a toxoplasmosis challenge than intradermal-immunised sheep (whose sera harbor specific IgG). Therefore we could conclude that IgG level is not correlated to protective response.

Some mechanisms, however, are linked to protection by antibodies including neutralisation and inhibition of parasite cell invasion (Correa et al., 2007). We tested the role of vaccinated squirrel monkeys' serum in inhibition of parasite multiplication but the lack of correlation between the presence of antibodies and the percentage inhibition suggests a lesser role in parasite control. Moreover, the IgG titre after vaccination did not reach that required to induce significant inhibition in naturally infected animals.

The cellular response is considered the most important mechanism responsible for the host defense, and studies examining host immune responses in various species have highlighted the importance of IFN-γ in protective immunity (Innes, 1997). Acquired resistance to infection depends mainly on IFN-γ therefore vaccination correlates of protection are based on this cytokine secretion. In our study, IFN-γ secretion by saimiri PBMCs was detected after two immunisations and reached a level, similar to naturally infected animals when tested 1 year after the full protocol. IL-17 secretion was also detected but at a low level and only 1 year after the last

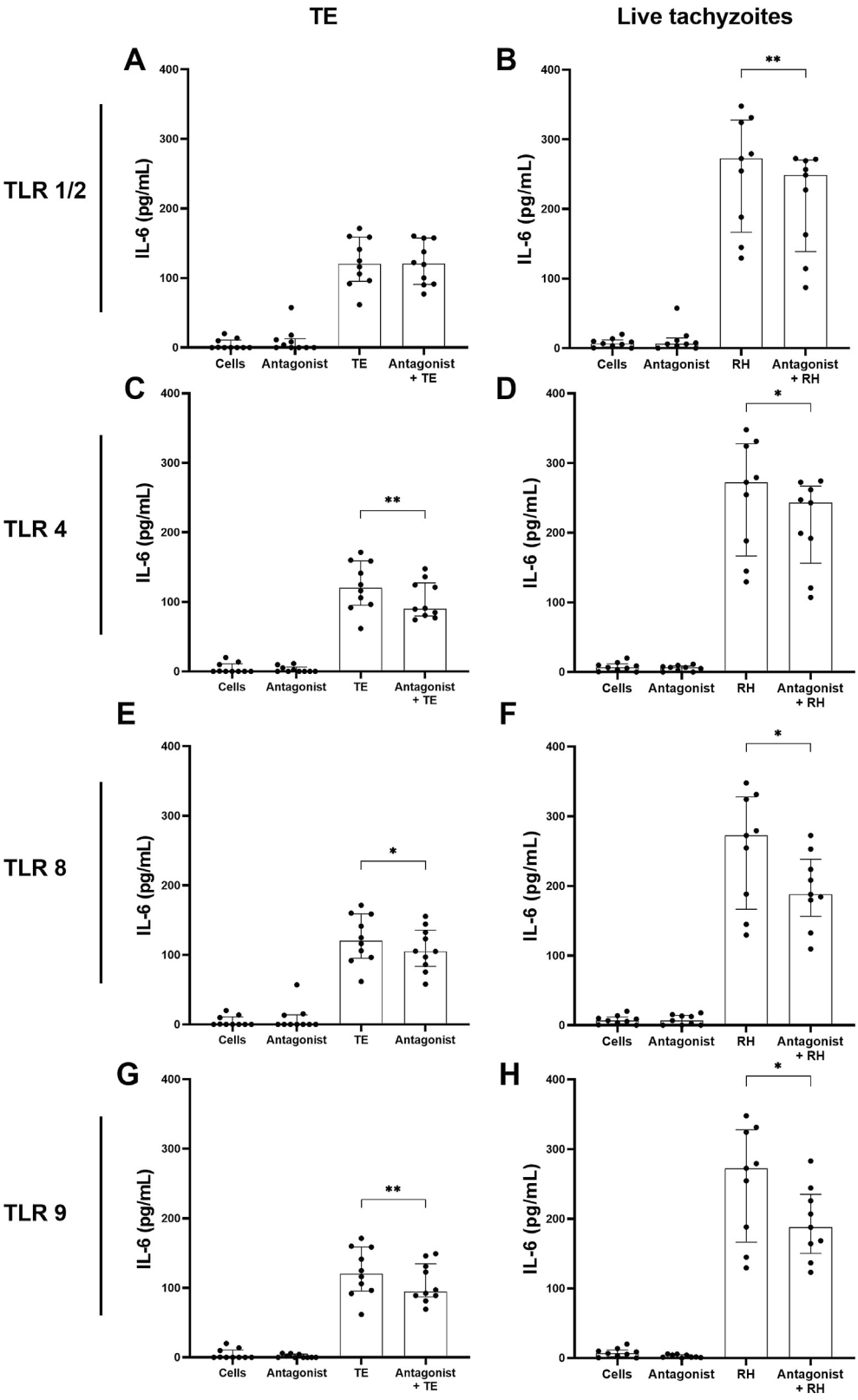

Fig. 5. Involvement of Toll-like receptors (TLRs) in *Toxoplasma gondii* recognition. The concentration of IL-6 was measured by ELISA in supernatants of 72 h cultures of peripheral blood mononuclear cell (PBMC) from naive squirrel monkeys stimulated with (A, C, E, G) total extract of *Toxoplasma gondii* proteins (TE) (n = 10) or (B, D, F, H) with *T. gondii* RH tachyzoites (n = 9), and with specific TLR antagonists. The left of the Figure shows the corresponding TLR evaluated by each specific antagonist (see Supplementary Table S2). Results, from two independent experiments, are represented as median with interquartile range and individual data point. Statistical analysis was performed using Friedman test and Wilcoxon post-test, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$. Cytokine detection threshold: IL-6 = 10 pg/mL.

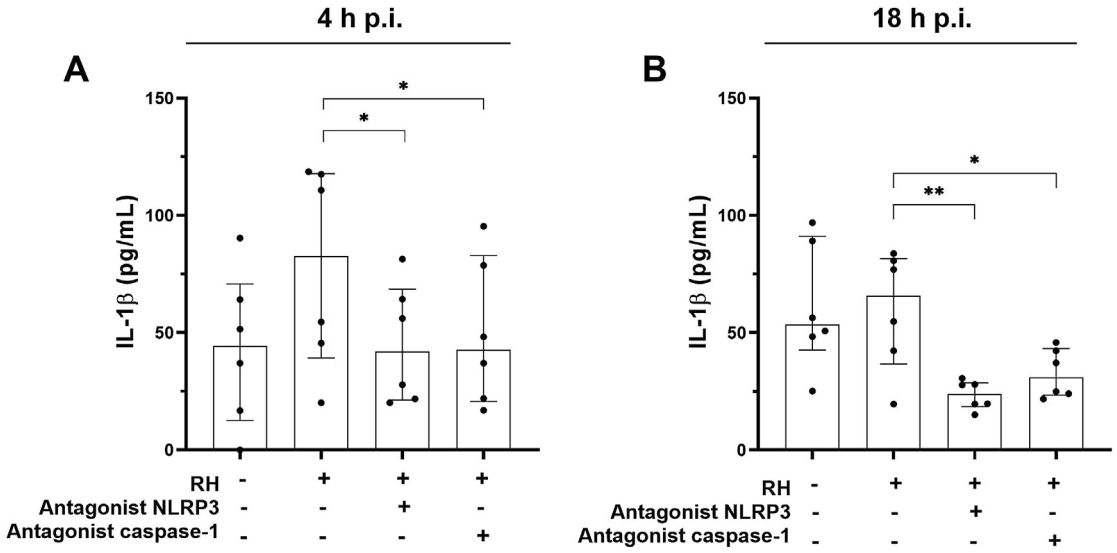

Fig. 6. Involvement of Nod-like receptors (NLRs) in *Toxoplasma gondii* recognition. Concentration of IL-1β produced by squirrel monkey peripheral blood mononuclear cells (PBMCs) (n = 6) after NLR activation by *T. gondii* was measured. PBMCs were first pre-activated by LPS before adding antagonists (MCC950 and Ac-YVAD-cmk). The infection by live tachyzoites (RH) at multiplicity of infection (MOI) = 1 was then performed and supernatants collected at (A) 4 h and (B) 18 h p.i. Statistical analysis was performed using ANOVA and paired T-test, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$. Cytokine detection threshold: IL-1β = 2.5 pg/mL.

immunisation. This Th1/Th17 profile was also observed in our previous experiments. Indeed, after three i.n. immunisations, IFN-γ and IL-17 were secreted by mice splenocytes and more specifically by CD4+ T cells (Dimier-Poisson et al., 2015). Unfortunately, we were unable to identify the IFN-γ-secreting population from squirrel monkey PBMCs. In sheep, a specific IFN-γ production was also observed in PBMCs, splenocytes and lymph nodes and no IL-17 could be detected (Ducournau et al., 2020). IL-17 has a well-recognized role in immune surveillance at mucosal surfaces and Th17 cells are critical for vaccine-induced memory immune responses against infectious diseases (Lin et al., 2010). Th17 cells are critical for vaccine-induced memory immune responses against infectious diseases especially against bacterial and viral infection (Lin et al., 2010). However, IL-17 has been associated with pathology in ocular toxoplasmosis in humans (Greigert et al., 2020) and ileitis in mice (Guiton et al., 2010). Moreover, in this work, IL-17 is not detected in naturally infected animals suggesting a minor role in long-term induced protection.

To conclude, the DGNP/TE vaccine would seem secure. No local reaction to the nose or injection site, or general reaction was noted after the vaccination (Veterinary thesis, Ferri-Pisani Maltot J., 2020, Oniris, France) including for three pregnant females who gave birth to healthy offspring. The protection induced by the vaccination is difficult to quantify in these field conditions. However, no mortality has been observed since the introduction of vaccination more than 4 years ago, contrary to the previous 8 years during which three parks were affected by recurrent episodes, recording between 1 to 15 deaths per year (Supplementary Fig. S3).

Referring to our previous work on experimental models and to the immune response induced after squirrel monkey's vaccination, this strategy could represent a hope for this extremely susceptible species. As the described immune responses are similar to well-known responses of other intermediate hosts, this work might be predictive of the feasibility of such vaccination against human toxoplasmosis.

The main hypothesis proposed to explain the high susceptibility of NWP is their arboreal lifestyle which does not allow for direct contact with feline faeces, and consequently the lack of co-evolutionary process of host-pathogen interaction. Such a co-adaptation of the host cells and *T. gondii* virulence effectors have been described in wild mice in comparison with inbred laboratory mice. Type 1 strains of *T. gondii* are highly virulent in laboratory mice because they are not destroyed by immunity-related GTPases (IRGs). In contrast in some wild-derived Eurasian mice, polymorphic IRG proteins inhibit the replication of this virulent strain (Murillo-León et al., 2019) demonstrating some co-evolutionary and co-adaptation at the molecular level.

Co-infection with other microorganisms could also participate to toxoplasmosis susceptibility. Most animals used in this study are either positive for HSV or CMV. These endemic viruses are very frequent in saimiris and their role in susceptibility to toxoplasmosis is not known. HSV2 is known to encode a viral homolog of IL-17 and to interfere with the transcription factor NF-κB in NWP (Yao et al., 2011). In humans, CMV possesses several genes with immunomodulatory functions (Jackson et al., 2011).

In humans, several single-nucleotide polymorphisms (SNPs) in innate immune system genes involved in the parasite detection have been associated with resistance or susceptibility to toxoplasmosis (McLeod et al., 2020). SNP in genes coding for TLR4, TLR9, NLRP1, the NLRP3 inflammasome activator purinergic receptor P2X7R and NOD2 have been associated with susceptibility to congenital infection and ocular toxoplasmosis (McLeod et al., 2020). Sensing of the pathogen-associated molecular pattern (PAMPs) by specific PRRs induces the development of the protective immunity. Among them, TLRs recognize a range of PAMPs and promote chemokines and cytokines production. In mice, innate recognition of *T. gondii* mainly involves TLR11/12 leading to high secretion of IL-12 (Yarovinsky et al., 2005). The role of endosomal TLR nuclear acid-sensing receptors was also suggested by Andrade et al., (2013), showing that mice carrying a mutation in UNC93B1, a chaperone for the endosomal TLR3, TLR7, and TLR9, are highly susceptible to *T. gondii* infection implicating these TLRs in the innate immunity to *T. gondii*.

The role of TLRs in parasite sensing in humans remains a matter of debate. TLR11 is not functional in primates and *tlr12* gene is not present in the primate genome. Moreover, the IL-12 response of human primary dendritic cells and monocytes requires direct contact with the parasite and phagocytosis of the live tachyzoite (Tosh et al., 2016). The work of Andrade et al., (2013) demonstrated that human PBMCs if primed with IFN-γ and cultured with *T. gondii*

RNA and DNA, produce IL-12 and TNF-α suggesting that TLR 7, 8 and 9 may have a role in *T. gondii* recognition and resistance to the parasite in human cells. However, preliminary studies that used inhibitors of the endosomal TLRs suggest that these TLRs are not involved in the cytokine response of human monocytes infected with live tachyzoites (Sher et al., 2017).

TLRs in non-human primates (NHPs) are described but functional studies are mainly performed in cynomolgus and rhesus macaques (Thompson and Loré, 2017). In humans and NHP, TLR9 is expressed in B-cells and plasmacytoid dendritic cells (pDC). C-type oligodeoxynucleotides (ODNs) stimulate B cells to secrete IL-6 and pDC to produce IFN-γ. Functionality of TLR4 and TLR9 have already been assessed in responses to their respective' agonists in squirrel monkeys (Tougan et al., 2013; Nehete et al., 2020) in vitro and in vivo. Nehete et al., (2020) show that TLR9 of squirrel monkey PBMCs responds to specific agonist stimulation in vitro by secretion of IL-12, IFN-α and IFN-γ.

In the present work, TLRs antagonists have been used to study the role of squirrel monkey TLRs in *T. gondii* sensing. IL-6 secretion was observed when PBMCs from naive animals were cultured with live *T. gondii* or soluble extract. A partial inhibition of IL-6 secretion was obtained after adjunction of each specific TLR1/2, TLR4, TLR8 and TLR7/9 antagonist. This showed that these TLRs may be involved in recognition of *T. gondii* by squirrel monkey cells. Surprisingly, soluble extract was able to activate endosomal TLRs 7, 8 and 9. Fisch et al., (2019) already reported that soluble extract transfection in THP-1 cells led to IFN-γ-induced cells death. They identified the component in soluble extract responsible for the cell death as DNA, suggesting that DNA is still present in total antigen extract and may activate TLR9. Our results showed that, like in mouse and human, TLR2, 4, 7, 8, 9 of squirrel monkey can recognize and be activated by *T. gondii* at least under in vitro conditions.

Activation of inflammasome is one of the additional pathways involved in *T. gondii* sensing. These multiprotein complexes contain sensor proteins that activate caspase-1-mediated processing of IL-1α, IL-1β and IL-18 in their bioactive forms. In murine macrophages both NLRP1 and NLRP3 cytosolic sensors are implicated in the control of infection (Gorfu et al., 2014). In humans, infection of monocytes with *T. gondii* induces activation of NLRP3 and release of IL-1β without pyroptosis (Gov et al., 2017). We used a specific NLRP3 inhibitor MCC950 that directly binds to the ATPase domain of the protein and that was described in mouse and human macrophages, to block NLRP3 inflammasome activation and IL-1β production (Zahid et al., 2019). NLRP3 protein sequence of *Saimiri boliviensis boliviensis* has been added recently in GenBank (**XP_010344136.1**). Our results are consistent with the data obtained in mouse and human and we demonstrated a reduced release of IL-1β in *T. gondii* infected-PBMCs treated with this NLRP3 inhibitor. Consistent with the results in human and mouse, we did not observe cell pyroptosis after infection.

A cell-permeable, selective and irreversible inhibitor of caspase-1 induced significant inhibition but at a similar level as observed with the NLRP3 inhibitor in our culture conditions. This suggests that the other canonical inflammasomes may not have a role in *T. gondii* sensing and control in squirrel monkey cells in contrast to rodents and human cells. Alternatively, inhibition may only be weaker than in mouse and human cells, due to the lack of specificity or affinity of this inhibitor for squirrel monkey caspase-1. NLR sensor-like NLRP1 and other non-NLR sensors (like AIM2) can also form inflammasomes. All these sensors initiate the assembly of canonical inflammasomes by recruiting caspase-1. Both NLRP1 and AIM2 are activated after *T. gondii* infection. Witola et al., (2011), demonstrated that silencing of the expression of *NLRP1* gene alters the *T. gondii*-induced expression profiles of proinflammatory cytokines and enhances proliferation of *T. gondii* in a human monocytic cell line. Polymorphisms in the *NLRP1* locus of Lewis rats are also associated with resistance to *T. gondii in vivo* (Cavaillès et al., 2006; Cirelli et al., 2014). IFN-inducible guanylate-binding protein (GBP1) was shown to promote the lysis of *T. gondii* vacuoles and parasite membranes, releasing DNA into the cytosol leading to cell apoptosis and control of the parasite *via* AIM2 (Fisch et al., 2019, 2020). Bolivian squirrel monkey GBP1; 2; 4 are known to target and destroy *Shigella flexneri* (Kohler et al., 2020). All these pathways are closely linked, and eventually are all used by the cells to control the parasite at an early phase of infection.

Innate detection of *T. gondii* by TLRs and NLRs which is the first step that drive the development of protective immunity seems to be similar in squirrel monkey cells to the processes described in mouse and human cells. However, the present findings should be interpreted with caution, because only selected cross-reactive reagents were included in this study and limited volume of blood can be collected. The extreme susceptibility to toxoplasmosis remains to be further investigated using specific and more sensitive squirrel monkeys' reagents. However, the present study provided helpful base-results in the understanding of innate immune response against *Toxoplasma gondii* in squirrel monkeys. In conclusion DGNP/TE vaccine appears to be the best known way to protect captive squirrel monkeys from toxoplasmosis reducing morbidity and mortality of highly susceptible species.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2023.02.003.

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