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Inactivated *Toxoplasma gondii* nanovaccine boosts T-cell memory response in a seropositive yellow-footed rock wallaby (*Petrogale xanthopus*) – A case report from Copenhagen Zoo

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ABSTRACT

Toxoplasma gondii is a ubiquitous parasite causing significant mortality in captive wildlife, especially marsupials. Historically, treatment has been unrewarding and no vaccine was available. An intranasal vaccine based on purified inactivated *T. gondii* was developed for toxoplasmosis prevention. A vaccination campaign started in early 2017 and was successful in preventing toxoplasma-related mortality in marsupials in many European and South American zoos. Amongst the vaccinated wallabies, about 30% were T. gondii seropositive before the vaccination, and no toxoplasma-related deaths were observed since the administration of the vaccine. The objective of this case study was to assess the potential effect of the vaccination on a seropositive wallaby. It is important to note that this vaccine doesn't induce any humoral response in sheep, and squirrel monkeys but induces a strong T-cell response. A *T. gondii* seropositive Yellow-footed rock wallaby (*Petrogale xanthopus*) from Copenhagen Zoo received two doses of the aforementioned intranasal vaccine. Blood samples were collected before each vaccination and used for peripheral blood mononuclear cell isolation. The impact of the vaccination on the lymphocyte phenotype was characterized by flow cytometry. Cell size, represented by forward scatter, and granularity, represented by side scatter parameters were analyzed. Two doses of the vaccine induced a respective 30.1 and 25.6% increase in cell size and granularity in lymphocytes stimulated with *T. gondii* antigens, as assessed by flow cytometry. These changes were likely correlated with T-cell activation, which indicates that two doses of the vaccine might have boosted the already-existing T-cell memory response against *T. gondii* in a seropositive animal. No morphological changes were observed in lymphocytes from an unvaccinated seronegative wallaby. This is the first documented case of boosting an already-existing cellular immune response against toxoplasmosis by the vaccine in a seropositive Yellow-footed rock wallaby.

1. Introduction

Toxoplasma gondii is a widespread protozoan parasite considered to be one of the most common agents of zoonotic infections in humans (Dubey, 2021, p. 202). The parasite can infect virtually any warm-blooded species, but the sexual phase of its reproductive cycle is restricted to felids. Cats are the definitive hosts of the parasite, and other animals get infected as intermediary hosts. Among these intermediary hosts, some species, such as Old World primates, rats, cattle, and horses are relatively resistant, while others, i.e. macropods and other marsupials, ring-tailed lemurs, squirrel monkeys, meerkats, and Pallas' cats are extremely vulnerable to the infection (Denk et al., 2022). It is hypothesized that their vulnerability originates from their evolution separate from the parasite, making their immune responses maladapted to combat *T. gondii* infection efficiently. The data on *T. gondii* prevalence in wild Australian marsupials is scarce (Hillman et al., 2016), as clinical toxoplasmosis is rarely observed in free-ranging marsupials (Dubey et al., 2021). One report documents neurological problems and sudden

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death in a colony of allied rock wallabies (*Petrogale assimilis*) living in a peri-urban area of Magnetic Island, Australia (Bowater et al., 2024). The mortality was attributed to *T. gondii* and the outbreak of toxoplasmosis on the island was likely caused by an increased population of domestic cats in the area. Mortality in captive macropods is very common as documented in zoological parks and reserves in the United States (Guthrie et al., 2017; Spriggs et al., 2020; Anderson and Dennis, 2022), Argentina (Basso et al., 2007), Spain (Bermúdez et al., 2009; Fernández-Aguilar et al., 2013), and Hungary (Sós et al., 2012).

The clinical manifestations of the disease in captive macropods often include lethargy, gastrointestinal problems, ataxia, tachypnea, disorientation, blindness (Dubey and Crutchley, 2008), and often sudden death (Guthrie et al., 2017; Bowater et al., 2024; Jensen et al., 1985). Treatments with antibiotics, steroids, or intravenous fluids rarely improve the state of the animal (Jensen et al., 1985), but cases of a successful recovery after the treatment with trimethoprim/sulfadoxin (Hermosilla et al., 2010) or with Atovaquone (Dubey and Crutchley, 2008) have been documented.

Despite the high prevalence of *T. gondii* infection in zoo animals, there is currently no licensed vaccine to protect them against the disease caused by the parasite. The only veterinary vaccine approved for use in animals is Ovilis® Toxovax (MSD Animal Health), a live-attenuated vaccine (LAV) based on the tachyzoite form of *T. gondii*, administered *via* intramuscular injection (Katzer et al., 2014). Ovilis® Toxovax was licensed for use in sheep, aiming at reducing the economic losses caused by toxoplasma-caused abortions. The downside of this vaccine type is the use of an attenuated form of the parasite, which can be associated with a risk of a spontaneous reversal of the attenuated phenotype or insufficient attenuation for use in highly susceptible species. This, in turn, precludes the vaccine from its use in immunosuppressed, immunodeficient and older individuals, or in high-risk animals in which the immune response is yet to be characterized.

An intranasal vaccine composed of *T. gondii* purified inactivated parasite (PIP) formulated with maltodextrin nanoparticles for toxoplasmosis prevention was previously trialed in mice (Dimier-Poisson et al., 2015; Ducournau et al., 2017), sheep (Ducournau et al., 2020), and non-human primates (Ducournau et al., 2023; Fasquelle et al., 2023). The advantages of this inactivated vaccine are numerous. The killed parasite is devoid of the risk of causing the disease, and upon formulation with nanoparticles it can stimulate an efficient Th1 cellular immune response in the examined species. Inactivated vaccines are also easier to produce than LAVs – their generation and processing consist of fewer steps involving a live pathogen, making the process safer, easier and requiring less specialized facilities. Using a killed parasite is also advantageous when it comes to vaccine storage, as it is easier to preserve the properties of a PIP than a live parasite.

In 2017, vaccination campaigns using this Inactivated *T. gondii* nanovaccine were started in various zoos in France, and other European countries. Copenhagen Zoo is one of the participants of the vaccination campaigns, and wallabies are among the species vaccinated. With over 24 primate species (i.e. lemurs, New World Primates), various marsupials (i.e. quolls, kangaroos, Tasmanian devils, wombats, wallabies), meerkats, and Pallas' cats vaccinated, no toxoplasmosis-related mortality in vaccinated animals was observed since the beginning of the vaccinations (Vaxinano, 2024). Upon the characterization of the immune response induced by the vaccination in squirrel monkeys, a strong induction of a protective Th1-dominated response was observed, without vaccine-associated antibody production in this species (Ducournau et al., 2023; Fasquelle et al., 2023). In this study, the immune response induced by the vaccination was evaluated in a vaccinated *T. gondii*-seropositive Yellow-footed rock wallabies at Copenhagen Zoo, and compared with an unvaccinated seronegative wallaby. It is important to note, that approximately 30% of the vaccinated wallabies included in the aforementioned campaign were seropositive for *T. gondii* prior to the vaccination.

2. Materials and methods

2.1. Animals and vaccination

A *T. gondii*-seropositive female Yellow-footed rock wallaby aged 2 years and 10 months from Copenhagen Zoo received two doses of the intranasal vaccine constituted by 50 μg of *T. gondii* PIP associated with 150 μg of maltodextrin nanoparticles, one month apart. A seronegative 3 year and 2 month-old conspecific female was also vaccinated. Both animals were anesthetized with medetomidine (30 mg/ml Pharmacie Glostrup, 2600 Glostrup, Denmark, 0.1–0.15 mg/kg IM), ketamine (100 mg/ml Ketador Vet, VetViva Richter GmbH, 4600 Wels, Austria, 2.1–2.9 mg/kg IM), butorphanol (10 mg/ml Butomidor, Richter Pharma AG, 4600 Wels, Austria, 0.1–0.15 mg/kg IM), methadone (10 mg/ml Insistor, Salmfarm, 6000 Kolding, Denmark, 0.1–0.15 mg/kg IM) and midazolam (5 mg/ml Hameln Pharma GmbH, 31787 Hameln, Germany, 0.05–0.07 mg/kg IM), by remote delivery for the vaccination to take place. Anaesthesia was reversed with atipamezole (5 mg/ml Antisedan, Orion Pharma Animal Health, 2300 København S, Denmark, 0.47–0.59 mg/kg IM) and naltrexone (50 mg/ml APL, 14175 Kurva, Sweden, 1.6–1.9 mg/kg IM). Blood samples were collected before each dose of the vaccine, transported in lithium-heparin-coated tubes, and processed within 6 h. The serological status of the animals was determined by an immunochromatographic IgG-IgM test (Toxoplasma ICT IgG-IgM, LDBio, France). Vaccination of susceptible animals at Copenhagen Zoo was initiated in December 2021 and procedures to study the reaction of the animals to this vaccination were approved by the Institutional Animal Use and Care Committee (approval number 21-01).

2.2. PBMC isolation

Peripheral Blood Mononculear Cells (PBMCs) were isolated from the blood of one vaccinated seropositive yellow-footed rock wallaby, and one unvaccinated seronegative yellow-footed rock wallaby *via* density gradient centrifugation. Briefly, heparin-stabilized whole blood samples were diluted 1:1 with phosphate-buffered saline (PBS, SSI) and layered on SepMate™ tubes (StemCell Technologies, cat.: 85450) with Lymphoprep™ (StemCell Technologies, cat.: 07861) and centrifuged at 1200g for 20 min at room temperature. The cells were washed twice with PBS +2% Fetal Bovine Serum (FBS, Gibco, France) at 300g, counted and frozen with 1-3 $\times$ 10^6 PBMCs/vial in 50% FBS/40% RPMI 1640 (Thermo Fisher Scientific, cat.: 11875093)/10% DMSO (Invitrogen, France).

For the isolation of PBMSc from human donor blood samples sourced from Etablissement Français du Sang (EFS, convention PLER-UPR/2024/083), whole blood samples were diluted 1:1 with phosphate-buffered saline (PBS, Gibco, France) and carefully layered over an equivalent volume of room-temperature Histopaque®-1077 (Sigma-Aldrich Germany). The samples were centrifuged at 400g for 30 min at room temperature with gentle acceleration and deceleration. Then, the layer constituted by PBMCs was aspired and washed with PBS. Residual red blood cells were lysed *via* 30s incubation in ultrapure water and then the cells were washed once again. PBMC pellets were resuspended in RPMI 1640 culture medium (Gibco, France) + 10% Fetal Bovine Serum (FBS, Gibco, France), 1% penicillin-streptomycin (Gibco, France) and 0.001% 2-mercaptoethanol (Gibco, France). Cells were counted and frozen in the culture medium supplemented with 10% DMSO (Invitrogen, France) and thawed on the day of antigen stimulation.

2.3. Flow cytometry

PBMCs isolated from the vaccinated *T. gondii*-seropositive Yellow-footed rock wallaby and one unvaccinated seronegative animal of the same species were seeded in V-shaped 96 well-plates at a density of 2E+05 cells/well. Cells were stimulated for one week with either 1 μg of *T. gondii* PIP or 0.5 μg phytohemagglutinin (PHA, Sigma Aldrich

Germany, L2769-2 mg) (positive controls, data not shown). After a week of incubation at 37 °C, cells were harvested, washed with PBS supplemented with 2% FBS, marked with TO-PRO-3 (ThermoFisher Scientific France) viability marker and sorted using an Attune NxT Acoustic Focus Cytometer (Invitrogen, France). Based on their size and granularity, the population corresponding to lymphocytes was gated on the FSC-A/SSC-A plot. Then, single live cells were selected on FSC-H/FSC-A plots, and the changes in the cell size (FSC parameter) and granularity (SSC parameter) between unstimulated and stimulated cells were measured. The same procedure was performed to stimulate and sort PBMCs from human donors.

3. Results

Without wallaby-specific reagents to monitor cell-mediated immune parameters, we developed a method based on changes in size and granularity of PBMCs stimulated with *T. gondii* PIP. The method was first validated on *T. gondii* PIP-stimulated human PBMCs. Upon analyzing FSC and SSC parameters in stimulated PBMCs from a *T. gondii*-seronegative donor, no changes in lymphocyte size (FSC-A) and a slight, 4.6% increase in granularity (SSC-A) parameter were observed (Fig. 1A–B). On the contrary, a significant increase in both parameters was observed in PBMCs from seropositive human donors. Indeed, 40.5 and 17.8% rise in FSC-A, and 65.1 and 51.7% increase in SSC-A parameters occurred in donors n°2 and n°3, respectively (Fig. 1C–D). Upon analyzing FSC and SSC parameters in an unvaccinated *T. gondii*-seronegative wallaby, no changes in lymphocyte morphology were observed when stimulated with the PIP (Fig. 2A–B). On the contrary, lymphocytes from a vaccinated *T. gondii*-seropositive wallaby stimulated with the PIP showed a corresponding basal 13.0 and 9.6% increase in FSC-A and SSC-A before the vaccination. A corresponding increase of 30.1 and 25.6% in FSC-A and SSC-A parameters was observed after the first two doses of the intranasal vaccine in this animal (Fig. 2C–D). Upon analyzing the changes in lymphocyte count between unstimulated and stimulated PBMCs from the seropositive wallaby, we observed an even increase in the cell number after the first dose of the vaccine (Fig. 3). Indeed, 4.57 and 4.24-fold increase in the lymphocyte count was observed after a single vaccination in unstimulated and stimulated cells, respectively. While the number dropped in unstimulated cells to 2.44-fold of the initial value, it increased to 5.68-fold of the initial value in stimulated cells in the samples from 11 moths after the second vaccination.

4. Discussion

Recent studies point to a rising trend in suspected toxoplasmosis cases in zoo animals (Denk et al., 2022), in which *T. gondii* represents a significant mortality threat. A retrospective review of pathology records at Copenhagen Zoo, from 2010 to 2020, before vaccination of susceptible species against the parasite was initiated, revealed 6 confirmed and 5 suspect cases of the disease as the cause of mortality in zoo animals, three of them macropods. Toxoplasmosis is one of the lead causes of mortality in captive macropods, particularly in Bennett's wallabies (*Macropus rufogriseus*) (Anderson and Dennis, 2022; Basso et al., 2007; Dubey et al., 2021; Dubey and Crutchley, 2008; Guthrie et al., 2017) and red kangaroos (*Osphranter rufus*) (Anderson and Dennis, 2022; Dubey et al., 2021; Spriggs et al., 2020). Indeed, 90% mortality caused by acute toxoplasmosis was observed in a mob of 10 Bennett's wallabies affected by a newly described strain of *T. gondii* (Guthrie et al., 2017). The animals were seronegative before the transport from New Zealand to the United States of America and died of toxoplasmosis on average 224 (59–565) days after the move. Toxoplasmosis was also the third most prevalent cause of death in Bennett's wallabies in Cleveland Metroparks Zoo between 1995 and 2016, where 7 in 64 animals died following infection with the parasite (Anderson and Dennis, 2022). Florida Zoo reported significant mortality due to systemic toxoplasmosis in their macropods: three different species of wallabies (agile wallaby, Bennett's wallaby, and Tammar wallaby), as well as red kangaroos, died of infection (Spriggs et al., 2020). The occurrence of deaths was correlated with heavy rainfall and flooding of the macropod exhibit, emphasizing the role of transmission of *T. gondii* oocysts by water. Deaths from toxoplasmosis in Bennett's wallabies have also been reported in Argentina (Basso et al., 2007). The first peer-reviewed reports of toxoplasmosis in captive wallabies in Europe date from 2009 (Bermúdez et al., 2009). In Spain, a mob of 7 Bennett's wallabies died over a 17-month period (Fernández-Aguilar et al., 2013). Upon the examination of one of the deceased animals, *T. gondii* infection was detected. A

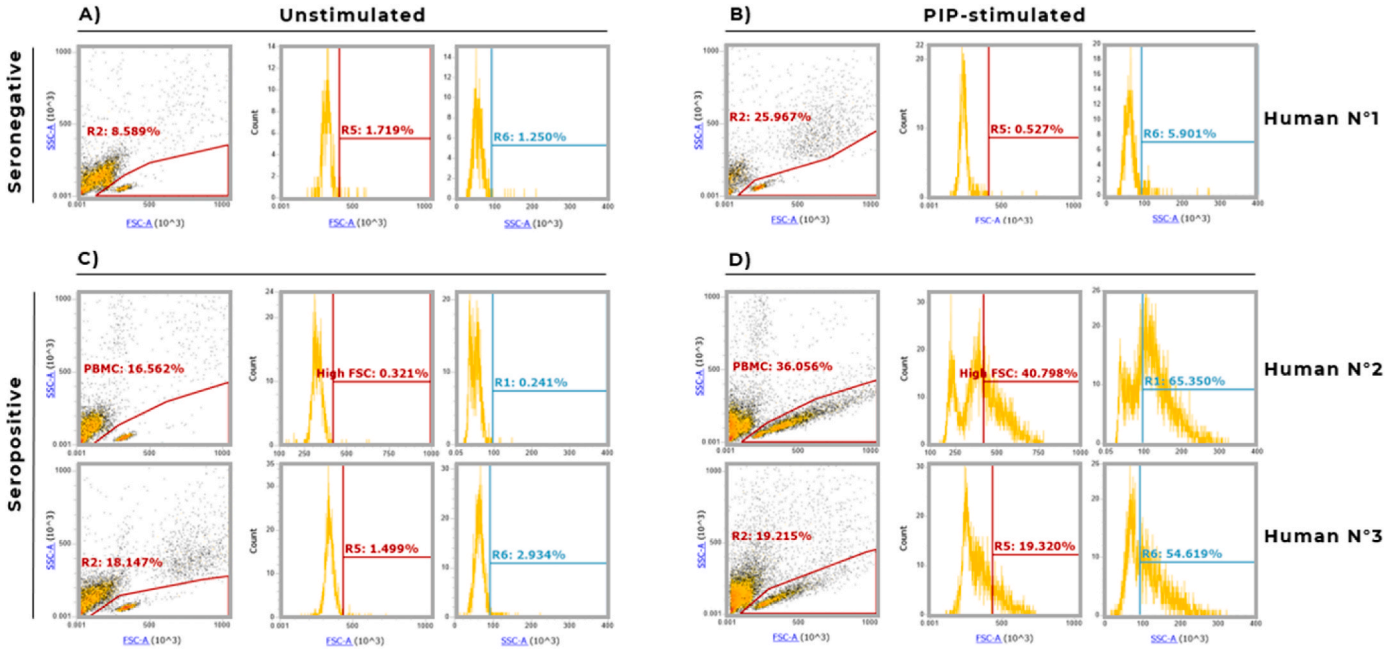

Fig. 1. Changes in cell size (count/FSC-A) and cell granularity (count/SSC-A) in unstimulated (A, C) and T. gondii PIP-stimulated (B, D) PBMCs from a T. gondii seronegative (A–B) and a seropositive human donor (C–D).

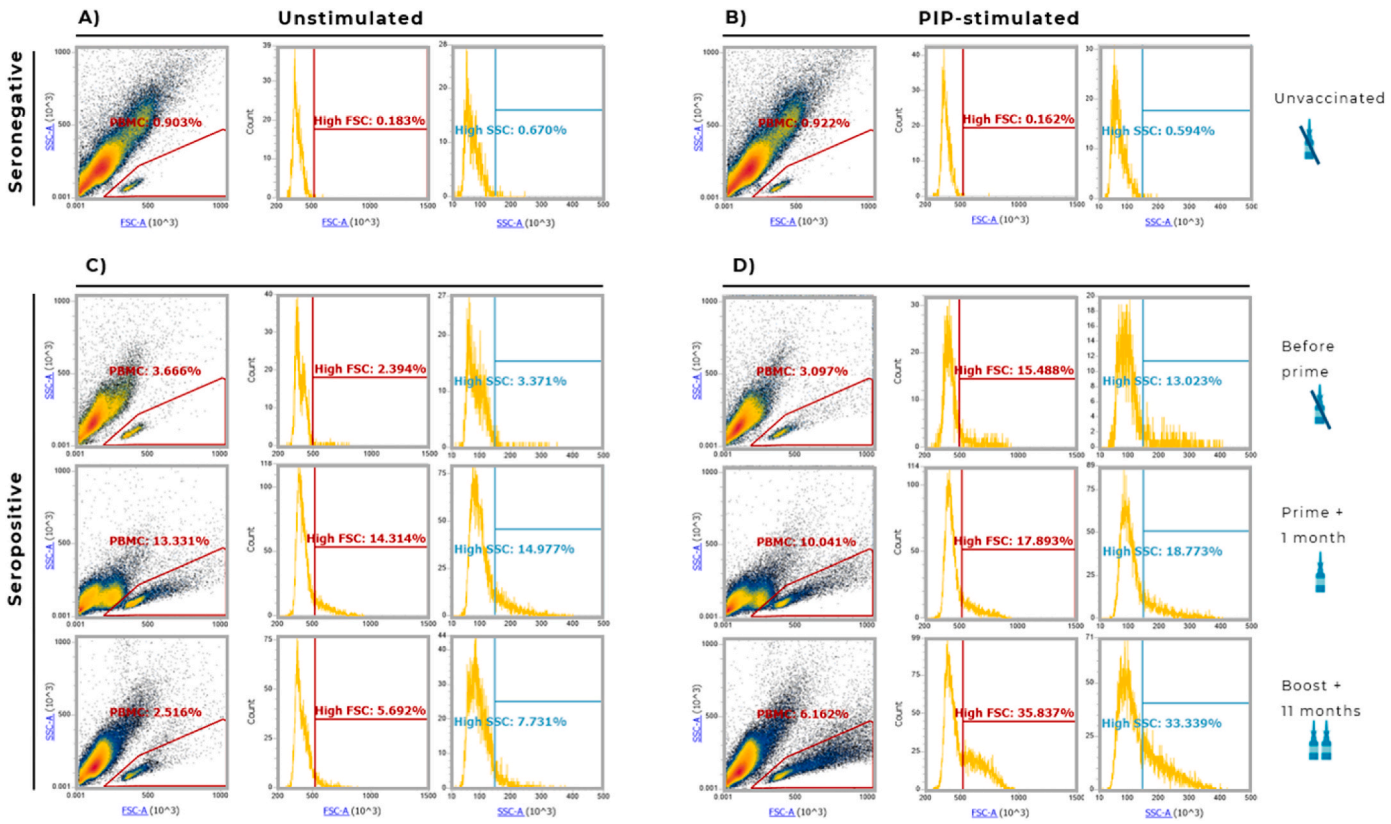

Fig. 2. Changes in cell size (count/FSC-A) and granularity (count/SSC-A) in unstimulated (A, C) and T. gondii PIP-stimulated (B, D) PBMCs from an unvaccinated seronegative wallaby (A–B) and a vaccinated seropositive wallaby (C–D).

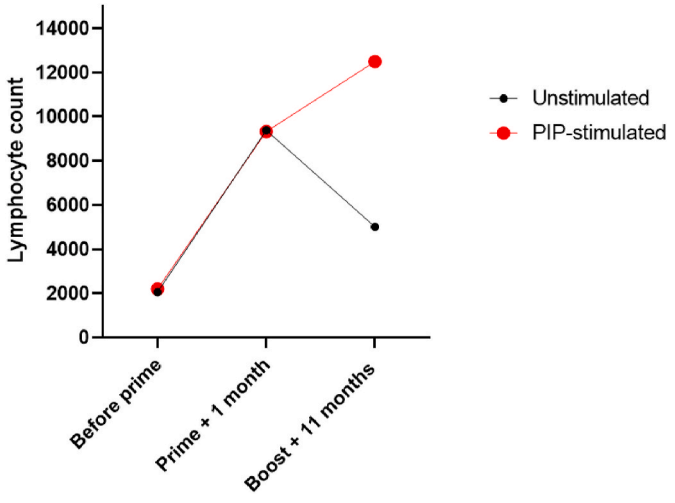

Fig. 3. Changes in a lymphocyte count in a sample from a seropositive wallaby.

mortality event in which twenty-one Tammar wallabies (*Notamacropus eugenii*) died from toxoplasmosis was also described in Budapest Zoo in Hungary (Sós et al., 2012).

Many species of wallabies, including species in the genus *Petrogale*, are endangered, and captive breeding programs are an important tool in the preservation of rare taxa (Read and Ward, 2009, 2011). However, high toxoplasmosis-related mortality in captive wallabies can compromise the success of such programs. Australian marsupials are very vulnerable to *T. gondii* infection, likely as a consequence of having evolved in isolation from the parasite. Since both acute and chronic forms of toxoplasmosis can result in sudden death, strategies for preventing the first infection or preventing parasite reactivation are warranted. Sadly, the sole marked-approved veterinary vaccine to date, Ovilis® Toxovax (MSD Animal Health) is not advised for those high-risk animals. An alternative vaccine administered in the form of intranasal spray was used to immunize Yellow-footed rock wallabies in the Copenhagen Zoo. As many of the vaccinated wallabies were *T. gondii* seropositive, an attempt was made to characterize the immune response in a seropositive Yellow-footed rock wallaby after receiving two first doses of the three-dose vaccination regimen.

In contrast to the humoral immune response, which can be easily measured by quantifying serum and mucosal antibodies against the parasite, studying the cellular component of the immune system is challenging. Most of the assays that allow for the characterization of lymphocyte T-cell responses are based on species-specific antibodies and are available for species broadly used in research, i.e. mice, sheep, rhesus macaques, but not marsupials. The intranasal vaccine was described to induce a Th1-biased immune response, characterized by T-cell activation and the associated IFN-γ release, without stimulating the antibody production in squirrel monkeys (Ducournau et al., 2023; Fasquelle et al., 2023). IFN-γ production by PBMCs is one of the hallmarks of T-cell activation and a Th1-polarised immune response. Changes in this cytokine's level can be easily measured by ELISA, ELISPOT or intracellular flow cytometry in species for which the antibodies are available. Since the measure of IFN-γ production using species-specific antibodies was impossible, the changes in morphological parameters, namely cells size (FSC) and granularity (SSC), of the *T. gondii* PIP-stimulated lymphocytes from the vaccinated seropositive and seronegative wallabies were analyzed *via* flow cytometry. Changes in the forward scatter (FSC) parameter of flow cytometry light scattering have been correlated with T-cell activation in human PBMCs (Böhmer et al., 2011). Furthermore, the alterations in side scatter (SSC) parameter have also been described as a useful measure of changes in the ratios between

different lymphocyte subtypes (Wu et al., 2023). The changes in total lymphocyte count in the samples were also analyzed.

T. gondii PIP stimulation of PBMCs isolated from both seropositive humans and wallabies induced a significant increase in both cell size and granularity of the stimulated lymphocytes. The phenomenon was restricted to the antigen-experienced cells, as none or marginal changes in those parameters were observed in *T. gondii*-seronegative individuals, human or wallaby. The response was, therefore, antigen-specific. Increased lymphocyte size and cell complexity are considered hallmarks of T-cell activation (Böhmer et al., 2011), and are often accompanied by IFN-γ release. In the immune response to *T. gondii*, IFN-γ activates various antiparasitic mechanisms inhibiting parasite growth and is mainly produced by $CD4^+$ T cells and NK cells in the acute stage and by $CD8^+$ cytotoxic T lymphocytes during chronic infection thus helping to contain latent infection and prevent re-activation (Ihara and Yamamoto, 2024). IFN-γ release upon stimulation with *T. gondii* antigens was previously documented in PBMCs from *T. gondii*-seropositive human donors showing either no symptoms or suffering from ocular toxoplasmosis (García-López et al., 2024). Since no IFN-γ detection kits for wallabies are available, changes in FSC and SSC parameters were the only measurable markers of lymphocyte activation upon exposition *T. gondii* antigens.

Similar to the seropositive human sample, the PIP-stimulation of PBMCs from the seropositive wallaby increased FSC by 13.0% and SSC by 9.6% prior to the vaccination while no increase was observed in the seronegative wallaby (Fig. 2C–D). This confirms the presence of a basal memory immune response established by the T. gondii infection in the seropositive animal. Interestingly, the FSC and SSC values observed in PIP-stimulated PBMCs from the seropositive wallaby increased by 17.1 and 15.9%, respectively, after two doses of the vaccine compared to the basal values from before the vaccination (Fig. 2C–D). This may indicate that the vaccination re-activates and boosts the cellular component of the adaptative memory immune response already established by the natural infection. This observation is confirmed by the analysis of the lymphocyte count in the sample from the seropositive wallaby after double immunization (Fig. 3). Indeed, changes in the lymphocyte number revealed the presence of a lymphocyte population that proliferates selectively in reaction to T. gondii antigens.

Cellular memory response involving cytotoxic $CD8^+$ T-cells, and helper $CD4^+$ T-cells, is directly involved in maintaining the latency of the parasite during the chronic phase of infection (Ihara and Yamamoto, 2024). Beyond a preventative effect of vaccination prior to infection, it is thus plausible that the intranasal vaccination prevents the reactivation of dormant parasites in *T.gondii*-infected animals by inducing a boost of an existing memory T-cell response. This hypothesis is supported by the fact that approximately one-third of the wallabies vaccinated during the vaccination campaigns were seropositive and no *T. gondii*-related deaths occurred in those animals since the beginning of the vaccination. Indeed, the seropositive animal presented in this study was euthanized 2 years following vaccination due to alveolar periostitis. Histopathological examination of all major organs revealed a single bradyzoite-filled cyst in the myocardium but no other histological features of systemic infection with T. gondii, indicating that the infection had been contained.

5. Conclusions

As observed in the seropositive wallaby and seropositive human donors, stimulation of lymphocytes with *T. gondii* PIP can reactivate the already-existing antigen-specific memory T-cell response, and morphological changes in cell size and structure that accompany this activation can be measured by flow cytometry. A marked increase in this T-cell activation was observed in the seropositive animal after two doses of a toxoplasma vaccine. The vaccine might protect seropositive animals from parasite reactivation by boosting the memory T-cell responses, which can also help to contain the infection and/or reduce the symptoms of disease in seropositive animals in an event of recrudescence. The latter may explain the lack of mortality due to toxoplasmosis in vaccinated seropositive wallabies, since the beginning of the vaccinations. This is the first documented case of boosting a T-cell memory response by intranasal inactivated toxoplasma nanovaccine in a T. gondii-seropositive wallaby.

CRediT authorship contribution statement

Daniela Ogonczyk-Makowska: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **François Fasquelle:** Writing – review & editing, Validation, Project administration, Methodology, Investigation, Conceptualization. **Anaïs-Camille Vreulx:** Methodology, Investigation. **Angelo Scuotto:** Writing – review & editing, Validation, Project administration. **Amélie Degraeve:** Investigation. **Bryan Thiroux:** Investigation. **Louise Françoise Martin:** Writing – review & editing, Investigation. **Stamatios Alan Tahas:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Jeanne Toft Jakobsen:** Investigation. **Gregers Jungersen:** Writing – review & editing, Validation, Methodology, Conceptualization. **Didier Betbeder:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following conflict of interest:

Daniela Ogonczyk-Makowska, Anaïs-Camille Vreulx, Angelo Scuotto, Amélie Degraeve, Bryan Thiroux are employed by Vaxinano and involved in toxoplasma nanovaccine development. François Fasquelle is a former employee of Vaxinano. Didier Betbeder is the founder of Vaxinano.

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